Journal of Global Biosciences
ISSN 2320-1355
Volume 6, Number 6, 2017, pp. 5070-5076
Website: www.mutagens.co.in

Research Paper

SCREENING, ISOLATION AND IDENTIFICATION OF ANTIBIOTICS PRODUCING MICROORGANISMS FROM POTENTIAL SOIL SAMPLES OF WESTERN REGIONS OF MUMBAI

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Abstract

Antibiotics are one of the most important commercially exploited secondary metabolites produced by bacteria and employed in a wide range. Bacteria are easy to isolate, culture, maintain and to improve their strain. Bacillus species being the predominant soil bacteria because of their resistant endospore formation and production of vital antibiotic like bacitracin etc. are always found inhibiting the growth of the other organisms. In the present research study, soil bacteria with the antibiotic activity was screened and isolated using primary screening method i.e. Crowded Plate Technique. The colonies showing a zone of inhibition were screened using the secondary screening method i.e. Wilkins's Overlay Method where the inhibitory activities of the micro-organisms were checked against some of the important opportunistic micro flora like *Staphylococcus aureus* and *Escherichia coli.* Some antibiotic producing organisms and some organic acid producing organisms were isolated using these two techniques. The bacteria isolated were: Gram positive cocci, Gram negative coccobacilli and Gram positive rods. The result so obtained was that antibiotic producing organisms were isolated from the soil sample which may be useful for scientists to carry out research work and cure diseases which are now prevalent.

Key words: Antibiotic producers, primary and secondary screening methods.

INTRODUCTION

In the beginning of 20th century, the idea of growth inhibition of one micro-organism present in the vicinity of other one came into existence. Later, it was demonstrated that growth inhibition of micro-organism was mediated by secretion of certain toxic metabolites which was termed as antibiotic. In nature, there is universal distribution of antibiosis among the micro-organisms owing to which they are involved in antagonism [1].

Those micro-organisms which have capacity to produce more antibiotics can survive for longer time than the other producing antibiotics in fewer amounts. However, antibiotics produced by micro-organisms have been very useful for the cure of certain human diseases caused by bacteria, fungi and protozoa.

There are so many different potential sources where antibiotics can be discovered, such as medicinal herbs [2] and soil. However, soil, which is a naturally occurring loose mixture of mineral and organic particles [3] still remains the most important target for most researchers in their efforts to discover novel antibiotics which have pharmaceutical values. This is because many microbes especially bacteria that reside in soil have the ability to produce biologically active secondary metabolites such as useful antibiotics. In their natural habitats, bacteria utilize the antibiotics they produce as protective substances by rendering the invasion of other bacterial species. Protection is not the only function of antibiotics. Hence, according to Linares *et al.*, antibiotics also act as signalling molecules that bacteria use as a means of communication between cells.Antibiotic producers are isolated from soil for the production of new varieties of antibiotics for the welfare of human beings. Screening is the use of highly selective procedures to allow the detection and isolation of only those micro organisms of interest from among a large microbial population.

Primary screening allows the detection and isolation of micro organisms that possess potentially interesting industrial applications. The Crowded Plate Technique is the simplest screening technique employed in detecting and isolating antibiotic producers. It consists of preparing a series of dilutions of the source material of the antibiotic producing micro organisms, followed by spreading the dilution on the agar plates. Colonies showing antibiotic activity is indicated by the presence of a zone of inhibition surrounding the colony. Such a colony is sub-cultured to a similar medium, and purified.[4]

Primary screening is usually followed by a secondary screening to further test the capabilities of and gain information about these micro organisms. Secondary screening can be qualitative or quantitative in its approach. The secondary screening technique used for the isolation of antibiotic producers from soil is the Wilkin's Overlay Method. A Wilkin's medium contains a pH indicator dye called Bromo-thymol blue which is green in colour at neutral pH but yellowish at acidic pH and blue in alkaline pH. This method differentiates antibiotic producers from the acid producers. Those colonies that produce antibiotics give zone of inhibition against sensitive organisms without changing colour surrounding it while in case of acid producers we may find zone of inhibition due to fall in pH along with yellowish area due to high acid production.

The number of multi-drug resistance pathogenic microbes has increased over time and there are only limited therapeutic drugs that are applicable to combat these pathogens. Therefore, there is a need for continuous discoveries of new antibiotics in order to make treatments under antibiotics remain effective. This study is an attempt to discover novel antimicrobial agents from soil. Therefore, the aim of the present study was to screen and isolate antibiotic producing micro-organisms from soil samples in western region, Mumbai. The outcome of this finding may be important to give direction for researchers and for future treatment of multidrug resistant human pathogens.

MATERIALS AND METHODS

Study area and period

The study area was located at different regions of western Mumbai. 10 sites were randomly selected for study shown in TABLE 1. Study period: September 2016 – January 2017.

TABLE 1 - Description of soil samples from different sites in the western region

Soil	Area	Location	Depth
Sample 1	Rhizosphere of papaya tree	Society garden in Andheri west	10 cm
Sample 2	Botanical garden	Vile parle west	5 cm
Sample 3	Agricultural field	Vasai	20 cm
Sample 4	Rhizosphere of banyan tree	Society garden of Borivali west	15 cm
Sample 5	Field near an industry	Virar	10cm
Sample 6	Plantation field near industrial area	Palghar	15 cm
Sample 7	Agricultural field	Virar	12 cm
Sample 8	Garbage dumping ground	Vile parle west	10 cm
Sample 9	Botanical garden	Santacruz west	11 cm
Sample 10	Rhizosphere of ashoka tree	Mira road east	7 cm

Sample Collection:

The debris from the soil surface of the sampling sites was first removed before commencement of sampling. A hand trowel sterilized with 70% ethanol was used for the collection of soil samples from the rhizosphere region of different regions of western Mumbai. The sample was collected in a sterile polythene bag and taken to the laboratory for analysis. The soil sample was sieved to extract fine soil particles which were then serially diluted for isolating micro-organisms that are potential antibiotic producers.

Screening Of Soil Isolates For Antibiotic Activity:

Primary Screening: Crowded Plate Technique One gram of soil was weighed and mixed in 10ml of sterile distilled water to get 1:10 dilution, then thoroughly mixed by vigorous shaking. After allowing the sediment to settle, supernatant was used for subsequent dilutions. Dilutions were prepared by taking 1ml of stock solution (having 1:10 dilution) and transferring into 9ml sterile distilled water in another test tube to give 1:100. This process of transfer from preceding tube continued till 1:100000 dilutions were achieved. 0.1ml of soil inoculums from each dilution was taken and inoculated separately onto petri plates with nutrient agar media of pH 7-7.2. Plates were incubated at room temperature for 2 days in inverted position. Colonies that produced zone of clearance were sub cultured in nutrient agar and their pure cultures were stored in 4 ^{o}C until further use.

Secondary Screening- Wilkins Overlay Method

For Wilkin's agar technique, the plates from Crowded Plate Technique showing colonies with zone of inhibition were selected and were overlaid with molten Wilkins's agar with 0.2 ml of 24-hours old culture of *S.aureus* and *E.coli* and then were incubated at 37

°C for 24 hours The colonies showing zone of inhibition against the test organism were isolated. Its morphological and cultural characteristics were studied.

Characterization and Identification of antibiotic producers Isolates:

The characterization and subsequent identification of isolates were carried out based on macroscopic and microscopic observation.[5]. (TABLE 2)

RESULTS

Crowded Plate Technique: Antibiotic producing micro-organisms were screened by Crowded Plate Technique and were observed for the presence of any colony with a clear zone around it. Plates with approximately 300-400 colonies were selected that showed crowd but well demarcated colonies. Out of 10 soil samples which were selected for the present study 4 soil samples showed four different types of colonies showing clear zone of inhibition around them (TABLE 2).These colonies were isolated and their pure cultures were stored at 4 °C till further use. **Wilkins Overlay Method:** The four soil samples which were screened using the primary screening technique that is Crowded Plate Technique showed zone of inhibition against the test organism *Staphylococcus aureus* and *E.coli* on doing the secondary screening technique that is Wilkins Overlay Method. Yellow colouration due to a change in pH was observed in the Wilkin's agar due to organic acid producing organisms in dilution 10^{-4} of sample 1 (TABLE 2).

Characterisation of bacterial isolates – In the screening of the soil sample, three bacterial species were identified. The bacterial isolates include: Gram positive cocci, Gram negative coccobacilli and Gram positive rods (TABLE 3).

TABLE 2- Comparison of the antagonistic activity of isolates against the test organism during secondary screening

SOIL SAMPLE	DILUTION	CROWDED PLATE TECHNIQUE	WILKINS OVERLAY METHOD
SAMPLE 1 Rhizosphere of Papaya Tree	10^{-1}	Antibiotic producers were obtained on primary screening showing zone of inhibition.	Antibiotic producers screened using crowded plate technique showed zone of inhibition against test organism *E.coli*.
	10^{-2}	Antibiotic producers were obtained on primary screening showing zone of inhibition.	Antibiotic producers screened using Crowded Plate Technique showed zone of inhibition against test organism *S.aureus*.
	10^{-4}	No antibiotic producers were found.	Some zones of inhibition could also be due to presence of organic acids producers that were obtained
SAMPLE 2 Botanical Garden	10^{-1}	Antibiotic producers were obtained on primary screening showing zone of inhibition.	Antibiotic producers screened using Crowded Plate Technique showed zone of inhibition against test organism *S.aureus* .
SAMPLE 9 Botanical Garden	10^{-1}	No antibiotic producers were found	–
	10^{-2}	Antibiotic producers were obtained on primary screening showing zone of inhibition.	Antibiotic producers were screened using crowded plate technique showed zone of inhibition against test organism *E.coli*.
	10^{-3}	Antibiotic producers were obtained on primary	Antibiotic producers were

		screening showing zone of inhibition.	screened using crowded plate technique showed zone of inhibition against test organism *E.coli.*
	10^{-5}	Antibiotic producers were obtained on primary screening showing zone of inhibition.	Antibiotic producers were screened using crowded plate technique showed zone of inhibition against test organism *E.coli.*
SAMPLE 10 Rhizosphere of Ashoka Tree	10^{-1}	Antibiotic producers were obtained on primary screening showing zone of inhibition.	Antibiotic producers were screened using crowded plate technique showed zone of inhibition against test organism E.coli.
	10^{-2}	Antibiotic producers were obtained on primary screening showing zone of inhibition.	Antibiotic producers were screened using crowded plate technique showed zone of inhibition against test organism *S.aureus.*

KEY: "-" means no inhibition was seen against the test organism

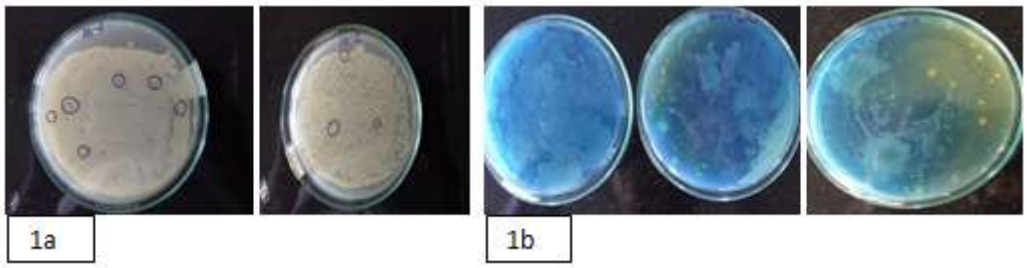

Figure 1:

(a) Shows zone of inhibition by antibiotic producing micro-organisms in Crowded Plate Technique in 10^{-1} to 10^{-2} dilutions of the soil sample 1.

(b) Shows zone of inhibition by antibiotic producing micro-organisms in 10^{-1}and10^{-2} dilution and organic acid producing micro-organisms in 10^{-4} dilution in Wilkin's Overlay Method of the soil sample 1.

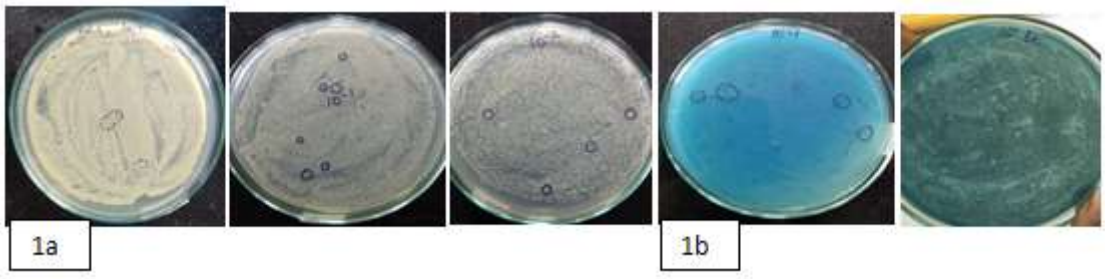

Figure 2:

(a) Shows zone of inhibition by antibiotic producing micro-organisms in Crowded Plate Technique in 10^{-1} to 10^{-2} dilutions of the soil sample 10.

(b) Shows zone of inhibition by antibiotic producing micro-organisms in 10^{-1}and10^{-2} dilution and organic acid producing micro-organisms in 10^{-4} dilution in Wilkins Overlay Method of the soil sample 1.

TABLE 3- Characteristic features of the antibiotic bacterial isolates from soil.

COLONY CHARACTERISTICS	ISOLATE-1	ISOLATE-2	ISOLATE-3
MACROSCOPIC			
Colour	Colourless	White	Buff
Size	1mm	2mm	1mm
Shape	Circular	Concave	Circular
Opacity	Opaque	Opaque	Opaque
Texture	Smooth	Smooth	Smooth
Consistency	Butyrous	Butyrous	Butyrous
Margin	Entire	Entire	Entire
MICROSCOPIC			
Gram nature	Gram positive	Gram negative	Gram positive
Morphology	Cocci	Coccobacilli	Rods

DISCUSSION

In searching for new antibiotics, relatively simple and rapid methods have been developed for screening micro-organisms for antibiotic producing ability. Soil samples are commonly employed in the antibiotic producing organism. Antibiotic isolated may be bactericidal or bacteriostatic in nature. Production of antibiotic by micro-organisms from soil is affected by many factors including nitrogen and carbon source. Temperature variation also affects the synthesis of antibiotic. Antibiotic is synthesis during the idophase stage of the growth and there is no correlation between the bacteria sporulation and antibiotic production. Antibiotics are the most important bioactive compounds for the treatment of infectious diseases. But now, because of the emergencies of multi-drug resistant pathogens, there are basic challenges for effective treatment of infectious diseases. Thus, due to the burden of high frequency of multidrug resistant pathogens in the world, there has been increasing interest for searching effective antibiotics from soil micro-organisms in diversified ecological niches [6].

In the present study, the randomly selected soil samples from western region of Mumbai were taken for isolation of antibiotic producing micro-organism by primary and secondary screening methods and three antibiotic producing bacterial isolates were obtained from soil. The successful isolation of micro-organism from environmental samples requires an understanding of the potential soil sample areas and environmental factors affecting their growth. Previous studies showed that selection of different potential areas such as rhizosphere soil samples were an important activity for isolation of different types of potent antibiotic producing soil micro-organism [7].

The present study of primary screening using Crowded Plate Technique and secondary screening using Wilkins Overlay Methods indicated that the three bacterial isolates showed potential antimicrobial activity against test organism. This result (30%) is higher than 21.88%and less than 59.09% from previous reports [6].

Soil samples were chosen as samples for analysis in this study based on the likelihood of the presence of native micro-organisms that have not previously been studied by other researchers. These native soil micro-organisms have higher probability to produce novel antimicrobial substances. In this study, a total of 10 samples from different depths were successfully isolated via preliminary screening and selection. Among these isolates, only 3 were selected to undergo secondary screening. The reduction in the isolates number was because some of the isolates were appeared to be the same species based on their physical appearances and growth morphologies. From this study, it was

noticed that the number of microbes that could be obtained were decreasing with the depth of the soil. This indicated that the depth of the soil and its organics constituents did influence the population and distribution of soil microbes [8,9]. According to Sewell[6] bacteria can be found most abundantly in the first three inches from the soil surface with the number decreasing and only few amount of bacteria presence when the depth reached six feet. Besides, the results obtained from this study has shown to be similar with the research conducted by [7,10] where they found out that there was greater amounts of bacterial and fungal populations in the top soil (0-10 cm) if compared to that of other depths. This might be due to the higher organics.

INNOVATIONS AND APPLICATIONS

Thus, this study has screened antibiotic producing bacteria from different soil samples which may give an important clue and lead towards finding novel secondary metabolites (antibiotics) which can be up scaled or optimized for treatment of drug-resistan tpathogens. The results of this study may initiate many researchers in this particular area to look for different sources of samples for searching new bioactive secondary metabolites like antibiotics.

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