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RESEARCH ARTICLE

ISOLATION AND IDENTIFICATION OF SECONDARY METABOLITES PRODUCING BACTERIA ISOLATED FROM SOIL TERMITES

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ABSTRACT

In the present investigation *Bacillus* sp. were isolated from subterranean termites gut. *Bacillus* sp. was identified based on the morphological and molecular characterization. 16SrRNA of *Bacillus* sp. sequenced and submitted to Gen Bank. Antibacterial activity of *Bacillus* sp. was screened. It inhibits the growth of *S.typhi, E.coli* and *S.aureus*. The bioactive compounds were analyzed by UV-Visible spectroscopy and thin layer chromatography. The highest peak was observed between 240 to 280 nm. In the cytotoxic assay 27.307% cell death was observed in 20µl concentration of the sample and 9.790% cell death was observed in 5µl concentration of the sample. In the GC-MS analysis totally 8 compounds were recorded. These compounds may be responsible for the anticancer activity.

INTRODUCTION

Termites are insects that are classified at the taxonomic rank of infra order Isoptera or as epifamily Termitoidae within the cockroach order Blattodea. Termites were once classified in a separate order from cockroaches, but recent phylogenetic studies indicate that they evolved from close ancestors of cockroaches during the Jurassic or Triassic. However, the first termites possibly emerged during the Permian or even the Carboniferous. About 3,106 species are currently described, with a few hundred more left to be described. Although these insects are often called white ants, they are not ants. The termites gut consist of four gut (which includes the crop and muscular gizzard), the tubular mid gut (which as in other insects is key site for secretion of digestive enzyme and relatively, a voluminous hind gut (which is also a major site for digestion and for absorption of nutrients). The phenotic diversity of the termite gut microbiota is remarkable and has been documented for the lower and higher termites. Antimicrobial activity of the termite species most commonly used by the South Indian tribes for treating diseases likely to be associated with microorganisms. The antibacterial activities of 90% alcohol extracts of three species of subterranean termites (Solavan, 2007). *Bacillus* sp. are effective against Gram-positive and Gram-negative bacteria (Mirac Yilmaz *et al.,* 2006).

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MATERIALS AND METHODS

Sample collection

The termites sample were collected from Echanari, Coimbatore, Tamil Nadu, India.

Extract preparation

1gram of termites was taken and crushed the termites. Added 5 to 10ml of sterile water incubates for 1 hour.

Isolation of bacteria

20 ml of nutrient Agar was prepared. 1ml of sample ware using spread plate technique. Incubated for 24 hrs. After 24 hrs the isolated bacteria was used for the production of secondary metabolites.

Identification of organism

Skim milk agar and starch agar medium used as a specific medium. The culture ware streaked in skim milk and starch agar, and incubate for 24hours.

DNA Isolation

Culture was added to the microfuge tube centrifuged at 5000rpm for 5minutes collect the pellet and supernatant was discareded. Mix well the pellet and incubate 30minutes. Added 150µl of 10% SDS buffer. Mix well and incubate 65˚c for 30 minutes. Then added to chloroform-160µl, phenol180µ and isoamylalcohol -10µl (in the radio of 25:24:1) mixed and centrifuge the 10000 rpm for 10 minutes. Collect the aquas layer and added 0.5 volume of sodium acetate and 2 volume of isoprophenol. Mixed well centrifuged at 10000 rpm for 10 minutes. To the pellet 500 µl of 100%of ethanol added and centrifuge at 6000 rpm for 6 minutes then the pellet was air dried and dissolved with TE buffer. Another step for repeated to centrifuge for 70%of ethanol for 6000 rpm in 6 minutes. Finally centrifuge at 5000 rpm for 5 minutes. Collect the pellet and air dry in added to 1X TE buffer.

Agarose gel electrophoresis

1% of agarose gel were used to visualize the DNA. The gel was prepared in 1x TAE buffer and was treated EtBr was added to visualize the DNA in UV transilluminator, after planning the comp in gel casting tray the allowed to solidified for 15-30 minuts, then the sample loaded and run the sample

PCR

PCR (Polymerase Chain Reaction) prepared to DNA 20µl, two primers (SHV) Reverse primer, Forward primer. PCR master mix 6µl, PCR buffer 6µl, and distilled water 6µl, the total volume of 25. In the culture was poured to ependrpff tupes. It produced to the 5 steps. Initial denaturation 94˚C for 1 min, Naturation 94˚Cfor 30 sec, Anneling 50˚C30 sec , Extention 72˚C for 1 min, and final extention was 72˚C for 30 sec. in the cycles are repeated to 20 times.

Gene sequencing

A DNA fragment of the 16S rRNA gene was amplified from genomic DNA with the forward primer 9F (5″-AGA GTT TGA TCC TGG CTC-3″) and the reverse primer 926R (5″-CCG TCA ATT CCT TTG AGT T-3″) (BioServe, India) by PCR (Wang *et al.*, 2007). The PCR mixture contained 2.5 µL 10x PCR buffer; 2.5 µL of 2 mMdNTP; 2.5 µL of25 mM MgCl2; 2 µL genomic DNA (50-100 ng); 0.4 µL 5unit *Taq*polymerase and 1 µL each primer (10 pmol), supplemented with 14.1 µL distilled water to a final volume of 25 µL. The thermocycler conditions were as follows using Applied Biosystem 96 well plate Gradient PCR machine.

Phylogenetic analysis

The sequence of 16S rRNA of selected bacterial strains were compared against the sequence available from GenBank using the BLASTIN program and were aligned using CLUSTAL W software developed by Higgins *et al.,* (1992). The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model (Kimura, 1980). Bootstrap analysis was done based on 1000 replications. All these analysis were performed by MEGA4 package (Tamura *et al*., 2007)

Restriction site analysis

The restriction sites in 16S rDNA gene was analysed by using restriction mapping program (nc2.neb.com/NEBcutter2).

Antibacterial Activity

60ml of Mueller Hinton agar was prepared and poured on 4 sterile petri plates and allowed to solidify. Four different cultures namely, *E.coli, S,aureus, s.typhi* and termite extract were spreaded using a sterile cotton swab. Three wells were cut using gel cutter. 10µl sample, 20µl sample and 20µl DMSO were inoculated into each wells and one antibiotic disc was placed (NX10) to analyze the antibactertial activity. The zone of inhibitions ware measured.

Extraction of compound and UV- Visible

After the production of secondary metabolite the broth was aseptically transferred to sterile centrifuge tubes and centrifuged at 5000rpm for 30 mins. The supernatant was collected in sterile conical flask and the pellet was discarded. The supernatant was transferred to the separating funnel and mixed with ethyl acetate in the ratio 1:3 (supernatant: ethyl acetate). After 15 mins, separating funnel was shaked and kept undisturbed for b 10-15 mins for separation of two layers. Both organic and aqueous phases were tested for antimicrobial activity. Organic phase was concentrated by rotary evaporation at 40oC.This crude extract was also tested for its antimicrobial activity by agar diffusion method. After incubation collect the extraction compound and UV Scaning for 200 to400nm.

Thin layer chromatography

The aqueous termites extracts were added as spot using capillary tubes on the one end of the thin layer plate at above 1 cm. Plate was allowed it for air dry, then it was placed in a beaker containing solvent Methanal, acetic acid, water and chioroform in the ratio of 6: 4. The samples were allowed to run towards the other end of the plate. The sheet was removed and allowed it to air dry and 2% of ninhydrin was sprayed and again allowed to air dry for 10 minutes. The plate was then visualized under the UV light and violet colour spot was absorbed plate.

GC-MS Analysis

In the GC-MS analysis Thermo GC - Trace Ultra ver: 5.0, Thermo MS DSQ II equipment was used. DB 35-MS capillary were used as column. The Non - Polar Column Dimension is 30 Mts, ID : 0.25 mm, FILM : 0.25 µm. Helium as a carrier gas. Initially 1 µl of sample was injected. The temperature increased from 70ºC to 260 ºC. Using computer searches on a NIST Ver.2.1 MS data library and comparing the spectrum obtained through GC-MS-MS compounds present in the extracts were identified.

MTT Assay

MTT (3- [4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) is a yellow coloured water soluble tetrazolium dye. Mitochondrial enzyme lactate dehydrogenase, produced by

metabolically active cells reduces MTT to water-insoluble formazan crystals. When dissolved in appropriate solvent, these formazan crystals exhibit purple colour.

Cytotoxicity Assay

He La Cells were grown in RPMI-1640 medium (Hi Media, Mumbai) supplemented with 10% fetal bovine serum (FBS) (Hi Media, Munbai), 100 U/ml penicillin and 100 µg/ml streptomycin (Hi Media, Mumbai) Cells were incubated in a humidified incubator contain 5% CO2 at 37 ºC. After 24hrs the cells were seeded in to 96 well The cell culture suspension was washed with 1 X PBS (Phosphate Buffered Saline) and then added with 200 µl MTT [3-(4, 5-Dimethyl thiazole-2yl)-2, 5-diphyhyl tetrazolium Bromid solution to the culture flask. It is then incubated at 37°C for 3 hours, removed all MTT solution, washed with 1 X PBS and added with 300 µl DMSO to each culture flask and incubated at room temperature for 30 minutes until all cells get lysed and homogenous color was obtained. The solution was then transferred cell debris. Debris was dissolved using DMSO. OD was measured at 540 nm using DMSO blank. Then the percentage viability was calculated using the percentage of viability formulated.

RESULTS

Identification of Bacteria

After 24 hours incubation bacterial colonies were isolated. Based on the colony morphology, grams staining and biochemical tests the bacterial colonies were identified as *Bacillus* sp. (Plate 1).

Plate 1: *Bacillus* sp. on starch agar and Skim milk agar medium

Molecular characterization

The bacterial DNA fragment of the 16S rRNA gene was amplified from genomic DNA with the forward primer 9F(5″-AGA GTT TGA TCC TGG CTC-3″) and the reverse primer 926R (5″- CCG TCA ATT CCT TTG AGT T-3″) (BioServe, India) by PCR. Based on the molecular characterization, bacterial culture was identified as Bacillus sp. VSCBNR7. The sequence data was then assembled and submitted to the NCBI GenBank.

Phylogenetic tree of *Bacillus* sp.VSCBNR7

The sequence of *Bacillus* sp.VSCBNR7 maximum homology with *Bacillus thuringiensis* KF550913.1 and *Bacillus* sp. KM6759651 (Fig. 1)

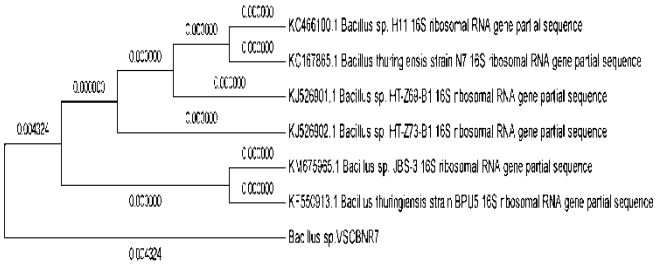

Figure 1. Phylogenetic tree of *Bacillus* sp.VSCBNR7

Restriction site analysis

Totally 46 restriction sites were identified. 54% GC contents were recorded and 46% AT contents were recorded (Fig. 2).

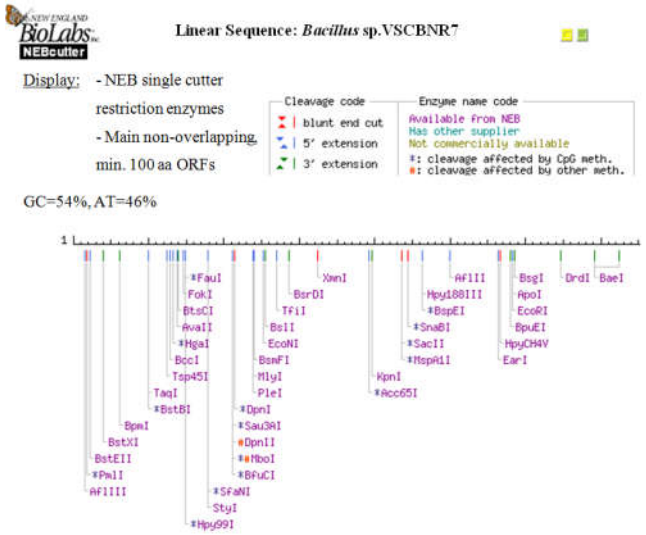

Figure 2. Linear Sequence: *Bacillus* sp.VSCBNR7

Antibacterial activity of *Bacillus* sp. VSCBNR7

Bacillus sp. VSCBNR7 inhibits the growth of *S.typhi, E.coli* and *S.aureus*. Maximum zone of inhibition 7mm was observed in 20µl sample against *E.coli. Bacillus* sp. VSCBNR7 inhibits *S.typhi* with inhibition zone of 7mm in 10µl sample. It inhibits the growth of *S.aureus* with 6mm zone in 20µl sample. Minimum zone of inhibition (2mm) was observed against *S.aureus* in10µl sample. Inhibition zone was completely absent in DMSO and antibiotic disc (Plate 2& Table 1).

Table 1. Antibacterial activity of *Bacillus* sp. VSCBNR7

S.No.	Organism	10µl Sample	20µl sample	DMSO	Antibiotic Disc
1.	*Salmonella typhi*	7mm	5mm	-	-
2.	*E.coli*	3mm	7mm	-	-
3.	*S.aureus*	2mm	6mm	-	-
4.	Extract	-	-	-	-

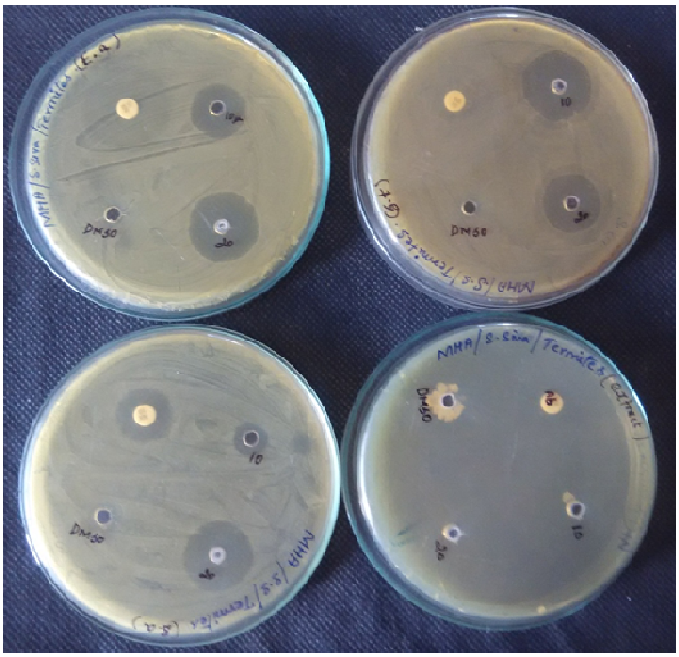

Plate 2. Antibacterial activity of *Bacillus* sp. VSCBNR7

Extraction of Compound

The compounds were extracted with ethyl acetate. The bioactive compounds present in the extracts were identified using UV- visible spectroscopy at 200 to 400nm. The highest peak was observed between 240 to 280 nm (Fig 3).

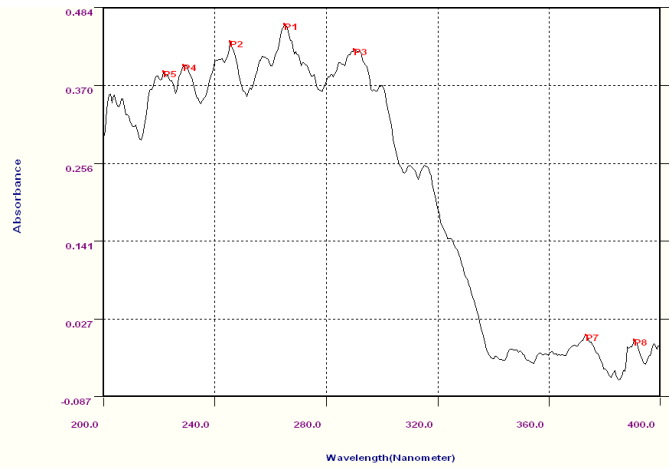

Fig. 3. UV- Visible spectroscopic analysis of *Bacillius* sp.

Thin Layer Chromotography

The blue colour spot was observed with an Rf values of 0.84 (Plate 3).

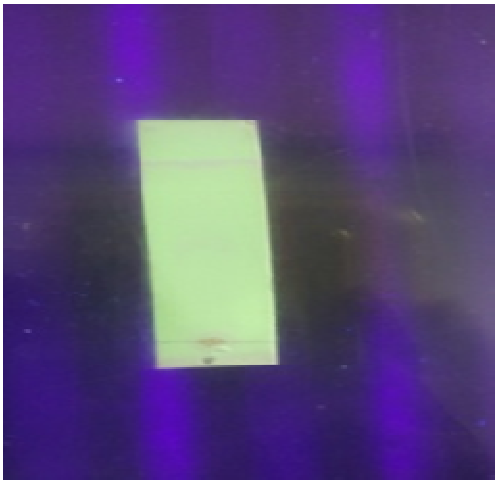

Plate 3. Thin Layer Chromotography

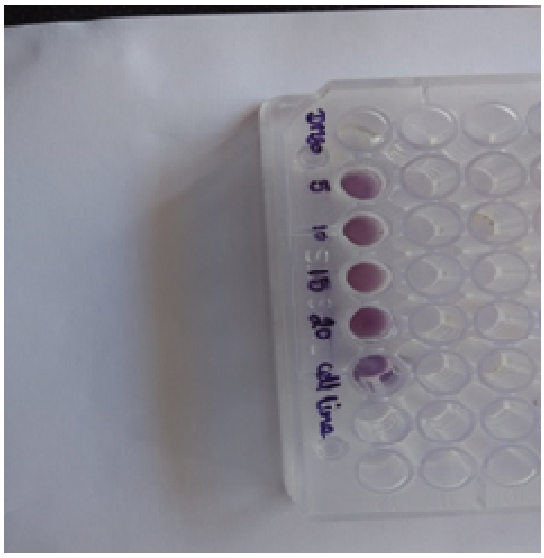

Plate 4. MTT Assay

Cytotoxic assay for *Bacillus* sp

In the Cytotoxic assay maximum cell death was recorded in 20µl concentration of sample and minimumcell death was recorded in 5µl concentration of sample (Plate 4 & Table 2).

Table 2. Cytotoxic assay for *Bacillus* sp

SAMPLE	CELL DEATH
5µl	9.790
10µl	13.496
15µl	23.181
20µl	27.307

GC-MS analysis of *Bacillus* sp. VSCBNR7

In the GC- MS analysis totally 8 compounds were isolated. They are Tetradecanoic acid, 9- Octadecenamide, (Z)- (Oleamide) ,Coumarin-6-ol, 3,4-dihydro-4,4,5,8-tetramethyl, à Terpineol, 3-Eicosyne ,Octadecanoic acid, methyl ester (CAS) 1,2-Octanediol 2-O-benzoyl ester and Dehydroherbarin. Dehydroherbarin contain anticancer activity. Remaining compounds have antibacterial, antifungal, andtiinflamatory and antiacne properties.

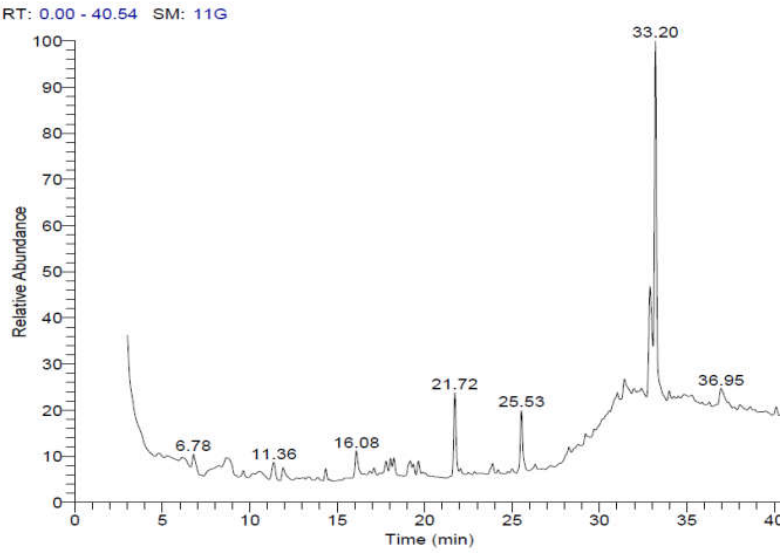

Fig. 4. GC-MS analysis of Bacillus sp. VSCBNR7

Table 3. GC – MS analysis of *Bacillus* sp. VSCBNR7

No.	Name of the compound	Molecular Formula	Compound Nature	Activity
1.	Tetradecanoic acid	C14H28O2	Fatty acid	Antioxidant Cancer preventive Cosmetic Hypercholesterolemic Nematicide
2.	9- Octadecenamide, (Z)- (Oleamide)	C20H40O2	Amide	Antimicrobial
3.	Coumarin-6-ol, 3,4-dihydro-4,4,5,8-tetramethyl	C13H16O3	Aldehyde	Antimicrobial, antifungal activity
4.	à Terpineol	C10H18O	monoterpene alcohol	antimicrobial, antispasmodic and immunostimulant properties
5.	3-Eicosyne	C20H38	Ester	Antiinflammatory, antiandrogenic cancer preventive, dermatitigenichypocholesterolemic, 5-alpha reductase inhibitor, anemiagenicinsectifuge, flavor
6.	Octadecanoic acid, methyl ester (CAS	C19H38O2	Polyenoic fatty acid	Antiinflammatory, Hypocholesterolemic, Cancer preventive, Hepatoprotective, Nematicide, Insectifuge Antihistaminic, Antiarthritic, Anticoronary, Antieczemic, Antiacne, 5-Alpha reductase inhibitor Antiandrogenic
7	1,2-Octanediol 2-O-benzoyl ester	C15H22O3	Alkaloids	Anitinflammatory, anti ulcer
8	Dehydroherbarin	C16H14O5		Anti cancer

DISCUSSION

In the present investigation *Bacillus* sp. VSCBNR7 inhibits the growth of *S.typhi, E.coli* and *S.aureus*. Maximum zone of inhibition 7mm was observed in 20µl sample against *E.coli. Bacillus* sp. VSCBNR7 inhibits *S.typhi* with inhibition zone of 7mm in 10µl sample. Similarly Mirac Yilmaz *et al.,* 2006 determined that *B. brevis* M6 showed an inhibition zone diameter of 16 mm against *S. aureus* ATCC 25923 and that *B. cereus* M15 showed an inhibition zone diameter of 6.4 mm against *M. flavus*. Perez *et al*., 1993 reported that *B. subtilis* MIR 15 strain displayed antimicrobial activity against *P. aeruginosa, E. coli* and *M. luteus.* Oscariz *et al*. (1999) isolated and identified a bacteriocin-producing strain of *B. cereus* from a soil sample. The strain was active against most Gram-positive but not Gram-negative bacteria. The findings of the present study indicate that *Bacillus* isolates have antimicrobial effects particularly against the Gram-positive test bacteria. However, *B. cereus* M15 has inhibitory affect both against Gram-positive and Gram-negative bacteria. In the present study bioactive compounds were identified using thin layer chromatography. The blue colour spot was observed with an Rf values of 0.84. This was agreed with Judith reinhard (2001) reports.

Catherine brasseur (2016) reports were supported with our findings. In the present study GC- MS analysis totally 8 compounds were isolated. The compounds have the ability to inhibit the growth of bacteria and fungi. Some of the isolated compounds having antioxidant and anticancer properties (Table 3& Fig 4).

Conclusion

Termites are consumed in many regions globally, but this practice has only become popular in developed nations in recent years. Termite species are used as food by humans or are fed to livestock, and the termite gut has inspired various research efforts aimed at replacing fossil fuels with cleaner, renewable energy sources. Recent research is focused on the termite gut associated with microorganisms. These microbes having wide number of bioactive compounds and these compounds had antibacterial, antiviral and anticancer activity.

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