MOLECULAR CHARACTERIZATION AND ANTIBACTERIAL ACTIVITY OF ACTINOMYCETES FROM EARTHWORM GUT (*Eisenia foetida* - Savingny, 1826)

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Abstract

Earthworms are natural invertebrates of agro ecosystem belonging to the Phylum Annelida, Class – Chaetopoda and Order Oligochaeta and dominant in the temperate and tropical soils. They are the first group of multicellular eucoelomate invertebrates to have succeeded to inhabit terrestrial environment. *Eisenia foetida* worms are used for vermicomposting. They are epigeic earthworms. The earthworm guts may be considered as favorable habitats for bacteria, because several studies showed increased microbial numbers in the guts versus the soil, in which earthworms were living. The earthworm gut microbial community is qualitatively not much different from the microbial community in the surrounding soil. The molecular characteristics of actinomycetes were carried out by PCR amplification of 16S rRNA gene sequencing. The D12 actinomycetes culture were highly zone formation and antibacterial activity of uropathogens bacteria. So further DNA sequencing using this culture. The sequences of the D12 isolates were compared with sequences of already existing species of *Streptomycetes* from EMBL database to determine the phylogenetic relatedness using Neighbor joining tree method. It was revealed that, the finally resulted the species name *Streptomyces fulvissimus.*

Keywords: Actinomycetes, Earthworm gut, Antibacterial activity, Uropathogens, *Streptomyces fulvissimus*

I. Introduction

Earthworms are hermaphrodite, meaning each individual possesses both male and female reproductive organs. (Sherman, 2003) *Eisenia foetida* known under various common names such as redworm, brandling worm, panfish worm, trout worm, tiger worm, red wiggler worm, red californian earthworm, etc., is a species of earthworm adapted to decaying organic material. These worms thrive in rotting vegetation, compost, and manure. They are epigeic earthworms. *Eisenia foetida* worms are used for vermicomposting. Earthworm's varieties influence this decomposition by enhancing the structure and dynamics of the microbial population inside their gut as an efficient bioreactor (Shankar *et al.,* 2011).

Earthworms prime the symbiotic gut microflora with secreted mucus and water to increase their degradation of ingested organic matter and the release of assimilable metabolites (Pramanik *et al.,* 2007). Thus the micro-organisms and earthworms act symbiotically to accelerate and enhance the decomposition of organic matter and as a consequence, mineralization and humification takes place resulting in the availability of nutrients for plants (Lee, 1985; Edwards and Bohlen, 1996; Chaioui *et al.,* 2003).

The actinomycetes (sing. actinomycete) are a large group of gram-positive bacteria, high G-C percentage that form branching filaments or hyphae and asexual spores. These bacteria closely resemble fungi in overall morphology. Presumably this resemblance results partly from adaptation to the same

habitat. Studies of the fine structure of actinomycetes spores during germination have been confined to the genera *Streptomyces* (Kalakoutswl and Agre, 1973).

Actinomycetes possess many properties that make them good organisms for application in bioremediation of soils contaminated with organic pollutants. They play an important role in the recycling of organic carbon and are able to degrade complex polymers (Goodfellow and Williams, 1983). Some reports indicated that *Streptomyces flora* could play a very important role in degradation of hydrocarbons (Radwan *et al.,* 1998, Barabas *et al.,* 2001). In some contaminated sites Actinomycetes represent the dominant group among the degraders (Johnsen *et al.,* 2002). Actinomycetes are a vital cluster of microorganisms, not only as degraders of organic matter within the natural environment, but also as producers of antibiotics and other valuable compounds of commercial interest (Saugar *et al.,* 2002; Bentley *et al.,* 2002 and Basilio *et al.,* 2003).

In the *Streptomyces* species studied, the spores had a two-layered wall and the inner one extended to form the germ-tube wall. It is not clear if this layer is newly synthesized during germination or if it is formed by reorganization of wall material existing in the dormant spore. Ultra structural changes during the germination of fungal spores have been studied more extensively (Bartnicki-Garcsi, 1968).

Molecular science, which has each classification and identification, has its origin within the early super molecule crossbreeding studies, however has achieved a new standing following the introduction of super molecule sequencing techniques (Donnell *et al.,* 1993). Importance of phylogenitic studies supported 16S rRNA sequences is increasing within the science of bacterium and actinomycetes (Yokota, 1997). Sequences of 16S rRNA have provided actinomycetologists with a phylogenic tree that enables the investigation of evolution of actinomycetes and conjointly provides the premise for identification.

Analysis of the 16S rRNA begins by analytic DNA (Hapwood *et al.,* 1985) and amplifying the gene coding for 16S rRNA exploitation the enzyme chain reaction (Sivakumar, 2001). The refined DNA fragments are directly sequenced. The sequencing reactions are performed exploitation DNA sequence so as to work out the order during which the bases are organized at intervals the length of sample (Xu *et al.,* 1999) and a computer is then used for finding out the sequence for identification exploitation phyletics analysis procedures. Though, analysis of 16S rRNA generally allows us to identify the organism up to the genus level only.

II. Materials and methods

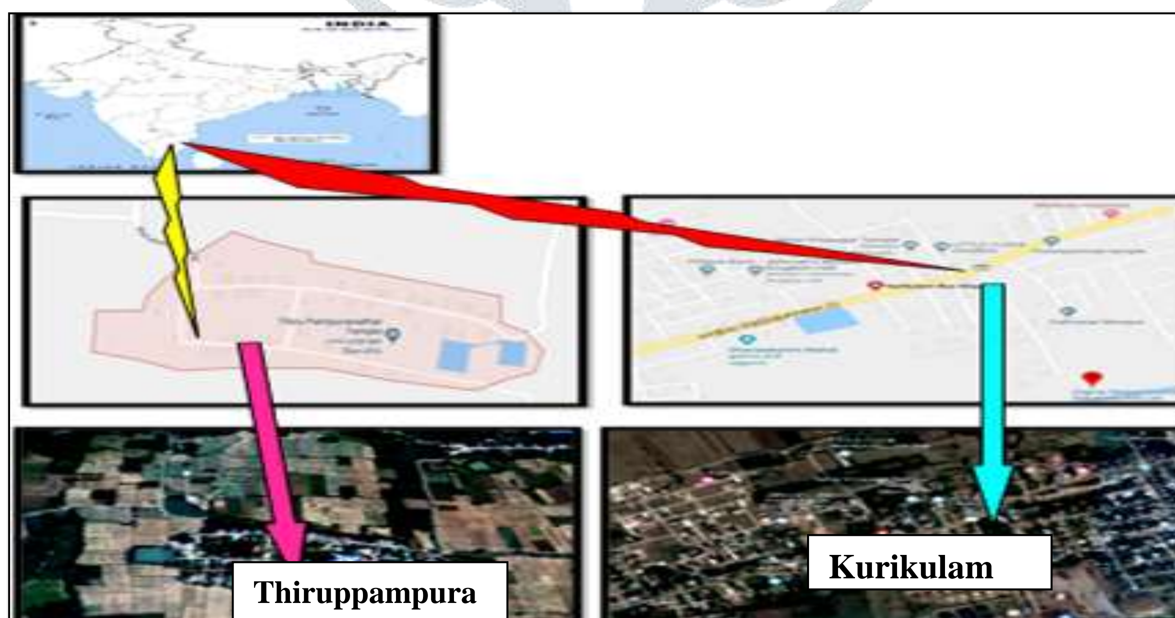

Figure 1. MAP SHOWING THE STUDY AREA THIRUPPAMPURAM VILLAGE AND KURIKULAM DUMPING SITE

2.1. Collection of sample

The samples were collected from two different sites in paddy field Thiruppampuram Village, Thiruvarur district, and Municipal Solid Waste dumping site at Kurikulam, Kumbakonam Thanjavur District, Tamil Nadu, and India. The samples were collected digging and hand-sorted method as per the techniques of Edwards and Lofty (1977). Collected worms were washed in freshwater. The earthworms and soil were stored in perforated polythene bags and were brought to the laboratory for their further studies.

2.2. Isolation of actinomycetes from earthworm (*Eisenia foetida*) gut and soil

A healthy sexually mature, clitellated worm was taken, washed with tap water and were then cleaned externally with 70% ethanol. Sterile dissecting pins were used to hold the earthworm down on a board and an incision was made longitudinally along the earthworm. The gut was then freed from surrounding blood vessels and nephridia and separated into gut sections. The gut sections were washed in sterile distilled, weighed and homogenized for 5 minutes with a pistal mortal in sterile 0.85% of (0.85g NaCl mixed with 100 ml distilled water to prepare 0.85% sterile saline) NaCl solution. The gut and soil homogenate was serially diluted (10^{-1}, 10^{-7}) and settle for 1 hour and plated on to (Petri plates were cleaned methanol and sterilized in autoclave with 20 minutes in 120 psi). The isolation of actinomycetes from starch casein agar was supplemented with amphotericin B, and streptomycin of each 0.25µg/250 ml medium to inhibit the normal bacterial and fungal flora. The plates were incubated at 23^0C / 28^0C and observed for 7 days for the growth and sporulation of actinomycetes.

2.3. Identification of Actinomycetes

Pin-point powdery like structure colonies which are the characteristic of actinomycetes with clear zone of inhibition around it were observed in the plates. The pinpoint colonies with inhibitory or clear zone of inhibition were selected and the suspected colonies were selectively isolated and transferred to Starch Casein Agar medium with the help of loop inoculation in streak plate method (Hamaki and Suzuki *et al.,* 2005).The plates were kept for incubation at 25 to 30^0 C for 7-14 days in bacteriological incubator an inverted position. The actinomycetes isolates were purified by pure culture techniques. The colonies were refrigerated in Starch Casein Agar slants by frequent sub culture for further studies.

To characterize the taxonomic position of the selected earthworm gut actinomycetes isolate, a range of tests were carried for morphological, physiological, biochemical and molecular identification out according to the guidelines of Bergey Manual of systematic bacteriological (Locci, 1989), *International Streptomyces Project* (Shirling and Gottlieb, 1966) and Williams *et al.,* (1983).

2.4. Molecular identification

DNA preparation method: Growth from mature slant culture of the actinomycetes were inoculated aseptically into 250 ml Erlenmeyer flasks each containing 50 ml of Starch Casein Agar medium and incubated in a rotary shaker at 30°C for 3-5 days at 180 rpm. Cultures were centrifuged at 14,000 rpm for 20 minutes. The 0.1 g of mycelium was transferred into sterile porcelain dish and crushed with liquid nitrogen. The crushed mycelium was transferred into fresh tube containing 500µl of TE buffer supplemented with lysozyme (20mg/ml). The tube was incubated at 37°C for 30 minutes.

Added 20µl of 10% SDS (w/v) and 20µl of proteinase K into the tube and incubated at 55°C for 30 minutes. The lysate was cooled down and extracted once with equal volume of phenol: chloroform solution (v/v, 1:1) at 10,000rpm for 5 minutes. The aqueous phase was transferred carefully to a fresh tube and DNA was precipitated by adding 70-90% ethanol and keeping at -20°C for 30 minutes. The pellet was formed by centrifuging at 10,000 rpm for 10 minutes.

The pellet was washed twice with 90% ethanol and dissolved the pellet in TE buffer. To obtain RNA free DNA added 20µl of RNA ase solution (20µg/ml) and then incubated at 37°C for 1hr. The sample was once again extracted with equal volume of phenol: chloroform and precipitated as above. The purity and concentration was checked in Bio photometer PCR amplification, sequencing and restriction analysis PCR amplification of the 16S rRNA of the *Streptomyces* sp. was performed using two primers: 8F (5'AGAGTTTGATCCTGGCTCAG3') and 1541R (5'AAGGAGGTGATCCAGCCGCA3'). The final volume of reaction mixture of 25µl contained Taq DNA polymerase is supplied in 2X Taq buffer, 3.2mM of MgCl2, 0.4µM of each dNTP, 0.02% Bromophenol blue, 20 pico-moles of each primer, and 2.5U of Taq DNA polymerase and 100mg of template DNA.

The amplification was performed on Eppendorf Thermo-cycler 96, according to the following profile: an initial denaturation step at 95°C for 2 min. followed by 30 amplification cycles of 95°C for 1 min, annealing 55°C for 1 min, and a final extension step of 72°C for 2 min. The PCR product was detected by Agarose gel electrophoresis and was visualized by ultraviolet (UV) fluorescence after Ethidium Bromide staining. The restriction digestion of 16S rRNA was performed according to the method described previously. Prior to sequencing, amplified products were purified using a HiPurATM PCR product purification spin kit according to the manufacturer s instructions. Sequencing reactions of PCR products were perform with the ABI PRISM® BigDyeTM Terminator version 3.1 Cycle Sequencing Kit according to the manufacturer instructions using 1541R primer.

Single-pass sequencing was performed on each template using below 16s rRNA universal primers. The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were resuspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer.

III. Result and Discussion

Earthworm guts may be considered as favorable habitats for bacteria, because several studies showed increased microbial numbers in the guts versus the soil, in which earthworms were living (Bassalik, 1913; Parle, 1963; Pedersen *et al.,* 1993; Schonholzer *et al.,* 1999; Wolter *et al*., 1999). In contrast, an increase in microbial biomass by the gut passage was not always observed (Devliegher *et al*., 1995; Daniel *et al*., 1992, Scheu, 1987). While some early studies proposed that the earthworm gut microbial community is qualitatively not much different from the microbial community in the surrounding soil (Bassalik, 1913; Parle, 1963).

A total of 22 isolates were isolated from earthworm gut and soil samples. The numbers of samples and isolates in each sample were presented in Table 1, 2. Twenty two isolates exhibiting typical features of actinomycetes were selected for further study and maintained on slants of respective media. It was found that SCA (30%) and AIA (30%) were the ideal media for isolation of maximum number of actinomycete isolates from the soil and earthworm gut samples followed by AGS (18%), KMM (11%) and CA (11%). The twenty two isolated actinomycetes were further used in antibacterial activity.

Figure 2. *Eisenia foetida*

Aerial mycelium Substrate mycelium

Figure 3. D12 – CULTURE PLATE OF ACTINOMYCETES SPECIES

Table 1 - TOTAL NUMBER OF ISOLATES ACTIVE AGAINST SENSITIVE STRAINS DURING PRIMARY SCREENING

Sl. No	Sample source	Number of isolates screened	Number of isolates active
1	Paddy soil – Thiruppampuram Village	6	1
2	Paddy soil – Earthworm Gut	8	2
3	Dumping site soil – Kurikulam, Kumbakonam	3	-
4	Dumping site – Earthworm Gut	5	1
Total		22	4

Table 2 - COLONY CHARACTERISTICS OF ACTINOMYCETES ISOLATES

Sl. No.	No. of isolates	Sample source	Growth	Sporulation	*Aerial mycelium colour*	Substrate mycelium colour	Diffusible pigment	Melanoid Pigment
1	D1	P S	Good	Poor	White Yellow	Yellow	-	-
2	D2		Good	moderate	Yellowish	Yellow	-	-
3	D3		Good	Good	White Yellow	White Yellow	-	-
4	D4		Good	Good	Blackish grey	Pale Yellow	-	-
5	D5		Good	Good	Grey	Brown	-	-
6	D6		moderate	Poor	Grey	Pale Yellow	-	-
7	D7	P S E G	Good	Good	Brownish	Brown	-	Dark brown
8	D8		Good	Good	Rough White	White	-	-
9	D9		Good	Good	Blue	Blue	-	-
10	D10		Poor	Poor	Green	Green	-	-
11	D11		Good	moderate	Jelly Yellow	Light Yellow	-	-
12	D12		Good	Good	Yellow White	Yellow	-	-
13	D13		Good	Good	Whitish grey	Brown	-	Brown
14	D14		Good	Good	White	Pale Yellow	-	-
15	D15	D S E G	Good	moderate	White	White	-	-
16	D16		Good	Good	Grey	Brown	-	Brown
17	D17		Good	Poor	Grayish brown	Brown	-	-
18	D18		Good	Good	White	Pale Yellow	-	-
19	D19		Good	Poor	Grey	Dark Brown	-	Reddish
20	D20	D S	Good	Good	Grey	Grey Black	Black	-
21	D21		Good	Poor	Yellow	Yellow	-	-
22	D22		Good	Good	Grey	Black	Black	-

* **PS** – Paddy Soil, **PSEG** - Paddy Soil Earthworm Gut, **DSEG** – Dumping Site Earthworm Gut, **DS** – Dumping Site Soil

3.1. Primary Screening

Results of screening for antibacterial activity by cross streak method. Nine actinomycete isolates inhibited gram positive bacteria *S. aureus.* In case of gram negative bacteria, *V. cholera* were inhibited 6 actinomycetes isolates and *S. bodyii* were inhibited by 8 isolates and 10 isolates were found to be antagonistic to *K. pneumoniae,* 12 isolates were inhibited in *E. coli* and *P. vulgaris* was inhibited by 11 isolates. Among all tested bacteria, were found to be more inhibited by 4 (D7, D12, D14, D15) number of isolates.

3.2. Secondary Screening

The four antagonistic activity actinomycetes cell supernatant culture filtrates were subjected to secondary screening by using well diffusion method and exhibited significant inhibitory effect of tested bacteria. The potent actinomycetes were characterized by morphological and biochemical methods. Four isolates showed activities against the all tested bacteria. Actinomycetes (isolate D12) had maximum activity against the all tested bacteria in comparison with others based on larger zone of inhibition and were selected molecular identification.7

Table 3 - ANTIBACTERIAL ACTIVITIES OF ACTINOMYCETES BIOACTIVE COMPOUND IN ZONE FORMATION (CM)

Sl.No	Bacterial Pathogens	Zone of inhibition (cm)			
		D7	D12	D14	D15
1.	*Staphylococcus aureus*	0.9	2.5	0.8	1.2
2.	*Escherichia coli*	1.4	2.2	1.3	0.8
3.	*Klepsella pnemoniae*	1.7	2.0	2.1	1.6
4.	*Protease vulgaris*	1.9	2.7	1.1	1.7
5.	*Vibrio cholarae*	0.7	1.7	1.4	0.9
6.	*Shigella bodyii*	1.2	2.8	1.2	2.0

Figure 4. SECONDARY ANTIBACTERIAL ACTIVITY

3.3. Molecular identification

Current identification and classification of actinomycetes are based a polyphasic approach, comprising morphological, physiological and molecular studies (Goodfellow *et al.*, 2012) based on each taxon should be described and differentiated from related taxa. The sequencing of highly conserved macromolecules, notably 16S rRNA genes, has provided valuable data for constructing phylogenies and above the genus level (Ludwig and Klenk, 2005). The DNA: DNA relatedness, molecular fingerprinting and phenotypic techniques are methods of choice for delineating taxa and below the rank of species (Rossello-Mora and Amann, 2001). Distinguishing phenotypic differences are required for the description of a new species (Wayne *et al.,* 1987). Exploring the diversity of endophytic actinobacteria is indispensable for screening of beneficial strains and understanding their ecological niche.

Sequence obtained after Sequencing of 16S rRNA gene

Streptomyces fulvissimus **(Paddy Soil Earthworm Gut Actinomycetes) 16S rRNA**

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>contigD12
GATCGCAAGACCTTGCACTATTAGAGCGGCCGATATCGGATTAGCTAGTTGGTGGGGT
AACGGCTCACCAAGGCGACGATCCGTAGCTGGTTTGAGAGGACGACCAGCCACACTG
GGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGACAAT
GGGGGAAACCCTGATCCAGCCATCCCGCGTGTGCGATGAAGGCCTTCGGGTTGTAAAG
CACTTTTGGCAGGAAAGAAACGTTCCGGGTTAATACCCCGGGAAACTGACGGTACCTG
CAGAATAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCAAG
CGTTAATCGGAATTACTGGGCGTAAAGCGTGCGCAGGCGGTTCGGAAAGAAAGATGT
GAAATCCCAGAGCTTAACTTTGGAACTGCATTTTTAACTACCGAGCTAGAGTGTGTCA
GAGGGAGGTGGAATTCCGCGTGTAGCAGTGAAATGCGTAGATATGCGGAGGAACACC
GATGGCGAAGGCAGCCTCCTGGGATAACACTGACGCTCATGCACGAAAGCGTGGGGA
GCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGTCAACTAGCTGTTG
GGGCCTTCGGGCCTTGGTAGCGCAGCTAACGCGTGAAGTTGACCGCCTGGGGAGTAC
GGTCGCAAGATTAAAACTCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGATGAT
GTGGATTAATTCGATGCAACGCGAAAAACCTTACCTACCCTTGACATGTCTGGAATGC
CGAAGAGATTTGGTAGTGCTCGCAAGAGAACCGGAACACAGGTGCTGCATGGCTGTC
GTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCAT
TAGTTGCTACGAAAGGGCACTCTAATGAGACTGCCGGTGACAAACCGGAGGAAGGTG
GGGATGACGTCAAGTCCTCATGGCCCTTATGGGTAGGGCTTCACACGTCATACAATGG
TCGGGACAGAGGGTCGCCAACCCGCGAGGGGGAGCCAATCCCAGAAACCCGATCGTA
GTCCGGATCGCAGTCTGCAACTCGACTGCGTGAAGTCGGAATCGCTAGTAATCGCGGA
TCAGCATGTCGCGGTGAATACGTTCCCGGGTCTTGTACACACCGCCCGTCACACCATG
GGAGTGGGTTTTACCAGAAGTAGTTAGCCTAACCGCAAGGAGGGCGATTACCACGGTA GGATTC
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The molecular characteristics of actinomycetes were carried out by PCR amplification of 16S rRNA gene and their sequencing (Plate D12). The D12 actinomycetes culture were highly zone formation and antibacterial activity of uropathogens bacteria. So further DNA sequencing using this culture. The sequence of D12 isolates were compared with sequences of already existing species of *Streptomycetes* from EMBL database to determine the phylogenetic relatedness using Neighbor joining tree method. It was revealed that, the finally resulted the species was concluding in *Streptomyces fulvissimus.*

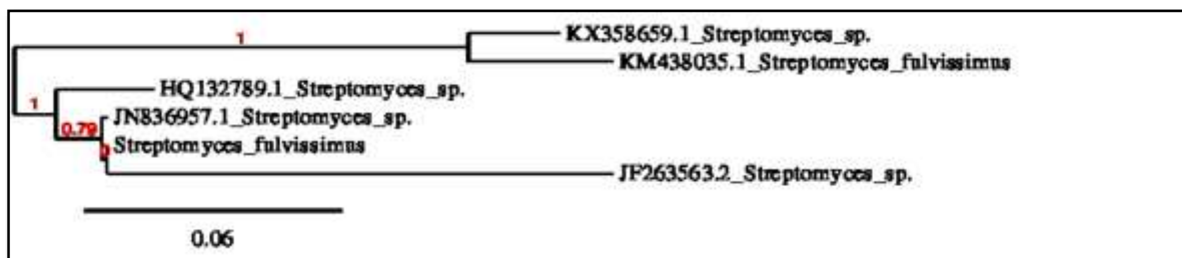

Figure 5. NEIGHBOR – JOINING PHYLOGENETIC TREE OF *Streptomyces fulvissimus* SPECIES BASED ON THE 16S rRNA GENE SEQUENCE DATA. THE FIGURES ON THE NODES INDICATE THE BOOTSTRAP VALUES AND SCALE INDICATES THE GENETIC DISTANCE

Based on the BLAST analysis,>99% similarity and 100% sequence coverage with *Streptomyces fulvissimus* (KM438035.1) in the Gen bank nucleotide database, the present studied species was confirmed as *Streptomyces fulvissimus.* The genetic distance was very high between the ancestors as seen in Fig. 5. At same time, minimal genetic distance was observed between the individuals of *Streptomyces fulvissimus.* From all the molecular and phylogenetic analyses it was confirmed that the present studied species belonging to *Streptomyces fulvissimus.*

The most important considerations for the isolation of new actinomycetes from the earthworm gut. It is anticipated that isolation, molecular characterization and study of actinomycetes can be useful in the discovery of novel species of actinomycetes. Actinomycetes are the most important resources for the secondary metabolites. Recent advances of molecular genetics in this genus have enabled us to elucidate not only the organization of biosynthetic genes for their secondary metabolites but also regulatory mechanisms closely linked to the cellular differentiation processes.

The present study concluded DNA result reported that the paddy field earthworm gut actinomycetes were *Streptomyces fulvissimus* are produced biologically active compounds when compare with the dumping site earthworm gut has the better activity and it has been observed that numerous numbers of colonies has been seen.

The screening and isolation of promising strains of actinomycetes with antibacterial properties which are relatively poorly investigated has increased the interest of researchers in both basic and applied fields. Clearly, more research on the formulation, development of novel technologies and methodologies is needed for employing them in the agricultural, medical and pharmaceutical fields. An extensive characterization and identification of the diverse population of actinomycetes associated with paddy field earthworm gut may also provide greater insight into the interaction and evolution of mutualism. It is also important to understand the mechanism that enables these *Streptomyces fulvissimus* species to interact with their antibacterial activity may be of biotechnological potential.

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