Isolation of Bacillus Velezensis from Leepuram Sea Sediment and Its Bioactive Compound and Antimicrobial Activity

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Spectroscopy, Bacillus velezensis.

ABSTRACT
Research into marine bacteria in India for their potential active chemicals is essential for the creation of medications derived from marine sources. The objective of this research was to collect Bacillus velezensis from Leepuram Sea Sediment, test their antibacterial efficacy with the agar diffusion method, and find secondary metabolites by GC-MS spectroscopy.Traditional microbiological methods and phylogenetic analysis of 16S rRNA sequences were used to successfully identify Bacillus velezensis from sediment samples obtained in the Leepuram Sea, Kanyakumari. Using the agar well diffusion method, we tested the isolated metabolites for their antibacterial activity against Gram-positive and Gram-negative bacteria. The results showed that the extract had a strong antibacterial effect, inhibiting the growth of harmful bacteria with zones of inhibition as large as 19 mm for E. coli and Pseudomonas.Bacillus velezensis produces secondary metabolites with pharmacological potential, according to this study. These metabolites may have useful uses in biocontrol, agriculture, and medicine. The implications of these results for human health and the creation of new antimicrobial drugs are likely to grow substantially when more worldwide research is carried out.

1. Introduction

There is a danger to society from the increasing difficulty of treating infections caused by bacteria and other microbes that are resistant to antibiotics. An estimated one million people each year lose their lives due to AMR-related illnesses (de Kraker et al., 2016, Droz et al., 2019). The incorrect and unchecked use of antibiotics, insufficient regulation, and a dearth of research and new antibiotic discoveries are three major contributors to the increase of microbes that are resistant to antibiotics (Ferri et al., 2017). Discovering secure substitutes for conventional antibiotics has risen to the forefront of scientific agenda considering the pressing necessity to safeguard the planet and attain sustainable development. Antimicrobial peptides, phage, nanoparticles, as well as photodynamic therapy are some of the promising new alternatives that are presently being researched. Because of their wide range of structural and functional features, as well as their fast and specific effects, antimicrobial peptides have a lot of promise as alternatives to conventional antibiotics (Amerikova et al., 2019). The antimicrobial and antifungal properties of lipopeptides have been the subject of substantial research (Lei et al., 2019).

There are many industries, including agriculture, health, and manufacturing, that greatly benefit from bacteria belonging to the genus Bacillus. Their usefulness arises from characteristics including being easily isolated and cultivated, having the ability to form biofilms, and being resilient to harsh environmental conditions by means of spore development (Guo et al., 2019; Ben Slama et al., 2019). Different types of Bacillus velezensis have recently been the subject of a great deal of study (Chen, 2017; Baptista, 2018; Cheffi et al., 2019). All sorts of things, from soil and water to air and the plant rhizosphere, as well as in humans, animals, as well as fermented foods, are home to these helpful bacteria. Their functions in stimulating plant growth and biologically controlling phytopathogens, such as oomycetes, nematodes, bacteria, viruses, and fungi, are vital (Nguyen et al., 2019; Xu et al., 2019). The genome of the type of strain of B. velezensis, FZB42, was sequenced in 2007 after it was obtained in 2005 from the Vélez River in Málaga, Spain (Ruiz-Garcia et al., 2005; Chen et al., 2007). The availability of more than 200 B. velezensis genome sequences in GenBank have allowed researchers to gain a better grasp of the gene clusters that produce secondary metabolites, most notably bacteriocins, polyketides, as well as lipopeptides (Dunlap et al., 2015; Palazzini et al., 2016; Cai et al., 2017). Furthermore, genes associated

with biocontrol and plant growth improvement have been discovered in B. velezensis strains using genome mining techniques (Chen et al., 2007; Stein et al., 2005).

Numerous strains of B. velezensis have been studied extensively due to their distinct properties, which have been isolated from different substrates throughout time. Sequence similarity above 99% in the 16S rRNA gene were the first basis for classifying B. velezensis as B. amyloliquefaciens (Chen et al., 2017, Hong et al., 2021). Nevertheless, the taxonomic differences between both species have been elucidated by sophisticated genomic investigations that target complete genomes. B. velezensis is quite likeB. amyloliquefaciens subsp. plantarum, B. methylotrophicus, as well as B. oryzicola, according to phylogenetic studies (Sudarmono et al., 2019). These strains were reclassified in 2017 as part of a "B. amyloliquefaciens operational group" that also includes B. siamensis, B. velezensis, and B. amyloliquefaciens (Deleu et al., 2008).

A secondary metabolite was isolated and studied in this study from the marine Bacillus velezensis organism that was found in the Leepuram Sea Sediments. The potential of the crude extract as an antioxidant, antimicrobialand antibiofilmwas also evaluated in vitro.

2. Materials and Methods

Sample collection and bacterial isolation: Sea sediments were collected; Bacteria wereisolated using the serial dilution technique (Hayakawa et al., 1987).

Bacterial Isolate Identification:

Sediment obtained at Leepuram seashore in Kanyakumari district.

Culture inoculation:

Preparation of media nutrient agar has been done for plating. Serial dilution has been carried out for cultures grown from the sample and the last 5 series (10^{-6}- 10^{-10}) were inoculated in the nutrient agar media and the series of 10^{-3},10^{-4}, using spread plate method and the plates were incubated at room temperature.

Isolation of Marine Bacillus:

The samples (0.5g) were titrated, suspended with sterile seawater and spreaded on the entire surface of 1/10 Marine Agar Medium. The medium were prepared by adding the components of peptone (0.12g), Yeast extract (0.025 g), Fepo4 (0.025 g), Agar (3.75 g), samples (5 ml). After incubation at 25°c for 2 days, all colonies with different morphology were chosen for bacterial isolation (Sakemi et al., 1988).

Identification of Marine Bacillus:

The isolated marine Bacillus was identified based on the colony morphology, gram staining property and biochemical characteristics. Finally, they were confirmed by their growth on Marine agar medium.

Preparation of Bacillus cultures and crude extracts:

300 ml of marine broth was prepared. The medium was prepared by adding the components of Peptone (5g), Yeast extract (1g), FePO4 (0.1g), Seawater (1 L). The marine Bacillus was cultured in 300 ml of marine broth in 500ml of conical flask for the production (Blunt et al., 2004).
Extraction:

To extract compounds from bacterial cultures. The microbes should be cultured in a suitable growth medium till they produce enough biomass. After that, transfer the culture to a separator funnel. Add ethyl acetate to the culture as well as mix thoroughly to allow the solvent dissolve the desired metabolites. Allow the liquid to settle after mixing, which will result in the upper layer becoming ethyl acetate. Carefully drain or siphon the ethyl acetate layer, that holds the extracted chemicals. The last step in making a concentrated extract is to drop the pressure and let the ethyl acetate evaporate. This approach successfully separates bioactive compounds for further research and characterisation (Effendi et al., 2004).

Thin layer chromatography:

Thin layer chromatography (TLC) was used to separate crude extracts from various solvents (Sani et al., 2018). The crude fraction (50 µL) was spotted on the TLC plate, and chromatography was done using solvent systems hexane: ethyl acetate (1:1, 3:7, 7:3 v/v). Spraying with p-anisaldehyde revealed the presence of spots. The eluted

plates were thoroughly dried, and the chromatograms were seen under UV at long and short UV (365 nm and 254 nm), and the movement of the phytochemical along with the solvent was quantified (Rf value). Molecular identification of Bacillus velezensis

A single cell was picked up from the solid surface medium and suspended in 50 µL water (nuclease-free). The extraction of genomic DNA was performed using the Nucleo Spin® Tissue Kit (Macherey-Nagel) following the instructions provided by the manufacturer. The reaction mix for a PCR reaction consists of a 5 µL DNA sample, 1.5 µL of Forward and Reverse Primers, 5 µL of deionized water, along with 12 µL of Taq Master Mix, making a total of 25 µL. The reaction is subject to thermal cycling conditions. Initially, the DNA template is subjected to a 2-minute denaturation at 95°C, which disrupts inter-strand hydrogen bonds and results in the formation of single-stranded DNA. The filaments are further separated by a second 30-second denaturation at 95°C (Ramyaet al., 2024).

Table 1 Primer used for Bacteria [5]

Target	Primer Name	Sequence Details	Number of Base
16S rRNA	16S-RS-F	5'CAGGCCTAACACATGCAAGTC 3'	19
	16S-RS-R	5' GGGCGGWGTGTACAAGGC 3'	20

6 µL of PCR samples were analyzed using 0.32 g of agarose dissolved in 40 mL of 1 X TAE

buffer, then added 4 µL of red Gel. Gel electrophoresis wasrun at 110 volts for 1 hour. The electropherogram wasviewed and photographed under UV light in the Geldocumentation system. Bands of the isolate were comparedto the 1 kb DNA standard to verify PCR results. PCRproducts are purified and then sequenced using 2 primersSequencing data analysis was carried out using the BioEdit software program. Sequence alignment analysis was carried out by comparing the sequences obtained from sequencing with those already in GeneBank using BLAST (Basic Local Alignment Search Tool) on the site http://blast.ncbi.nlm.nih.gov/Blast.cgi). Phylogeny analysis was performed using the PhyML 3.0 tool and the aLRT (approximate Likelihood Ratio Test). The HKY85 model of nucleotide substitution was employed. PhyML is well-known for its rapidity and precision in phylogeny analysis, as seen by compared with various existing programs using simulated data, and it provides ideas into the evolutionary links between aligned sequencing.

Compound Identification Using Gas Chromatography Analysis:

Ethyl acetate fraction was subjected to GC-MSanalysis, and the conditions used for the GC-MS analysisare presented in Table 1. The mass spectra of the chemicalcomponents were compared to the known chemicalcompounds in the National Institute Standard andTechnology (NIST) library (Chaudhary and Tripathy., 2015).

Table 2. GC-MS condition

GCprogram	
Column	Rtx5(fused silica), 30m×250µm×0,25µm.
Equipment	GCMS-QP2010SSHIMADZU
Carrier gas	Heliumgas0,5 mL/min
Pressure	13.7kPa
Detector	Mass detector
Sampleinjection	3µl
ColumnOventemperature	70°C
Injectiontemperature	300°C
Total GCruntime	80min
MSprogram	
Inletlinetemperature	250°C
Sourcetemperature	300°C
Electronenergy	70Ev
Massscanm/z	28-600amu

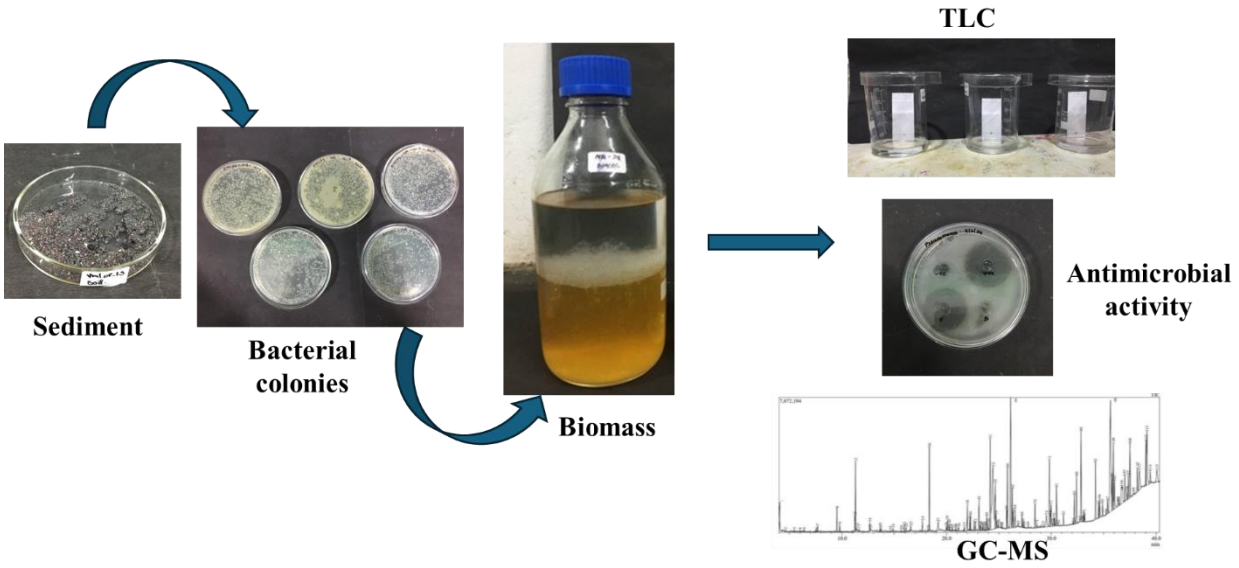
Antibacterial activity:

The crude extracts were tested for antimicrobial activity using the agar well diffusion technique (Antunes et al., 2010). Plant extracts were dissolved in Dimethyl Sulphoxide (DMSO 100% v/v) to obtain working concentrations (20mg/mL). Standardized broth cultures of test bacterial isolates (E. coli, Staphylococcus aureus, Pseudomonas, and Klebsiella) were distributed aseptically onto the surfaces of Mueller Hinton Agar (MHA)

plates with sterile cotton swabs. All culture plates were set aside to dry for about 5 minutes before agar wells were created using a sterile cork-borer (8 mm in diameter). The wells were filled with 200 µL of crude extracts and controls. After that, the plates were incubated at the ambient temperature for 1 hour to let the agents penetrate the agar substrate. In the antibacterial test, positive controls included gentamycin (50 µg/mL) while negative controls included DMSO (100% v/v). The MHA plates were subsequently incubated at 37°C for 24 hours. The sizes of the inhibitory zones (IZDs) were determined.

Statistical analysis.

Each assay was performed in triplicate. The experimental data are presented as the mean ± standard deviation.



3. Results and Discussion

In the present study, marine sediment samples were collected from Leepuram sea, Kanyakumari

Analysis of Physico - chemical parameters of the marine samples

Analysis of the physico - chemical parameters of the marine water samples collected fromtwo different sites. The parameters such aspH, temperature, electrical conductivity,dissolved solids, salinity, zinc, copper, iron,nickel, cobalt, total mercury, total cyanide,total lead,selenium, total silver, nitrate, nitrite,ammonia, inorganic sulphide and sulphatewere analyzed using the standard methods (Strickland., 1972).

Table 3 Physico - chemical parameters of the marine samples

S.No:	Name of the Parameters	Leepuram
1.	Moisture (%)	62.71
2	PH	7.2
3	Temperature(`c)	24
4	Nitrogen(kg)	0.03± 0.01
5	Potassium(kg)	0.06± 0.03
6	Phosphorus(kg)	0.04± 0.01
7	Calcium(ppm)	4.3±1.22
8	Carbon (%)	4.7±2.83
9	Zinc(ppm)	3.1±1.07
10	Copper(ppm)	1.9±0.04
11	Iron(ppm)	2.2±1.02
12	Manganese(ppm)	5.3±3.02

Screening, isolation, and identification of bacterial isolates

Based on their colony morphology, eight marine sediment bacterial isolates were selected for screening Bacillus velezensis. The Bacillus velezensisproduced by one of these marine bacteria, which was isolated from the Leepuram sea. The isolated colonies morphological, and physiological tests revealed it to be a Gram-positive coccus, Gram-positive and negative rod. White circular transulent, white circular opaque, fluorescent circular transulent texture, dull colony surface texture was seen (Table 1 and Figure 1).

Table 4: Colony morphology from Leepuram sea sediments

S.No	Dilution	Colony formed	Colony Morphology
1.	10^{-6}	3	White Circular Transulent White Circular Opaque Fluorescent Circular Transulent
2.	10^{-7}	3	White Circular Transulent White Circular Opaque Fluorescent Circular Transulent
3.	10^{-8}	3	White Circular Transulent White Circular Opaque Fluorescent Circular Transulent
4.	10^{-9}	3	White Circular Transulent White Circular Opaque Fluorescent Circular Transulent
5.	10^{-10}	3	White Circular Transulent White Circular Opaque Fluorescent Circular Transulent

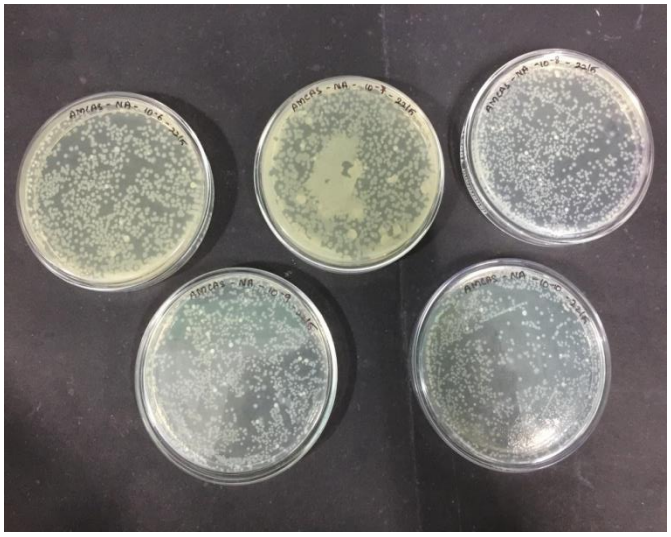


Figure 1: Colony morphology from Leepuram sea sediments

The crude extract of bioactive compound was exhaustively extracted with 200ml of ethyl acetate using soxhlet apparatus. The extracted crude bioactive compound was carried out using silica gel coated TLC sheet. Crude extract were spotted at the bottom of TLC sheet using capillary tube and placed in a glass tank with solvent system. After running the chromatography, the TLC plate was air dried and placed on closed visualize the separated compounds as spots. The crude extractions of bioactive compounds were exhaustively extracted with ethyl acetate using soxhlet apparatus. The extract was stored at 4 ◌̊ c in air-tight plastic vials for further studies. The bioactive compounds were carried out using readymade silica gel coated TLC sheet. The crude extract was spotted at the bottom of TLC sheet using capillary tube and placed in a glass tank with solvent system. After running the chromatography, the TLC plate was air dried and placed on closed chamber to clearly visualize the separated compounds as spots. The visualized separated compounds were showed in the Figure 2.

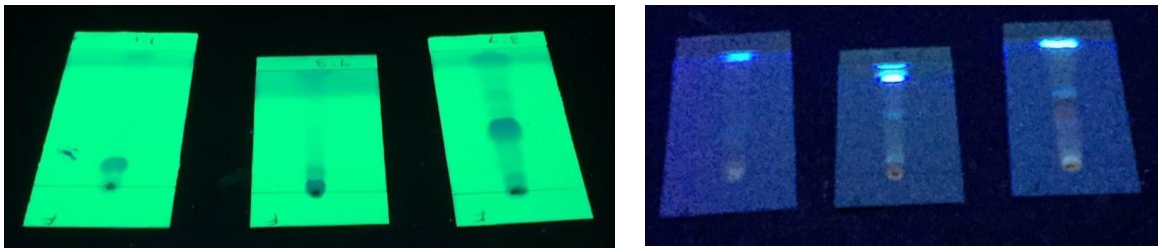


Figure 2: TLC for crude extracts of marine sediments.

Table 5: Rf values for crude extracts of marine sediments

Sample	Solvent	Mobile phase	Band	Rf Value
Bacteria -Colony	Ethyl Acetate	1:1	09	0.06,0.24,0.32,0.64,0.72,0.76,0.88,0.94,1.0
		7:3	10	0.11,0.24,0.62,0.66,0.68,0.71,0.84,0.91,0.95,1.0.
		3:7	07	0.05,0.11,0.46,0.53,0.92,0.94,0.98.

For the identification and authentication of the endophytic organisms, 16S rRNA sequencing was conducted. Bacterial colonies were initially identified based on colony morphology and Gram staining, followed by authentication through Sanger Sequencing Analysis of the 18S DNA. The BLAST analysis of the sequencing results involved a comparison with the National Centre for Biotechnological Information (NCBI) database. Among the 36 endophytes, 5 bacterial endophytes were identified and taxonomically categorized under the phylum Bacillota. The detailed identification and classification of these strains, including their closest matches in the NCBI database, are provided in Table 2 and depicted in Figure 2. It is worth noting that the remaining endophytes could not be identified due to limited sequence homology in the gene bank database. The percentage of identity was determined among the closest species, with Bac_01, Bac_02, Bac_03, Bac_04, and Bac_05 exhibiting 100% similarity with Bacillus velezensis, Bacillus anthracis, Bacillus thuringiensis, Priestia megaterium, and Bacillus subtilis, respectively, as illustrated in Figure 2.

Table 6: Six endophytic Bacterial strains identified by 16sr RNA Sequencing

Isolates Name	Closest Relative[a]	Accession No[b]	%Identity[c]
BAC_01	Bacillus velezensis	OM541328.1	100%
BAC_02	Bacillus anthracis	ON063211.1	100%
BAC_03	Bacillus thuringiensis	ON063216.1	100%
BAC_04	Priestia megaterium	ON063226.1	100%
BAC_05	Bacillus subtilis	OP020696.1	100%

A Closest species which high % identity in BLAST Analysis, bNCBI Gene bank accession number in website (http://www.ncbi.nlm.nih.gov/pubmed), cGen Bank accession no.of our strains deposited on NCBI website (http://www.ncbi.nlm.nih.gov/pubmed), d% identity of strain based on BLAST Analysis

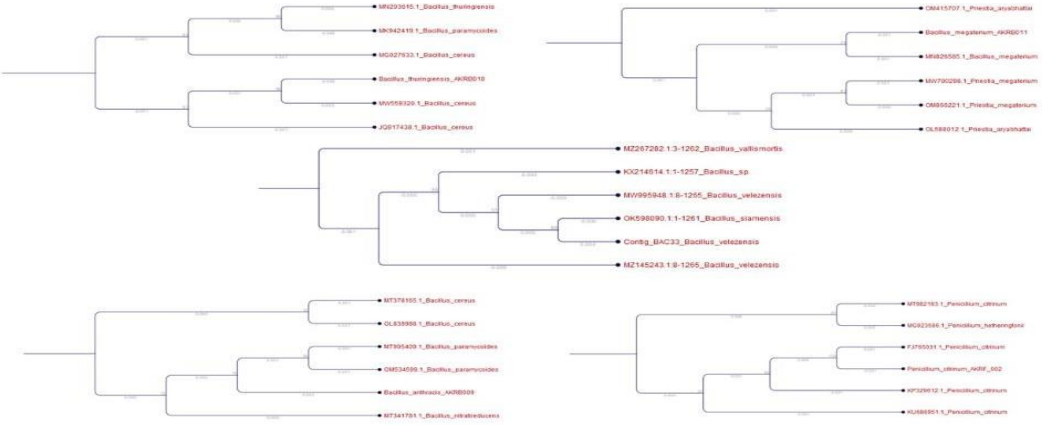


Figure 3: Phylogenetic tree of isolated endophytic Bacteria

The chemical compounds in this extractwere identified by using a GCMS analysis (Figure 5). The major compounds of theextract were 3,5,5-Trimethyl-2-Cyclohexen-1-One (3.12%), Benzene ethanol (2.83%),1-Hexanol, 2-Ethyl- (2.04%), Pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro- (1.98%), Phenol (1.92%), Azulene (1.67%), Sulfide, Allyl Methyl (1.45%), 2,7-Dimethyl-4,5-Octanediol (1.35%) and other minorcompounds were also presented in Table 3. The variouschemical compounds may contribute to antimicrobialactivity.

Table 7. Compounds identified using GC MS

Compounds identified using GC MS	Yield (%)
Butanoic Acid, 2-Methyl	0.58
3-Pentanol (Pentan-3-ol)	1.12
Sulfide, Allyl Methyl	1.45
3-Hydroxyisovaline	0.76
Phenol	1.92
1-Hexanol, 2-Ethyl-	2.04

2,7-Dimethyl-4,5-Octanediol	1.35
Benzene ethanol	2.83
3,5,5-Trimethyl-2-Cyclohexen-1-One	3.12
2-Piperidinone	0.95
Azulene	1.67
Hexatriacontane	0.89
Cyclononasiloxane, Octadecamethyl-	1.22
Pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro-	1.98

Alkane has alsobeen reported to have antibacterial activities (Boussaada etal. 2008) and antifungal activities (Ahsan et al. 2017).Several other compounds were reported to have antimicrobial activity (Nahid Rahbar2012; Amer et al. 2019).

The antimicrobial effects extract was evaluated against the growth of one-Gram positive and two-Gram negative bacteria. The extract exhibited antimicrobial activity for Gram -ve rather than Gram +ve bacteria (Table 1).

Table 8: Zone of inhibition of Bacillus velezensis

Culture	+ve	-ve
Pseudomonas	17mm	18mm
Staphylococcus aureus	17mm	18mm
E. coli	17mm	19mm
Klebsiella pneumonia	8mm	12mm

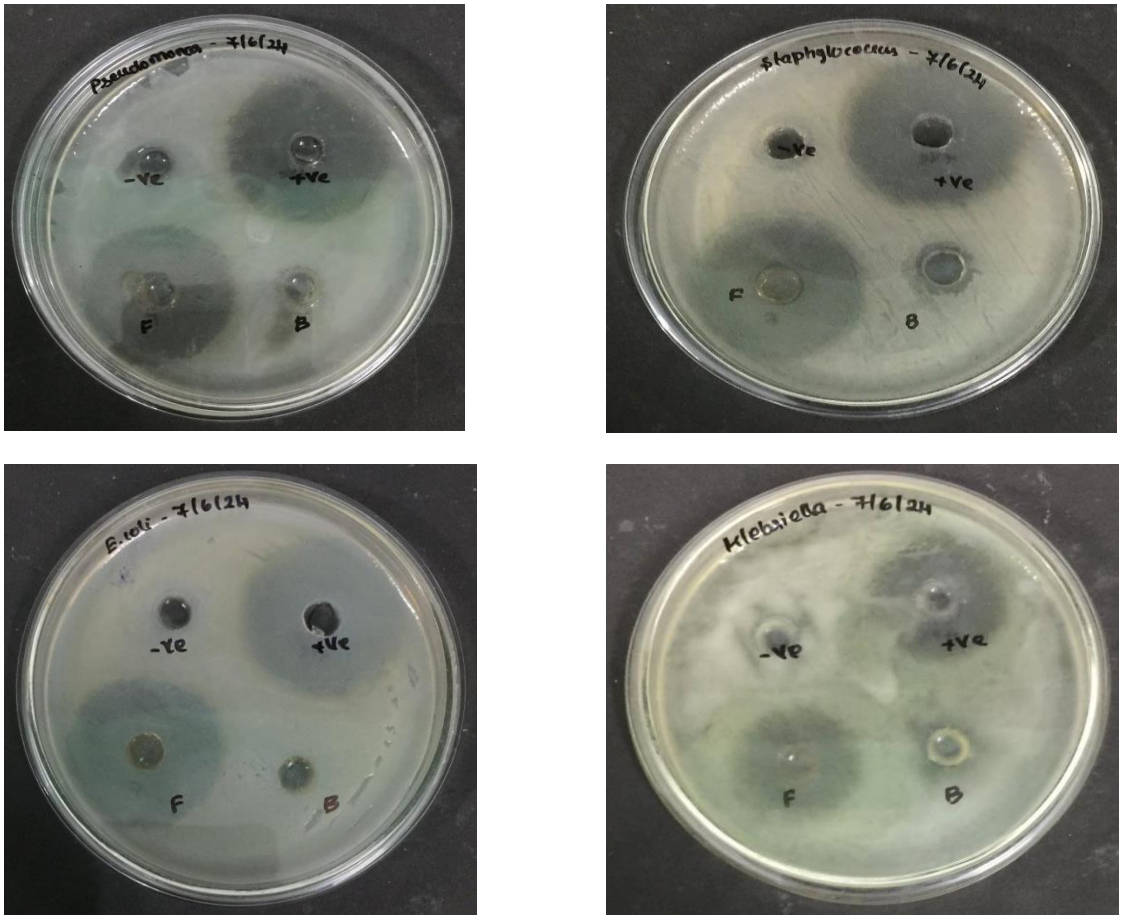


Figure 4: Antimicrobial activity of Bacillus velezensis

Antimicrobial activity by Bacillus spp. was proven in previous studies (Gram et al., 2010, Balakrishnan et al., 2014). The results obtained in the present study indicated the potential production of bioactive compounds by marine Bacillus sp. Anand et al., 2006 reported the production of a highly active metabolites by marine Bacillus sp. Mohana et al., 2016 stated that Bacillus sp. isolated as sponge endosymbiotic bacteria showed antimicrobial activity with broad range against virulent marine fish pathogens such as Vibrio alginolyticus,Vibrio vulnificus, Vibrio parahaemolyticus, Aeromonas salmonicida, Flavobacterium sp., Edwardsiella sp., Proteus mirabilis and Citrobacter brackii with zones of inhibition (16-23 mm). Many scientists isolated Bacillus sp. from diverse group

of invertebrates and confirmed the production of novel active metabolites against aquatic fish pathogens (Phelan et al., 2011,Liu et al., 2012,Wefky et al., 2009).

4. Conclusion

Bacillus velezensis, a marine bacterium recently isolated from Leepuram Sea Sediment, shows promise as a source of bioactive chemicals with powerful antibacterial action. Based on our research, it appears that the metabolites that were extracted have antioxidant and antibacterial capabilities. This means that they can effectively combat a range of harmful microbes. B. velezensis may have uses in biocontrol, the agricultural sector, and medicines due to its capacity to synthesize these bioactive chemicals. Complete characterization of the active ingredients, clarification of their action mechanisms, and investigation of their potential uses in reducing antibiotic resistance require additional research. This study adds to what is already known about B. velezensis as its bioactive metabolites, which could lead to long-term replacements for antibiotics.

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