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Isolation and Identification of Actinomycetes with Antibacterial Activity from Soil Samples Around Kandy, Sri Lanka

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ABSTRACT

Commercially exploited antibiotics are originally isolated from Actinomycetes. Even so, Actinomycetes are mostly underestimated for their antibacterial potential. This study aimed to isolate and identify Actinomycetes with antibacterial activity from soil samples around Kandy Sri Lanka. Soils were taken from five different habitats (waste disposal (WS), originally cultivated (OR), riverbanks (RB), pasture (PS), and rhizospheric (RH)). Serially diluted samples were grown on an Actinomycetes isolation agar medium and screened for antibacterial activity against *Escherichia coli* (ATCC25922), *Staphylococcus aureus* (ATCC25923), *Pseudomonas aeruginosa* (ATCC27853) and *Klebsiella pneumoniae* (ATCC13883) using perpendicular streak method. Isolates with inhibitory activity were subjected to secondary screening. 19 actinomycetes isolates were identified of which 10 isolates showed inhibitory activity during preliminary screening. During the secondary screening, two isolates from RB site and one isolate from OR site expressed broad-spectrum antibacterial activity while the other five isolates from RB, OR, WS, and PS sites inhibited either *Escherichia coli* or *Staphylococcus aureus*. The soil samples are a promising source of novel antibacterial compounds and thus these findings can be used for further investigations in developing broad-spectrum antibiotics for therapeutic targets in the future.

Key words: Actinomycetes; soil; antibacterial activity; *S. aureus*, *E. coli*

1. INTRODUCTION

There are many natural compounds in nature. They are produced by various organisms like bacteria, fungi, and plants as secondary metabolites. 5% out of that over 1 million natural compounds are based on microbial origin.[1] The natural compounds that are produced by microbes are the major sources of currently used antibiotics .[2] Antibacterial compounds can treat the infections of bacteria. In general, these chemical compounds can inhibit or suppress growth and also destroy bacteria. The natural origin of most antibiotics is soil bacteria and fungi.[3] Commonly used antibacterial compounds in the current health sector, are originally isolated from bacterial actinomycetes, specifically, from the genus Streptomyces. For most of the antibiotics that were screened between the years 1945 and 1978, over 55% of novel antibacterial compounds originated from Streptomyces.[4] Actinomycetes can be known as inexhaustible origin antibiotics.[5] Actinomycetes are organisms that are prokaryotic, contain single cells, and are categorized as bacteria but named in the order Actinomycetes.[4] They can survive in a wide range of ecosystems. Actinomycetes are dissimilar compared to bacteria, whereas the normal bacteria are in rod shape or spherical shape but actinomycetes are in oval shape.[6] Actinomycetes are also members of the gram-positive bacteria type. They contain spores, a high fraction of Guanine and Cytosine in DNA, and synthesis of aerial and substrate mycelium.[7] According to their filamentous morphology, they look like fungi. So, they are considered transitional states of fungi.

Despite the achievements of the discovery of antibiotics and the progress in the process of antibiotic development, still, the second leading cause of death worldwide is the diseases of various infections caused by microbes. 17 million deaths are incurred annually due to bacterial infections.[8] Antibacterial therapy can protect millions of lives and considerably reduce the rate of premature death from bacterial infection.[9]

So, inquiry and discovery of new antibacterial compounds are needed to fight against new diseases and pathogens that are resistant to existing antibiotics.

Discovering a new antibiotic is a difficult task. Actinomycetes still contribute as a treasure chest to the identification of new antibiotics. Secondary metabolites that are produced by actinomycetes mostly consist of antibacterial properties. So far, two-thirds of the existing antibiotics are contributed by actinomycetes, using Streptomyces.[10]

A considerable number of studies have been performed in many countries such as Malaysia, South India, India, Northeast India, Chennai, Ethiopia, and the Philippines to identify antibiotics from actinomycetes in soil samples. In general, these studies are limited to being performed mainly in Asian and African countries. As for the soil samples investigated, they are from diverse sources like tropical soil sediments[11] forest soil samples[12], freshwater sediments[2], garden soil[13], rhizosphere and agricultural soil[14] green spaces[15], bio waste soil[16], desert soil[17], rainforest soil[18], lake oil and so on. The distribution of actinomycetes in different soil samples is important. Nasrabadi et al performed a study on "Distribution of actinomycetes in different soil ecosystems and effect of media composition on extracellular phosphate activity" in 2013 showing that actinomycetes are more abundant in irrigated soil that is being watered properly and regularly. Then the diversity is gradually decreased in forest soil, rainfed, and then pasture soil.[19] Almost all the studies showed antibacterial activity against test organisms through preliminary and secondary screening.[20] All the studies have undergone pretreatments for collecting soil samples to reduce the growth of unwanted microbes naturally occurring in soil.[20] Furthermore, after the identification of the antibacterial activity of soil samples, most of the studies were able to identify the antibacterial compound and the responsible genes.[11] In contrast, only a handful of studies have been performed in Sri Lanka on the same topic. The University of Colombo, Sri Lanka has carried out a study[21] that explains the development of a biofertilizer. Another study conducted by the University of Kelaniya, Sri Lanka[22] shows that actinomycetes isolated in a solid medium gave antimicrobial action against selected pathogens.

Antibacterial compounds are magical drugs. However, the issue is continuous resistance among key pathogens, making it less effective. Therefore, the need for new antibiotics is immediate for the purpose of reversing persistent infections. Even though resistance is caused by the abuse or overuse of antibiotics, it is also affected by the lack of investments in the discovery and development of novel antibiotics.[23] Actinomycetes in the soil are a never-ending source of antibiotics. Most of the research studies suggest that the prime source of antibiotics is the actinomycetes because of their unique set of enzymes which will lead to synthesizing new antibiotics for therapeutic targets.[24] So, this study aimed to identify novel antibiotic(s) from actinomycetes that can be used for therapeutic purposes.

2. MATERIALS & METHODS

2.1 Sample Collection

Five different sites were randomly selected around Kandy, Sri Lanka. Selected sites were waste disposal (WS), originally cultivated (OR), riverbanks (RB), pasture (PS), and Rhizospheric soil (RH). A global Positioning System (GPS) was used to indicate the collection sites and recorded accordingly. On the same day of sample collection pH and temperature of the soil were measured.

2.2 Isolation of Actinomycetes from Soil

Pretreated and serially diluted soil samples were grown on Actinomycetes Isolation agar (Sigma, Aldrige) plates and were incubated at 37 ^{0}C for 2 days.[25]

2.3 Colony Identification

Different morphological colonies were identified under a high magnifying lens with respect to the color of the colony, morphology form/surface, elevation, consistency, colony margin, reverse side appearance, aerial mycelium, and substrate mycelium. Colony number was also observed for all the samples for the characterization of different isolates, and Gram staining was performed for all isolates.[26]

2.4 Primary Screening-Perpendicular Screening

Each of the actinomycetes isolates was streaked as a straight line on Muller Hinton Agar (MHA) medium and incubated at 37 ^{0}C for 4 days. After 4 days, different strains of test bacteria (*Escherichia coli* (ATCC25922) *Staphylococcus aureus* (ATCC25923), *Pseudomonas aeruginosa* (ATCC27853), and *Klebsiella pneumoniae* (ATCC13883)) were streaked at a right angle, not touching each other, and then the plates were incubated at 37 ^{0}C for 24 hours. The test organism that is susceptible to antibiotic compounds produced by actinomycetes will not grow near the actinomycetes streak. Zones of inhibition were observed and recorded.[27]

2.5 Secondary Screening-well Diffusion Method

Ethyl acetate crude extracts were prepared from the isolates that showed inhibitory activity against the test organisms during primary screening and secondary screening was performed using the well diffusion method.[28] The wells were made on the surface of the MHA. Each plate was made of three wells on related portions. To seal the wells approximately 0.1 ml agar solution was

added. The wells were filled with ethyl acetate crude extract (50 microliters per well), positive control (Gentamycin 0.025 mg/ml), and negative control (sterile distilled water). All plates were sealed using parafilm and were incubated at 37 ^{0}C in an upright position for 24 hours. The zones of inhibition were measured by getting the average diameter of 3 directions perpendicular to each other. All experiments were performed in three replicates.[29]

3. RESULT

3.1 Chemical and Physical Analysis of the Soil Samples

Out of the five sites that were investigated four were alkaline sites while one was acidic. The temperature of all the soil samples was observed to be in the range of 20 ^{0}C - 25 ^{0}C.

3.2 Isolation of Actinomycetes

A total of 21 isolates were identified, 6 from the WS site, 6 from the OR site, 6 from RB site, 2 from PS site, and 1 from RH site. Of these, 19 isolates were identified as actinomycetes by gram staining and morphological identification while 2 were identified as gram-positive bacilli. Characteristic features of the actinomycetes isolates are shown in Table 1.

3.3 Preliminary Screening

All the 21 isolates were subjected to preliminary screening. Only 8 isolates expressed inhibitory action against the test organisms ((5 against *S. aureus*,5 against *E. coli* and 2 against *P. aeruginosa*). None of the isolates expressed an inhibitory effect against *Klebsiella pneumoniae*. The zones of inhibition during preliminary screening are given in Table 2.

3.4 Secondary Screening

Colonies that gave inhibitory action during preliminary screening were used to prepare an ethyl acetate crude extract. The secondary screening was performed using these extracts by performing a well-diffusion assay against *S. aureus* and *E. coli* as test microorganisms. The OR4 gave the highest zone of inhibition against *E. coli* and *S. aureus*. OR2 gave the next highest inhibition against *S. aureus*. RB1 and WS3 gave approximately similar inhibitory zones against *S. aureus*. Inhibitory zones observed for Actinomycetes isolates are given in Figures 1 and 2.

4. DISCUSSION

Actinomycetes are one of the most unique microorganisms that produce versatile antibacterial compounds and essential secondary active metabolites.[2] The secondary metabolites that are produced by the actinomycetes have the potential to inhibit the growth and development of infectious bacteria. Currently, available antibacterial compounds are becoming resistant to infectious organisms and antibacterial resistance has become a universal health problem. The World Health Organization stated that antimicrobial resistance is one of the huge and ever-growing health problems that humans face among 10 global public health threats. Therefore, it is essential to investigate new compounds that can produce antibacterial activity.

Actinomycetes in the soil provide a never-ending source of antibiotics. Most of them are under-investigated. In this study, a total of 21 strains were isolated from 5 different geographical areas in Kandy district, Sri Lanka. All the sites are heterogeneous in nature. Out of these 21 isolates 19 isolates were identified as actinomycetes and 2 as bacillus. All the isolates were fast growing on the Actinomycetes Isolation Agar medium and diverse in colony morphology.

This study investigated the antibacterial activity of the isolates against the gram-negative and gram-positive test organisms in primary and secondary screening. During primary screening, 8 isolates showed antibacterial activity (5 against *S. aureus* and 5 against *E. coli*). None of the isolates expressed an inhibitory effect against *Klebsiella pneumoniae* while two isolates expressed an inhibitory effect against *Pseudomonas aeruginosa*. The potency of antibacterial compounds may not be sufficient to inhibit these test organisms. 14.28% of the isolates showed a broad spectrum of antibacterial activity by giving an inhibitory effect against gram-negative and gram-positive bacteria. In a study conducted by Kumar N.et al 12.82% of isolates showed an inhibitory effect out of 117 different actinomycetes isolates[28] whereas another study done by Wahab A. et al indicated that only 6 gave inhibitory action against gram-positive bacteria while 4 strains expressed activity against gram-negative bacteria.[20]

According to Shirling and Gottlieb[30] the reason for the observation of varying degrees of inhibition may be the structural differences between the gram-positive and gram-negative bacteria. There is an outer polysaccharide membrane as a protective layer which makes it more difficult for the antibiotic to be penetrated in gram-negative organisms while gram-positive bacteria consist of an outer peptidoglycan layer which does not make penetration difficult.

In the secondary screening, all the isolates expressed an inhibitory effect as same as the inhibition observed in preliminary screening, except in the case of the inhibitory effect against *Pseudomonas aeruginosa*. Singh L.S. et al showed ethyl acetate extract of *S. sannanensis* SU 118 was able to produce a powerful antibiotic against gram-positive bacteria.[33] Another similar kind of study done by Nurkanto A. et al showed that extract of the ethyl acetate and methanol gave effective antibacterial activity rather than the aqueous extracts. The solubility of the extracellular

antibiotic produced by actinomycetes in the organic layer is better than the solubility in the aqueous layer.[33]

The heterogeneous site where the soil samples were taken may explain the different confinement, growth factors, and diversity of the population of the actinomycetes in different habitats. Temperature and pH factors impact sporulation, adaptation, morphological differences, and secondary product secretion. However, the isolates identified from the soil were in acidic condition and the isolates from riverbanks gave the highest isolate number that inhibited the test organisms. This could be an adaptation of actinomycetes to survive in the harsh acidic condition.

The soil samples taken in this study were potential sources of antibiotic-producing actinomycetes. Further investigation and isolation of the compound/s with antibacterial activity is needed in the future.

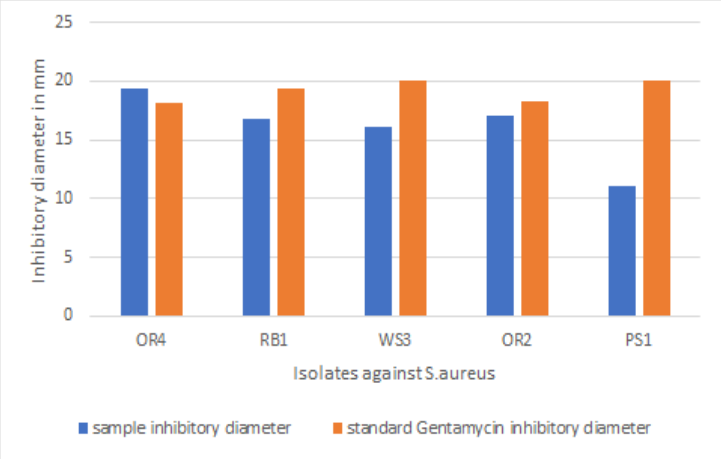

Figure 1: Inhibitory zones observed against S. aureus

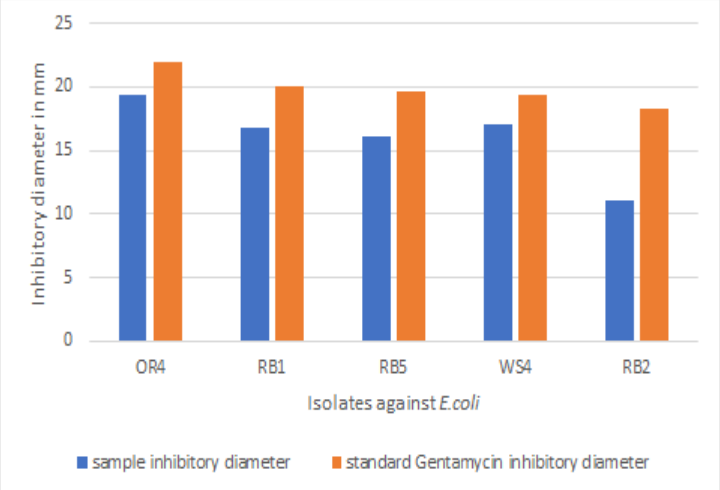

Figure 2: Inhibitory zones observed against *E. coli*

5. CONCLUSION

21 microbial isolates were obtained from 5 different sites. Out of these 21 isolates 19 isolates were positively identified as actinomycetes according to the microscopic morphological characteristics. During the primary screening, 8 isolates (OR4, RB1, RB5, WS3, WS4, OR2, RB2 and PS) showed inhibitory zones against four test organisms.

During the secondary screening using the ethyl acetate crude extracts, the OR4 gave the highest zone of inhibition against *Escherichia coli* and *Staphylococcus aureus* thus warranting further investigation of this sample in the future.

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Table 1: Characteristic features of the Actinomycetes isolates

Isolate No./Characteristics	Gram staining	Color	Morphology form/surface	elevation	consistency	margin	Reverse side	Aerial mycelim	Substrate mycelium
WS1	+	Off white	Smooth, cotton filamentous	Elevated	Dry	Filamentous	Light brown	Off white	Light brown
WS2	+	Black and white	Hard, thick	Flat	Dry	Round	Brown	White	Black
WS3	+	Muddy color	Smooth and thick	Flat	Dry	Round	Brown	Muddy	Brown
WS4	+	Watery	Smooth limited to one area	Flat	Dry	Round	Watery	Watery	Watery
WS5	+	Watery	Smooth and thick	Flat	Dry	Filamentous	Elevate	Watery	Watery
WS6	+	Colorless	Smooth, watery	Thick	Dry	Round	watery	Watery	Watery
OR1		Off white	Smooth, cotton filamentous like	Elevate	Dry	Filamentous	Light brown	Off white	Light brown
OR2		Black and white	Hard, thick	Flat	Dry	Round	Brown	White	Black
OR3		Muddy	Smooth and thick	Flat	Dry	Round	Brown	Muddy	Brown
OR4		Watery	Smooth, limited to one Ares	Flat	Dry	Round	Watery	Watery	Watery
OR5		Watery	Smooth and thick	Flat	Dry	Filamentous	Elevate	Watery	Watery
OR6		Colorless	Smooth, watery	Thick	Dry	Round	watery	watery	watery
RB1	+	Muddy	Hard and thick	Flat	Dry	Round	Muddy	Muddy	Muddy
RB2	+	Pure white	Hard, colony is spreaded by margins chalky	Flat	Dry	Round	Black	White	Black
RB3	+	Off white	Smooth, cloudy	Flat	Dry	Filamentous	Off white	Off white	Off white
RB4	+	Creamy	Hard, colony is surrounded	Flat	Dry	Cracky	Creamy	Creamy	Creamy

			by white margins						
RB5	+	Off white	Smooth, cloudy	Falt	Dry	Filamentous	Creamy	Off white	Creamy
RB 6	+	Creamy	Cotton like filamentous around colony	flat	dry	Round	Dark creamy	Creamy	Dark creamy
PS1		Light green	Hard, cloudy	Flat	Dry	Round	Pale yellow	Creamy	White
PS2		Greenish brown	Hard, chalky	Flat	Dry	Round	Brown	Creamy	White
RH1		Light green cottonlike	Smooth, cottonlike	Elevated	dry	round	Light green	Light green	White

Table 2: Inhibitory zones observed for Actinomycetes isolates during preliminary screening.

Isolate number	**Zone of inhibition against *Staphylococcus aureus* in cm/0.1**	**Zone of inhibition against *Escherichia coli* in cm/0.1**	**Zone of inhibition against *Pseudomonas aeruginosa* in cm/0.1**
OR4	0.7	0.8	-
RB1	0.9	1.0	-
RB5	0.2	0.3	0.1
WS3	0.2	-	-
WS4	-	0.9	0.1
OR2	0.2	-	-
RB2	-	0.2	-
PS1	0.1	-	-

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