

SCREENING OF ACTINOMYCETES FROM EARTHWORM CASTINGS FOR THEIR ANTIMICROBIAL ACTIVITY AND INDUSTRIAL ENZYMES

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Submitted: March 14, 2011; Returned to authors for corrections: June 22, 2011; Approved: August 30, 2011.

ABSTRACT

Actinomycetes from earthworm castings were isolated and screened for their antimicrobial activity and industrial enzymes. A total of 48 isolates were obtained from 12 samples of earthworm castings. Highest numbers of isolates were recovered from forest site (58.33 %) as compared to grassland (25%) and agricultural land (16.66%). The growth patterns, mycelial coloration of abundance actinomycetes were documented. The dominant genera Identified by cultural, morphological and physiological characteristics were *Streptomyces* (60.41%) followed by *Streptosporangium* (10.41%), *Saccharopolyspora* (6.25%) and *Nocardia* (6.25%). Besides these, other genera like *Micromonospora*, *Actinomadura*, *Microbispora*, *Planobispora* and *Nocardiopsis* were also recovered but in low frequency. Among the 48 isolates, 52.08% were found active against one or more test organisms. Out of 25 active isolates 16% showed activity against bacterial, human fungal as well as phytopathogens. Among 48 isolates 38, 32, 21, 20, 16 and 14 produced enzyme amylase, caseinase, cellulase, gelatinase, xylanase and lipase respectively while 10 isolates produced all the enzymes. More interestingly 2, 3, and 1 isolates produced amylase, xylanase and lipase at 45°C respectively. In the view of its antimicrobial activity as well as enzyme production capability the genus *Streptomyces* was dominant. The isolate EWC 7(2) was most promising on the basis of its interesting antimicrobial activity and was identified as *Streptomyces rochei*. The results of these findings have increased the scope of finding industrially important actinomycetes from earthworm castings and these organisms could be promising sources for industrially important molecules or enzymes.

Key words: Antimicrobial activity, enzymes, earthworm castings, actinomycetes

INTRODUCTION

The development of multidrug resistance in pathogenic organisms is continuous problem faced by currently used

antibiotics (3). Therefore, the availability of potent drug is becoming particularly an acute problem (30). In this scenario, microorganisms have been intensively screened from soil as a source of therapeutically important molecules over a half

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century (11) however; the frequency of discovery of structurally new compounds is apparently decreasing these years. This trend seems to imply that the easily accessible microorganisms in soil had been exhausted and there is a need to seek unutilized microorganisms from unexplored sources (16). It is likely that the diversity of secondary metabolites relies more or less on the isolation source, namely, the habitat of the producers (16). On the basis of above facts new actinomycetes strains that generate active compounds have been recently isolated from novel sources including saline, ocean, mangrove forests and niche habitats such as caves, pristine forests, lakes, rivers, and other wetlands (29, 31). To cope up with the demand for new pharmaceutical compounds and to combat the antibiotic resistant pathogens, researchers have been forced to look for novel microorganisms in unusual and unexplored environments.

The earthworm casting has rarely been explored for actinomycetes having antimicrobial activity and industrial enzymes. Extensive literature survey revealed that the casting activity led to nutrition and microbial enrichment (36). Hence, the number of total bacteria, siderophore producing bacteria and fluorescent Pseudomonads were greater in casts as compare to the soil without casting activity (7) but there was no report on actinomycetes. The interaction between earthworm, beneficial soil microorganisms and root pathogens had been studied by Doube et al (8) and found that earthworms act as vectors for beneficial soil bacteria. Hence, there is an immense possibility to identify new actinomycetes in the earthworm casting to discover novel bioactive compounds. Accordingly, the present study was aimed to screen industrially important actinomycetes in the earthworm castings with the ultimate objective of discovering novel bioactive compounds.

MATERIALS AND METHODS

Sampling area

The study area covered the Doon valley (Dehradun) of Uttarakhand, India. The latitude and longitude of Dehradun is 30°20'N and 78°04'E respectively. It is 660 meters above the

sea level. In summer the temperature is minimum of 16.7°C and maximum of 36°C. The average rain fall is 2073.3 mm.

Collection of samples

A total of 12 earthworm (*Pheretima posthuma*) castings were collected during rainy season in the month of August 2008 from different places of Doon valley, Uttarakhand, India. Four Samples were collected from each site e.g. forest, agricultural land and grassland (Table 1). The earthworm castings were carefully taken with spatula and kept in sterile polypropylene bags. The collected earthworm castings were taken to the laboratory for isolation of actinomycetes.

Measurement of pH

The pH of all the samples was determined by pH meter (Toshcon, Ajmer, India). Ten grams of each sample was suspended in 20 mL of distilled water and allowed to stand for 20 minutes with occasionally stirring to reach equilibrium. After being left to settle, the pH was measured.

Isolation of actinomycetes

Actinomycetes were isolated using both selective and non selective methods. For non selective isolation, 1.0 g of soil from each sample was dissolved in 10 mL sterile normal saline and serially diluted up to 10^{-3} and plated (1 mL) on yeast malt extract agar medium (yeast extract, 4.0 g/L; malt extract, 10.0 g/L; dextrose, 4.0 g/L; agar, 20 g/L (HiMedia, India), ISP-2 (37) containing cycloheximide (HiMedia, India) and nystatin (50 µg/mL each, (HiMedia, India) in order to minimize fungal contamination. The plates were incubated for 2 to 3 weeks at 27°C. Selective isolation was done by 3 methods i.e. (a) 1g of sample was pre-treated at 80-85°C for 1h and cooled down to room temperature. Preparation of serial dilution and plating was done according to method mentioned in non selective isolation. (b) 1 g of soil sample was dissolved in 10 mL sterile distilled water and serially diluted up to 10^{-3} and plated on Humic acid Vitamin (HV) agar (12, 13). (c) Soil sample was pre-treated at 120°C for 1 h serially diluted and plated on HV agar supplemented with nalidixic acid (14, 15).

Identification of actinomycetes

The appearance and growth of actinomycetes were observed everyday on their respective plates. The isolates, which showed good growth in 4 days, were considered as fast growers and those that showed good growth between 4 and 7 days were classified as moderate growers and the slow growers took more than 7 days for their growth. All the actinomycetes colonies were picked up, purified, sub cultured on ISP-2 slants and incubated at room 27°C for 3-4 weeks. The well sporulated cultures were preserved in 20% glycerol vials at -80 °C. All strains were characterized morphologically and physiologically according to the methods described in the International *Streptomyces* project (37) and Bergey's Manual of Systematic Bacteriology (25). The cover slip culture technique was used to study the morphological characteristics such as substrate and aerial mycelia, spores in chains, forms of chain i.e. rectiflexibilis, retinaculiperti, spirals etc. The cell wall diaminopimelic acid isomers and whole cell sugars were determined according to the method described in IMTECH Laboratory manual (17). Important isolates were identified on the basis of 16S rDNA sequence homology as described previously (24)

Screening of actinomycetes for their antimicrobial activity

All the actinomycete isolates were screened for antibacterial and antifungal activity by agar plug method (9). The actinomycete isolates were spread over the entire surface of ISP- 2 medium. As soon as the actinomycete isolates developed, agar discs were cut out by the cork borer (6 mm diameter). These plugs were transferred to the surface of Mueller Hinton agar plates seeded with the human bacterial pathogens (*Staphylococcus aureus* MTCC2940, *Bacillus subtilis* MTCC441, *Escherichia coli* MTCC739, *Pseudomonas aeruginosa* MTCC424) and on sabouraud dextrose agar plates seeded with human fungal pathogens (*Candida albicans* MTCC1637, *Microsporium canis* MTCC2820 and *Trichophyton rubrum* MTCC296, *Aspergillus fumigatus* MTCC3070) and plant pathogens (*Macrophomina phaseolina*,

Fusarium oxysporum and *Rhizoctonia solani*). The Petri dishes were then kept in incubator at 37°C for bacterial pathogens and 27°C for fungal pathogens at 24, 72 h respectively. If the antibiotic produced by the organism inhibits the growth of the test organism, a clear zone is formed round the discs. The plant pathogens were obtained from Department of microbiology, Sardar Bhagwan Singh P.G. Institute of biomedical sciences and research, Balawala, Dehradun. The experiments were conducted in triplicates and mean reported as results.

Screening of actinomycetes for their extracellular enzymes production

Lipolytic activity was detected using the method described by Sierra (38). Xylanase production was determined in basal salts agar medium containing oat spelt xylan (0.5%, HiMedia, India). The plates of basal medium salt with oat spelt xylan were inoculated with actinomycetes and incubated at 28°C for 1-2 weeks. The developed colonies were assayed for xylanase activity by Congo red staining method (5). Plates were flooded with Congo red (0.1%, HiMedia, India) for 15 min and then washed with 1M NaCl (HiMedia, India), the colonies showing halos around them were recorded positive for xylanase activity. Detection of extracellular cellulase production by actinomycetes was checked by way of plate assay (20). The production of caseinase gelatinase and amylase was determined according to standard methods (1).

RESULTS AND DISCUSSION

The history of new drug discovery processes shows that novel skeletons have, in the majority of cases, come from natural sources (4). This involves the screening of microorganisms and plant extracts, using a variety of models (34). Today, the emphasis is on the exploration of unusual and previously ignored ecosystems (6). In this scenario, it is important to screen actinomycetes from different unexplored habitats, which may prove to be the important sources of potent molecules. Among bacteria, the actinomycetes are the most important source of bioactive compounds and many clinically

relevant antibiotics in use today, and may continue to be so (3). This study was focused on earthworm castings because earthworms redistribute organic matter within the soil, increase soil permeability and increase microbial activity by their burrowing and feeding activities (40). Hence, an effort had been made to explore the actinomycetes from earthworm castings for their antagonistic activity and industrial enzymes. A total of 12 earthworm casting samples were collected from different locations of Doon valley, India (Table 1). The pH of the earthworm casting was ranged from 7.8-8.5, which is suitable for actinomycetes (41). A total of 48 actinomycetes were isolated from earthworm castings, out of which 28 (58.33%), 12 (25%) and 8 (16.66%) from samples of forest, grass land and agricultural land respectively (Table 1). Moreover, highest numbers of isolates i.e. 34 were recovered from non selective methods as compared to selective isolation methods. The heating of soil samples at 120 °C or 100 °C and

dry-heat in combination with chemical pretreatments, reduced the numbers of filamentous bacteria and streptomycetes on isolation plates, resulting in the selective isolation of various rare actinomycetes genera (12, 13, 32). All the isolated actinomycetes from castings were checked for growth rate on ISP-2 medium. It was observed that out of 48 isolates, 14 (29.16 %) were fast growers, 30 (62.5 %) exhibited moderate growth and 4 (8.33 %) were slow growers (Figure 1). All the isolates were also grouped on basis of colour series. Four main classes of colour were observed, often with colour intergrades seen within a class. The main colours were white (45.83 %), gray (35.41 %), red (6.25%), and yellow (4.16%) while 8.33% isolates were without any distinct colour (hyaline) (Figure 2). Occurrence of these colours has been documented previously (23, 37). The variations in the colours of the aerial mycelia of the isolates may be an indication contributing to diversity or variability of the isolated actinomycetes.

Table 1. Details of sampling site and isolation of actinomycetes from the Earthworm castings (*Pheretima posthuma*)

Location	Collection site	pH	No. of isolates
Dehradun	Forest	8.5	28
	Grassland	8.0	12
	Agricultural land	7.5	8
Total			48

Four samples were collected from each site

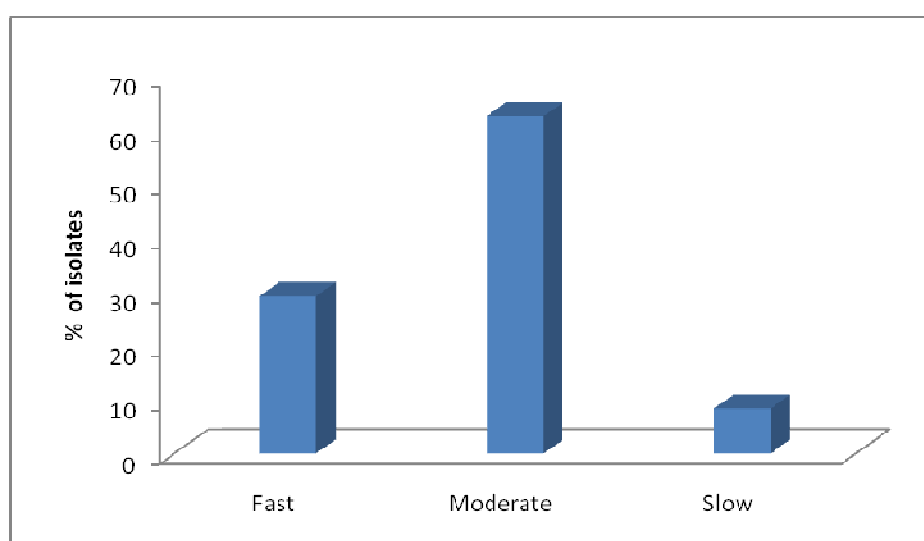


Figure 1. Growth characteristics of actinomycetes isolated from the earthworm castings

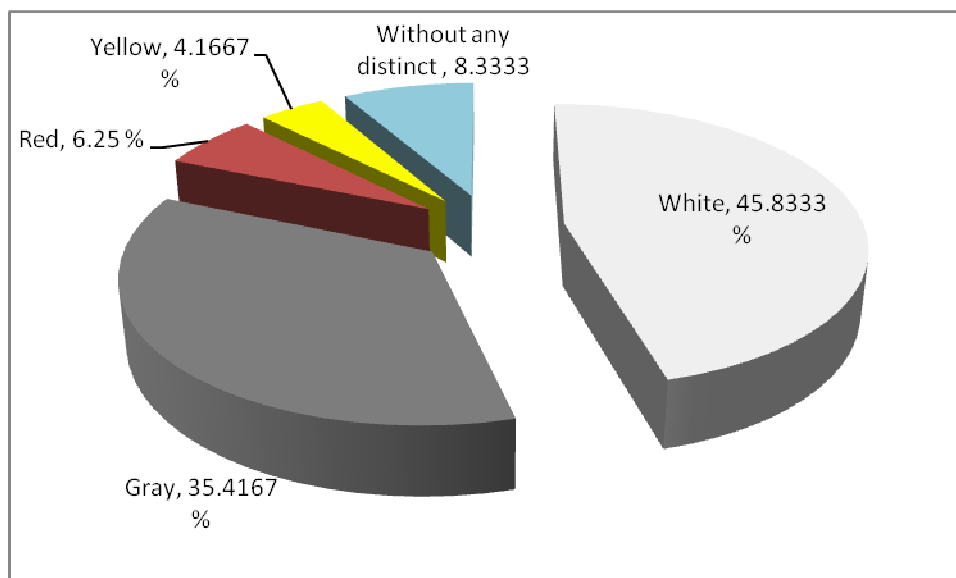


Figure 2. Mycelial coloration of actinomycetes isolated from earthworm castings

On the basis of cultural, morphological and physiological characteristics, the predominant genera were *Streptomyces* (60.41%, n=29) followed by *Streptosporangium* (10.41 %, n=5), *Saccharopolyspora* (6.25 %, n=3) and *Nocardia* (6.25 %, n=3). Besides these, other genera like *Micromonospora*, *Actinomadura*, *Planobispora* and *Nocardiopsis* were also isolated but in low frequency (Figure 3). One isolate did not bear any spore characteristics hence could not be identified. Similarly, *Streptomyces albus*, *S. somaliensis*, *Nocardia asteroides*, *N. caviae* and *Saccharomonospora* were isolated from gut and castings previously (33) however, Jayasinghe and Parkinson (18) reported only the species of *Streptomyces* from castings that were antagonistic to the common litter and wood decomposer fungi. Mba (27) reported 9 strains of *Streptosporangium* from earthworm castings having phosphate solubilizing activity but there was no detail of their antimicrobial activity.

The degree of antimicrobial activity varied greatly. Among the 48 isolates, 25 (52.08%) were found active against one or more test pathogens. The percentage of isolates that showed antibacterial and antifungal activities only were equal (28% each). However, 6 (24%) isolates were found active

against both bacteria and fungus (human), and 1 (4%) exhibited activity against bacteria as well as phytopathogens (Figure 4a). More interestingly 4 (16%) isolates showed activity against bacterial, human fungal pathogens as well as phytopathogens. The detailed antimicrobial activity of actinomycetes had been given in Table 2. During the study it was recorded that the antagonistic potential of *Streptomyces* was dominant (64%, n=16) as compare to *Streptosporangium* (12%, n=3), *Actinomadura* (8%, n=2), *Nocardia* (8%, n=2), *Saccharoployspora* (4%, n=1), *Nocardiopsis* (4%, n=1) and *Planobispora* (4%, n=1) (Figure 4b.). This is in accordance with the previous reports that the *Streptomyces* cover around 80% of the total antibiotic products as compared to other genera (21). However, in recent years antibiotics like Meroparamycin (10), Chromomycins (45), milbemycin (44), JBIR-52 a new antimycin like compound (22) have been reported from *Streptomyces* species. Likewise antibacterials, antifungal agents have a wide application in human medicine, agriculture and veterinary medicine (28, 43). As antibiotic producers or hyperparasites of fungi, actinomycetes have played an important role in controlling soil-borne plant pathogens. The antagonistic impact of actinomycete species on

pathogenic fungi is well known and a few species have been used as biological control agents (26, 39, 46). Isolate EWC 7 (2) was found to be most prominent in the terms of desirable activities. It was found to have excellent antimicrobial potential against several bacterial and fungal pathogens. EWC 7(2) was identified as *Streptomyces rochei* (99%) on the basis of phylogenetic analysis of 16S rDNA sequences (GQ340692).

In recent years enzymes gained considerable attention in industrial process and replaced the chemical catalysts in various pharmaceuticals, textiles, paper, food industries etc. However, from terrestrial soil samples various enzymes have been reported by various workers (35). In this study we have also explored the actinomycete producing extracellular enzymes. During the study it was recorded that out of 48 isolates 38 (79.16%), 32 (66.66%), 21 (43.75%), 20 (41.66%), 16 (33.33%) and 14 (29.16%) produced enzyme amylase, caseinase cellulase, gelatinase, xylanase and lipase respectively (Figure 5). Similarly, Mba (27) isolated *Streptosporangium* from earthworm castings that produced cellulases. It was found that a total of 10 isolates (EWC 2, EWC 5, EWC 8, EWC 18, EWC 22, EWC 30, EWC 41, EWC 44, EWC45 and EWC 47) produced all the five enzymes. More interestingly, the isolates

such as *Streptosporangium* sp. EWC 5, *Streptomyces* sp. EWC 18 produced amylase, *Planobispora* sp. EWC 22, *Actinomadura* EWC 41, and *Streptomyces* sp. EWC 7(2) produced xylanase while EWC 7(2) showed lipolytic activity at 45 °C. The enzyme activity was experimented at high temperature because thermo tolerant enzymes are required for industrial purposes. The majority of actinomycetes isolates from earthworm castings produced amylase followed by caseinase cellulase, gelatinase, xylanase and lipase. Majority of enzymes were produced by the genera *Streptomyces* supporting the previous reports (2, 19). Likewise, an alkaline protease was reported from a salt- tolerant and alkaliphilic, *Streptomyces clavuligerus* strain MIT-1(42).

Actinomycetes from earthworm castings produced various metabolically active compounds and industrially important enzymes, hence reflecting its importance in view of its economic value. The study revealed that earthworm castings are potential source for a wide spectrum of antimicrobial and industrial enzyme producing actinomycetes. Moreover, it can be an imperative resource for bio prospecting novel/ rare *Streptomyces* spp., which could yield valuable bioactive molecules.

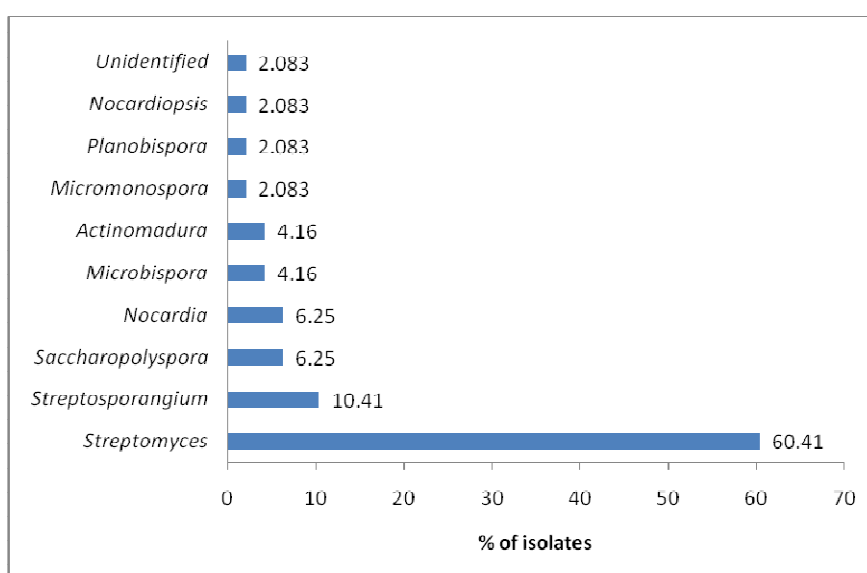


Figure 3. Distribution of actinomycetes in earthworm casting (Identified using cultural, morphological and physiological characteristics)

Isolates	Genera	Bacterial test organisms (IZD)*				Human fungal pathogens (IZD)*				Phytopathogens (IZD)*		
		SA	BS	EC	PS	CA	TR	MC	AF	MP	FO	RS
EWC 1	<i>Streptomyces</i>	15.6 ± 1.24	17.6 ± 0.47	12.6 ± 0.94	10.3 ± 0.47	14.0 ± 1.63	-	-	9.6 ± 0.47	-	-	-
EWC2	<i>Streptomyces</i>	19.0 ± 0.81	20.3 ± 1.24	9.0 ± 0.81	9.3 ± 0.47	-	-	-	-	17.6 ± 0.47	19.33 ± 0.94	17.3 ± 0.47
EWC 3	<i>Streptomyces</i>	16.6 ± 1.69	-	-	-	-	-	-	-	-	-	-
EWC 4	<i>Micromonospora</i>	-	-	-	-	20.0 ± 1.41	-	-	-	13.3 ± 0.47	16.3 ± 1.24	15.6 ± 1.69
EWC 5	<i>Streptosporangium</i>	26.6 ± 1.24	-	-	-	-	-	-	-	-	-	-
EWC 6	<i>Nocardia</i>	-	-	-	-	-	-	-	10.0 ± 0.00	-	-	-
EWC 7(2)	<i>Streptomyces</i>	25.0 ± 2.00	26.0 ± 0.81	24.0 ± 0.58	17.0 ± 0.58	20.0 ± 0.47	19.0 ± 0.81	18.3 ± 0.47	-	29.6 ± 0.47	21.3 ± 0.94	24.3 ± 0.94
EWC 8	<i>Streptomyces</i>	20.0 ± 0.81	16.3 ± 0.94	10.3 ± 0.47	9.6 ± 0.47	-	-	-	-	-	-	-
EWC 9	<i>Nocardiosis</i>	14.6 ± 0.94	-	-	-	-	-	-	-	-	-	-
EWC 11	<i>Streptomyces</i>	25.0 ± 1.63	20.6 ± 0.94	-	-	-	-	15.6 ± 0.94	-	-	-	-
EWC 12	<i>Streptomyces</i>	-	-	-	-	-	21.0 ± 0.81	-	-	-	-	-
EWC 14	<i>Nocardia</i>	19.33 ± 0.47	-	-	-	-	15.3 ± 0.47	-	-	-	12.0 ± 0.00	-
EWC15	<i>Streptomyces</i>	-	-	-	-	-	-	-	-	-	-	17.0 ± 1.87
EWC 18	<i>Streptomyces</i>	27.6 ± 0.94	23.0 ± 0.00	-	-	-	10.6 ± 0.47	-	-	-	10.00 ± 0.00	-
EWC 19	<i>Streptomyces</i>	13.6 ± 1.24	16.0 ± 1.41	-	-	17.6 ± 0.124	-	-	-	-	-	20.1 ± 0.62
EWC 20	<i>Streptomyces</i>	17.3 ± 1.24	-	-	-	-	15.6 ± 1.05	-	-	-	-	-
EWC 22	<i>Planobispora</i>	-	-	-	12.6 ± 0.94	-	16.0 ± 0.00	-	-	-	-	-
EWC 24	<i>Saccharopolyspora</i>	-	-	-	14.0 ± 0.81	-	-	-	-	-	-	-
EWC 30	<i>Streptomyces</i>	-	-	-	-	19.3 ± 1.02	-	-	-	-	-	-
EWC 41	<i>Actinomadura</i>	-	-	17.0 ± 1.41	-	-	-	-	-	-	-	-
EWC43	<i>Streptomyces</i>	-	-	17.3 ± 0.47	-	18.5 ± 0.70	-	-	-	-	-	-
EWC44	<i>Streptomyces</i>	-	-	-	-	15.0 ± 0.00	-	9.8 ± 1.54	-	-	-	-
EWC 45	<i>Streptomyces</i>	-	18.0 ± 0.81	-	-	-	-	-	-	-	-	-
EWC46	<i>Streptosporangium</i>	-	-	-	-	-	17.6 ± 0.62	-	-	-	-	-
EWC 47	<i>Streptomyces</i>	-	-	11.3 ± 1.24	-	-	-	11.1 ± 1.02	-	-	-	-

SA, *S. aureus*; BS, *B. subtilis*; EC, *E. coli*; PS, *P. aeruginosa*; CA, *C. albicans*; TR, *T. rubrum*; MC, *M. canis*; AF, *A. fumigatus*; MP, *M. phaseolina*; FO, *F. oxysporum*; RS, *R. solanii*; +, activity present; -, activity absent; IZD, inhibition zone diameter

*The values are average of triplicates ± standard deviation

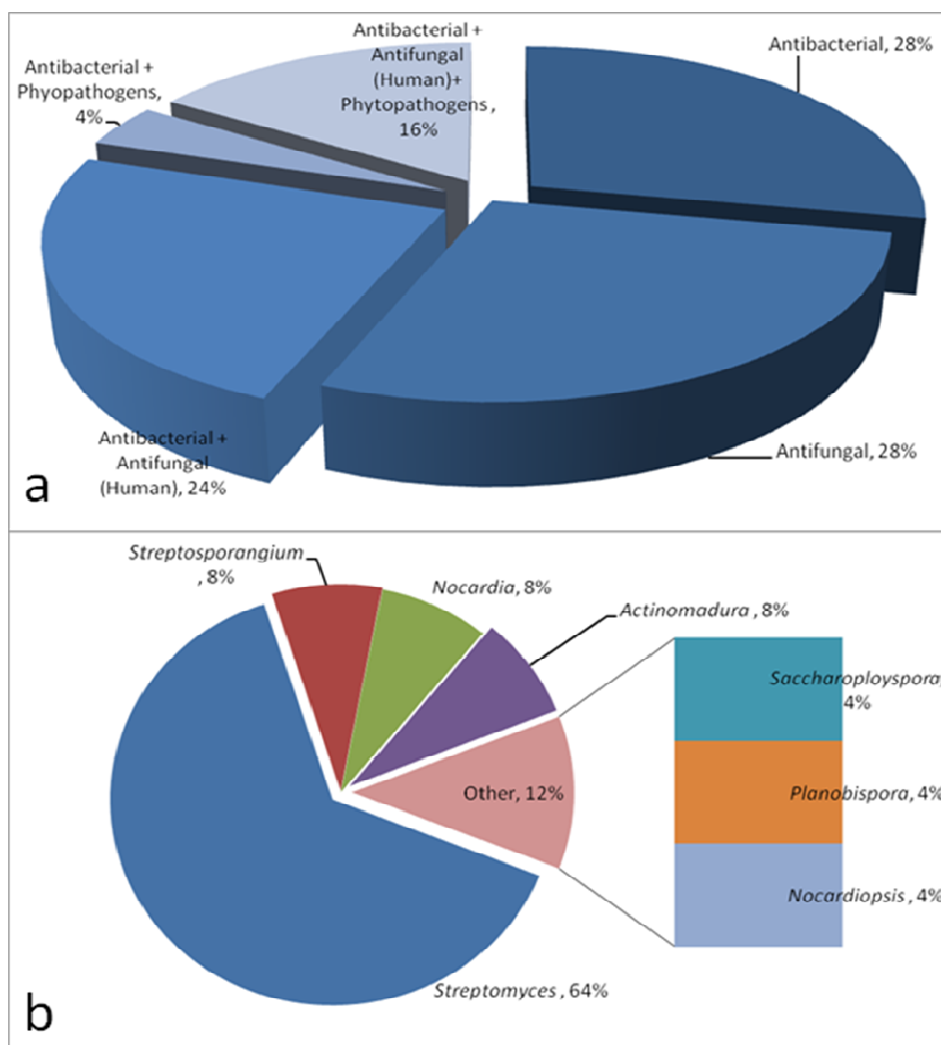


Figure 4. (a) Antimicrobial profile of actinomycetes isolated from earthworm casting (b) Antagonistic potential of genus *Streptomyces* as compared to other genera

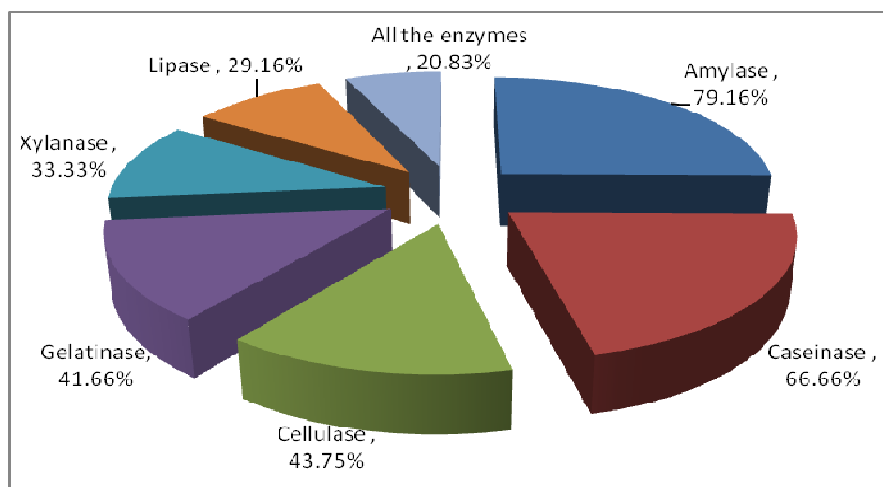


Figure 5. Extracellular enzymes production in actinomycetes isolated from earthworm castings (The data is not mutually exclusive)

ACKNOWLEDGEMENTS

Authors are thankful to Uttarakhand State Council for Science and Technology (UCOST), Government of Uttarakhand, India, for providing financial support (File No. UCS &T/R&D/LS/06-07/1158).

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