

Isolation and screening of soil *Actinomycetes* as source of antibiotics active against bacteria

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Abstract- A total of 117 actinomycetes strains were isolated from the wasteland alkaline and garden soil samples of the Ghaziabad and screened for their anti-bacterial activity. They were evaluated for their inhibitory activities on four test microorganisms. Fifteen actinomycetes isolate which exhibited antimicrobial activity against at least two of the test organisms and were characterized by conventional methods. The cultural characteristics of isolates were also studied in different culture media. The results indicated that six isolates were highly active against *Staphylococcus aureus* strains. Seven isolates were highly active with an inhibition zone more than 20 mm in diameter. Most of the isolates inhibited growth of the Gram negative bacteria tested. All the antibiotic producing actinomycetes were isolated at different temperatures from non agricultural wasteland alkaline soil and compost rich garden soil. Fifteen isolates showed activity against bacteria in which most of them from wasteland alkaline soil where the less interference by human for agriculture or other purpose. These microorganisms may have capability to produce some of the most important medicines ever developed.

Key words: Antimicrobial activity, antibiotics, drug resistance, soil sample, *Staphylococcus aureus*

Introduction

Actinomycetes are the most widely distributed group of microorganisms in nature which primarily inhabit the soil [1]. They have provided many important bioactive compounds of high commercial value and continue to be routinely screened for new bioactive compounds. These searches have been remarkably successful and approximately two thirds of naturally occurring antibiotics, including many of medical importance, have been isolated from actinomycetes [2]. Almost 80% of the world's antibiotics are known to come from actinomycetes, mostly from the genera *Streptomyces* and *Micromonospora* [3]. According to the World Health Organization, over-prescription and the improper use of antibiotics has led to the generation of antibiotic resistance in many bacterial pathogens. 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 [1]. Serious infections caused by bacteria have become resistant to commonly used antibiotics and become a major global healthcare problem in the 21st century [4]. *Staphylococcus aureus*, for instance, a virulent pathogen that is responsible for a wide range of infections, has developed resistance to most classes of antibiotics [5]. Clinicians and public health officials have faced hospital acquired drug resistant *S. aureus*, which

also bears resistance to many antibiotics. Hence there is need to rediscover new drugs active against these drug resistance pathogens. Majority of the actinomycetes in soil that are potential drug sources remain uncultivable, and therefore inaccessible for novel antibiotic discovery. Goodfellow and Haynes reviewed the literature on isolation of actinomycetes and suggested that only 10% of the actinomycetes are isolated from nature [6]. Most of the antibiotics in use today are derivatives of natural products of actinomycetes and fungi [7-8]. Actinomycetes can be isolated from soil and marine sediments. Although soils have been screened by the pharmaceutical industry for about 50 years, only a small fraction of the surface of the globe has been sampled, and only a small fraction of actinomycetes taxa has been discovered [9-10]. The present study was undertaken to isolate actinomycetes from the soil samples of wasteland and garden of Ghaziabad and to assess their anti-bacterial properties. The resistance problem demands that to discover new antibacterial agents effective against pathogenic bacteria resistant to current antibiotics. So we need to screen more and more actinomycetes from different habitats for antimicrobial activity in hope of getting some actinomycetes strains that produce antibiotics that have not been discovered yet and active against drug resistant pathogens.

Materials and methods

Soil sample collection:

Soil samples were collected from different wasteland and garden habitats of Ghaziabad. Each collection was made from 10-15 cm depth of the soil [11]. These were air-dried for 1 week [12], crushed and sieved. The sieved soils were then used for actinomycete isolation.

Isolation and culture condition:

For each collected sample, 1g of the soil were suspended in 100 ml of physiological water (NaCl 8.5 g/l) then incubated in an orbital shaker incubator at 28 °C with shaking at 200 rpm for 30 min. Mixtures were allowed to settle, and serial dilutions up to 10⁻⁵ were prepared using sterile physiological water and agitated with the vortex at maximum speed. An aliquot of 0.1 ml of each dilution from 10⁻² to 10⁻⁵ was taken and spread evenly over the surface of actinomycetes isolation agar and starch casein agar medium. The both media are added to rifampicin (2.5 mg/ml) and amphotericin B (75 mg/ml) to inhibit bacterial and fungal contamination, respectively. Plates were incubated at 28 and 37 °C, and monitored after 48, 72, and 96 h. Repeated streaking on starch casein agar plates led to purify bacterial colonies that showed an actinomycetes like appearance. The isolated strains are preserved at 4 °C during two months and maintained for longer period by serial subculture.

Gram staining

A smear of culture was taken in a clean glass slide and heated gently over a flame. The smear was covered with a thin film of crystal violet for 1 min and washed gently in slow running tap water. Gram's iodine solution was flooded over the smear for 1 min and washed with tap water. Alcohol was used to decolorize the smear until the violet color ceased to flow away. The slide was washed with water and counter stain safranin was flooded over the smear for 2 min, then the slide was washed, drained, air dried, and viewed under microscope. The culture retaining the violet color indicated that it was Gram-positive organism.

In vitro screening of isolates for anti-bacterial activity

Morphologically distinct actinomycete isolates were selected for anti-bacterial activity screening against the pathogenic test organisms by conventional spot inoculation method [13] and single line streak method [14] on agar medium. In spot inoculation method, pure actinomycetes isolates were spot inoculated on starch casein agar medium. The plates were incubated at 28 °C for six days, and then inverted for 40 min over chloroform in fumehood. Colonies were then covered with a 0.6% agar layer of nutrient agar

medium, previously seeded with one of the test organisms. The antimicrobial activity was observed after 24 h incubation at 37 °C. In single line streak method, pure actinomycetes isolates were inoculated in a single streak down the middle of a plate of screening media and incubated at 28 °C for 4 days for the production of any antibiotics. A single streak of each test organism was added perpendicular to the actinomycetes streak. Test organisms were placed perpendicular to culture streak. The plates were incubated for 24 h at 37 °C.

Test organisms

The test organisms used in present study were the Gram positive bacteria *Staphylococcus aureus* (MTCC 737), *Bacillus subtilis* (MTCC 121) and the Gram negative bacteria *Escherichia coli* (MTCC 118) and *Pseudomonas putida* (MTCC 1194).

Results and discussion

Soil samples were collected from wasteland alkaline soil and garden soil of Ghaziabad region of Uttar Pradesh, India. One gram of soil sample was dried and taken for isolation of actinomycetes. The suspected 117 actinomycetes were isolated at two different temperatures (28 & 37 °C) in which 71 (60.68%) from wasteland soil and 46 (39.31) from garden soil and inoculated on the starch casein agar medium for purification. And the pure colonies were maintained in the starch casein agar medium slant at 4 °C. All the 117 cultures were screened against bacteria but only the 15 isolates showed the antibacterial activity and were designated as WND1, 2, 3, 4, 5, WNA1,2,3,4 GRN1,2,3,4 and GPN1,2 (Table 1). They were also studied for culture characteristics (Table 2). This study was undertaken with an aim of isolation and screening of actinomycetes in wasteland soil and garden soil of Ghaziabad region at two different temperatures and selecting the isolates with antibacterial activity. Using the selective media and cultivation conditions described previously a total of 117 different actinomycetes isolates were recovered from 20 soil samples that were collected from wasteland soil and garden soil of Ghaziabad, Uttar Pradesh. The soil of wasteland from Dasna and Adhyatmik nagar gives the higher number of actinomycetes isolates (38 and 33 isolates, respectively) with respect to garden soils (Table 1). All isolates grew on starch casein agar media showing morphology typical of actinomycetes, since the colonies were slow growing, aerobic, glabrous, folded and with aerial and substrate mycelia of different colors. All actinomycetes isolates were Gram-stain positive. The cultural characteristics (Pigment production), morphological

characteristics of the different actinomycetes isolates are presented in Table 2. The colour of the substrate mycelium and aerial spore mass was varied. During screening of new isolates for drug discovery, many potentially interesting microorganisms might be excluded due to their morphological similarities, suggesting similar biochemical behavior; thus, many isolates are lost and only a few of them are finally tested. In this study, the total number of isolated actinomycetes (117) was screened on agar medium and antibacterial activity was observed in 15 (12.82 %) of the isolates and appeared promising (Table 1). In previous studies, it was shown that the isolation rate of actinomycetes with antimicrobial activity is higher than 40% [15] and in others less than 10% [16]. These results confirm that the actinomycetes are able to produce a wide variety of antibiotics with antibacterial activity. Thakur et al. also reported that a total of 110 actinomycetes isolates were isolated from the soil samples collected from the protected forest soil from two states in Northeast India [17]. These were then characterized by conventional methods and assessed for their antagonistic activity preliminary against test microorganisms. Results of the present study also indicate that the higher number of actinomycetes was isolated from wasteland alkaline soil active against bacteria, where the human activity is very less for agriculture or other purpose and these actinomycetes can be useful for many applications such as control of infectious diseases and drug discovery.

Conclusion

Fifteen isolates showed activity against bacteria in which most of them from wasteland alkaline soil where the less interference by human for agriculture or other purpose. These microorganisms produce some of the most important medicines ever developed. They are the source of lifesaving treatments for bacterial and fungal infections. In spite of the tremendous success of the past secondary metabolite research, the number of terrestrial antibiotics seems currently to approach a saturation curve with an apparent limit in the near future. The increasing number of duplications and the urgent demand for new leading antibiotics in pharmacology for the treatment of drug resistant infectious pathogens has enforced the search for metabolites in so far untouched habitats where the human activities are very less. Hence the wasteland alkaline soil is a goldmine of biodiversity has been amply justified by the richness of microbial diversity. Our studies will establish the rich actinomycete diversity of the region, especially the various niche habitats of

Ghaziabad and also help conserve and utilize them in bioindustry. Further intensive studies on the actinobacterial diversity of unique biotopes in Ghaziabad should form an important input into Indian biotech industry.

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Table 1- Total number of actinomycetes isolates with antibacterial activity isolated at different temperatures.

Origin	Isolation Temperature	Total strains isolated	Number of active isolates against bacteria (%)
Wasteland near Dasna	28 °C	22	4 (18.18)
	37 °C	16	1 (6.25)
Wasteland near Adhyatmik Nagar	28 °C	18	2 (11.11)
	37 °C	15	2 (13.33)
	Total	71	9 (12.67)
Garden soil Raj Nagar	28 °C	14	3 (21.42)
	37 °C	8	1 (12.5)
Garden soil Patel Nagar	28 °C	17	2 (11.76)
	37 °C	7	0 (0)
		46	6 (13.04)
Total		117	15 (12.82)

Table 2- Culture characteristics of selective isolates on starch casein agar medium

Origin	Culture code	Color	Mycelium type	Pigment production	Gram's reaction
Wasteland near Dasna	WND1	White	Aerial	Red	+
	WND2	Grey	Aerial	-	+
	WND3	Green	Substrate	Yellow	+
	WND4	White	Aerial	Red	+
	WND5	Yellow	Aerial	Yellow	+
Wasteland near Adhyatmik Nagar	WNA1	White	Substrate	-	+
	WNA2	Grey	Substrate	Red	+
	WNA3	White	Substrate	-	+
	WNA4	White	Aerial	-	+
Garden soil	GRN1	Green	Aerial	Red	+
Raj Nagar	GRN2	White	Substrate	-	+
	GRN3	White	Aerial	Red	+
	GRN4	Grey	Substrate	-	+
Garden soil Patel Nagar	GPN1	Yellow	Aerial	Yellow	+
	GPN2	White	Aerial	Red	+

Table 3- Antibacterial activity of isolates (single line streak and Spot inoculation method)

Culture Code	Tested microbes			
	MTCC 737	MTCC 121	MTCC 118	MTCC 1194
WND1	–	–	+	–
WND2	–	–	+	+
WND3	+	+	–	+
WND4	–	–	+	–
WND5	–	+	–	+
WNA1	–	+	+	+
WNA2	+	–	+	–
WNA3	+	+	–	+
WNA4	–	–	+	+
GRN1	–	–	+	–
GRN2	+	–	–	+
GRN3	–	+	+	–
GRN4	+	–	–	+
GPN1	–	–	+	–
GPN2	+	–	–	+

: no activity; +: activity.