Isolation, screening and antagonistic activity of *Bacillus pumilus* VNS 25 from Mangrove soil collected at Nizampatnam coastal area Andhra Pradesh

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ABSTRACT:

Mangrove and marine ecosystems are major sources of novel microorganisms which can produce potential bioactive compounds when compared with terrestrial ecosystems. Generally most of the microorganisms in the mangrove region are rich sources of secondary metabolites having strong enzymatic and metabolic activities because these ecosystems are saline and highly rich in organic matter. The present study was aimed to isolate and characterize the bioactive compounds producing bacteria from the soil sediments of Mangrove ecosystem in Nizampatnam coastal area situated in the south coastal area of Andhra Pradesh. Among all isolates 56 were selected and screened for antagonistic activity where 29 has shown poor, 21 has shown moderate and 6 isolates with potential activity. Further the strains with maximum antagonistic activity were purified and characterized by 16S rRNA and SEM analysis. The results confirmed that the purified samples are *Bacillus pumilus*-VNS 25 and could be useful for the discovery of the novel bioactive metabolites against broad range of Gram positive, Gram negative, fungi and plant pathogens.

Keywords: Bacteria, Mangrove ecosystem, bioactive metabolites, *Bacillus pumilus*.

INTRODUCTION:

The increase of rapid bacterial resistance to all clinically useful antimicrobial agents in the treatment of pathogenic diseases has been one of the most serious public health problems worldwide in the past decade (Atta and Radwan 2012). The increase in spreading of antibiotic resistance among bacterial pathogens, particularly methicillin–resistant *Staphylococcus aureus* (MRSA) and Vancomycin resistant *Enterococci* (VRE) causes serious bacterial and fungal infections which are gradually recognized as important cause of mortality and morbidity, especially among chronic deteriorated patients (Richards et al., 2005; Gullo 2009; Peleg and Hooper 2010). The increase in the frequency of multi-resistance pathogenic bacteria has raised an urgent demand in the pharmaceutical industry for more modern statistical methods and strategies for the screening of novel antibiotics with a broad spectrum of antimicrobial activity (Motta et al., 2004).

Mangrove ecosystems are well known potent areas for the existence and distribution of microbes found in several niche. (Gupta et al., 2007; Xu et al., 2009). The distribution of bacterial diversity inhabitating in the unique swamp of mangrove saline and anaerobic environment would progress the interactions of adaptability and functionality. (Kathiresan and Selvam 2006; Semenov et al., 1999). Among the estimated total number of microbial species only 10% is known from mangrove ecosystem where there is broad and diverse resource that can be tapped for useful products, such as antibiotics which possess novel mechanisms of action with diverse chemical structures (Bull et al., 1992).

Bacteria are known to be essential decomposers of organic matter among the different groups of microorganisms which control the reusing of essential nutrients in coastal sediments (Alongi, 1994). Bacteria are major participants in the nitrogen and phosphorus cycles in the mangrove sediment (Rojas et al., 2001) and also liable for maximum carbon transformation in the mangrove ecosystem which act as carbon basin (Holguin et al., 2001). Chemical compounds which are formed after the utilization of primary metabolites from the microorganism are known as secondary metabolites.

The bacteria in the marine and mangrove ecosystems undergo a variety of extreme conditions such as high altitudes, low oxygen (anaexia), high pressures, and dark conditions. Several physiochemical factors such as salinity, intensive ecological pressure and highly ecological adaptations influence species composition of microbial flora and fauna to produce biologically active compounds (Lozupone and Knight, 2007; Vieira et al., 2008; Silveira et al., 2011). The marine environment is a prolific source of novel microorganisms and possess a remarkable biosynthesis ability to produce metabolites with diverse biological activities (Yu et al., 2002; Vater et al., 2002; Pueyo et al., 2005) with *Bacillus* species is commonly recovered with antimicrobial production in a common mode. The halophiles are extremophile organisms that flourish in environments with very high concentration of salt, based on the

requirement concentration of NaCl they are termed as slightly, moderately or extremely halophiles (Ghosh et al., 2010). The saline environment is rich in organic matter due to microbial enzymatic and metabolic activities and they inhibit intertidal zones and can withstand a wide range of salinities (Liang et al., 2008).

Bacillus species are gram positive bacterial genus that is widely spread in nature due to their inherent property to form endospores and resistance to extreme conditions. Some species of *Bacillus* produces secondary metabolites that have a diverse chemical structure possessing biological activities against a wide range of pathogenic gram positive and gram negative bacteria. (Prieto et al., 2012; Stein 2005). Antimicrobial compounds produced by group of bacteria *Bacillus* strains has been steadily found to be suitable for various applications (Abriouel et al., 2011). The secondary metabolites of the *Bacillus* genus have shown antimicrobial, antifungal, bio insecticidal and probiotic activity (Oguntoyinbo, 2007; Ben Khedher et al 2011; Liu et al; Cutting 2011).

In the present work we describe the isolation of bacterial strains from mangrove soils, which can produces secondary metabolites. The bacterial strains were identified based on the morphology, cultural, biochemical characteristics and 16s rRNA sequencing.

MATERIALS AND METHODS:

Sample Collection:

Mangrove sediment samples are collected from different regions of Nizampatnam mangrove ecosystem (Latitude15° 54'0 N; Longitude 80° 40'0 E) situated along the south coast of Andhra Pradesh, India. The soil samples were collected from a depth of 6-10 cm, from the wetland as well as dry land and transferred into sterile bags and transported immediately to the laboratory and maintained at 4^0C.

Isolation of bacteria from mangrove soil samples:

Erlenmeyer (E.M) flask containing 100 ml of water was sterilized and 1gm of soil sample was added. The flasks were incubated on shaking incubator for 30 min for the detachment of soil particles and left to settle down for 30 min. The clear supernatant was subjected to serial dilution and 1ml of the dilution from 10^4 to 10^8 was inoculated on to the different mediums such as Zobells agar medium, Nutrient agar medium (2% to 4% Sodium chloride) and half strength sea water medium and incubated at 30 $^{\circ}$C for 1 to 2 weeks for the isolation of bacteria.

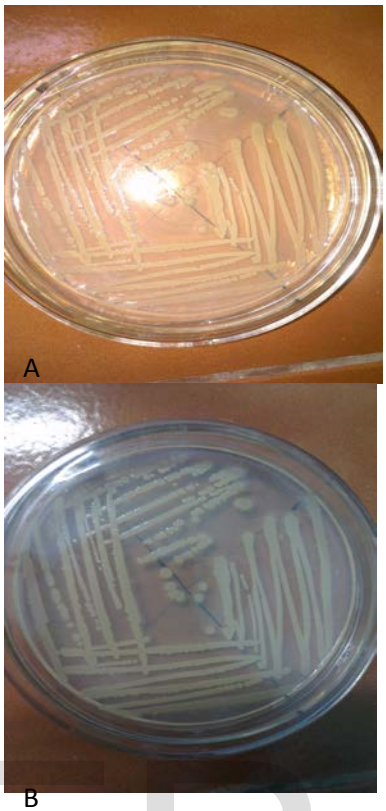


Fig:(A), (B) Colony morphology of the strain VNS-25

Identification of *Bacillus* isolates by conventional method and by 16S rRNA Gene sequence analysis:

Promising *Bacillus* isolates were characterized using the tests prescribed in Bergey's Manual of Systemic Bacteriology (Berkley et al., 1984; Claus and Berkley 1986; Sneath et al. 1986, and Collins and Lyne 1989). All *Bacillus* isolates were identified to species level by 16S rRNA gene sequence analysis using the taxonomy approach (Stackebrandt and Goebl, 1994, Guzman et al., 2009). The nucleotide sequence analysis of the 16S rDNA of the isolates was done at NCBI server using BLAST-n (www.ncbi.ncm.gov/blast) by aligning the partial sequences obtained with the primers mentioned above. Similarity search for the nucleotide sequence of 16S rDNA of the test isolates done online at the www.ncbi.nlm.nih.gov using BLAST search programme with default parameter for the nucleotide database of Genbank, revealed the tentative identification of the isolates.

Molecular identification:

Genomic DNA (Deoxyribose Nucleic Acid) used for the PCR was prepared from the single colonies grown on the nutrient agar medium for 24hrs at 150 rpm. The total genomic DNA was extracted from the potent strain. Bacteria was inoculated aseptically

from mature slant culture of bacteria in 30 ml (25x150) test tube (Riviera) containing nutrient broth medium (Beef extract 2g/L, yeast extract 2 g/l, peptone 5 g/l and sodium chloride 8 g/l, pH 7.0) and incubated in rotary shaker at 33°C for two days at 180 rpm. After growth culture was transferred in Oakridge tube aseptically and culture was centrifuged at 9000 rpm for 10 minutes. Supernatant was discarded and the pellet was collected in 1.5 ml eppendorf tube aseptically. The eppendorf tube was dipped in liquid nitrogen for 4-5 min to freeze the total mycelium. The bacteria was grinded using micro pestles. After grinding 500 µl of TE buffer (10mM Tris and 1 mM EDTA) was added the pellet was grinded till it become completely liquid. Tubes were incubated at 37°C for 45 minutes and 20 µl of % SDS (w/v) 20µl of proteinase- K was added and the tubes were incubated at 60°C for 20 minutes. The lysate was cooled down and extracted once with equal volume of Tris saturated phenol and chloroform solution (1:1) at 10K for 10 min at 16°C. The aqueous phase was transferred carefully to a fresh tube and 5-8 µL of RNase (10mg/ml) was added and incubated at 37°C for 40 minutes and again extracted once with equal volume of Tris saturated phenol and chloroform solution (1:1) at 10K for 10 min at 16°C. Transfer the aqueous phase carefully in to a fresh tube and DNA is precipitated by adding 0.6 volumes of isopropanol and centrifuge at 10K rpm for 8min at 4°C. Wash the with 70% ethanol. The pellet is finely dissolved in 80µL autoclaved double distilled water or TE buffer. The purity and concentration of DNA was checked by UV-spectrophotometer (Shimadzu) (Figure-2) and by subjecting the isolated DNA to electrophoresis (0.8% agarose gel) (Figure-3).

PCR Amplification:

Amplification 16SrDNA from bacteria was carried by using primers F- 3'GGATGAGCCCGCGGCCTA5' and

R-3' CGGTGTGTACAAGGCCCGGGAACG5'. Total volme of the reaction mixture is 25µl. table-1. Amplification using PCR (eppendorf pro)according to following programme, initial denaturation -94°C for 5 min, denaturation for 1 min and annealing at 62°C for 1 min and extension for 1 min with total 30 cycles. PCR product size was 1.4Kb.

S.No	COMPONENTS	REACTION VOLUME
1	DNA	1 µL (50ng/ µL)
2	Taq buffer	2.5 µL
3	DNTPs	1 µL (5mM)
4	Forward primer	0.5µL(15ppm)
5	Reverse primer	0.5µL(15ppm)
6	Taq enzyme	0.5 µL
7	Nuclease free water	19µL

Total volume = 25 µL

Amplified PCR product was visualized by electrophoresis using 1% agarose gel, samples are loaded in gel with 1kb ladder (Figure-3). Gel was checked on UV-gel documentation (syngene). PCR product was purified (Figure-4) by using Macherey-Nagel kit (MN, NucleoSpin Extract II, and Germany) according to the kit protocol and finally eluted product was sent for sequencing.

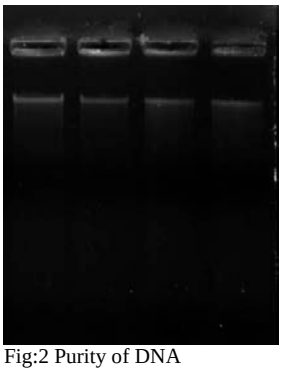


Fig:2 Purity of DNA

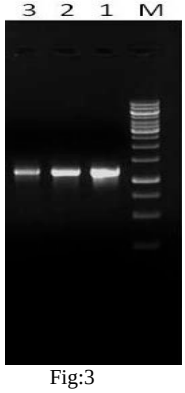


Fig:3

Fig:3 1, 2 and 3 lanes of Gel photograph showing amplified product of VNS-25 and M- basepair ladder

Nucleotide sequence accession number: The nucleotide sequence of 16S rDNA of *B. pumilus* strain VNS-25 has been deposited in the GenBank database and the GeneBank accession number KP001170.

Results and discussion:

To identify a potential bacterial antagonist, over 56 bacterial isolates from soil were screened for their ability to inhibit the pathogenic organisms. Isolated 56 microorganisms were screened for antagonistic activity, 21 showed poor, 29 showed moderate and 6 have shown potent activity against pathogenic bacterial and fungal cultures. Interestingly, bacterial isolate VNS-25 was a facultative anaerobe, gram-positive and has shown the strong zone of inhibition against the bacterial and fungal pathogens. Using the biological system and 16S rRNA gene analysis, the isolate was identified and belongs to the genus *Bacillus*. 16S rDNA sequence analysis showed that this *Bacillus* strain had 95% similarity with *B. pumilus*, and was designated *B. pumilus* VNS-25. Preliminary screening to study the spectrum of pathogenic bacterial growth inhibition by crude extracts from *B. pumilus* VNS-25 showed that it produced a potential antagonistic compound, which had significant activity against different bacterial and fungal pathogens (Table - 4).

The present study was designed to investigate the bacteria for the production of antimicrobial compounds with novel properties and unique skeletons from the mangrove ecosystem of Nizampatnam. For the exploration of novel antimicrobial compounds from mangrove ecosystem our continuous research was done in

isolation of morphologically distinct bacteria by employing serial dilution plate technique on different medium.

Table :1 Biochemical characters of VNS-25

S.No	Characteristics under study	VNS- 25	VNS-26
	Colour of the Bacteria	Yellow cream	Orange pigment
Physiological Characterization			
1	Gram reaction	Gram +ve	Gram +ve
2	Range of temperature for growth	25°C-50 °C	25oC-50 °c
3	Optimum temperature for growth	35°c	30 °c
4	Range of pH for growth	5-9	5-9
5	Optimum pH for growth	7.5	7.2
6	NaCl tolerance	0.5-5.0	0.5-5.0
7	Optimum NaCl concentration	3.0	2.5
Biochemical tests			
1	Catalase production	ND	
2	Urease production	+++	
3	Hydrogen sulphide production	---	
4	Nitrate reduction	+++	
5	Starch hydrolysis	+++	
6	Gelatin liquefaction	+++	
7	Methyl red test	++	
8	Vogues-proskauer test	+++	
9	Indole production	---	
10	Citrate utilization	+++	
11	Casein hydrolysis	+++	

Analysis of the 16s rDNA gene sequence of the strain VNS-25

The 16S rDNA sequence data supported the assessment of the isolate VNS-25 to the genus *Bacillus* and species *pumilus* (*Bacillus pumilus*). The 560 bp partial 16S rDNA sequence of the strain VNS-25 was obtained and submitted to the GenBank database under an accession number KP001170. The partial sequence was aligned and identical sequences are compared with all the 16S r RNA gene sequence accessible in the GenBank database by employing the multisequence advanced BLAST comparison tool that is available in the website of National Center for Biotechnology Information. The maximum 16S rRNA sequence similarity value of 95% was obtained for the *Bacillus Pumilus*. 16S rRNA gene sequence was aligned using the CLUSTAL W programme using the MEGA 5 Version. Phylogenetic tree was constructed using Molecular Evolutionary Genetics Analysis (MEGA) software Version 5 using maximum parsimony method (Tamura et al., 2007, 2011). The topologies of the constructed tree were evaluated by bootstrap analysis with 1000 resamplings by maximum parsimony tool. Sequence comparison of the strain VNS-25 with the corresponding sequences of the close representative strains of *Bacillus* from the GenBank data base indicates that this strain formed a close distinct phyletic line with clade encompassed by *Bacillus Pumilus*, *Bacillus Subtilis* and *Bacillus Cereus*. Differential characteristics of strain VNS-25 and the strains of closely related *Bacillus* sp. are represented in table 1.

Based on the morphological, physiological, biochemical, and cultural characteristics of the strain (table-2)has been included the genus *Bacillus* and deposited at NCBI genbank with an accession number KP001170.

Antimicrobial spectrum of *Bacillus* species VNS-25 is represented in table 4. The strain inhibited the growth of Gram positive and Gram negative bacteria, fungi and yeast suggesting a broad spectrum nature of the active compound.

The review of literature indicates the bioactive metabolite profile of *Bacillus* sp. includes antibacterial, antifungal, and anti trypnosomal compounds.

Utilization of the carbon sources:

S.No	NAME	INHIBITION
1	D-Glucose	+++
2	Sucrose	+++
3	Lactose	++
4	Maltose	++
5	Xylose	---
6	Arabinose	---
7	\Galactose	+++
8	Fructose	+++
9	Raffinose	+++
10	Ribose	+++

Table:2 : Utilization of the carbon sources. Excellent (+++), Moderate (++), Poor (-)

S.No	NAME OF ANTIBIOTIC	RESISTANT/SENSITIVE
1	Nalidixic acid	R
2	Streptomycin	S
3	Penicillin	R
4	Chloramphenicol	S
5	Rifampicin	S
6	Oxy tetracycline	S

Table:3 : Antibiotic susceptibility testing of VNS-25

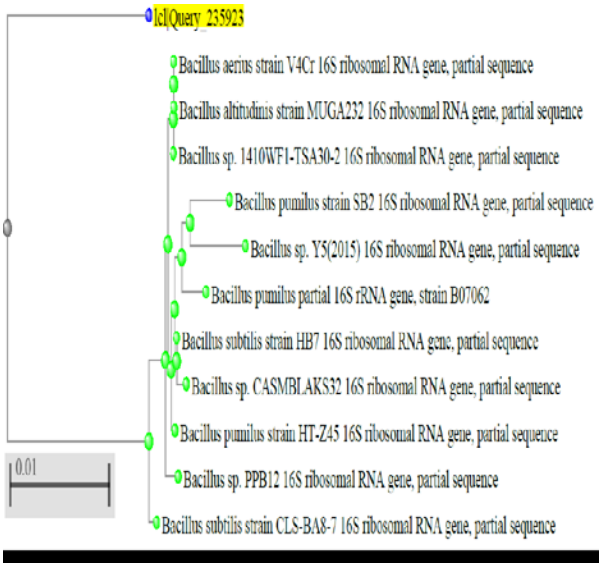
Antimicrobial activity:

S.No	Test organism	Diameter of Clear zone (mm)
1	*Bacillus subtilis* ATCC 6633	16 mm
2	*Bacillus megaterium*	17 mm
3	*Escherichia coli* ATCC 35218	20 mm
4	*Enterococcus faecalis* MTCC 439	17 mm
5	*Staphylococcus aureus* MTCC 3160	21 mm
6	*Streptococcus mutans* MTCC 497	18 mm
7	*Pseudomonas aeruginosa* ATCC 9027	16 mm
8	*Xanthomonas campestris*	15 mm
9	*Candida albicans* ATCC 1023	15 mm

Table:4 : Antimicrobial activity of Bacillus sp. VNS-25

Phylogenetic Analysis

This tree was produced using pairwise alignment USING Insilico tools by Tree method having max seq difference of 0.75



Conclusion:

The present study is mainly involved in the isolation and identification of bacteria based on the cultural, morphological, physiological, biochemical and molecular characteristics. Further studies are in progress on optimization, purification and active compound chemical structure elucidation. In the current attempt it is expected that the isolation and characterization of bacteria from mangrove soils of Nizampatnam will be useful for the identification of novel antimicrobial compounds effective against various clinical and plant pathogens.

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