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Screening of Secondary Metabolites Produced by Streptomyces Species from a Soil Sample that Can Produce Anti-Nematodal and Antiprotozoal Avermectins

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Authors' contributions

The work was carried out at department of Biotechnology. Author PM is a PG student and Authors TJ and SB assisted author PM. The work was carried out under the Guidance of author RS and the finances were borne by college.

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ABSTRACT

Avermectins are a group of secondary metabolites produced by *Streptomyces avermitilis,* which act on invertebrates. They activate glutamate-gated chloride channels in their nerves and muscles which in turn disrupt pharyngeal function and locomotion. Avermectin ingested insects are paralyzed and starve to death. Ten isolates identified as avermectin producers were characterized by morphological, colony characters and biochemical tests. Secondary screening leads to the identification of four isolates PM_2; PM_4; PM_7 and PM_{10} which produced 10, 8.4, 3.8 and 6.9mg respectively as identified by HPTLC. Bio-autography illustrated their anti-nematodal and antiprotozoal activity; the zone of clearance (turbid) was recorded 43, 24, 32 and 37 mm respectively for PM_2 through PM_{10}.

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1. INTRODUCTION

Soil is the habitat for several microorganisms which include bacteria, actinomycete, fungi, and algae. Soil microbes are an outstanding source for the isolation and identification of therapeutically important products [1].

Actinomycetes produce a large number of antibiotics. These Antibiotics have been reported to possess bactericidal, fungicidal, neuritogenic, anticancer, antipyretic, analgesic, anti-protozoal and anti- nematodal activities. These antibiotics can be purified from a single genus *Streptomyces* [2]. The genus *Streptomyces* was introduced by Waksman and Henrici in 1943 [3]. This Genus belongs to the Streptomycetaceae family [4]. More than 500 species of the genus *Streptomyces* have been described and most if not all *Streptomyces's* Species are antibiotic producers [5].

Streptomyces species are chemoorganotrophic, filamentous, Gram-positive organisms that are present in a wide variety of soil in rhizospere, dead and decay litter containing soil [6]. These are nonacid-alcohol fast; since these are present in dead and decay containing soil they share habitat with fungi, and appear very similar to fungi [7].

The colonies are slow-growing, having high G+C content 69-78% in their DNA [8]. These organisms have a soil-like odor due to the production of a volatile metabolite, geosmin. *Streptomyces* produces Avermectin. The avermectins are a potential candidate for the anti-helminthic and insecticidal agent. The same genus is also reported producing herbicidal, insecticidal, and nematocidal activities [9].

The avermectins were discovered in the early 1980s. Their selective toxicity towards nematodes and arthropods at very dilute doses, but relatively low or no toxicity to mammals make them potential future drugs. In invertebrates, they activate glutamate-gated chloride channels in their nerves and muscles and disrupt pharyngeal function and locomotion thereby causing paralysis. Avermectin ingested insects are paralyzed and starve to death. The selective toxicity that is, they do not harm vertebrates, has led to the conclusion that avermectins affect a specific cellular target which is either missing or out of reach in the resistant organisms [10].

Avermectin B1a is an indispensable drug in mass treatment programs to eradicate two widespread serious diseases namely river blindness and lymphatic filariasis, caused by nematodes, that affect millions of people worldwide [10].

2. METHODOLOGY

2.1 Isolation

Isolation of Streptomyces species was done from agricultural soil sample situated 18.150663 and 74.576782 with a GPS coordinates of 180 9' 2.3868" N and 740 34' 36.4152" E. The soil sample was collected at least 3-5 cm deep from thesoil.

The isolation and enumeration of *Streptomyces* were performed by serial dilution and spread plate method, yeast malt extract glucose agar medium (YMG agar) was used. Incubation was done at 28°C. Individual colonies growing on YMG agar were transferred on *Streptomyces* isolation agar medium for purification of *Streptomyces* [11].

2.2 Identification

The classification and identification of microorganisms are based on morphology, colony and biochemical characteristics [12]. This includes colony characters, detailed morphology, Gram's staining, motility, biochemicalcharacteristics.

To identify the organisms' morphology, colony characters, biochemical tests *viz:* fermentation of Carbon source - glucose, lactose, maltose, mannitol, potato starch hydrolysis, utilization of yeast extract, malt extract, peptone, urea hydrolysis, gelatin liquefaction, catalase, H_2S test wasperformed.

2.3 Fermentation Process

2.3.1 Seedmedium

The cultures were maintained on the YMG agar medium. Loop full culture of the different species was streaked on agar slant and inoculated into 50mL of yeast malt glucose (YMG) medium. The fermentation broth was incubated in an orbital shaker at 150rpm for 16-18 hours at 30°C [7].

2.3.2 Avermectin production

Production of avermectin from the soil isolates was studied in synthetic medium 2 (SM2) growths medium. Each production medium was inoculated with 5 mL of inoculum separately. After transferring the seed medium, each growth medium was incubatedat 30°C inashaker for 10days at 150rpm [6].

2.4 Elicitation

Since avermectins are secondary metabolites and produced in very low quantity and intracellular (Periplasmic space). Avermectins are produced in response to nematode growth in the territory of *Streptomyces* species. The nematodes were immobilized in 0.8% agarose and these beads were introduced in fermentation broth to elicit the production of Avermectins.

2.4.1 Extraction of avermectin

The fermentation broths were centrifuged at 4°C for 20 minutes at 6000rpm. the cell biomass was separated. The cell biomass in the form of the pellet was mixed with an appropriate amount of methanol to completely dissolve it. The mixture was centrifuged again and the supernatant was collected for avermectin analysis by high-performance thin-layer chromatography HPTLC[11].

2.5 Determined of Avermectin Concentration by High-performance Thin-layerChromatography

The ten purified samples were analyzed for avermectin drug content. The sample Application was done by Linomat 5. Five microliters of the ten samples were spotted onto the plate of pre-coated with silica gel 60F-254 (20cm×10cm) was used as a stationary phase. The Mobile phase was n-hexane: acetone: ethyl acetate (13:7: 0.2 v/v/v). The plated were developed in Twin trough chamber (CAMAG) Densitometeric scanning was done from 190nm to 500nm in absorbance mode with Camag TLC scanner III, using deuterium and tungsten lamp operated by win CATS software (Version 1.2.0). The slit dimension of 8.00 mm × 0.60 mm and a scanning speed of 20 mm s^{-1} were set [13].

2.6 Bio-autography

To evaluate the anti-nematode and antiprotozoal activity of avermectins the TLC plate was overlaid by 1% agarose containing the nematodes and protozoa and incubated at 37□C. the appearance of turbid spot indicated the death of nematodes and protozoa. The zone of turbidity wasmeasured.

3. RESULTS

3.1 Isolation

Initially, colonies were relatively smooth-surfaced but later they develop a mesh of aerial mycelium and appear floccose, granular.

Ten isolates were identified as *Streptomyces* these were coded as PM_1, PM_2, PM_3, PM_4, PM_5, PM_6, PM_7, PM_8, PM_9, and PM_{10} respectively till further identification.

3.2 Identification

Physical parameters such as their tolerance to pH, temperature were evaluated. The organisms' best grow at neutral pH ranging from 7 to 7.4 and temperature 28□C to 35□C. The other physical parameters and their results are tabulated.

Image 1: *Streptomyces* growing on YMG agar plates (PrimaryScreening)

Table 1. Physical parameters of *Streptomyces* growing on YMG agarplates

	Properties			
Selected isolates	**Spore morphology**	**Color of aerial mycelium**	**Color of substrate mycelium**	**Gramstaining**
PM_1	Round shaped	LightGrey	Grey	Gram-positive
PM_2	Ovalshaped	Darkgrey	Lightgrey	Gram-positive
PM_3	Spiral	Paleyellow	Yellowish	Gram-positive
PM_4	Spiral	DarkGrey	Grey	Gram-positive
PM_5	Branched	Yellowish	Whitish	Gram-positive
PM_6	Roundshaped	LightGrey	Lightgrey	Gram-positive
PM_7	Ovalshaped	Grey	Grey	Gram-positive
PM_8	Spiral	Paleyellow	Yellowish	Gram-positive
PM_9	Branched	Whitish	Whitish	Gram-positive
PM_{10}	Spiral	Offwhite	Whitish	Gram-positive

Table 2. Biochemical test of *Streptomyces* species

Biochemical Characteristics of the selected *Streptomyces* species										
Nitrogen sourceutilization										
	PM_1	**PM_2**	**PM_3**	**PM_4**	**PM_5**	**PM_6**	**PM_7**	**PM_8**	**PM_9**	**PM_{10}**
Yeastextract	+	+	+	+	+	+	+	+	+	+
Maltextract	+	+	+	+	+	+	+	+	+	+
Peptone	+	+	+	+	+	+	+	+	+	+
Urea	+	+	+	+	+	+	+	+	+	+
C-sourceutilization										
Glucose	+	+	+	+	+	+	+	+	+	+
Lactose	+	+	+	+	+	+	⊕	+	+	+
Maltose	⊕	+	+	+	⊕	+	+	+	+	+
Mannitol	+	+	+	+	⊕	+	⊕	+	+	+
Potatostarch	++	++	++	++	++	++	++	++	++	++
Othertests:										
Citrateutilization	_	+	+	_	_	+	_	+	_	_
Gelatinliquefaction	+	+	+	+	_	+	+	+	_	+
Catalasetest	+	+	+	+	+	+	+	+	+	+
H_2S productiontest	_	_	+	_	+	+	_	+	+	_

Similarly in an attempt to classify the organisms following Biochemical tests were performed – carbohydrate namely, glucose, lactose, maltose, potato starch. The ability to utilize citrate, liquefy gelatin. Catalase test, which evaluate the ability of organisms' respiration using oxygen, and protects them from toxic byproducts of oxygen metabolism i.e. reactive oxygen species (ROS). If the microorganism is capable of reducing the Sulphur compound to sulfides was determined by the H_2S productiontest.

A positive test was denoted by + sign and negative test by – sign, ++ was strongly positive and +sign within circle indicated the organism can ferment the sugar aerobically i.e. by producing acid and gas (in form of a bubble in

Durham's tube). Standard protocols were performed as mentioned in *Bergey's Manual.*

3.3 Fermentation Process

50 ml of synthetic medium 2 (SM2) was seeded by 5ml YGM media containing PM_1 to PM_{10} isolates in previously sterilized 330ml glass bottles. These were incubated at 30°C in orbitek shaker incubator at 105rpm.Turbidityindictedgrowthwhichwasassessedspectrophotometericallyat600nm.

HPTLC:

The HPTLC plate was scanned from 190 nm – 500 nm with 20mm s-1. A peak at 247nm indicated the presence of avermectins. PM2, PM4, PM7, and PM10 exhibited the Rf values of 0.66, 0.47, 0.49 and 1.57 that of avermectins.

The area under the curve as obtained by densitometry for pure avermectin and thatof an obtained peak was 875.8, 736.1, 334.0 and 550.1mm squared for PM_2, PM_4, PM_7, and PM_{10} respectively. Thus the concentrations of avermectins for the four isolates in the same sequence were 0.200, 0.168, 0.076 and 0.125mg/ml.

Thus 50ml broth contained 10, 8.4, 3.8 and 6.9mg avermectins for PM_2, PM_4, PM_7, and PM_{10} respectively. As shown in thegraph.

Image 2. Fermentation process for avermectin production(elicited)

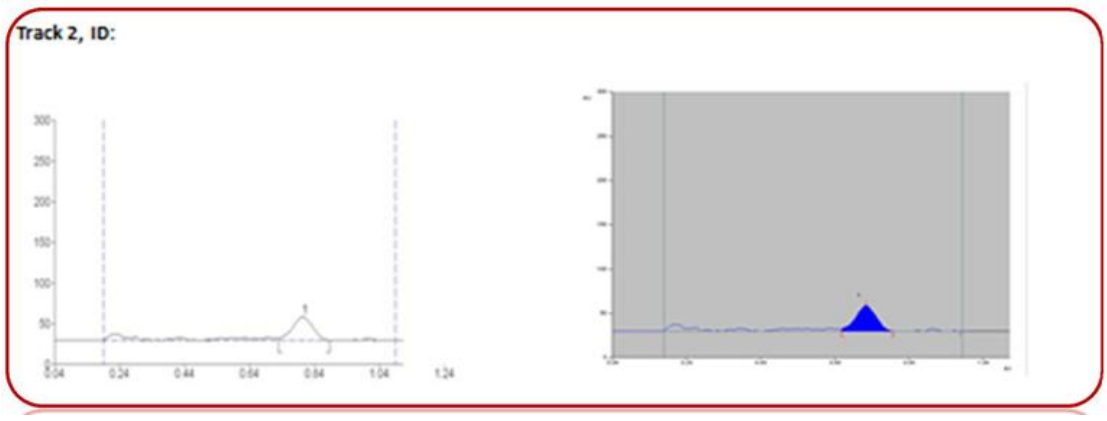

Image 3. Densitometric scanning of broth from 190nm-500nm

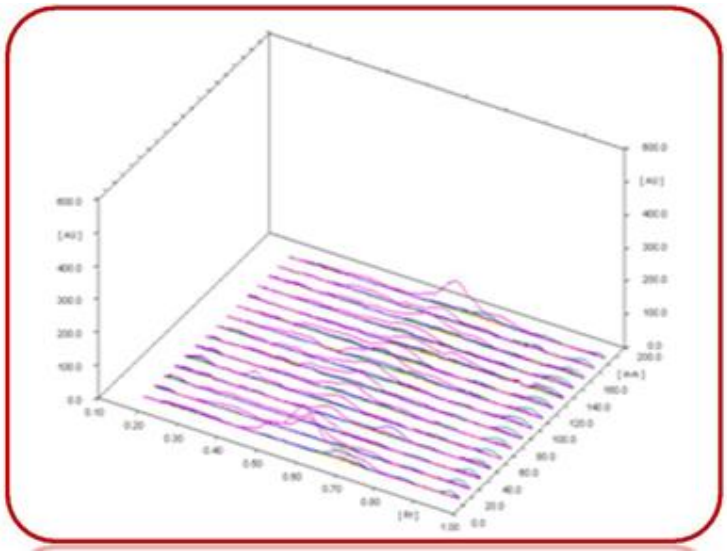

Image 4. HPTLC of PM2 avermectins resolved at Rf value0.66

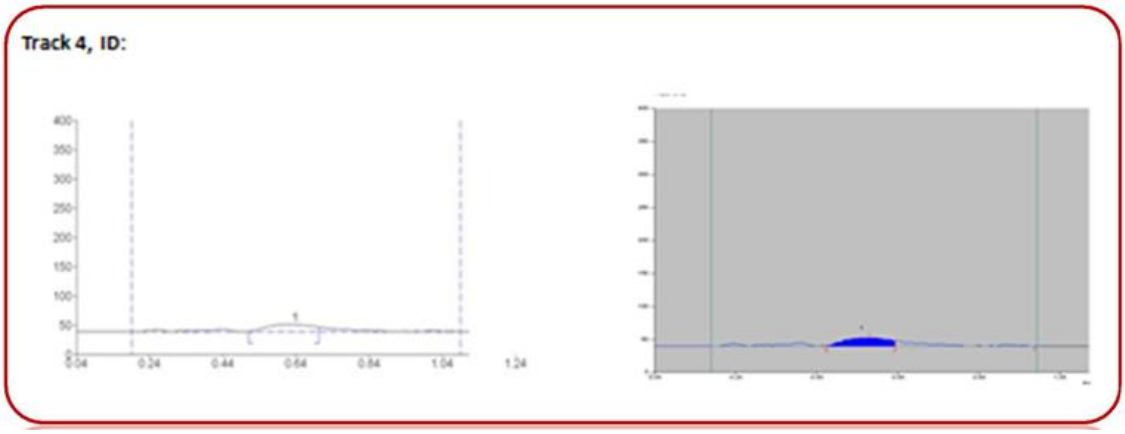

Image 5. HPTLC of PM4 avermectins resolved at Rf value0.47

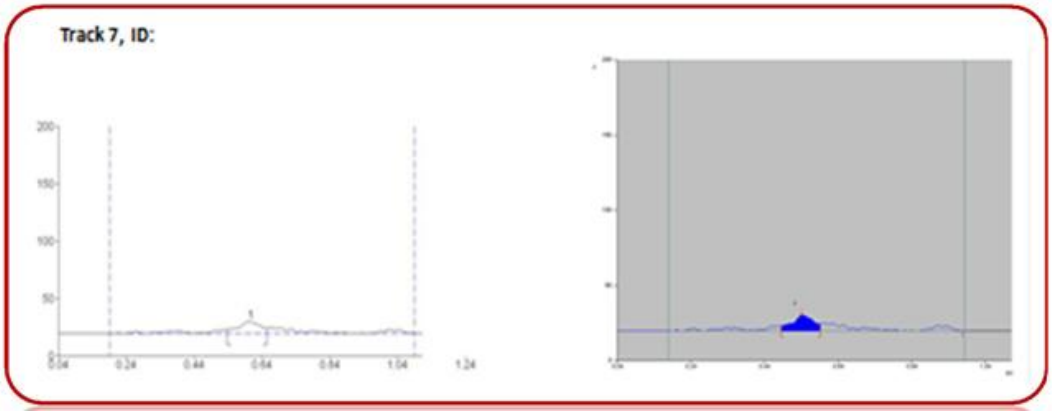

Image 6. HPTLC of PM7 avermectins resolved at Rf value0.49

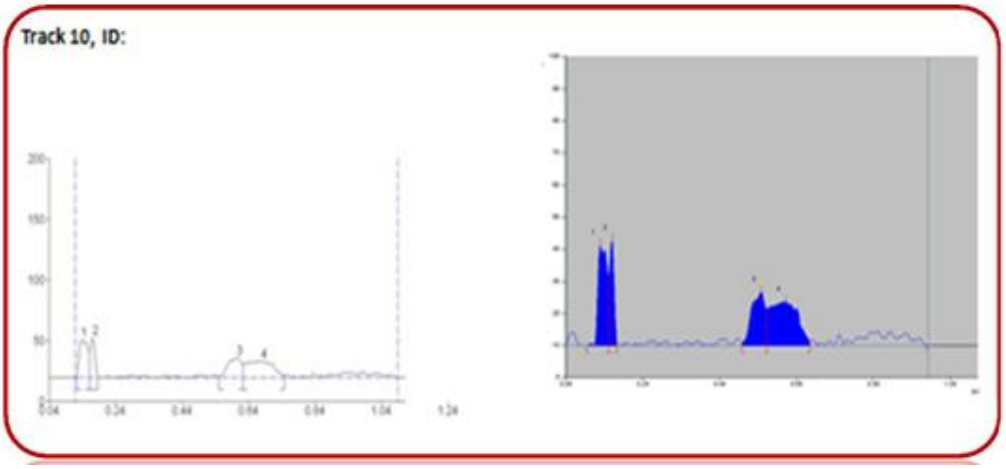

Image 7. HPTLC of PM_{10} avermectins resolved at Rf value0.57

Table 3. HPTLC analysis of isolates, Rf value, the area under curve andconcentration

Sr. no.	Isolates	RF value	Areaunder curved	Total avermectin accumulated mg/ml	Total avermectin of 50ml in broth
1	PM_2	0.66	875.8	0.200	10
2	PM_4	0.47	736.1	0.168	8.4
3	PM_7	0.49	334.0	0.076	3.8
4	PM_{10}	0.57	550.1	0.1258	6.29

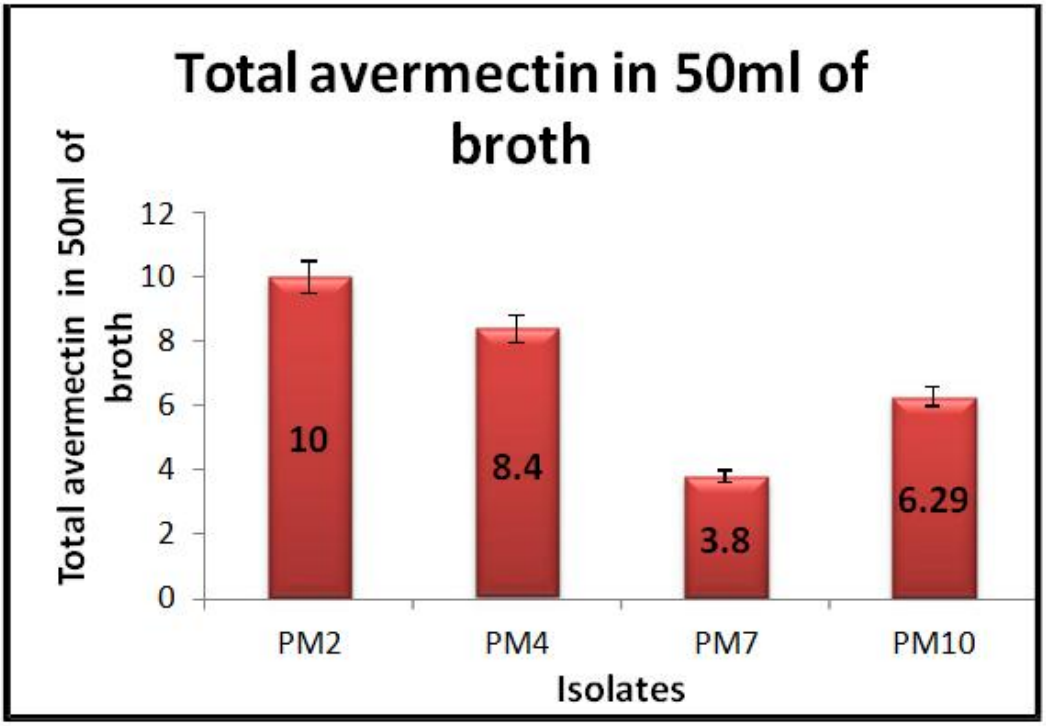

Image 8. Concentration of avermectins accumulated in 50 ml broth

Image 9. Bio –autographyresults

Table 4. Inhibition of nematodes and protozoa by avermectins

The TLC plate was overlaid by 1% agarose containing nematodes and protozoa and zone of clearance was measured, which was 43, 24,32,and, 37mm for PM2,PM4,PM7,and PM10 respectively.vermectin containing samples	Zone of clearance (turbid) mm indiameter
PM2	43
PM4	24
PM7	32
PM10	37

4. CONCLUSION

Ten isolates of *Streptomyces* were obtained after primary screening of soil, post fermentation process and HPTLC analysis revealed that isolates PM2, PM4, PM7, and, PM10 produced 10 mg, 8.4 mg, 3.8 mg and 6.9 mg of Avermectins/50 ml of fermentation medium. The anti- nematodalactivity was evaluated by Bioautography.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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