Prithvi Journal of Research and Innovation
[A Peer-Reviewed, Open Access Multidisciplinary Bilingual Journal; Indexed in NepJOL]
ISSN 2705-4888 [Print]; ISSN 2705-4896 [Online]; JPPS Star-Rated Journal
Volume 3; 15 December 2021; pp. 1-8
eJournal Site: http://ejournals.pncampus.edu.np/ejournals/pjri/

---------- *ORIGINAL RESEARCH ARTICLE*

A Comparison of Antibacterial Potential of Different Soil Actinomycetes from Pokhara

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Article History:
Submitted 18 October 2021
Reviewed 19 November 2021
Accepted 13 December 2021

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Article DOI:
https://doi.org/10.3126/pjri.v3i1.41625

Publisher:
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Prithvi Narayan Campus
Tribhuvan University
Pokhara, Nepal
[Accredited by UGC, Nepal]

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ABSTRACT

Actinomycetes are widely distributed in the environment and used for the production of several important secondary metabolites like antibiotics, immunosuppressive agents, enzymes and antitumor agents. Therefore, the main objective of this study is to isolate and assess antibacterial potential of different actinomycetes obtained from different soil samples. This study was conducted in the microbiology laboratories of Prithvi Narayan Campus and Lambda Food Lab Pvt Ltd, Pokhara. A total of nine soil samples were collected from different places of Pokhara (forest land, agriculture land and lake bank) and processed. Isolated actinomycetes were screened by primary and secondary screening for antibiotic producers against test organisms like *Staphylococcus aureus* (ATCC 25923), *Pseudomonas aeruginosa* (ATCC 27853) and *Bacillus* spp, *E coli* (ATCC 25922). This study isolated 27 actinomycetes in total, using the soil samples through spread plating on Starch Casein Agar (SCA) and by serial dilution. After incubation, actinomycetes colonies (rough, chalky) were selected for gram staining to observe thin thread-like mycelial and hyphal structures. The highest number of actinomycetes isolates were obtained from agricultural land's soil samples (14 out of 27 isolates i.e. 51.85%) whereas only 3 isolates were obtained from the lake soil. Primary screening was performed on Nutrient agar where test bacteria were streaked perpendicular to the isolated actinomycetes to observe antagonism. This showed 12 actinomycetes as active isolates inhibiting at least one test bacteria. The antibacterial compounds were extracted by ethyl acetate method and used in secondary screening. Secondary screening in Mueller Hinton agar (MHA) further revealed five isolates showed promising inhibitory capacity. In both screening methods higher sensitivity was observed towards Gram-positive

bacteria especially *S aureus* (ATCC 25923), and the least sensitivity towards Gram-negative bacteria especially *Pseudomonas aeruginosa* (ATCC 27853). Agricultural land was shown to harbor more actinomycetes than forest land and lake bank soil. Though variations were observed in primary and secondary screening, actinomycetes obtained from agricultural land demonstrated an inhibitory action against the Gram-positive and Gram-negative test organisms. As comparedt to Gram-negative bacteria, the Gram-positive had higher effects. These findings showed that soil of different locations of Pokhara valley found many actinomycetes strains, preventing the growth of pathogenic bacteria of certain kinds. The study suggested that further investigations need to be done that helped obtain new antimicrobial agents from actinomycetes, using various other sources.

KEYWORDS: Actinomycetes, Antibiotics, agricultural land, lake bank soil, forest land

INTRODUCTION

Antibiotics are bioactive secondary metabolites that can be obtained from microorganisms and can also be semi-synthesized or chemically synthesized (Balagurunathan & Radhakrishnan, 2010). Within the microbes, actinomycetes contributes most for antibiotic production. It contained actinomycetesare filamentous Gram-positive bacteria that included high G+C content, also known as Ray fungi.

Actinomycetes also produce other therapeutically useful secondary metabolites like antibacterial, antifungal, antiviral, antiparasitic, immunosuppressive, antialgal, anti-inflammatory, antitumor agents etc (Bérdy, 2005; Farnet & Zazopoulos, 2005; Balagurunathan & Radhakrishnan, 2010). Current researches in actinomycetes are focused in understanding their regulatory mechanisms, host interactions, their evolution and ecological characteristics and new specialized metabolites using modern molecular approaches (Prudence et al., 2020). However, in Nepal, most of the researches have been focused on accessing the antimicrobial properties of soil-derived Actinomycetes from various ecological niches ranging from soils of Kalapatthar, Everest region to Tarai region along with other sites like river banks, forests, etc. The distribution of Actinomycetes have also been accessed according to the altitude in various parts of Nepal and their potential has been tested against several pathogenic bacteria and fungi (Gurung et al., 2009; Pandey et al., 2004; Rai et al., 2018; Budhathoki & Shrestha, 2020; Sah & Lekhak, 2017). Actinomycetes isolates obtained from several sources were found more effective against Gram-positive bacterium when compared with Gram-negative bacterium (Wahab et al., 2015; Pandey et al., 2004; Sapkota et al., 2020; Budhathoki & Shresthaet al., 2020).

In this study, actinomycetes are widely distributed in sediments, water and soil to extreme environments such as the Himalayas and warm environments (Ventura et al., 2007; Gurung et al., 2009; Barka et al., 2016). It is necessary to discover new and novel antibiotics/antimicrobials in present context to combat issues like antibiotic resistance, comparatively delayed discovery of new antibiotics and inadequate screening of actinomycetes despite their diversified occurrence. Pokhara is rich in biodiversity with its topography and climate and it is supposed to harbor diverse and unique microbial flora including actinomycetes. Hence, this study was designed to explore and compare the actinomycetes useful for antibiotic production, from different soils of Pokhara valley.

METHODOLOGY

Collection of Soil Samples

To meet the objective of the study, nine soil samples in total were collected from sites such as forest land, agriculture land and lake bank of Pokhara valley. Around five

gram of soil samples were collected in a polyethylene bags containing one gram of $CaCO_3$ and they were mixed thoroughly. The samples were properly labelled and left at room temperature for about one week for drying. These samples were processed in the Microbiology laboratory of Prithvi Narayan Campus and Lambda Food Lab, Pokhara.

Isolation of Actinomycetes

In this study, actinomycetes were isolated from soil, which was conducted by serial dilution of soil samples. The activity was followed by spread plating on Starch Casein (SCA) agar medium. Then, a gram soil was mixed with nine ml of distilled water and was mixed vigorously with preheating at 50° C for 0.5 h. From the dilution tubes of 10^{-7} and 10^{-8}, 0.5 ml of suspension was used and spread on SCA media. Then, the plates were incubated at 27° C for seven days. After incubation, actinomycetes colonies, which were rough and chalky were selected from mixed culture and sub-cultured on fresh SCA agar plates. It was then incubated at 27° C for seven days again. Isolated cultures of actinomycetes were stored at 4° C for analysis (Oskay et al., 2004).

Microscopic Examination

The pure cultures of Actinomycetes were gram stained and observed microscopically for thin thread-like mycelial and hyphal structures.

Screening of Actinomycetes

The screening of actinomycetes was conducted, using two screening procedures: 1) primary screening and 2) secondary screening.

1) Primary Screening

The pure colony of actinomycetes was streaked in the central region of nutrient agar plate, incubating at 27° C for four days. After that, test organisms such as *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922 and *Bacillus* spp were then streaked perpendicular to actinomycetes strains. They were then incubated for a certain period of time. Following their incubation, the size of the inhibition zones was measured to determine antagonism (Madigan et al., 1997; Egorov, 1985).

Actinomycetes colonies demonstrating antibacterial potential were inoculated into the Yeast Extract-Malt Extract broth (ISP-2). They were then incubated in shaker at 28°c for eight days. Then, the broth was centrifuged at 4000 rpm for 30 minutes. An equal volume of ethyl acetate was added to the supernatant, making the mixture, which was shaken and poured into the separating funnel. It was then allowed to stand there. After this activity, the upper organic phase was collected to heat in a water bath at 40°C to evaporate ethyl acetate. Its residue was dissolved in phosphate buffer (pH 7) and stored at refrigerator temperature for use in the future (Ghorbani et al. 2013; Awasthi et al., 2014)

2) Secondary screening

In the secondary screening, active actinomycetes isolates were processed to have the results. Mueller Hinton agar (MHA) was lawn cultured with each test organisms after which wells of eight mm were made by sterile borer. In addition, 40 µl of ethyl acetate extracted antibiotic were loaded into the wells and the plates were left for 20-30 minutes. This was followed by their incubation at 37°C for a day. Having incubated, the zone of inhibition around the wells were measured if observed (Sharma et al., 2011; Gopinath et al., 2013).

Data Entry and Analysis

The findings obtained after the experiment were entered, tabulated and analyzed using Excel.

RESULTS

In this study, nine soil samples were collected from Pokhara, visiting three different locations. Higher number of actinomycetesie 14 among 27 was obtained from agriculture land and least from the lake bank soil (Table 1). Twenty-seven actinomycetes were isolated in total from which 12 isolates were found to have antagonistic properties as observed from primary screening. The study showed that *Staphylococcus aureus* (ATCC 25923) was prevented by the actinomycetes isolates and *Pseudomonas aeruginosa* as the list inhibited. Actinomycetes isolates obtained from agricultural land showed higher effectiveness against the test organisms followed by forest land soil and lake bank soils (Table 2). After secondary screening, only five isolates were able to show observable inhibition against test organisms. Higher inhibition was again observed among the actinomycetes isolates obtained from agricultural land soil and *Staphylococcus aureus* (ATCC 25923) was found to be most inhibited and *Pseudomonas aeruginosa* (ATCC 27853) as the least inhibited (Table 3).

Table 1

Description of Soil Samples

Soil sample source	No of actinomycetes isolated	No of active actinomycetes within isolated actinomycetes
Agriculture land	14	6
Forest land	10	4
Lake bank	3	2
Total	27	12 (44.4%)

Table 2

Result of Primary Screening

Soil sample source	No of active actinomycetes within isolated actinomycetes after primary screening	No of actinomycetes effective against			
		Gram-positive bacteria		Gram-negative bacteria	
		Staphylococcus aureus ATCC 25923	*Bacillus* species	*E coli ATCC* 25922	*Pseudomonas aeruginosa* ATCC 27853
Agriculture land soil	6	5	4	2	1
Forest land soil	4	4	1	1	1
Lake bank soil	2	1	0	1	0
Total	12	10	5	4	2

Table 3
Result of Secondary Screening

Soil sample source	No of active actinomycetes after secondary screening	No of actinomycetes effective against			
		Gram-positive bacteria		Gram-negative bacteria	
		Staphylococcus aureus ATCC 25923	*Bacillus* species	*E coli ATCC* 25922	*Pseudomonas aeruginosa* ATCC 27853
Agriculture land soil	4	3	1	2	1
Forest land soil	1	1	1	0	0
Lake bank soil	0	0	0	0	0
Total	5	4	2	2	1

DISCUSSION

Nine soil samples from three different localities including lakes, forests and agricultural land were collected for the study from which a total of 27 Actinomycetes were isolated after dilution and culture in Starch Casein Agar.

According to the findings of the study, 14 out of 27 isolates i.e. 51.85%, agricultural land was found to be the highest number of actinomycetes samples and least from the lake soil. Similar results can be observed in the research of Budhathoki & Shrestha (2020). Higher rates of actinomycetes from agricultural land is because of the reasons that lands with vegetation has higher number of actinomycetes population due to several symbiotic relationships with various roots no dulated plants and actinomycetes population also transformed from fertile land to moist lands (Klemmedson, 1979; Budhathoki & Shrestha, 2020; Ghorbani et al., 2013). Few actinomycetes in lakes might be due to diluting effect of water due to which aquatic actinomycetes have to produce more of bioactive compounds to be effective (Timothy et al., 2010). Contrasting results regarding distribution of actinomycetes in different soils of Chitwan has been observed in the findings of Rai et al. (2018). Variation in climatic condition in different geographical regions creates ecology harbouring distinct microbial flora with distinct characteristics. Similarly, several constraints like constraints of environment, growth, temperature, etc., affected the growth and diversity of actinomycetes. It is also found that temperature had an adverse effect on physiology, morphology, biochemistry, sporulation, metabolism including "antimicrobial metabolite production" of the cell (Sapkota et al., 2020).

Among total 27 actinomycetes isolates, 12 isolates (44.4%) were found to inhibit at least one test organism. Similar results were spotted in studies of Patel et al. (2014), Budhathoki and Shrestha (2020) and Sapkota et al. (2020). The distribution of actinomycetes that produced antibiotics in soil varies as per the quality and agricultural status (Rai et al., 2016).

Similarly, as compared to Gram-negative bacterium, isolated actinomycetes were found to be more active in contrary to Gram-positive bacterium. During the primary screening, within the Gram-positive bacteria, *Staphylococcus aureus* (ATCC 25923) was the most susceptible bacteria i.e. they were susceptible toward 10 actinomycetes isolates. *Bacillus* species was susceptible towards five isolates and *E coli* (ATCC 25922) towards four isolates of actinomycetes. *Pseudomonas aeruginosa* (ATCC 27853) was found as least susceptible with susceptibility towards only two isolates of actinomycetes. Similar

observations have been noticed in other findings also (Wahab et al., 2015; Pandey et al., 2004; Budhathoki et al., 2020; Sapkota et al., 2020). The reason for higher sensitivity towards *Staphylococcus aureus* (ATCC 25925) is due to the cellular morphological differences of cells of Gram-positive and Gram-negative and it might be also possible for the antibiotic compound from actinomycetes to be more functional and effective against Gram-positive than the Gram-negative cell. (Shirling & Gottlieb, 1966). Tyagi et al., 2014 have suggested that inhibitory potency of actinomycetes are higher against Gram-positive bacteria in their findings. Actinomycetes may produce multiple antibacterial metabolites and become an effective "inhibitor to both Gram-positive and Gram-negative bacteria" (Gurung et al., 2009). In primary screening, higher effectiveness against test organism is shown by actinomycetes obtained from agricultural land. It was found to be more effective against Gram-positive bacteria than Gram-negative bacteria. Effectiveness against Gram-positive and Gram-negative bacteria is also observed in actinomycetes that were obtained from the agricultural land soil.

In secondary screening only five isolates out of 12 isolates from primary screening i.e. 41.66% showed to have inhibitory effect against test organisms. For instance, *Staphylococcus aureus* (ATCC 25923) was more inhibited than *Pseudomonas aeruginosa* (ATCC 27853) as the least inhibited test organism. Six isolates were effective in contrary to Gram-positive bacteria and three in contrary to Gram-negative bacteria. These findings are found to vary according to other findings of Sapkota et al., 2020; Budhathoki & Shrestha et al., 2020. This indifference might be due to variation in extraction methods (Singh et al., 2014; Nurkanto et al., 2012). As compared to Gram-negative bacteria, actinomycetes obtained from agricultural land demonstrated an inhibitory action in contrary to Gram-positive and Gram-negative test organisms, which had higher effects against Gram-positive.

The variation in primary and secondary screening was observed as a result of the variation when it was composed with culture media, inoculum size, incubation period, etc. The other possibilities are that the broth inactivates active compounds as they were released by actinomycetes or modify chemically or the component of broth binds to the compound. Similarly, many actinomycetes appear to be poor fermenters (Gurung et al., 2009; Pandey et al., 2011). According to Bushell (1993), antibacterial activities of actinomycetes are often observed on agar but not in liquid media while screening the secondary metabolites.

CONCLUSION

These findings showed that the soil of different locations of Pokhara valley contain diverse actinomycetes strains. 12 out of 27 actinomycetes isolates showed efficient in producing antimicrobial substances. From primary and secondary screening of soil actinomycetes, it unveils that most of the actinomycetes isolates were more effective in contrary to Gram-positive than Gram-negative bacteria. Actinomycetes obtained from agricultural land demonstrated an inhibitory action in contrary to Gram-positive and Gram-negative test organisms. They had higher effects as compared to Gram-positive bacteria, in particular, *Staphylococcus aureus* (ATCC 25923).

ACKNOWLEDGEMENTS

We would like to thank the Centre for Research and Innovation (CRI), Prithvi Narayan Campus, Pokhara for a research grant. We are thankful to Microbiology laboratories of Prithvi Narayan Campus and Lambda Food Lab Pvt. Ltd. for providing space. We would like to acknowledge Mr. Samir Sirpali and Bishal Thagunna for their help in the sample collection.

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