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Insecticidal Activity of an Epibiotic *Bacillus kochii* from Gorgonian Coral, *Junceella juncea* (Pallas, 1766)

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Aim of the research is to isolate and identify bacteria from sea fan corals and analyzing its insecticidal activity against storage pest, *Sitophilus oryzae*. The bacterial strain WP3, was isolated from the coral *Junceella juncea* from Tuticorin coast, Gulf of Mannar region, south east coast of India. Insecticidal activity of pure compounds was assayed by bench top assay method. Different solvent extracts (hexane, acetone and methanol) of the culture broth (WP3) was done and its activity was noted. Methanol extract showed minimum LC_{50} when compared to acetone and hexane extracts. High performance liquid chromatography (HPLC) trace confirmed the presence of active compounds. Mass spectrophotometry (MALDI-TOF) analysis has shown that the mass of the molecules ranged from 1225 Da to 1927 Da. 16S rRNA sequencing and phylogenetic identification was done and the stain was identified to fell under the genera Bacillus. The result shows that the marine bacteria isolated from *Junceella juncea* corals are a potential source of novel bioactive agents and other natural products that may be utilized as pesticides.

Key words: *Junceella juncea, Bacillus kochii, Sitophilus oryzae,* Mass spectrophotometry.

According to a report by Food and Agriculture Organization (FAO), 120 billion dollars were lost worldwide due to 20–40% decrease in crop yield, because of the attack from pathogenic organisms and insect pests (Zhou *et al*., 2010). To ensure the constant and high productivity of crops, vast amount of pesticide were applied to control the pests. This has resulted in causing serious environmental pollution and accumulation of insecticide residues. Also, inducing a wide range of pesticide resistance was done (Jiang *et al.*, 2000). For better productivity without ill effects, biopesticides have gained increased attention and interest among those concerned with developing environmentally friendly and safe integrated crop management (Leonard *et al*., 2000). At present, microbial insecticides are the main part of the bio-pesticide industry (Shi *et al.*, 2000 and Xie *et al*., 1998). The taxonomic diversity of marine organisms is large as is their biochemical and behavioral diversity, this chemical diversity was found to contain pesticide substance close to DDT and Chlordane (Kennett *et al*., 1990).

Microorganisms possess exceptionally rich sources of drugs, including antibiotics, immune-suppressants etc (Chellaram *et al*., 2011). However, these drugs have been produced from a very small range of world's microbial diversity (Chelllaram *et al*., 2012). Actinomycetes have been found to be a best source of novel antibiotics and other bioactive compounds (Prem Anand, *et al*., 2012; Okami *et al*., 1979, Anbuselvi, *et al*., 2009; Prem Anand, *et al*., 2013 and Chellaram *et al*., 2013). The yield loss of crops was estimated to be 20 - 30%. An additional 10% of crop is lost due to post harvest storage and transportation (Duke *et al*., 1993). Cotton leafworm (*Spodoptera littoralis*), is considered one of the most affecting insect pests attacking crops, vegetables and fruit trees all over the world (Berlinger *et al*., 1997). It has the capability to develop resistance to most conventional insecticides. To prevent soil and

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environment pollution, biological or ecological control methods have been prioritized for limiting the destructive impacts of pest populations (Canaday *et al*., 1995, Hokkanen *et al*., 1995 and Nakasm *et al*., 1990). Reports indicated that actinomycetes play an important role in the biological control of insect including the cotton leaf worm *spodopetra liltoralis*, house fly *Musca domestica* (Hussain *et al*., 2002), *Culex quinquefasciatus* and *Drosophila melanogaster* (Gadelhak *et al*., 2005). In this paper, we have concentrated on isolation and identification of bacteria from sea fan corals and analyzing its insecticidal activity against *Sitophilus oryzae*. The isolated marine bacteria was identified as *Bacillus kochii* using 16s rRNA sequencing technique.

MATERIALS AND METHODS

Sample Collection

The coral (sea fan) *Junceella juncea* was collected by SCUBA diving from 5-10m depth 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 ethyl polythene 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. 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.

Preliminary screening of the isolates for insecticidal activity

Direct spray technique was used for preliminary insecticidal assay. 10 test insect, rice weevil was placed inside a petridish and 5ml quantity of the culture broth was sprayed over the insects using sprayer. The petridish was covered and the survivors were recorded after 24hrs. All experiments were carried out in triplicates and control was also maintained without the extract.

Insecticidal activity of pure compounds

Insecticidal activity of pure compounds was assayed by bench top assay method (Mc Laughlin *et al*., 1991). 1mg of each pure compounds were dissolved in 1ml of the solvents, and from these 100, 50 and 10µL was poured in separate petriplates in triplicates and allowed to evaporate overnight to obtain same concentration in µg/ml of compounds. Controls with solvents alone were taken in separate petriplates and allowed to evaporate overnight. Ten healthy adults of *Sitophilus oryzae* were introduced into each petridish and sufficient food was provided to the test organisms so that, death due to starvation is ruled out. After 24 hours, the number of dead insects was counted and percentage of mortality was noted. The efficiency of the compounds in killing the insects was determined.

Mass determination

Matrix assisted laser desorption/ ionization time-of-flight (MALDI-TOF) spectrum of crude and HPLC purified active fractions were acquired on Ultraflex Bruker mass spectrometer, equipped with a nitrogen laser of wavelength 337nm. Equal amounts of samples were mixed with the matrix solution (α-cyano-4-hydroxy cinnamic acid) saturated with 0.1% TFA and acetonitrile (1:1). Measured masses have an error of ~ ±3Da.

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 50µl of lysis solution (10mM Tris-HCl, pH 7.5; 10mM EDTA and 50µl/ml of proteinaseK). The mixture was incubated at 50°C for 15 minutes. ProteinaseK inactivation was done at 85°C for 10 minutes. The mixture was later centrifuged at 15,000 rpm at 4°C for 15 mins. Genomic DNA, present in the supernatant was directly used as template in PCR reaction. PCR amplification of almost full-length 16Ss rRNA gene was carried out with eubacteria specific primer set 16F27N (5'-CCAGAGTTTGATCMTGGCTCAG-3') and 16R1525XP (5'- TTCTGCAGTCTAGAAGGAGG TGWTCCAGGC-3') (Pidiyar *et al*., 2002). 10ng of the genomic DNA, 1X reaction buffer (10mM Tris-HCL, pH 8.8 at 25°C, 1.5mM $MgCl_2$, 50mM KCl and

0.1% Triton X-100), 0.4mM deoxynucleoside triphosphates (Invitrogen), 0.5U DNA Polymerase (New England Labs, UK) was used to perform a 25ml 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. PCR product of around 1.5 Kb was run by electrophoresis with 5µl of the PCR product on 1% agarose gel in 1X TBE buffer and stained with ethidium bromide 0.5 µl/ml. 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 5µl of sterile nuclease-free water. Later, one microliter (~ 50ng) 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 Bioinfomatics 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) using direct spray technique showed that the strain exhibits broad activity against *Sitophilus oryzae*

Three purified extracts of Bacillus strain (Acetone, Hexane and Methanol) was also tested against *Sitophilus oryzae*. LC_{50} of hexane, acetone and methanol extracts were found to be 151.2 µg/ml, 126 µg/ml and 100 µg/ml respectively (Fig.2). Percentage of mortality of the isolated extracts against the larva of *Sitophilus oryzae* in different concentrations was shown in the Table1. Concentration dependent mortality was observed. Table 2 shows the variation in between groups and within groups. P value was found to be 0.003 and *F-crit* value was found to be 2.86608

MALDI-TOF spectrums of the crude extract (Figure 3) gives the mass of active molecules. Mass of the crude extract molecules ranged from 1225Da to 1927Da.

Molecular identification and phylogeny

Sequence was obtained by 16s rRNA sequencing and related sequences were obtained from BLAST. Multiple sequence alignment was

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

Table 1. Percentage mortality and LC_{50} of marine *Bacillus* strain extracts against *Sitophilus oryzae*

Concentration of the extracts (µg/ml)	Mortality (%)		
	Hexane Extract	Acetone Extract	Methanol Extract
500	83.3	86.6	90
300	76.6	73.3	83.3
200	56.6	60	66.6
100	43.3	46.6	50
50	26.6	30	36.6
Control	0	0	0
LC_{50}	151.2	126	100

Table 2. ANOVA table for insecticidal activity of *Bacillus* strain

Source of Variation	SS	df	MS	F	P-value	F-crit
Between Groups	150162.758	4	37540.689	5.597	0.003	2.86608
Within Groups	134141.927	20	6707.096			
Total	284304.684	24				

done and phylogenetic tree was constructed using European Bioinformatics site (http:// www.ebi.eic.uk/ clustalw) and tree view 1.6.6. The strain WP3, was identified as a *Bacillus sp*. engaging 16Ss rRNA gene sequencing method. Phylogenetic analysis based on comparative analysis of the sequenced 16Ss rRNA indicated that the strain was closely related to *Bacillus kochii* strain (Fig. 4).

Reverse phase HPLC of the extract was carried out. Presence of active compounds was confirmed by the peak present around RT 15.0 in trace HPLC (Fig. 5). Further purification may result in the extraction of active compounds that are novel and efficient. Marine continue to provide the mankind with potential compounds that can be developed into an insecticide source.

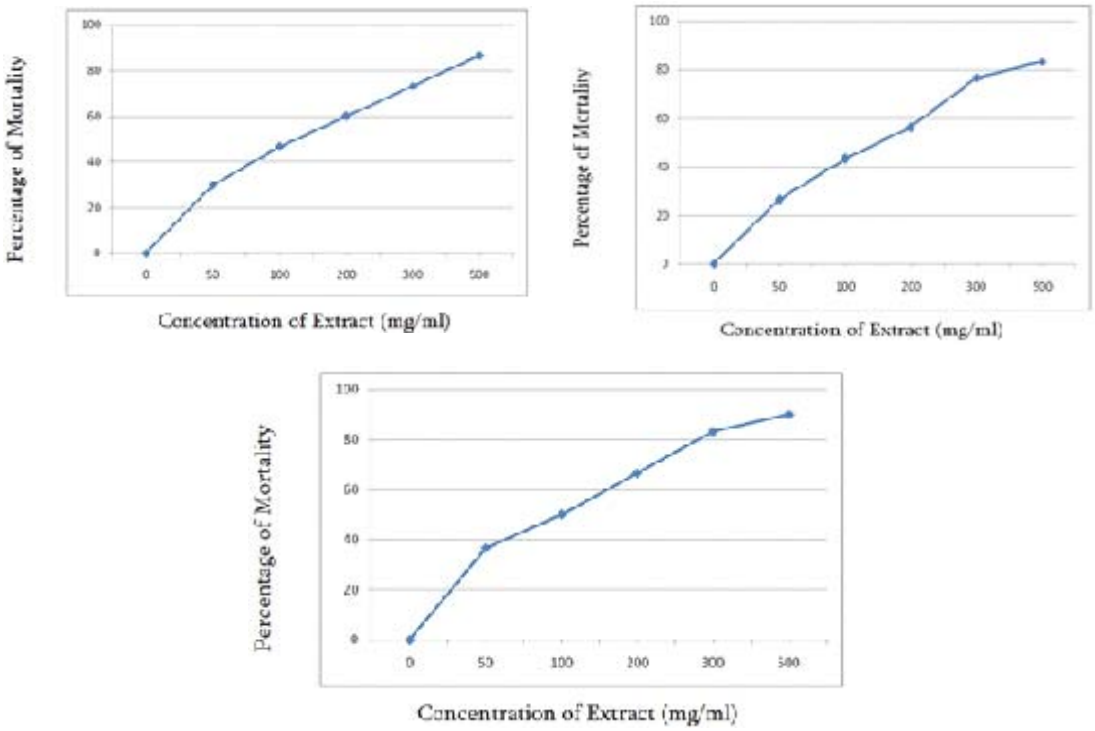

Fig. 2. LC_{50} of the isolated crude extract against *Sitophilus oryzae* larva. (A) Acetone (B) Hexane (C) Methanol

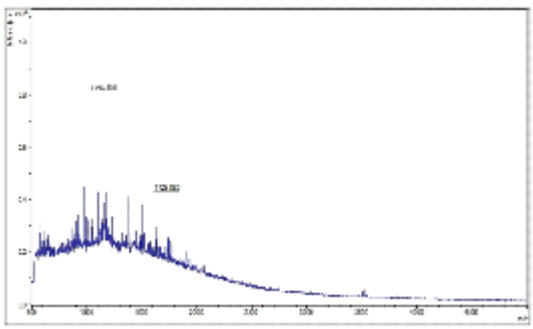

Fig. 3. MALDI- TOF data of crude extract of WP3 (Bacillus sp)

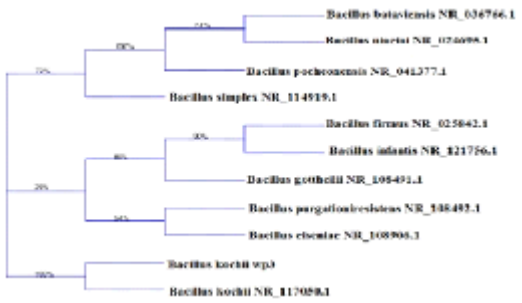

Fig. 4. Phylogenetic tree of the strain WP3 (*Bacillus sp*)

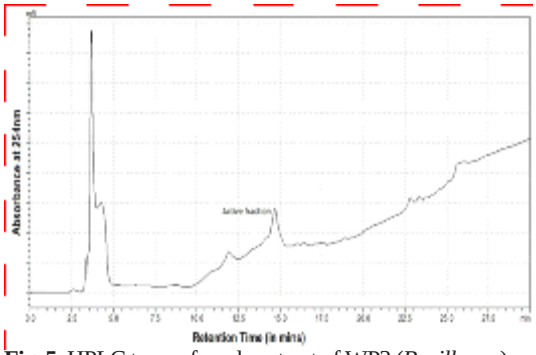

Fig. 5. HPLC trace of crude extract of WP3 (*Bacillus* sp)

DISCUSSION

Out of ten isolated strains tested for activist against *Sitophilus oryzae*, six strains showed high activity. The strain WP3 was chosen for further studies. Three extracts (hexane, acetone and methanol) were obtained and tested for its activity. LC50 of methanol extract was found to be minimum when compared to acetone and hexane extracts. This shows that the methanol extract of the strain showed efficient activity against *Sitophilus oryzae*. Earlier reports show that, the insecticidal compounds have been isolated form marine source such as macroalgae, sponge, coral and parts of mangrove tree (Chellaram *et al*., 2013). Kabaru and Gichia (2001) screened different parts of the mangrove tree *Rhizophora mucronata* for insecticidal activity and found that the extract of bark and pith exhibited high toxicity (Kabaru *et al*., 2001). 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). Anwarul Haque *et al.* (2013) studied the insecticidal activity of ethanolic crude extracts of marine *Streptomyces* sp. against larvae of *Sitophilus oryzae* and showed that, at a concentration of 24mg/ml, the isolate caused 100% mortality of the larvae.

CONCLUSION

This research has reported the insecticidal activity of methanolic crude extracts of marine *Bacillus* sp., which shows LC50 of 100 µg/ml. Further study is necessary for structure and functional group elucidation of the compound by using Nuclear Magnetic Resonance (NMR) and Infra-red (IR) spectroscopy to be used for biological advantage.

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