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T. Uma and Chowdappa Srinivas

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Identification of Secondary Metabolites Produced by *Streptomyces sp.* Isolated from Vermicompost

T. Uma[1] and Chowdappa Srinivas*[2]

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A B S T R A C T

Beneficial microorganisms provide first line defense for roots against diseases and promote plant growth. Actinomycetes in vermicompost aid in the breakdown of organic materials and the production of secondary metabolites. In the present investigation, actinomycetes were isolated from vermicompost prepared using *Arachis hypogeal*. Scanning electron microscope (SEM) images were used to study the morphological changes in actinomycetes. The SEM images revealed various stages of mycelia growth of the *Streptomyces sp.* during the growth period. Identification of bioactive compounds produced by *Streptomyces sp.* were analyzed through Gas Chromatography-Mass Spectrometer (GC-MS) the result revealed the presence of twenty major bioactive compounds, among them, Cetane, E-15-Heptadecenal and n-Hexadecanoic acid which are important for plant growth and disease suppression. Hence, actinomycetes established in vermicompost can be used for plant growth promotion, suppression of pathogens and synergistic interaction with other beneficial soil microorganisms.

***Key words*:** Vermicompost, *Streptomyces sp.,* Bioactive compounds, *Arachis hypogeal*

Vermicompost is also known as bio inoculant, due to the presence of diverse species of microflora [1]. There are two major sources of microbial flora in vermicompost, the initial population of microorganisms occur during the composting process. There is a varied succession of microbial populations in the composting period. Most of the pathogenic microorganisms are killed either by the heat generated due to microbial activities, by the antagonistic effect of other microorganisms or by attaining unfavourable conditions in the compost. Early microbial colonization is heterotrophs and dependent on the organic substrates [2-3]. Second, the source of microbial population possibly comes out from the gut of earthworm along with the vermicast and some potential pathogen indicators can be killed by the secreted coelomic fluid [4-5]. Earthworms play an important role in stimulating the microbial growth indirectly in vermicompost. Vermicasting consists of abundant beneficial microorganisms *viz*; bacteria, fungi and actinomycetes.

Actinobacteria are filamentous, aerobic, spore forming, Gram-positive bacteria with high G+C content [6-7]. Actinomycetes grow in various habitats viz; soil, compost, marine and in extreme environmental conditions. Growth parameters mainly get influenced by alkaline pH (range 6.5-8.0) and temperature [mesophilic actinomycetes (25-30°C) and thermophilic actinomycetes (55-65°C)]. Actinomycetes reproduce by transverse binary fission and the growth leads to formation of branched or unbranched sporulating mycelium [8].

The *Streptomyces sp.* within Actinobacteria phylum serves as a novel source for the production of metabolically active compounds [9-11]. The bioactive compounds produced by actinomycetes have significant economic value in agriculture and pharmaceutical industries as antibacterial, antifungal, anticancer, immunity suppression, anti-protozoan, anti-cholesterol, anthelmintic and antiviral agent [12-18]. The *Streptomyces sp.* has demonstrated biocontrol activity against various bacterial and fungal plant pathogens viz; *Alternaria sp.*, *Colletotrichum*, *Acremonium lactucum* and *F. oxysporum* [19].

There are two important mechanisms through which pathogen suppression can be reached directly by addition of microorganisms producing antagonistic agents or indirectly by adding soil amendments which could increase the microbial diversity creating an unfavorable condition for disease development. In the present investigation, actinomycetes were isolated from vermicompost prepared using *Arachis hypogeal*. The scanning electron microscope (SEM) images were used to study the morphological changes in actinomycetes. Identification of various bioactive compounds was done through GC-MS. The potential bioactive compounds from actinomycetes could sustainable agriculture.

* **Chowdappa Srinivas**
✉ csrinivas@bub.ernet.in

[1-2] Department of Microbiology, Biotechnology and Food Technology, Bangalore University, Jnana Bharathi Campus, Bangalore - 560 056, Karnataka, India

MATERIALS AND METHODS

Preparation of vermicompost

The organic substrates *Arachis hypogeal* shell, bulking agents (kitchen waste and garden waste) were collected and air dried. The organic substrates were mixed with the bulking agents in 1:1 ratio w/w making up to 10 kg. Cow dung slurry was added to each tub, mixed properly and allowed to decompose partially for three to four weeks [20]. After four weeks, proper turning was given to cool the substrates as well as to get homogenous compost. Water was sprinkled to maintain moisture level between 40-60% [21]. Earthworms (*Eudrilus eugeniae*) were procured from University of Agricultural Science (UAS), Bengaluru, vermicompost unit and were released at 50 numbers/10 kg of mixture and kept in shade for three to four months. By this process, earthworms multiplied 300 times within one-two months until the organic material was completely biodegraded. After complete decomposition of organic matter, the vermicompost was harvested and air dried in shade.

Isolation and identification of actinomycetes

The isolation of actinomycetes was carried out by serial dilution of the vermicompost. One gram of the vermicompost sample was diluted serially up to 10^{-4} dilutions and plated on starch casein agar (SCA), by spread plate technique and incubated at 27 ± 2°C for 5-7 days. The most prominent colonies were isolated and maintained on SCA slants at 4°C for further studies. The isolated actinomycetes were identified morphologically to check the arrangement of the conidiospore and arthrospore [22].

Morphological observation using scanning electron microscope

Morphological changes of actinomycetes were observed by using scanning electron microscope TESCAN-VEGA3 LMU (TESCAN, Czech Republic) with resolution of 3.0 nm at 30kV, magnification continuous from 4.5X to 1,000,000X, maximum field of view 0.08µm, accelerating voltage 200 V to 30 kV, electron gun tungsten heated cathode, probe current 1pA to 2 µA, scanning speed from 20 ns to 10 ms per pixel adjustable in steps or continuously and image size up to 8,192 x 8, 192 Pixels in 32-bit quality. *Streptomyces* sp. spores were mounted on a conductive substance such as aluminum and it is gold plated with vacuum. The secondary emission mode was used to visualize the 3-Dimensional (3-D) images of the actinomycetes. Compressive and split tensile strength was carried out under elevated temperatures and also at standard temperature [23].

Extraction of bioactive compounds from actinomycetes

Based on previous studies *Streptomyces* sp. was selected for further studies taken in 100 ml of ISP1 broth in 1000 ml of conical flask under sterile conditions and incubated at 30 ±2 °C for 7 days. After incubation, the culture medium was filtered using Whatmann filter paper and the culture filtrate was centrifuged at 10,000 rpm for 20 min to remove the cell biomass. The supernatant was mixed with equal volume of ethyl acetate (1:1v/v) and shaken vigorously for 1 h by using solvent extraction funnel. Solvent and filtrate mixture were stabilized for 24-48 h, after 48 h the solvent phase was separated from aqueous phase. The solvent phase was collected and evaporated by using rotary vacuum evaporator and concentrated sample was used for further studies [24-25].

GC-MS analysis of bioactive compounds

The Gas Chromatography–Mass Spectrometry (GC-MS) analysis was carried out to identify bioactive compounds in the crude ethyl acetate solvent extract of *Streptomyces* sp. GC-MS instrument used was Agilent technologies 5975, capillary column, column dimension was of 30.0m x 0.25 mm x 0.25 µ and helium gas was used as carrier gas at ml/min. Temperature of the column was programmed to 50 ^{0}C initially and gradually increased up to 300°C / min and an isothermal period was kept for 5 min [26].

RESULTS AND DISCUSSION

Isolation and identification of Actinomycetes

Vermicomposting is the process of transformation of recent organic matter in stabilized organic matter through the action of earthworms along to the flora that lives inside their digestive dealings [27]. In the gut of earthworms, the not digested organic matter along to that organic matter that was not assimilated are expelled together with earth particles in the form of an organic compound rich in nutrients, easily assimilated by plants, which receives the name of coprolites [28-29].

The vermicompost harvested was granular, black and odorless, with efficient water holding capacity. A total of five actinomycetes isolates were isolated from the prepared vermicompost. Initially, the morphology of the colonies was relatively smooth surfaced, but later they develop a tuft of aerial mycelia that may appear floccose, granular, powdery, or velvety. Most of the colonies formed were hard, leathery or fluffy round colonies with discrete edges. The morphology and arrangements of mycelia was observed as branched filament [30-31]. Based on the morphological structure and cultural characteristics most of the isolates were identified to belong to *Streptomyces* sp. (Fig 1). Further molecular identification of *Streptomyces* sp. by 16S rRNA showed 99% similarity with *Streptomyces cavourensis* strain producing significant alignments from BLAST [32].

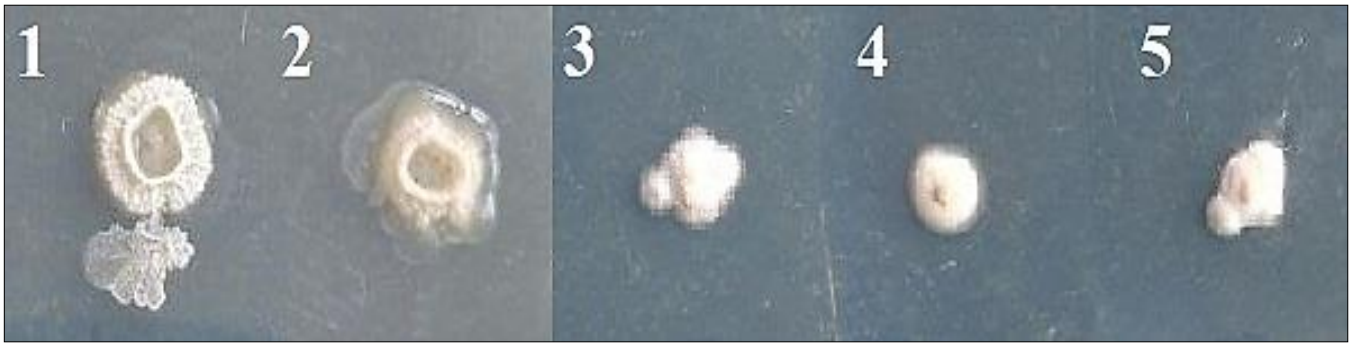


Fig 1 Actinomycetes species isolated from *Arachis hypogeal* shell used for vermicompost

Actinomycetes showed mycelium like filament which could form spores. Microscopic images observed by SEM revealed the morphological changes during the growth period of *Streptomyces sp.* The image at the initial stage of growth

showed rod shaped bacteria ranging up to 1µm. As the culture was further incubated the cell elongated, branched and curled up to the tip of the mycelium. Aerial conidia or spore like structure also was seen at the end of the mycelia. On the seventh day of growth, branched, spiral shaped mycelia were observed. Cells width measured approximately ranging from 565.68 ±11.82 nm to 0.56±0.04 µm and length ranging from 969.44±1.2 nm to 0.83±0.06 µm from day 1 to day 7 respectively (Fig 2). Based on the morphological characteristics and microscopic identification very prominent isolate was selected for identification of bioactive compounds.

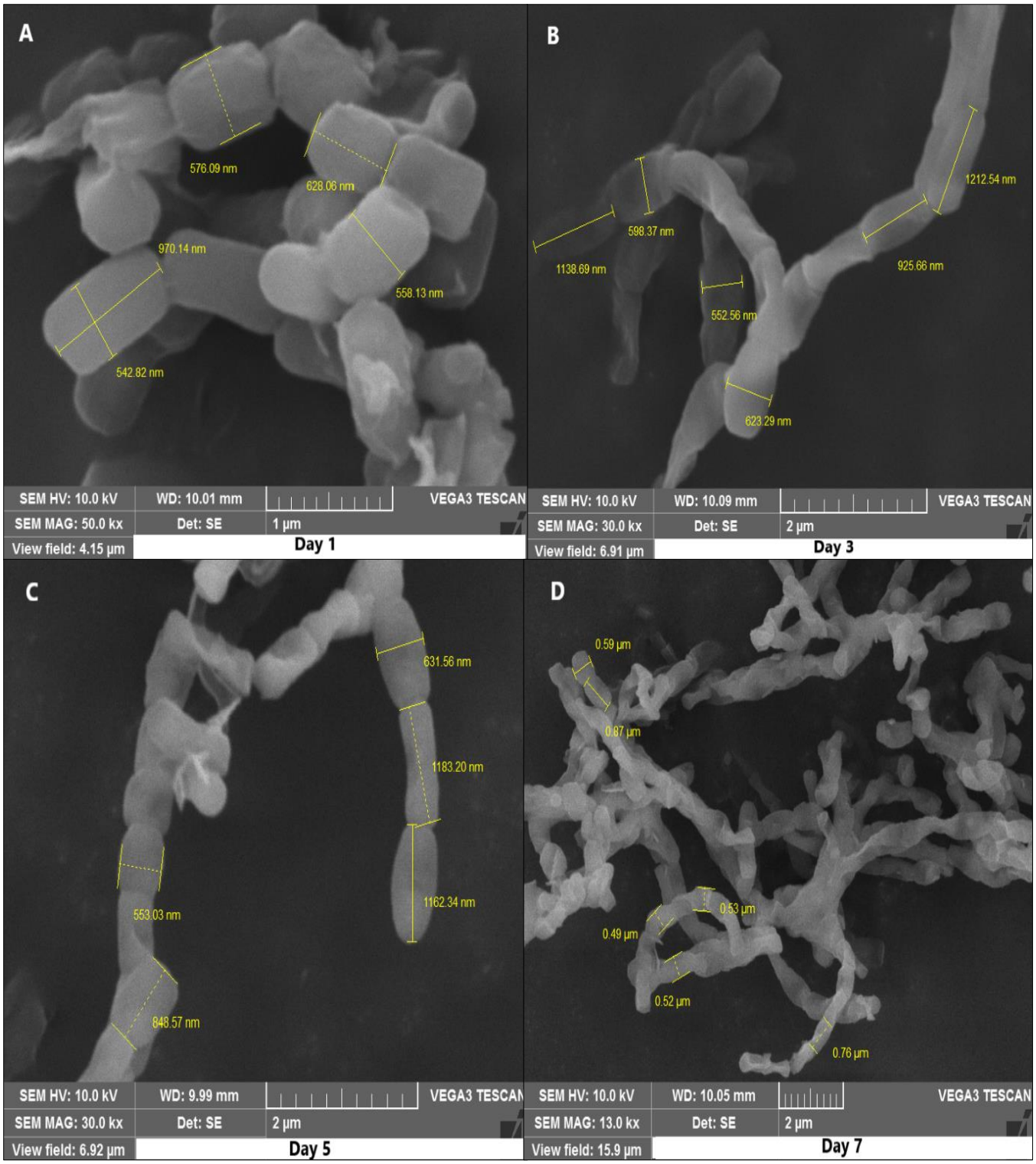


Fig 2 Scanning electron microscopic image of *Streptomyces* sp. on different days of incubation, A- day 1, B- day 3, C- day 4, D-day 7 days of incubation

GC-MS analysis for bioactive compounds

The GC-MS chromatogram revealed a total of 21 bioactive compounds from ethyl acetate extract of the *Streptomyces* sp. listed in the table (Table 1). Amongst that 11 major bioactive compound (Fig 3, 3a and 3b) were found to exhibit important biological activities such as antibacterial, antifungal and antioxidant GC-MS analysis gives information about the molecular weight of each identified compound rom ethyl acetate extract of the *Streptomyces* sp. [33-35].

Table 1 List of bioactive compounds identified from *Streptomyces sp.* by GC-MS analysis.

S. No.	Retention time (min)	Compound name	Molecular formula	Molecular weight	Biological activities
1	5	Dodecane	$C_{12}H_{26}$	170.203	Anti-oxidant
2	2	1-Dodecane	$C_{12}H_{24}$	168.187	Antibacterial, antifungal
3	20	1-Tetradecene	$C_{14}H_{28}$	196.219	Antimicrobial diuretic, anti-tuberculosis
4	2	Cetane	$C_{16}H_{32}$	224.250	Antifungal, antibacterial and antioxidant properties
5	15	Acetic Acid, Phenylmethyl ester	$C_9H_{10}O_2$	150.068	Flavoring agent
6	2	E-15-Heptadecenal	$C_{17}H_{32}O$	252.245	Antioxidant and antibacterial activity
7	10	1-Docosene	$C_{22}H_{44}$	308.344	Antibacterial
8	4	Phenol,2,4-bis(1,1-dimethylethyl)	$C_{14}H_{22}O$	206.167	Antibacterial activity
9	15	Phthalic acid, hept-4-yl isobutyl ester	$C_{19}H_{28}O_4$	320.198	Antimicrobial, Emulsifier
10	5	1,2-Benzenedicarboxylic acid, bis (2-methylpropyl ester)	$C_{16}H_{22}O_4$	278.151	AntimicrobialFlavoring agent
11	35	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256.240	Antimicrobial and antioxidant, hypocholesterolemic, nematicide, antiandrogenic flavour, hemolytic, 5- Alpha reductase inhibitor, antimalarial activity, Anti Inflammatory, cancer preventive, hepatoprotective, insectifuge, antihistamine, antieczemic, anticancer,,antiandrogenic, anti-coronary, antiarthritic

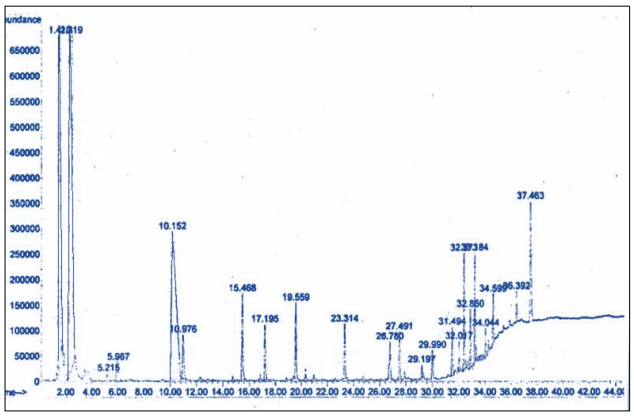
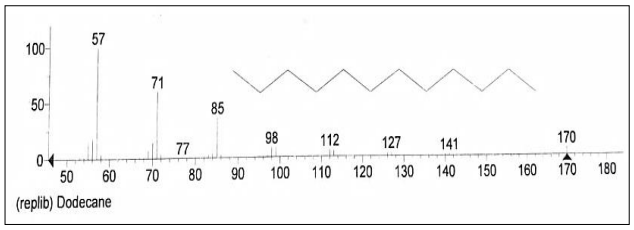
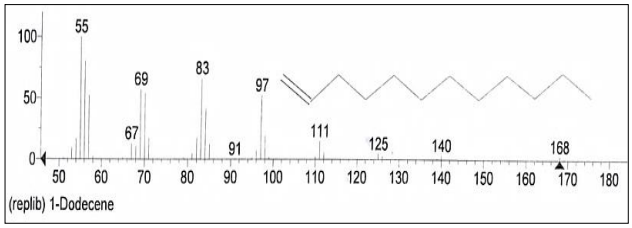
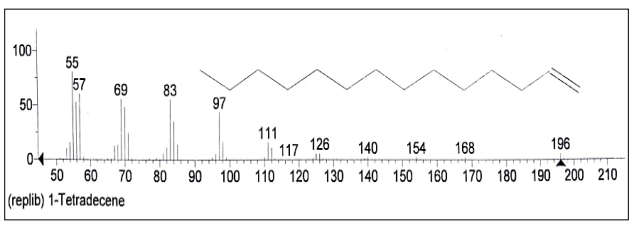
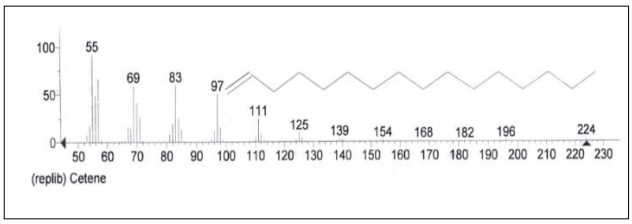


Fig 3 GC-MS chromatogram of bioactive components identified in the ethyl acetate extract of *Streptomyces cavourensis*









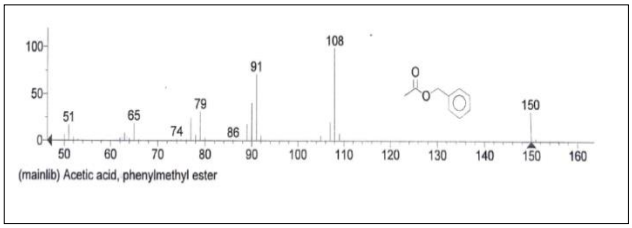
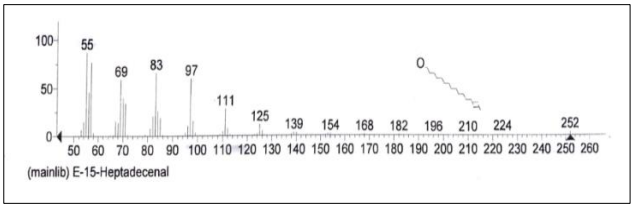
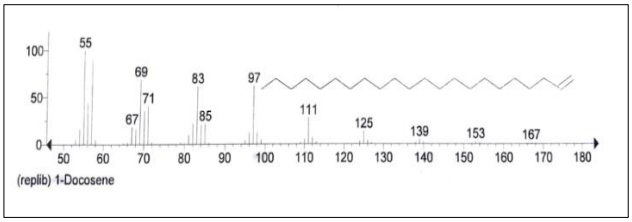
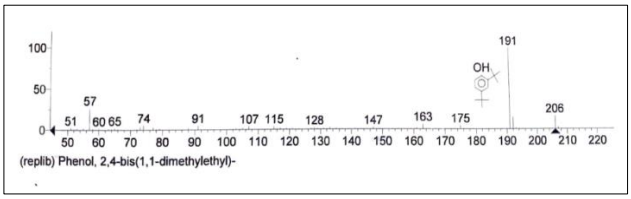


Fig 3(a) Mass spectrum of bioactive compounds identified from *Streptomyces* sp. (1) Dodecane (2) 1-Dodecene (3) 1-Tetradecene (4) Cetene (5) Acetic acid, phenylmethyl ester







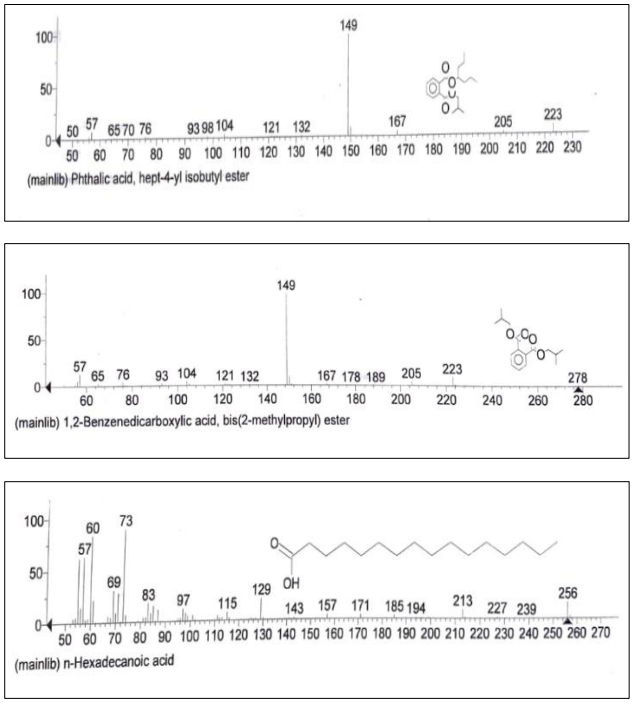


Fig 3(b) Mass spectrum of bioactive compounds identified from *Streptomyces* sp. (6) E-15- Heptadecenal, (7) 1- Docosene, (8) Phenol,2,4-bis(1,1-dimethylethyl, (9) Phathalic acid, hept- 4-yl isobutyl ester, (10) 1,2-Benzenedicarboxylic acid, bis (2-methylpropyl ester, (11) n- Hexadecanoic acid

Actinomycetes from earthworm castings are potential sources for wide-spectrum metabolically active compounds and industrially important enzymes, reflecting the importance of its economic value. Several strains of actinomycetes have been found to protect plants against plant diseases. There is a growing interest in the use of secondary metabolites, such as toxins, proteins, hormones, vitamins, amino acids and antibiotics from microorganisms, particularly from actinomycetes, for the control of plant pathogens as these are readily degradable, highly specific and less toxic to nature [36]. The crude ethyl acetate solvent extract revealed a total of 20 bioactive compounds with various biological activities as reported by [37].

The major bioactive metabolites examined in *Streptomyces sp.* extract are listed in Table I. These bioactive compounds were previously reported in microbes which was accountable for pharmacological activities. Amongst the various bioactive compounds identified, n-Hexadecanoic acid revealed major biological activities viz; Antimicrobial and antioxidant, hypocholestrolemic, nematicidal, antimalarial activity, Antiinflammatory, cancer preventive, hepatoprotective, insectifuge, antihistamininic, antieczemic, anticancer, antiandrogenic, anticoronary, antirthiritic. [38] revealed that spherical silver nanoparticles synthesized *Streptomyces sp.* produce antimicrobial, antioxidant and larvicidal. Antioxidant properties were also identified by Cetane and E-15-Heptadecenal.

The bioactive compound like Phenazine derivative from *Streptomyces sp*. isolated from *Neesia altisima* showed antagonistic activities against Gram positive bacteria [39]. Apart from antioxidant properties most of the bioactive compounds identified showed antibacterial and antifungal properties such as 1-Dodecane, Cetane, E-15-Heptadecenal, 1-Nanodecene, Phenol,2,4-bis(1,1-dimethylethyl), Diethyl Phthalate, 1-Docosene, Phthalic acid, hept-4-yl isobutyl ester, Phthalic acid, isobutlyl 2-pentyl ester, Phthalic acid, butyl 2-pentyl ester, Dibutyl phthalate, 1,2-Benzenedicarboxylic acid, bis (2-methylpropyl ester), n-Hexadecanoic acid and Diisooctyl phthalate. *S. cavourensis* isolated from *Cinnamomum cassia* Prels in Yen Bai province (21°53'14″N; 104°35'9″E) of northern Vietnam also showed antimicrobial and cytotoxic effect against methicillin-resistant *Staphylococcus aureus* ATCC 33591 and methicillin-resistant *Staphylococcus epidermidis* ATCC 35984 [40].

There are reports on the agricultural implications of these organisms in biological control of plant pathogens [41-43] and triggering of signal transduction in host plants to initiate defense responses to cope with the stresses at cell, tissue and organ level following inoculation of these organisms [44]. A novel antifungalmycin N2 bioactive compound produced by *Streptomyces* sp. N2 also showed effective biocontrol activity against *Rhizoctonia solani* by significantly reducing the sheath blight infection in rice plants [45]. Most studies have utilized *Streptomyces* as potential biological control agents of soil-borne fungal plant pathogens [46-47].

Charcoal rot in sorghum (and *Fusarium* wilt in Chickpea *(Cicer arietinum L.)* can be significantly controlled by treating it with *Streptomyces sp.* indicating its antagonist effect and [48-49]. *Streptomyces sp*. is also reported to be a potential bioenhancer and biocontrol agent against the disease and increasing plant growth in turn increasing the yield [50]. Similar studies revealed that *S. griseocarneus* exhibits biocontrol and plant growth promotion of chilli (*Capsicum annuum*) by preventing the development of anthracnose or reducing the lesion size and enhancing the growth of plant aerial part resulting in actinobacteria mediated growth promotion [51]. *Streptomyces sp.* was also found to be effective against *F. oxysporum f. sp. cubense* causal agent of wilt disease in banana [52]. Actinobacteria apart from employing the antagonist property it also helps the plant to overcome abiotic stresses [53].

CONCLUSION

Microbial communities in vermicompost can be dependent on the organic substrate they feed on and the species of earthworm used. Actinomycetes established in vermicompost can be potential source of industrially important secondary metabolites. Most of the actinomycetes are saprophytic and has the ability to decompose complex organic substrate. Vermicompost being one of the best sources of organic fertilizer, the presence of beneficial microorganism in vermicompost can lead to increased crop productivity and support sustainable agriculture. Actinomycetes being the predominating population in vermicompost, the secondary metabolites released outside the cell can increase the quality of the vermicompost by acting as antimicrobial agents and plant growth promotion. Actinomycetes could not only help in decomposition of organic substance but also help in production of novel secondary metabolites applicable in agriculture.

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