

ISOLATION AND PARTIAL CHARACTERIZATION OF ANTAGONISTIC ACTINOMYCETES FROM EARTHWORM GUT

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ABSTRACT

Nowadays, the drug resistant strains of pathogen emerge more quickly than the rate of discovery of new antibiotics. Because of this, many scientists and pharmaceutical industry have actively involved in the isolation and screening of actinomycetes from different untouched habitats, for the production of antibiotics. Earthworms are precious and low cost source of numerous bioactive molecules, which could find place in human and veterinary medicine. The main objective of the study was to isolate and screen for antagonistic actinomycetes from an earthworm gut. Out of six actinomycetes isolated from the earthworm gut, one isolate possessed high broad spectrum antibacterial activity in cross streak method and was selected for further studies. It was inoculated into the fermentation medium and by solvent extraction procedure using Ethyl acetate the metabolite was extracted to its full

form. The extract was highly active against selected bacterial pathogens used in the test and was further analyzed by thin layer chromatography. The UV spectra of the ethyl acetate extract for the active isolate showed an absorbance peak at 316 nm. The isolated strain was characterized for physiological and biochemical properties, nutritional uptake and the isolate fitted the *Streptomyces* genus. Based on the results of our study it was confirmed that *Streptomyces* isolated from earthworm gut was found to be a promising source for extracting novel antibiotics for treating bacterial infections in human.

KEYWORDS: Actinomycetes, antibiotics, antibacterial activity, cross streak method, thin layer chromatography, *Streptomyces*.

INTRODUCTION

Microbial natural products are notable not only for their potent therapeutic activities but also for the fact that they frequently possess the desirable pharmacokinetic properties required for clinical development ^[1]. Actinomycetes are the most widely distributed group of microorganisms in nature which primarily inhabit the soil ^[2]. Actinomycetes are gram positive bacteria frequently filamentous and sporulating with DNA rich in G+C from 55-75% ^[3]. They have provided many important bioactive compounds of high commercial value and continue to be routinely screened for new bioactive compounds ^[4]. Actinomycetes are the most biotechnologically valuable prokaryotes responsible for the production of about half of the discovered bioactive secondary metabolites including antibiotics ^[5]. Among actinomycetes, around 7,600 compounds are produced by *Streptomyces* species ^[6].

The present scenario demands the discovery of new antimicrobial agents which can be of a better potential and activity than the previously discovered agents. Resistance of bacteria to the effects of antibiotics has been a major problem in the treatment of diseases. Infectious diseases are still the second leading cause of death worldwide ^[7]. Nowadays, the drug resistant strains of pathogen emerge more quickly than the rate of discovery of new drugs and antibiotics. Because of this, many scientists and pharmaceutical industry have actively involved in isolation and screening of actinomycetes from different untouched habitats, for their production of antibiotics ^[2]. At present the literature contains sufficient evidence of the presence of actinomycetes in the gut of soil invertebrates (in particular earthworms) ^[8]. Many scientists have studied the microbial community in the gut of earthworms ^[9]. Mostly the population density of actinomycetes in the intestinal tract of invertebrates was studied by ^[10]. In the present study we focused on the isolation of earthworm (*Eudrilus eugeniae*) gut flora and partial characterization of Actinomycetes exhibiting broad range of Antagonistic activity against selected human pathogens.

MATERIALS AND METHODS

Isolation of Earthworm Gut Flora

Dissection of Earthworms: The earthworm species *Eudrilus eugeniae* used for the present study was collected from the Vermiculturing Unit maintained by the Department of Microbiology, NASC, Coimbatore. 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 three sections: foregut, mid gut and hindgut.

Isolation of Microbes from Earthworm Gut

The gut sections were washed in sterile distilled water, weighed and homogenized for 5 minutes with a vortex mixer in sterile 0.85% NaCl solution. Each gut sections were individually plated on to Nutrient agar, Actinomycetes isolation agar and Sabouraud's dextrose agar for bacteria, actinomycetes and fungi respectively and kept for incubation. Three replicates were maintained from each dilution.

Isolation and Purification of Actinomycetes

Whitish pin-point 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 actinomycetes isolation agar medium with the help of loop inoculum method [11]. The plates were kept for incubation at 25 to 30°C for 2-3 days in an inverted position. The actinomycetes isolates were purified by pure culture techniques. The colonies were refrigerated in starch casein agar slants by frequent sub culturing for further studies. Among the isolated actinomycetes, the potential strain for further study was selected owing to its antagonistic activity in primary screening.

Screening of Antibiotic Producing Strains

Preparation of Preliminary Test Organisms

The test organisms used for preliminary screening were *Escherichia coli*, *Klebsiella sp.*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Serratia marcescens*, *Aeromonas hydrophila*, *Staphylococcus aureus*, *Bacillus sp.* and *Candida albicans*. Test bacteria were grown in 4.5 ml Nutrient broth for 18 hrs, and then standardized to Mc Farland standard of 0.1 at OD 600 nm. *Candida albicans* was grown on Potato dextrose agar (PDA) plate for 48 hr, then re-suspended in Nutrient broth and standardized to McFarland standard of 0.1 at OD 600 nm before use.

Primary Screening by Cross streak Method

Preliminary screening of antibiotic producing strains against human pathogens was done by cross-streak method [12]. The antagonistic activities of the actinomycetes against pathogens were analyzed by determination of the size of inhibition zone. Single streak of isolated

actinomycetes strains were made on surface of the Actinomycetes isolation agar and incubated at 28°C for 5 days. After observing a good ribbon like growth of the actinomycetes on the plates, the overnight pathogenic bacterial strains were streaked at right angles to the original streak of actinomycetes and incubated at 37°C for overnight. A control plate was also maintained without inoculating the actinomycetes, to assess the normal growth of bacteria. After incubation antagonistic activity of the actinomycetes against pathogens were screened and the antibiotic producing strains were isolated.

Isolation of Antibacterial Metabolites

The isolate showing the broad spectrum antimicrobial activity was grown in submerged culture in 250 ml flasks containing 50 ml of Starch casein nitrate broth aseptically. It was shaken at 28-30°C at 250 rpm for seven-ten days. The visible pellets, clumps or aggregates and turbidity in the broth, will confirm the growth of the organism in the flask. The contents of the flask were filtered through sterile Whatman No. 1 filter paper aseptically. The filtrate was subjected for solvent extraction method to recover antibacterial metabolites in pure form. Ethyl acetate was added to the filtrate in the ratio of 1:1 (v/v) and shaken vigorously for 1 h for complete extraction. The ethyl acetate phase which contains antibiotic substances was separated from aqueous phase. It was evaporated to dryness in water bath and the residue obtained was used to determine antimicrobial activity^[13].

Secondary Screening

Secondary screening was performed by well diffusion method^[14] using purified extract obtained by the evaporation of the ethyl acetate extract. The antimicrobial activity of the actinomycete isolate was detected as clear zone of inhibition around wells and it was measured in millimeters (mm).

Thin Layer Chromatography and Bioassay of Antibiotic

Ten micro-liters of the ethyl acetate fractions and reference antibiotics were loaded on to the silica gel plates and the chromatogram was developed using chloroform: methanol (4:1) as solvent system. The plates were run in duplicate; one set was used as the reference chromatogram and the other was used for the bioassay of antibiotic. The spots in the chromatogram were visualized in the iodine vapor chamber and UV chamber^[15].

UV/Visible absorption of extract

The structure elucidation of the compound was performed by using UV Spectroscopy. The

UV-Visible absorption spectrum of the extract was tested by using Shimadzu UV-2550 spectrophotometer at 300 - 340 nm to determine the λ maximum of the band ^[16].

Identification and Characterization of Actinomycetes

The isolated Actinomycetes strains were identified on the basis of their phenotypic characterization, physiological characteristics and biochemical characteristic which includes some basic tests, aerial mass color, reverse side pigment, melanoid pigments and spore chain morphology ^[17].

1. Macroscopic Identification

The isolated actinomycetes colonies were observed under high power magnifying lens. The colony morphology with respect to color, aerial mycelium, size and nature of colony and reverse side color were observed and identified ^[18].

2. Microscopic Observation

Microscopic observation of the potent isolate was performed using Wet mount technique ^[19] and Cover Slip Culture Technique ^[20]. The observed structure was compared with Bergey's Manual of Determinative Bacteriology ^[21] and the organism was identified.

3. Biochemical characterization

The various biochemical tests like Starch hydrolysis, Triple Sugar Iron (TSI) agar, Indole test, methyl red test, Vogus- Proskauer test, Citrate utilization test, Catalase test were performed for the identification of the potent isolate ^[22].

RESULTS

Isolation and Purification of Actinomycetes: A total of 6 actinomycetes colonies were isolated from earthworm gut by using serial dilution agar plating method (Fig.1).



Fig. 1: Actinomycetes Isolate P2

Screening of Antibiotic Producing Strains

Primary screening by Cross Streak Method

The isolated actinomycetes were screened against common pathogens by cross-streak method. Out of 6 actinomycetes isolated, four isolates namely P1, P2, 3, 4 showed promising antibacterial activities after screening against test organism (Table 1). Inability of test organisms to grow in the presence of actinomycete suggests antibiotic production by the actinomycete. Among them isolate P2 which possessed high broad spectrum activity against bacteria in comparison to other isolates was selected for further studies (Fig.2).

Table (1): Cross Streak Assay.

S. No.	Bacterial Pathogens	Antagonistic activity of Isolated Actinomycetes Strains			
		Isolate P1	Isolate P2	Isolate 3	Isolate 4
1.	<i>S. aureus</i>	+	+++	++	+++
2.	<i>Bacillus</i> sp.	++	++	-	-
3.	<i>Enterococcus</i> sp.	-	-	-	+
4.	<i>E. coli</i>	+	+++	+++	++
5.	<i>Klebsiella</i> sp.	+	+++	+++	++
6.	<i>P. aeruginosa</i>	-	++	-	-
7.	<i>Salmonella</i> sp.	++	+++	+++	-
8.	<i>S. marcescenes</i>	+++	+++	-	+++
9.	<i>Candida</i> sp.	-	+	-	+
10.	<i>Aeromonas</i> sp.	+	+++	-	+

+++ Better inhibition, ++ Good inhibition, + Moderate inhibition, - No inhibition

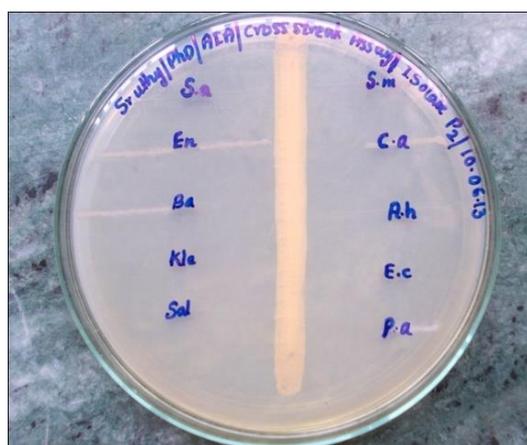


Fig. 2: Cross Streak Assay Isolate P2

Secondary Screening

The isolate P2 was inoculated into the fermentation medium and the antimicrobial components purified by solvent extraction procedure were assessed for their antibacterial properties against clinical pathogens by agar well diffusion method. It illustrated that ethyl

acetate extract of isolate (P2) showed a very broad spectrum of antimicrobial activity against both gram positive and gram negative bacteria used in the test; the result of the same can be seen in the Fig.3 below. The antibacterial activity of the extract was highest against *S. aureus* with a zone of inhibition of 27.7 ± 0.88 mm (Fig.4).

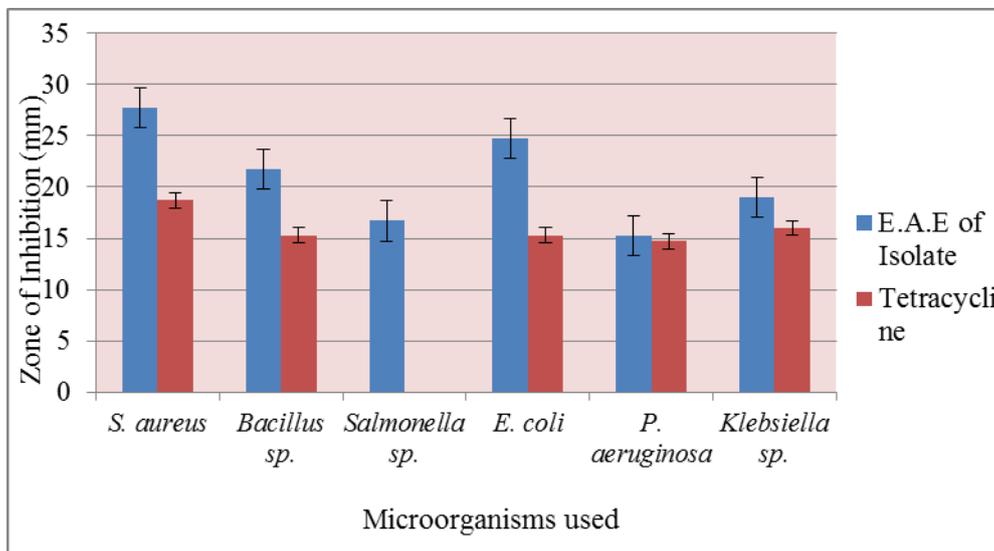


Fig. 3: Antibacterial activity of Extract against Clinical pathogens.

E.A.E- Ethyl acetate extract

Values are presented as the mean \pm SEM (standard error of mean); n = 3 for all groups.

***P<0.001, *P<0.05, considered significant.

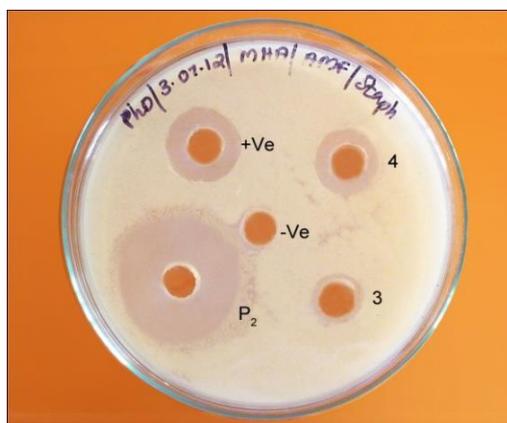


Fig. 4: Antibacterial Assay – *S. aureus*

Thin Layer Chromatography and Bioassay of Antibiotic

Thin layer chromatography of Ethyl acetate fractionated antibiotic was carried out to assess its chemical nature. When visualized under iodine vapor two brown spots were detected in the silica gel plates, one at the point of loading itself and other near to the solvent front on the chromatogram with Rf value of 0.89.

Structural analysis of the antibiotic by using UV Spectroscopy

The structural elucidation of the compound was performed by using U.V. The λ max of antibacterial compound was found to be 316 (Fig.5).

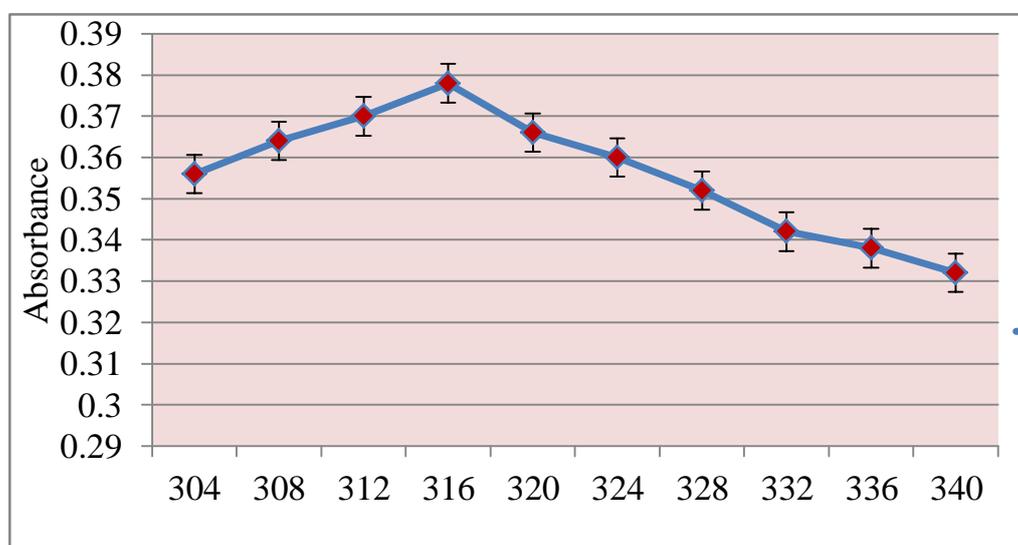


Fig. 5: Determination of λ Max of Antibiotic.

Identification and Characterization of Actinomycetes

The actinomycetes were identified based on its morphological, cultural characters and cover slip culture techniques. Aerial mass color of the substrate mycelium was determined by observing the plates after 7 to 10 days. It was done only after observing the heavy spore mass surface. The isolate P2 showing maximum zone of inhibition was stained with gram's staining procedure and was found to be gram positive rod in chain. The colonies were rough and elevated and the morphological observation in light microscope reveals chains of spores which is a specific characteristic of *Streptomyces* species.

The cultural and microscopic characterizations of actinomycetes are presented in table 2. The strain grew well on Actinomycetes isolation agar medium and produced grayish white color aerial mycelium. According to their morphological characteristics isolate P2 was found to belonging to the genus *Streptomyces*. Biochemical test were performed like Indole, MR, VP, Citrate, Nitrate reduction, Urease, Catalase, Oxidase, Starch Hydrolysis, Gelatin Hydrolysis, Lipid Hydrolysis, Casein Hydrolysis, TSI and the results were tabulated (Table 3). Based on morphological, physiological and biochemical characterizations, the active isolate was identified as *Streptomyces* sp.

Table-(2): Morphological and Cultural characteristics of Isolate P2.

S. No	Morphological & Cultural Characteristics	Isolate P2
1.	Growth on Culture medium	Excellent
2.	Aerial Mycelium	Grayish White
3.	Substrate Mycelium	Pale Yellow (-)
4.	Melanoid Pigmentation	Negative
5.	Type of Spore	Long chain of spores
6.	Gram Stain	Gram Positive rods

Table (3): Biochemical Characteristics of Isolate P2.

S. No	Name of the Test	Properties
1.	Indole	-
2.	Methyl Red	+
3.	Voges Proskauer	-
4.	Citrate Utilization	+
5.	Nitrate Reduction	+
6.	Urease	+
7.	Catalase	+
8.	Oxidase	+
9.	Starch Hydrolysis	-
10.	Gelatin Liquefaction	+
11.	Casein Hydrolysis	+
12.	Triple Sugar Iron Agar	alk/alk

+ Positive, - Negative

DISCUSSION

In the present scenario microbial natural products appear as the most promising sources for developing future antibiotics. Actinomycetes are the most biotechnologically valuable prokaryotes responsible for the production of about half of the discovered bioactive secondary metabolites including antibiotics [5]. In the present study out of 6 actinomycetes isolated from earthworm gut, isolate P2 possessed high broad spectrum activity. Organic solvents with different polarities have been used by many researchers for the extraction of antimicrobial compounds from actinomycetes [23]. The antimicrobial compounds from the isolate P2 showed broad spectrum of activity against both Gram positive and Gram-negative organisms which supports the popular notion that actinomycetes can be sources of potent antibacterial agents that can be of value in the treatment of infections especially those caused by clinically resistant bacteria, such as *Staphylococcus aureus* and *Klebsiella pneumoniae* [2]. The antibacterial activity of the actinobacteria isolated from the molluscans samples was studied by [24], who reported that the tested ten actinobacterial isolates were active against gram positive and gram negative bacteria. The results of the primary and secondary screening

reveals that Ethyl acetate extract of isolate (P2) showed a very broad spectrum of antimicrobial activity against both gram positive and gram negative bacteria used in the test, which were in parallel with the results of [25]. According to [26], the extracts from ethyl acetate showed maximum antimicrobial activity against bacteria and fungi, other solvent extracts showed moderate activity against test organism which is similar to the present study. The results of the present study clearly indicated that the antimicrobial activity of potential strain is due to the production of extracellular bioactive compounds. The published literature stated that most of the antibiotics from actinomycetes are extracellular in nature [27].

The earthworm gut is favorable for the development of actinomycetes due to neutral pH, optimal humidity and temperature. In addition, the increased organic carbon and nitrogen content in earthworm gut may also stimulate microbial activity [9]. The actinomycetes isolate P2 grew well on Starch casein nitrate agar medium and produced grayish white aerial mycelium and pale yellow coloured substrate mycelium. Starch casein agar was reported as a suitable medium for the isolation of actinobacteria from soil, water and less explored ecosystems such as mountain, forest, desert ecosystems [28]. The consistency of the isolate was powdery in nature. The mycelial color difference between different strains of Actinomycetes may be due to the synthesis of various secondary metabolites [29]. The colonies were rough and elevated and the morphological observation in light microscope reveals chains of spores which is a specific characteristic of *Streptomyces* species.

The isolated strain was characterized for physiological and biochemical properties, nutritional uptake and the isolate fitted the *Streptomyces* genus as reported by several investigators [30]. *Streptomyces* are Gram positive filamentous bacteria which belong to the order Actinomycetales. [31,32] reported that the only genera *Streptomyces*, the members of Actinomycetales account for the approximately 93% producing secondary metabolites. Compared to other Actinomycetes, *Streptomyces* species showed efficient antagonistic activity [33]. The most intriguing trait of *Streptomyces* is the capacity to produce secondary metabolites including antibiotics and bioactive compounds valued in human and veterinary medicine, agriculture, and unique biochemical tools [34]. Thus the results of present investigation revealed that actinomycetes isolated from earthworm gut are the potent source of novel antibiotics and were found to have potential antagonistic activity against test organisms.

CONCLUSION

In order to meet the increasing demand of natural medicines in recent years, bioactive components with medicinal values from earthworms have already provoked increased interest. The results obtained from the present study indicated that earthworm gut is promising and could be a vital source of habitat possessing antimicrobial activity. The immense scope of actinomycetes for the exploration of therapeutically active biomolecules has been recognized recently. In the future, there is a possibility of finding new antibiotics from gut flora of earthworm. For proper identification of the antimicrobial compounds, it is essential to obtain in pure form which requires a series of purification procedures and different chemical analysis. Still a little effort was made in this approach.

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