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## Herbicidal Activity of an Epibiotic *Bacillus* Strain WP3 from Sea Fan Coral

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### ABSTRACT

Marine organisms continue to be a huge source to produce unique and potential bioactive compounds that cure deadly disease. This research investigates the herbicidal properties of bacteria isolated from surface of the coral, *Junceella juncea* (Pallas, 1766). Two hundred and thirty four different bacterial strains were isolated from the coral from Tuticorin coast, Gulf of Mannar region, south east coast of India. The strain WP3 was found to prove excellent herbicidal property when tested against *Lemna minor*. The 16S rRNA sequencing and phylogenetic identification shows that the stain WP3 was identified to fall under the genera *Bacillus*. The assay was carried out for crude extract and column purified extract. Mass spectrophotometry (MALDI-TOF) analysis has shown that the mass of the molecules ranged from 1148-1729 Da. Thus the marine bacteria isolated from corals are a potential source of novel bioactive agents and other natural products.

**Key words:** Bioactive compounds, *Junceella juncea*, phylogenetic, mass spectrophotometry

### INTRODUCTION

World's oceans provide vast source of biological compounds that are highly significant for the discovery of therapeutic agents (Wright, 1998). The oceans represent the most diverse resource of life with huge dimensions and extreme variations in pressure, salinity and temperature. A unique adaptation strategy has to be acquired to sustain these extreme conditions, leading to new natural products (Faulkner, 2000). In marine environment, bacteria grow on submerged biotic and abiotic surfaces (Chellaram *et al.*, 2012a; Dunne, 2002). In particular, a diverse community of surface attached bacteria was found on the surface of marine invertebrates (Rohwer *et al.*, 2002; Chellaram *et al.*, 2012b). Several factors including surface-specific interactions account for the diversity of these organisms (Rohwer *et al.*, 2002; Taylor *et al.*, 2005). Weeds reduce crop yield by competing for water, light, soil nutrients, space and carbon dioxide. Weeds also pose additional problems such as reducing crop quality by contaminating the commodity, interfering with harvest and serving as hosts for crop diseases. Nowadays the appropriate application of herbicides has brought about a great reduction of labour and increase in crop yield to support the world population (Anand *et al.*, 2012). Active compounds from marine sources can be developed into herbicides to aid agriculture. Natural products minimizes environmental pollution, thus highlighting the need for bioactive compounds from marine sources. The methanol extract of salt marsh *Salicornia brachiata*, *Sesuvium portulacastrum* and *Suaeda maritima* were found to have

herbicidal activity against *Lemna minor* (Anand *et al.*, 2014). They isolated bacterial stains from the surface of sponges, seaweeds, crabs, ascidians and cephalopod eggs and reported that the bacterial broth extracts exhibited 90% inhibition against *L. minor* (Anand *et al.*, 2012).

Here, our aim is to isolate and identify bacteria from sea fan and analyze its herbicidal activity. The isolated marine bacteria was identified as *Bacillus kochii* using 16S rRNA sequencing technique. The marine bacterium was found to possess bioactive compounds that provides herbicidal activity.

## MATERIALS AND METHODS

**Sample collection:** Sea fan coral, *Junceella juncea* was collected from a depth of 5-10 m at Tuticorin coastal waters, Gulf of Mannar region, south east coast of India. A single branch of the coral was gently cut off and care was taken not to disturb the whole organism. The collected samples were then placed inside sterile ethylpolythene bags underwater and transferred to the laboratory aseptically in iceboxes.

**Isolation of bacteria:** The coral sample was first washed gently with sterile seawater to remove sand particles. Isolation of epibiotic bacteria was done by swabbing a small area of the coral surface with a sterile cotton swab. The swab was then directly swabbed on Zobell Marine Agar (ZMA) plates. The ZMA plates were incubated at room temperature for six days and from the fifth day on colonies of different morphotypes were isolated and repeatedly streaked on Zobell marine agar plates to obtain pure cultures. The pure cultures were then stored at 4°C in marine agar slants until further studies.

**Cold-ethanol precipitation:** The cold-ethanol precipitation of the culture broth was carried out following the slightly modified method of Schubert and Finn (1981). The *Bacillus* strain WP3 culture was centrifuged at 7500 rpm at 4°C for 15 min. To the supernatant two volumes of ice-cold ethanol was added gradually simultaneously agitating with a magnetic stirrer. When the solvent addition was complete, the culture was agitated at 4°C for at least 60 min. The culture was then placed in an ice bucket and left overnight inside a cold room (4°C). The precipitate was separated from the supernatant by centrifugation at 7000 rpm for 30 min in 4°C. The precipitate was dried in room temperature to remove the ethanol and then dissolved in 5 mL of MilliQ water. The herbicidal activity of the ethanol precipitate was carried out.

**Herbicidal activity of pure compounds:** Herbicidal activity was studied by using *Lemna minor* L. (duckweed) as test plant. *Lemna minor* L. is a miniature aquatic monocot, Lemna plant which consists of a central frond or mother frond with two attached daughter fronds and a filamentous root. Under normal conditions, the plant reproduces exponentially with budding of daughter fronds from pouches on the sides of the mother fronds.

*Lemna* plants are sensitive to bioactive compounds. These plants are used to identify anti-tumor and phyto-toxic compounds. Search for new herbicidal compounds from marine sources is limited. Scientist have been working on marine herbicidal products from recent years. The herbicidal activity of pure compounds was assayed against *Lemna minor* L. following a bench top bioassay. To assay the herbicidal activity, 2 mg of the pure compound was dissolved in 20 mL of suitable solvent. From this solution, 100, 50 and 10 µL was pipette into dram vials corresponding to 0.01, 0.005 and 0.001 µg mL<sup>-1</sup>, respectively in triplicates. Controls were added with appropriate solvents alone without the compound. The solvents of the control and test vials were allowed to

evaporate overnight and 2 mL of E-medium (consisting of  $\text{KH}_2\text{PO}_4$ -680 mg,  $\text{KNO}_3$ -1515 mg,  $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ -1180 mg,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ -492 mg,  $\text{H}_3\text{BO}_3$ -286 mg,  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ -3.62 mg,  $\text{FeCl}_2 \cdot 6\text{H}_2\text{O}$ -5.40 mg,  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ -0.22 mg,  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ -0.22 mg,  $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ -0.12 mg and EDTA-11.2 mg in 1 L of distilled water) was added into each vial. A single healthy *L. minor* plant containing a rosette of three fronds was introduced into each vial and was placed in glass chamber with about 2-4 cm water at the bottom to maintain the moisture content of the chamber and was sealed with glass plate. The fronds per vial was counted daily upto 10 days and symptoms of damage to the frond such as yellowing and decaying was noted.

**Mass determination:** Matrix Assisted Laser Desorption/Ionization Time-Of-Flight (MALDI-TOF) spectrum of crude and 7HPLC purified active fractions were acquired on Ultraflex Bruker mass spectrometer, equipped with a nitrogen laser of wavelength 337 nm. Equal amounts of samples were mixed with the matrix solution ( $\alpha$ -cyano-4-hydroxy cinnamic acid) saturated with 0.1% TFA and acetonitrile (1:1). Measured masses have an error of  $\sim \pm 3\text{Da}$ .

**Molecular identification and phylogenetic analysis of *Bacillus* strain WP3:** Single colony of the strain WP3 was taken from the agar plate. The strain was suspended in 50  $\mu\text{L}$  of lysis solution (10 mM Tris-HCl, pH 7.5; 10 mM EDTA and 50  $\mu\text{L mL}^{-1}$  of proteinase K). The mixture was incubated at 50°C for 15 min. Proteinase K inactivation was done at 85°C for 10 min. The mixture was later centrifuged at 15,000 rpm at 4°C for 15 min. Genomic DNA, present in the supernatant was directly used as template in PCR reaction. The PCR amplification of almost full-length 16S rRNA gene was carried out with eubacteria specific primer set 16F27N (5'-CCAGAGTTTG ATCMTGGCTCAG-3') and 16R1525XP (5'-TTCTGCAGTCTAGAAGGAGGTGWTCAGGC-3') (Pidiyar *et al.*, 2002). Ten nanogram of the genomic DNA, 1X reaction buffer (10 mM Tris-HCL, pH 8.8 at 25°C, 1.5 mM  $\text{MgCl}_2$ , 50 mM KCl and 0.1% Triton X-100), 0.4 mM deoxynucleoside triphosphates (Invitrogen), 0.5 U DNA Polymerase (New England Labs, UK) was used to perform a 25  $\mu\text{L}$  reaction volume PCR.

An automated Gene Amp PCR system 9700 thermal cycler was used to perform PCR under the following conditions. The amplification condition was given as follows: 94°C for 1 min (denaturation), 55°C for 1 min (annealing), 72°C for 1.30 min (elongation) at and 72°C for 10 min final elongation. The PCR product of around 1.5 Kb was run by electrophoresis with 5  $\mu\text{L}$  of the PCR product on 1% agarose gel in 1X TBE buffer and stained with ethidium bromide 0.5  $\mu\text{g mL}^{-1}$ . The PCR product was precipitated by PEG-NaCl (20% PEG in 2.5 M NaCl). Precipitation was done at 37°C for 30 min. Centrifugation of reaction mixture was done again at 12,000 rpm for 30 min at room temperature. The resultant pellet was washed twice with 70% ethanol. The pellet was later dried and resuspended in 5  $\mu\text{L}$  of sterile nuclease-free water. Later, one microliter ( $\sim 50$  ng) of purified PCR product was sequenced (Pidiyar *et al.*, 2002). The sequence analysis was done at NCBI server (<http://www.ncbi.nlm.nih.gov/BLAST>). The alignment of the sequence was done using CLUSTALW programmed at European Bioinformatics site (<http://www.ebi.eic.uk/clustalw>). Phylogenetic tree was constructed using the MEGA Software version 3.1. The sequence of the 16S rRNA gene of the *Bacillus* strain WP3 was deposited in GenBank.

## RESULTS

**Bioactivity:** Testing of *Bacillus* strain WP3 (Fig. 1) following bench top bioassay showed that the strain exhibits broad activity, inhibiting the growth of *Lemna minor* plant. The epibiotic strain



Fig. 1: Strain WP3 (*Bacillus* sp.)

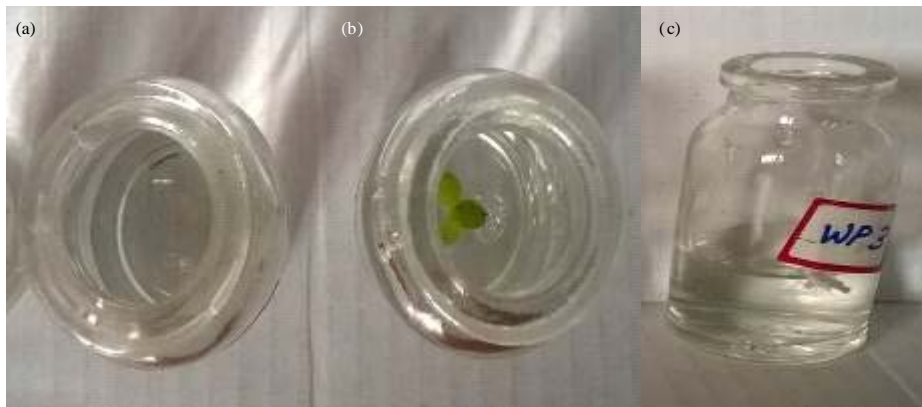


Fig. 2(a-c): Herbicidal activity of strain WP3 (*Bacillus* sp.) (a) WP3, (b) Control and (c) WP3

Table 1: Herbicidal activity of strain WP3 against *Lemna minor*

WP3 Conc. (mg mL <sup>-1</sup> )	Days																	
	1			2			3			4			5			6		
	H	EA	M	H	EA	M	H	EA	M	H	EA	M	H	EA	M	H	EA	M
Control		GH			GH			GH			GH			GH			GH	
500	G	LY	LY	LY	Y	Y	D	Y	D	D	D	D	D	D	D	D	D	D
300	GH	G	GH	G	G	G	LY	Y	Y	D	D	D	D	D	D	D	D	D
200	GH	GH	GH	G	G	G	G	G	G	Y	LY	LY	D	Y	Y	D	Y	D
100	GH	GH	GH	GH	GH	GH	G	G	GH	LY	LY	G	Y	Y	G	Y	Y	LY
50	GH	GH	GH	GH	GH	GH	GH	GH	GH	G	G	G	G	LY	G	LY	Y	LY

H: Hexane, EA: Ethyl acetate and M: Methanol. GH: Green and Healthy, G: Green, LY: Light yellow, Y: Yellow and D: Dead

might have secreted bioactive compounds (Fig. 2). The crude extract (ethanol) was also found to be active against *L. minor*. MALDI-TOF spectrums of the crude extract (Fig. 3) give the mass of active molecules. Mass of the crude extract molecules ranged from 1148-1729 Da (Fig. 4). Table 1 shows

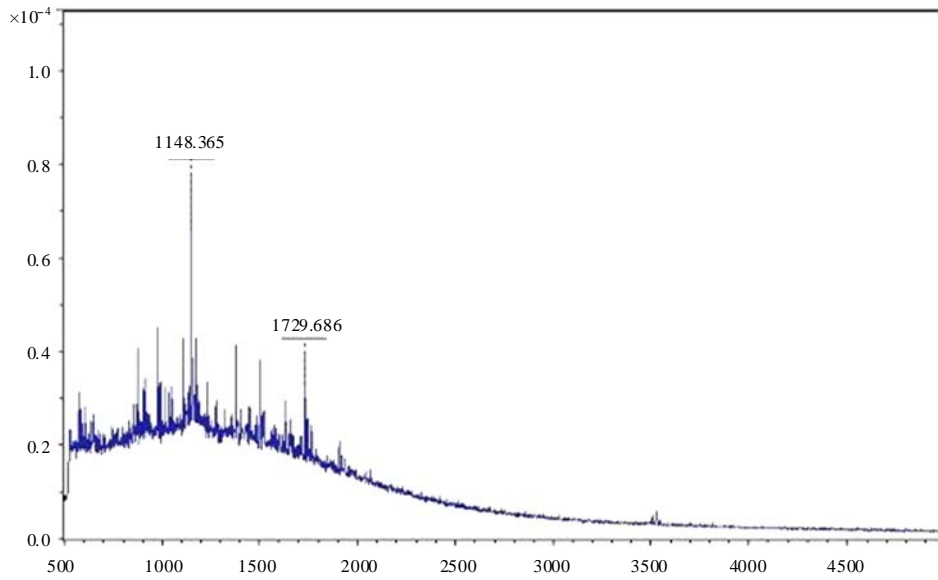


Fig. 3: MALDI-TOF data of crude extract of WP3 (*Bacillus* sp.)

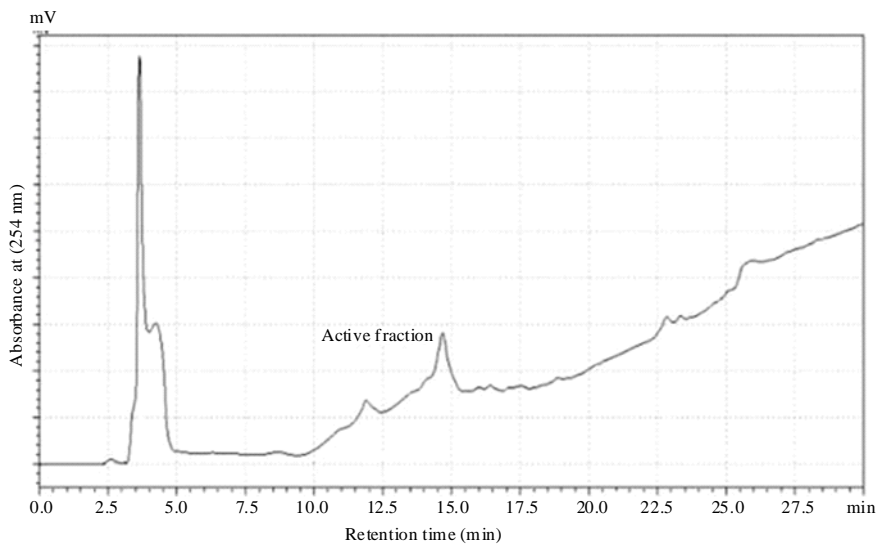


Fig. 4: HPLC trace of crude extract of WP3 (*Bacillus* sp.)

the data for strain WP3 in different concentrations against *L. minor* for six days. Hexane and methanol extract of *Bacillus kochii* inhibited the growth of *L. minor* at 200-500 mg mL<sup>-1</sup> on 6th day. As the concentration increases, mortality rate increases, proving the presence of active compounds in the extract.

**Molecular identification and phylogeny:** The strain WP3, was identified as a *Bacillus* sp. engaging 16S rRNA gene sequencing method. Phylogenetic analysis based on comparative analysis of the sequenced 16S rRNA indicated that the strain was closely related to *Bacillus kochii*

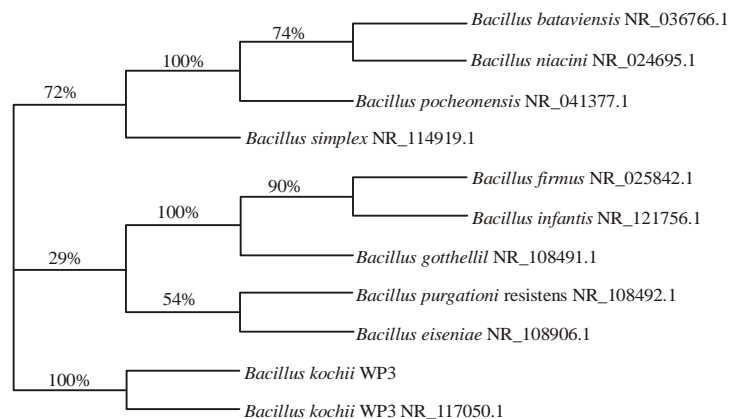


Fig. 5: Phylogenetic tree of the strain WP3 (*Bacillus* sp.)

strain (Fig. 5). Sequence was obtained by 16S rRNA sequencing and related sequences were obtained from BLAST. Multiple sequence alignment was done and phylogenetic tree was constructed using European Bioinformatics site (<http://www.ebi.ac.uk/blast/>) and tree view 1.6.6. Further purification may result in the extraction of active compounds that are novel and efficient. Marine source continue to provide humanity with potential compounds that can be developed into drug against deadly diseases. These compounds can also be tested for its activity against deadly diseases like cancer, malaria, etc.

## DISCUSSION

In the present study, herbicidal activity of epibiotic strain, *Bacillus kochii* was studied and the strain inhibited the growth of *Lemna minor* plant at the concentration of 200-500 mg mL<sup>-1</sup> Marine chemicals often possess quite novel structures which in turn lead to pronounced biological activity and novel pharmacology. The study of such chemicals therefore is a very promising endeavor (Blunt *et al.*, 2005). Microorganisms will automatically acquire their resistance towards common antibiotics by altering their metabolism and genetic structure. These microbial compounds are most prominent source for discovery and production for new drugs (Zhang *et al.*, 2009; Yuan *et al.*, 2010; Chellaram *et al.*, 2013). This study showed that the isolated bacterium *Bacillus kochii* has excellent herbicidal activity. It is evident that *Bacillus kochii* constitutively produces a novel compound responsible for herbicidal activity. Further study is necessary for structure and functional group elucidation of the compound using Nuclear Magnetic Resonance (NMR) and Infra-red (IR) spectroscopy, in order to practice it for agricultural advantage.

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## REFERENCES

- Anand, T.P., C. Chellaram and F.C. Shanthini, 2012. Herbicidal and Growth promoter activity of marine bacteria. *Int. J. Pharma Biosci.*, 3: 359-368.
- Anand, T.P., C. Chellaram and M.M. Praveen, 2014. Herbicidal and growth promoter activity of three saltmarsh plants from Pulikat coast. *Int. J. ChemTech Res.*, 6: 1051-1054.

- Blunt, J.W., B.R. Copp, M.H.G. Munro, P.T. Northcote and M.R. Prinsep, 2005. Marine natural products. *Nat. Prod. Rep.*, 22: 15-61.
- Chellaram, C., S. Venkatesh, T.P. Anand, G. Kuberan and A.A. John *et al.*, 2012a. Antimicrobial properties of insect gut associated bacteria. *World J. Med. Sci.*, 7: 260-263.
- Chellaram, C., T.P. Anand, N.R. Shailaja and D. Kesavan, 2012b. Herbicidal effects of marine animal, *Trochus tentorium* from Gulf of Mannar, Southeastern India. *Asian J. Anim. Vet. Adv.*, 7: 250-255.
- Chellaram, C., P. Raja, A.A. John and S. Krithika, 2013. Antagonistic effect of epiphytic bacteria from Marine Algae, Southeastern India. *Pak. J. Biol. Sci.*, 16: 431-434.
- Dunne, Jr. W.M., 2002. Bacterial adhesion: Seen any good biofilms lately? *Clin. Microbiol. Rev.*, 15: 155-166.
- Faulkner, D.J., 2000. Highlights of marine natural products chemistry (1972-1999). *Nat. Prod. Rep.*, 17: 1-16.
- Pidiyar, V., A. Kaznowski, N.N. Badri, M. Patole and Y.S. Shouche, 2002. *Aeromonas culicicola* sp. nov., from the midgut of *Culex quinquefasciatus*. *Int. J. Syst. Evol. Microbiol.*, 52: 1723-1728.
- Rohwer, F., V. Seguritan, F. Azam and N. Knowlton, 2002. Diversity and distribution of coral-associated bacteria. *Mar. Ecol. Prog. Ser.*, 243: 1-10.
- Schubert, P.F. and R.K. Finn, 1981. Alcohol precipitation of proteins: The relationship of denaturation and precipitation for catalase. *Biotechnol. Bioeng.*, 23: 2569-2590.
- Taylor, M.W., P.J. Schupp, R. de Nys, S. Kjelleberg and P.D. Steinberg, 2005. Biogeography of bacteria associated with the marine sponge *Cymbastela concentrica*. *Environ. Microbiol.*, 7: 419-433.
- Wright, A.E., 1998. Isolation of Marine Natural Products. In: *Methods in Biotechnology, Natural Products Isolation*, Cannell, R.P.J. (Ed.). Humana Press Inc., New Jersey, ISBN: 978-0-89603-362-7, pp: 305-408.
- Yuan, L.J., Y.Q. Zhang, L.Y. Yu, C.H. Sun and Y.Z. Wei *et al.*, 2010. *Actinopolymorpha cephalotaxi* sp. nov., a novel actinomycete isolated from rhizosphere soil of the plant *Cephalotaxus fortunei*. *Int. J. Syst. Evol. Microbiol.*, 60: 51-54.
- Zhang, W., Z.Y. Li, X.L. Miao and F.L. Zhang, 2009. The screening of antimicrobial bacteria with diverse Novel Nonribosomal Peptide Synthetase (NRPS) genes from South China Sea sponges. *Mar. Biotechnol.*, 11: 346-355.