Research Article

Isolation of Antibiotic Producing Bacteria from Soil Samples Collected from a Biodiversity Hotspot of North East India

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ABSTRACT

Soil is considered as one of the most suitable environment for microbial growth. The microbial population in soil is very diverse. There is great opportunity for discovering new groups of microorganisms in soil which may possess industrial and clinical importance. In this present study Rani Reserve forest, a Biodiversity Hotspot of North East India had been identified as the site for sample collection. The selected area is away from human settlement and hence the natural diversity is expected to undisturbed. The soil of Rani Reserve forest is expected to be a natural habitat for a large number of microbial population of varied strains. 10 bacteria were isolated from the soil of Rani Reserve forest by serial dilution followed by spread plate method. The bacterial samples were identified by conventional methods of Colony characteristics, staining techniques and biochemical test. After that they were tested against two Gram +ve (*Staphylococcus aureus* and *Streptococcus pyogenes*) and three Gram –ve (*E.coli, Pseudomonas aeruginosa, Klebsiella*) pathogens as they are found to be commonly associated with secondary infections. Among the isolates 7 strains showed positive results against the test strains. For correlation and confirm identification the isolates which showed antagonistic activity against the test strains were identified by VITEK 2 instrument.

Keywords: *Staphylococcus aureus*, *Streptococcus pyogenes, E.coli, Pseudomonas aeruginosa, Klebsiella,* Antibacterial activity, Bio-chemical test, VITEK[R] 2.

INTRODUCTION

Microorganisms are the dominant component of any ecosystem on Earth and many ecosystems are exclusively microbial. Microbes are found in a wide range of environmental conditions. Soil is a complex and dynamic biological system, which inhabits diversified forms of microorganisms. The most abundant group of organisms in the soil are the bacteria, exceeding both in numbers and in the variety of their activities. Bacteria that are isolated from soil are used in different field such as Agriculture, Industry and for the production of antibiotic and medicines.

Antibiotics are antimicrobial agents produced by bacteria, fungi or of synthetic in nature. Production of antimicrobial compounds seems to be a general phenomenon for most bacteria. If the number of antibiotic producing bacteria can be increased, then it will be beneficial for the mankind coming from medical field to the scientific researchers.

The isolation and screening of bacteria from diverse habitats had led to the discovery of many novel and useful secondary metabolites. The increasing spectrum of antibiotic resistance is a major cause of concerned in the field of health science. More emphasis is required for discovery of newer antibiotics. Therefore isolation of new antibiotic producing bacteria will be beneficial for medical science.

In this present study Rani Reserve forest is choosen as target site because the Rani Reserve forest is a tropical rain forest and the human population is less near this area. As the probability of human disturbances is less, the soil of Rani Reserve forest is expected to inhabit a large number and large variety of microorganisms.

Objective of the Study

1. Isolation of bacteria from soil sample

2. Identification and characterization of isolated bacteria by standard protocol

3. Antibacterial assay of isolated bacteria against Gram positive bacteria (*Streptococcus pyogenes, Staphylococcus aureus*) and Gram negative (*Escherichia coli, Pseudomonas aeruginosa, Klebsiella sp.)* test strains

MATERIALS AND METHODS

Bacterial Strains

List of pathogenic strains against which antimicrobial test was performed.

Sl. No.	TEST STRAINS	ACCESSION NO
1	*Staphylococcus aureus*	MTCC96
2	*Streptococcus pyogenes*	CP008926
3	*E. coli*	MTCC739
4	*Klebsiella*	KT001920
5	*Pseudomonas aeruginosa*	MTCC2453

Media

Media used for various experiments

Sl. No.	Media	Company	Purpose
1.	Nutrient agar (N.A)	Hi media	Growth and isolation of colony
2.	Nutrient broth (N.B)	Hi media	Growth
3.	Muller Hinton Agar (MHA)	Hi media	Growth and antibacterial activity
4.	Peptone broth	Hi media	Bio chemical test

Chemicals

Chemicals used for various biochemical experiments

Sl. No.	Name of chemical	Purpose
1	Iodine solution	Starch hydrolysis
2	Methyl red	MR test
3	Kovak's reagent	Indole test
4	H_2O_2	Catalase test
5	Gram's iodine	Gram test
6	Malachite Green	Endospore stain
7	Indian ink	Capsule stain

METHOD

Collection of Sample

Soil samples were collected from four different sites of Rani Reserve Forest. Soil samples were collected in the sterile specimen containers and were transported to the laboratory.

Sl. No.	AREA	DEPTH (cm)
1	Dry	0-30
2	Wet	0-30
3	Dry	60-100
4	Wet	60-100

Isolation of Bacteria from the Soil Samples

Bacterial colonies were isolated from soil sample by serial dilution method followed by spread plate and streak plate method.

Identification of bacteria by staining and biochemical test

The isolated bacterial species were identified by different staining technique such as Gram stain, capsule staining and endospore staining. They are also characterized by different biochemical test such as Indole, Methyl red, Citrate, Catalase, Triple sugar ion and Starch hydrolysis test.

Antibacterial assay

The antibacterial assay was performed by Agar well diffusion method (2).

In this test nutrient broth is prepared. The bacterial cultures were inoculated in the broths and incubated for 24 hrs.

For accomplishing antibiotic assay, Muller Hinton Agar (MHA) medium was prepared and sterilized and poured into petri plates under aseptic conditions. After solidification test organisms were swabbed on the MH plates with a sterile cotton swab and left for 10 min. After drying the wells were made on the plates and the broth cultures were loaded into the wells with the capacity of 50µl in each well. The plates were incubated 24 hrs. at 37°c. After 24 and 48 hrs the inhibition zones were observed and recorded.

RESULTS

Isolation of bacteria from soil sample

A total of 11 bacterial isolates were obtained from the soil samples collected from the four locations of Rani reserve forest by serial dilution followed by spread plate techniques. Further individual colonies with distinctive colony characteristics were streaked onto separate plates for obtaining pure culture and were names R1, R2, R3, R4, R5, R6, R7, R8, R9, R10, R11After incubation it was observed that R5 did not survive in the Nutrient agar plate. Therefore R5 couldn't be studied and a total of 10 isolated were processed for further studies

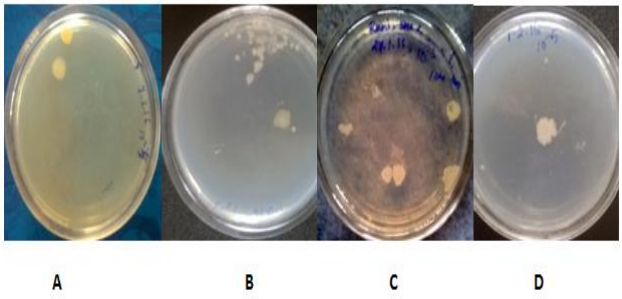


Figure 1: (A-D) Mixed culture of bacterial Colonie isolated from the dilution of 10^{-3}, 10^{-4} and 10^{-5}

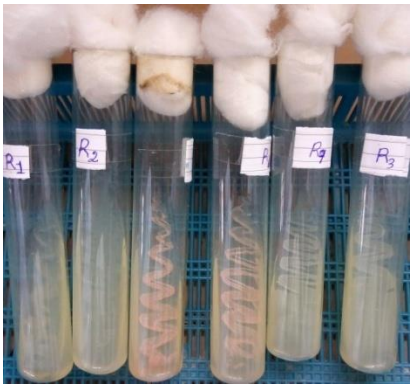


Figure 2: Pure culture Slant preparation of isolated bacteria

Antibacterial activity

Bacterial samples R1, R2, R4, R6, R7, R8 and R10 showed antibacterial activity against test bacterial strains and are as tabulated in table 1 and table 2.

Table 1: Bacterial sample showing results against Gram positive test bacteria

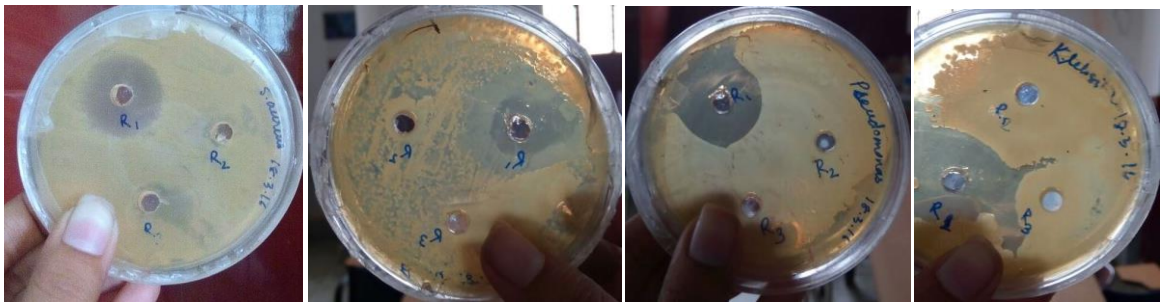
Sl.no	Sample name	*Staphylococcus aureus* (mm in hrs.)	*Streptococcus pyogenes* (mm in hrs.)
1.	R1	23 (24hrs) 28 (48hrs)	NZ
2.	R2	NZ	40 (36 hrs)
3.	R3	NZ	NZ
4.	R4	25 (48 hrs)	NZ
5.	R6	27 (48 hrs)	NZ
6.	R7	NZ	48 (24 hrs)
7.	R8	NZ	28 (36 hrs)
8.	R9	NZ	NZ
9.	R10	19 (24 hrs)	47 (24 hrs)
10.	R11	NZ	NZ

* Note: NZ: No Zone

Table 2: Bacterial sample showing results against Gram negative test bacteria

Sl. No	Sample name	*E.coli* (mm in hrs.)	*Klebsiella sp.* (mm in hrs.)	*Pseudomonas aerogenosa* (mm in hrs.)
1.	R1	25 (24hrs)	20 (24hrs)	25 (24hrs) 28 (48 hrs)
2.	R2	NZ	NZ	NZ
3.	R3	NZ	NZ	NZ
4.	R4	NZ	NZ	NZ
5.	R6	NZ	NZ	NZ
6.	R7	NZ	NZ	17 (48 hrs)
7.	R8	NZ	NZ	NZ
8.	R9	NZ	NZ	NZ
9.	R10	NZ	NZ	NZ
10.	R11	NZ	NZ	NZ

*Note: NZ – No Zone



A B C D

Figure 3: (A-D): Results showing antibacterial activity of R1 sample against S.aureus (A), E.coli (B), P.aeruginosa (C) and Klebsiella (D)

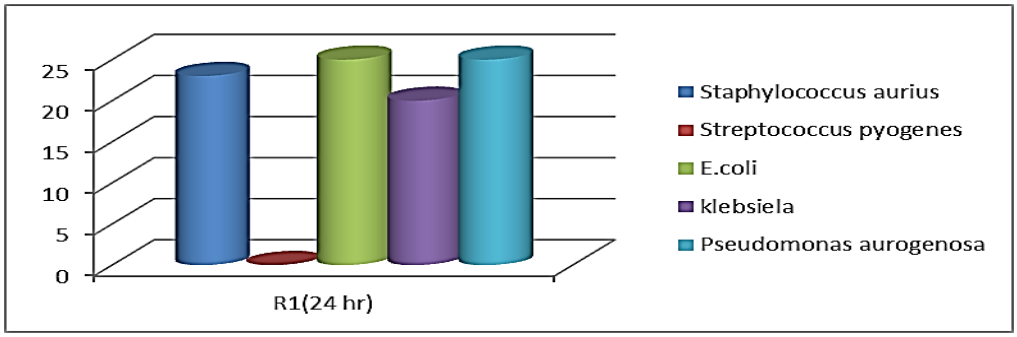


Figure 4: Graphical representation of antibacterial activity of R1 sample against *S.aureus, E.coli, P.aeruginosa* and *Klebsiella*

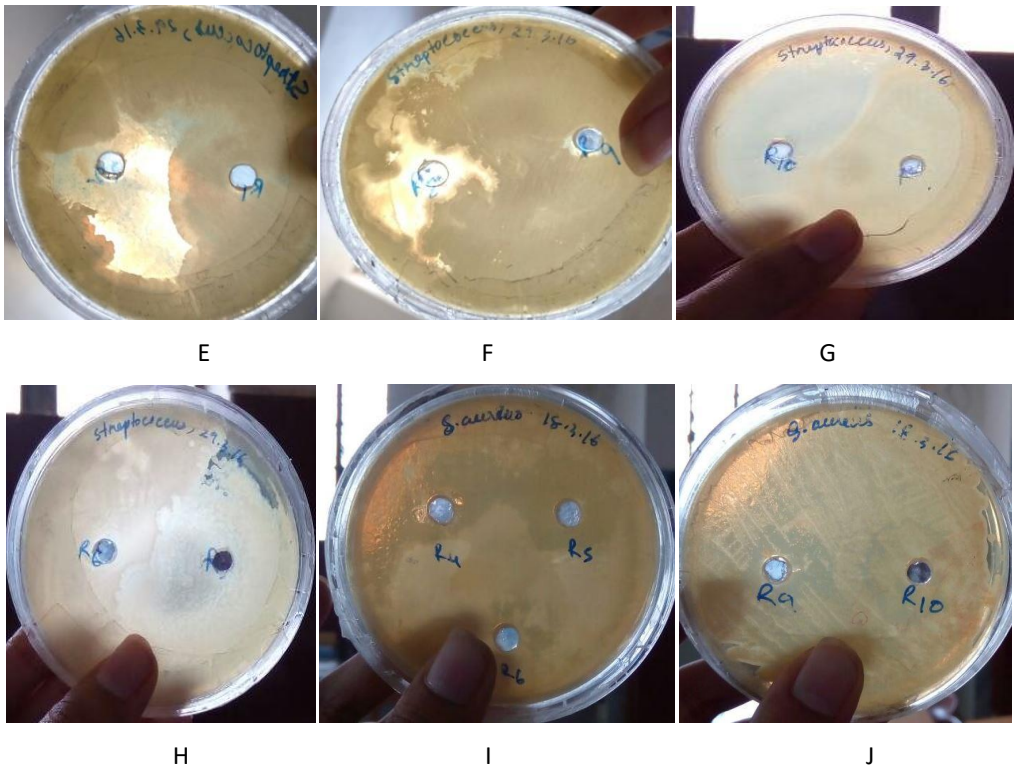


Figure 5: (E-H): Showing antibacterial activity of R2, R8, R10 and R7 against *S.pyogenes. I-J):* Showing antibacterial activity of R4, R6, and R10 against *S.aureus*

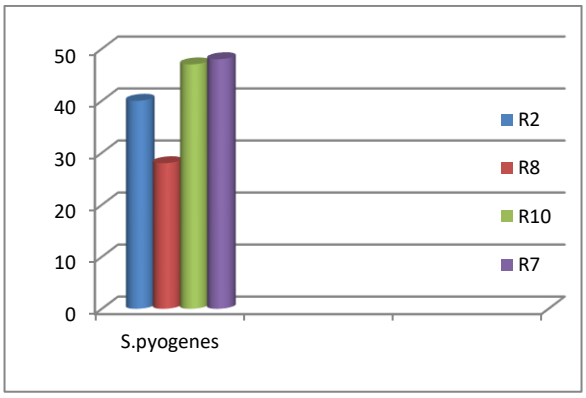


Figure 6: Graphical representative

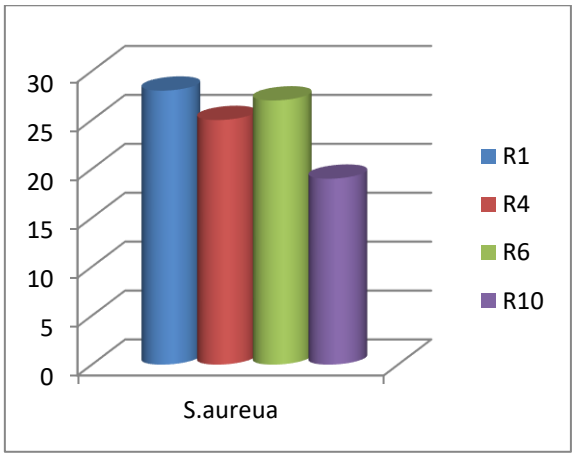


Figure 7: Graphical representative showing antibacterial activity of R1, R4, R6 and R10 against *S.aureus*. antibacterial activity of R2, R8, R10 and R7 against *S.pyogenes*

Identification of isolated bacteria

A total of 10 isolates were isolated from the soil samples collected from the four sites and 7 out of the 10 isolates were seen to possess antagonistic activity against the test strains. In this study, various biochemical tests were performed like indole test, MR test, citrate test, TSI test, catalase test for the identification of bacterial samples. Basic staining techniques such as Gram stain, negative stain and endospore staining were done for their characterization. For confirmed identification Bacterial isolates which showed antibacterial activity against pathogenic test strains were identified by VITEK 2 instrument at microbiology lab of down town hospital. The organisms are identified as

R1- *Bacillus subtilis*

R2- *Staphylococcus gallinarum*

R4- *Kocuria kristinae*

R6- *Bacillus sp.*

R7- *Staphylococcus klossii*

R8- *Bacillus sp*.

R10- *Bacillus megaterium*

DISCUSSION

Antibiotics are antimicrobial agents produced by bacteria, fungi or of synthetic in nature. Production of antimicrobial compounds seems to be a general phenomenon for most bacteria. Soil is the major reservoir of microorganisms that produce antibiotics. Considering

that soil is densely packed with microorganisms, it is not a wonder that many bacterial and fungal species have evolved over the eons to develop ways of inhibiting their neighbors for the benefit of their own growth. An antibiotic produced by a microbe can inhibit many other microbes. The bacterial genera *Bacillus* and *Streptomyces* along with the fungal genera *Penicilium* and *Cephalosporium* are commonly found in soil. Soil has historically been used to find new antibiotic producers, at present many of the 'old' antibiotics are being manipulated in the laboratory and chemically modified to form new versions of older antibiotics. The isolation and screening of bacteria from the soil of diverse habitats had led to the discovery of many novel and useful secondary metabolites. The increasing spectrum of antibiotic resistance is a major cause of concern in the field of health science. More emphasis is required for discovery of newer antibiotics. Therefore, isolation of new antibiotic producing bacteria will be beneficial for medical science.

The present study is aimed at isolation and characterization of antibiotic producing bacteria from soil samples collected from Rani Reserve forest. The Rani Reserve forest has been selected as target site keeping the following point of view:

a. It is a tropical rain forest

b. The human population near this area is less

c. As it is rich in nutritional source for microbial growth, it favors growth and multiplication of diverse group of microorganisms including saprophytic bacteria.

In this present study, nutrient agar (NA) medium has been used for isolation of bacteria from the soil sample of Rani Reserve forest. After serial dilution of the sample collected from 4 different site at Rani reserve forest, 10 different colonies were obtained which are shown in the Figure 1(A-D). They are named as R1, R2, R3, R4, R6, R7, R8, R9, R10 and R11.They were tested by agar diffusion method on the Muller Hinton agar (MHA) medium against 5 pathogenic test bacterial strains for their ability to produce any antibacterial substance. The results were recorded based on the ability of the isolates to inhibit the growth of the test strains. Among all these 10 isolates, R1, R2, R4, R6, R7, R8 and R10 isolates showed antibacterial activity against the test strains of pathogenic bacteria that are *S.aureus, S.pyogenes, E.coli, P.aeruginosa and Klebsiella*, which are shown in the figure3 and figure 5.

In an earlier study of exploration of antibiotic producing bacteria M.E.Umasankar et.al isolated variety of bacteria from the soil of Amirthi Forest, Vellore. In their study 10 soil samples were collected from different area and 50 sp. were isolated. The isolate AF7 and AF8 showed high inhibition against tested Gram negative organisms.

In this study, R1 sample showed zone of inhibition of 28mm against *S.aureus*, 25mm against *E.coli*, 28mm against *P.aeuroginosa* after 48 hrs and 20 mm against *Klebsiella* sp. after 24 hrs. These are shown in the table 1,2 and Figure 3 (A-D). R4, R6 and R10 sample showed 25mm, 27mm and 19mm zone of inhibition respectively against *S.aureus*. R2, R7, R8 and R10 sample showed 40mm, 48mm, 28mm, and 40mm zone of inhibition respectively against *S.pyogenes*.

Bacillus subtilis showed maximum activity against *S.aureus*, *E.coli*, *P.aeruginosa* and *Klebsiella*. It was isolated from the soil of wet area, (depth 0-30 c.m). *Staphylococcus gallinarum*, *Staphylococcus klossii*, *Bacillus sp*. and *Bacillus megaterium* showed activity against *S.pyogenes*. *Kocuria kristinae*, *Bacillus sp*. and *Bacillus megaterium* showed activity against *S.aureus*.

CONCLUSION

Rani Reserve forest was selected as the target site for this study and soil samples of various depths had been taken for isolation of bacteria. 10 bacterial strains were isolated as pure culture from soil samples of Rani Reserve forest. Isolated colonies showed variation indicating microbial diversity at the selected site. Antibacterial activities of the isolates were carried out against the test strains. Among them 7 strains (R1, R2, R4, R6, R7, R8 and R10) showed positive results against Gram positive and Gram negative test strain that are *S.aureus, S.pyogenes, E.coli, P.aeruginosa* and *Klebsiella*. Since the targeted site is undisturbed wild habitat deprived of human population, further studies can lead to identification of more diverse forms of microorganisms with antimicrobial activity. As the bacterial strains isolated from soil from targeted site i.e., Rani Reserve forest showed significant antibacterial activity against the test strains further studies are required for characterization and identification of the component of the isolates that are responsible for their antibacterial activity. Proper identification of such components can lead to development of newer antibacterial drugs in the future which would boast the present day crisis of increasing rate of antibiotic resistance.

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