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A brief research study on novel antibiotic producing isolate from VIT Lake, Vellore, Tamil Nadu

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ABSTRACT

The soil ecosystem has an enormous amount of microorganisms, and some of these microorganisms can potentially be useful to us. *Bacillus* and *Actinomycetes* are the most abundant microorganisms present in soil and both are known to produce antibiotics and inhibit the growth of other microorganisms. However, there are other potential microorganisms which may have such beneficial properties and are unknown to us. In this study, various microorganisms in the soil were screened to isolate an antibiotic producing microorganism. Soil was collected from VIT lake and serially diluted followed by crowded plate technique to screen for antibiotic producing colonies. The colony which was suspected to produce antibiotics was selected and sub-cultured by streaking. Antibiotic-sensitivity test was performed on Mueller-Hinton Agar and the inhibitory effect of the isolated microorganism towards the growth of bacteria was observed. The growth curve of the isolated microorganism was found out. Characterization was done by performing various biochemical tests and staining methods. Antibiotic production media was prepared, and the antibiotic production of the microorganism was plotted against time. From the antibiotic production curve, it was found that the 6th day showed the maximum antibiotic productivity.

INTRODUCTION

Soil is a very diverse ecosystem which supports the growth of countless organisms, be it plants, animals, humans, or microbes. The major biotic component of soil comprises of the microbiota. It harbors millions of microorganisms like bacteria, fungi and *Actinomycetes*. They are the an integral part of every food chain, as they decompose the dead and decaying organic matter in the soil, into simple molecules, which they use for their nutrition. Soil provides optimum conditions such as moisture, temperature, pH and organic matter which are favorable for the growth of microorganisms. These microorganisms produce primary metabolites which are required for various cellular functions like growth and metabolism. They also produce secondary metabolites in the latter stages of their growth, which are needed for their defense and survival. Antibiotics are important secondary metabolites that are produced by certain microorganisms. They have immense importance for us humans as they help fight many bacterial infections and diseases. Predominantly, *Bacillus* and *Actinomycetes* are present in soil. They are known for producing inhibitory substances like Bacitracin and Actinomycin, respectively (Abdulkadir and Waliyu, 2012).

Another important soil microorganism is *Streptomyces griseus*, which produces the bactericidal compound Streptomycin which combats several pathogenic bacteria like *Mycobacterium tuberculosis* (Thakur *et al.*, 2007). They have been widely utilized over the past decades to fight several bacterial infections. But one major problem that we face today is the growing resistance of pathogens towards the already available antibiotics (Eddie, 2013). Hence, there is a dire need to find out alternatives and discover new antibiotics to fight these resistant pathogens (Nithya and Pandian, 2010). There are many other potential antibiotic producing microorganisms present in the soil that have yet to discovered or utilized. Since soil harbors a wide range of microflora, there is a possibility of finding out new species and strains that have this characteristic property. Thus, this was the main aim of the study – to screen and isolate an antibiotic producing microorganism from the soil near VIT Lake.

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MATERIALS AND METHODS

Collection and preparation of soil sample

Soil sample was collected from the lake area in VIT University, Vellore, Tamil Nadu, India. The moist soil near the plants was collected. About 30g of this soil was taken and it was stored in sterile plastic bag.

Then, 9 test tubes were taken, marked and labeled, and 9ml of saline was poured in each test tube. The tubes were subject to sterilization in an autoclave, 121°C for 15 minutes. 1g of the soil sample was dissolved in 10 ml of water, and from 1 ml of this solution was transferred to the first test tube. Similarly, 1 ml of the solution in the first test tube was transferred to the second, second to third and so on. Hence the soil sample was serially diluted. This was done entirely in sterile conditions inside a laminar air flow.

Isolation of the microorganism

Chemicals and glassware were provided by the general laboratories of VIT University, Vellore. Nutrient agar medium was prepared by adding 2.8g of nutrient agar and 2g of agar powder to 100ml of distilled water. The medium along with 2 glass petri dishes were sterilized in an autoclave at 121°C for 15-20 minutes. After sterilization, the media was slowly cooled to 50°C. Meanwhile, two sterilized glass petri dishes were taken and labeled. Crowded plate technique (Fig1) was used to isolate the antibiotic producing colonies. 0.5 ml of the 0.1 and 0.01 dilution test tubes (the first and second test tubes) were taken and added to the two labeled petri dishes. 20 ml of the media was poured into each of these petri dishes. Then, after the media solidified, the petri dishes were inverted and kept for incubation at 37 °C for 24 hours.

After the period of incubation was over, the petri dishes were observed for clear zone of inhibitions. The microorganism colony responsible for the clear zone was then picked up and sub-cultured on nutrient agar plates and slants by the method of streaking (Fig. 2). Streaking was done using a sterile nichrome loop inside a laminar air flow. After the streaks were done, the plates and slants were kept for incubation at 37 °C for 24 hours.

Antibiotic Sensitivity test

Mueller Hinton Agar was prepared, sterilized, and poured into two sterilized petri dishes. After the media solidified, culture of *Escherichia coli* and *Staphylococcus aureus* were swabbed onto the two plates separately with a sterile cotton bud. Discs of Whatman filter paper no. 3 were made and sterilized, and the isolated microorganism was coated onto the discs. The discs were placed in each petri dish, along with discs of other standard antibiotics such as Streptomycin, Rifampicin, Bacitracin and Ampicillin. In another Mueller Hinton agar plate, *Escherichia coli* were swabbed and the isolated microorganism was directly streaked onto the swabbed culture. The plates were incubated at 37 °C for 24 hours. After the incubation period, the plates were observed for zone of inhibitions.

Characterization and Staining methods

Gram staining and spore staining were performed on the microorganism that was isolated and sub-cultured. In Gram staining, a smear was prepared on a clean glass slide by air drying and heat fixing it. To the smear, a drop of Crystal violet solution was added and allowed to stand for 60 seconds, and washed with distilled water. A few drops of Gram's Iodine was added and left for 30 seconds, and decolorizer was added and the slide was tilted. Finally, Safranine was added and the slide was allowed to stand for 60 seconds. Similarly, spore staining was done by adding Malachite green on a smear, followed by exposure to steam for 5 to 7 minutes and then finally adding Safranine as the counter stain for 60 seconds. Then the slide was washed, dried and viewed under the microscope under 10X and 40X magnifications. Hanging-drop method was performed to check whether the microorganism was motile or not. A drop of the culture was placed on a cover slip, with the sides coated with petroleum jelly. The cavity slide was then placed on it in such a way that the drop was in the center of the cavity. The slide was then rapidly inverted, leaving the drop to "hang" from the cover slip. This was then viewed under 10X magnification under the light microscope.

Plotting the growth curve

Nutrient broth was prepared in two Erlenmeyer flasks and was then subjected to sterilization by autoclaving. After the media was cooled to room temperature, one colony from the isolated culture was picked up using a sterile inoculating loop and dropped into one of the flasks. The flask which was not inoculated served as the control as well as the blank for colorimeter. Then it was incubated in a shaker incubator for the next 24 hours. The colorimeter was set to 600 nm and the OD was recorded, first 3 times after every half hour, followed by recording the OD after every one hour for the next 24 hours. Then the OD values were plot against the time (in hours) to obtain the growth curve for the isolated microorganism.

Biochemical Tests

IMViC tests and TSI agar test were performed. For the Indole test, one colony was inoculated into a test tube containing tryptophan broth and then incubated for 24 hours. After incubation, Kovac's reagent was added to the test tube and then it was observed for color change. MR-VP test was performed by taking two test tubes, and filling them both with MR-VP broth and then sterilizing them. The colony was inoculated, and similarly it was incubated again. After incubation, in one test tube, a few drops of Methyl red was added, and in the second test tube, alpha-napthol and potassium hydroxide was added. Both the test tubes were observed to see if there was any color change. Citrate utilization test was done by preparing Simon's citrate agar slants and then inoculating them with the isolated microorganism. It was incubated for 24 hours and then was observed for color change. TSI (Triple Sugar Iron) test was done to check for carbohydrate utilization. TSI slants were prepared and the microorganism was inoculated. These were then incubated for 24 hours, and after

incubation, the slants were observed for color change. The culture was also streaked on Macconkey agar to check if it was a lactose fermenter or not.

Antibiotic Production

Antibiotic production media was prepared by addition of 3g glucose, 0.6g sodium nitrate, 0.1g potassium dihidro-phosphate, 0.5g of potassium chloride, 0.02g of magnesium sulphate, 0.01g of ferrous sulphate, 5g of peptone and 3g of beef extract in 100 ml of distilled water. This was then sterilized by autoclaving at 121°C for 15-20 minutes. After sterilization, the broth was cooled and inoculated with the isolated colony and then kept in a shaker incubator for 24 hours. The spectrophotometric method was used to obtain the optical density of the inoculated broth. The optical density of the mixture, taken in a 1:64 dilution with distilled water, was measured every day for seven days using a UV-visible spectrophotometer at 280 nm wavelength.

RESULTS AND DISCUSSION

The soil was collected from the lake soil of VIT University. Vellore has a semi-arid climate with high temperatures and little rainfall. The soil sample that was used was moist, loamy soil near the banks of the lake (Vuppu *et al.*, 2012).

The colonies that were observed in the crowded plate were counted using a colony counter and the morphology of the colonies was observed. The clear zones were observed in the crowded plate, and the colony near one of the clear zones was selected and then sub-cultured onto a fresh agar medium by the streaking method. The streak-plate was incubated for 24 hours at 37°C. The next day, the streaks of the selected culture were obtained, and the antibiotic sensitivity test was performed. The further characterization methods like staining and biochemical tests were performed.

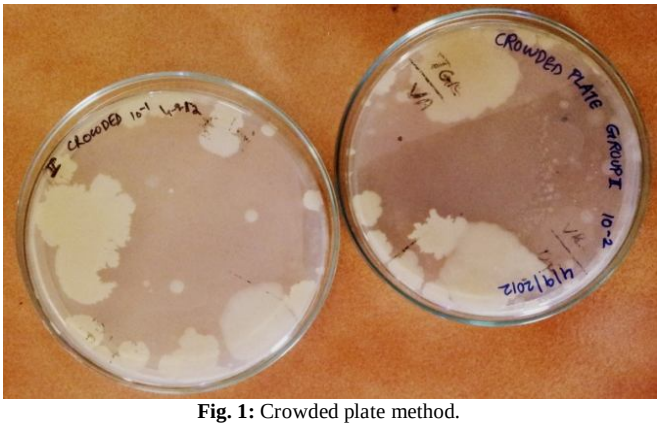

Fig. 1: Crowded plate method.

The zones of inhibition were observed when the antibiotic sensitivity test was performed (Fig 3). The isolated microorganism successfully grew on a media swabbed with a test microbe *Escherichia coli* and inhibited the latter's growth. When directly streaked on a Mueller-Hinton agar plate swabbed with *E. coli*, a distinct clear zone could be seen in the area that was streaked, hence confirming the antibiotic producing nature of the isolated microorganism (Fig 4).

Fig. 2: Sub-culture of colony by streaking.

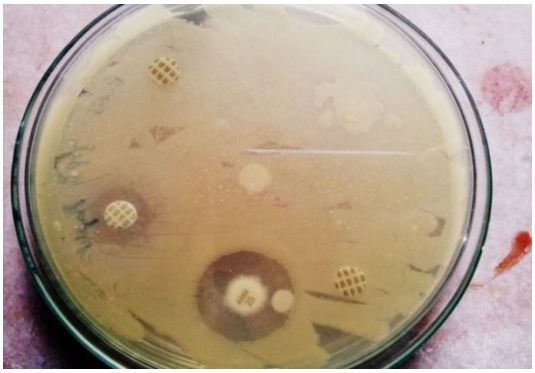

Fig. 3: Antibiotic sensitivity test.

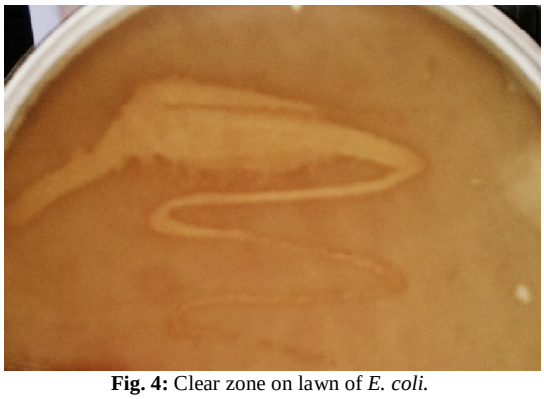

Fig. 4: Clear zone on lawn of *E. coli.*

After performing Gram's staining and spore staining, the morphological features of the bacterial cells were clearly visible. The bacteria were single, rod shaped cells. They were Gram negative (Fig 5) and non-endospore forming bacteria. After performing the hanging drop technique, the bacteria were seen swarming to the edge of the drop, indicating that they were motile and possessed flagella. The bacterial growth curve was measured by taking the OD values at hourly intervals for 16 hours using a UV-visible spectrophotometer. It was plotted against the time (Fig 6).

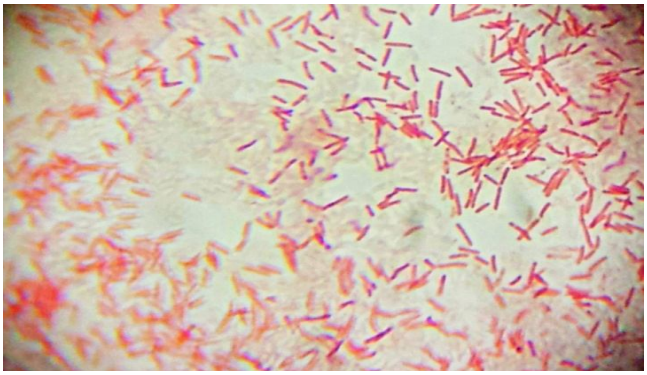

Fig. 5: Gram staining; Gram negative rods.

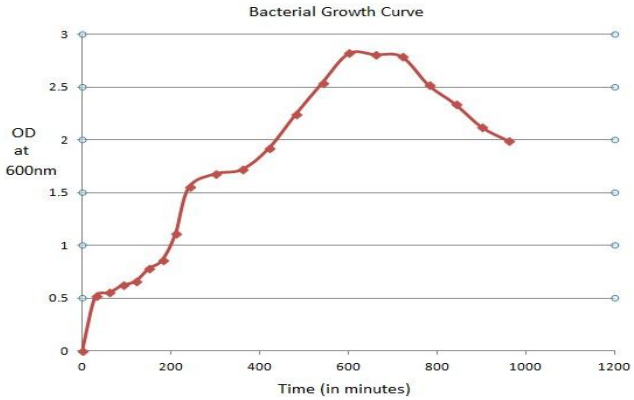

Fig. 6: Standard growth curve.

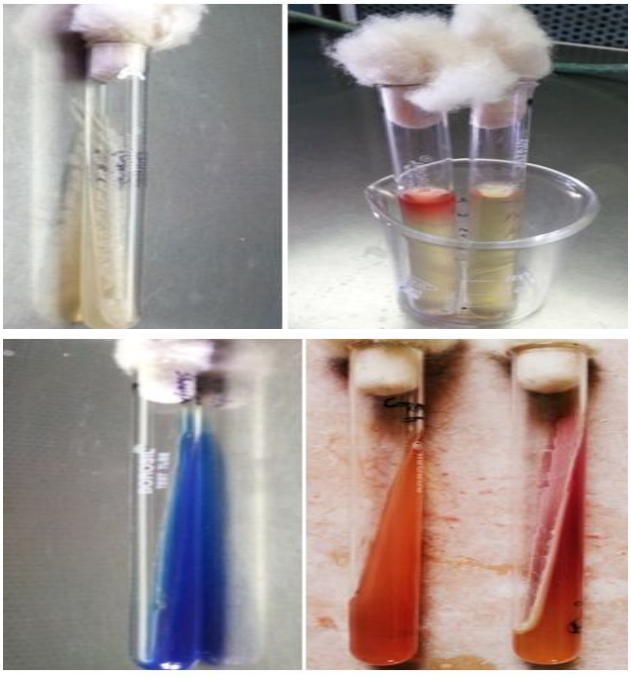

Fig. 7: Biochemical tests: Indole, Methyl red, Voges-Proskauer, Citrate Utilization and TSI tests.

The various biochemical tests (Fig 7) that were performed gave the following results – Indole (-), MR (+), VP (-), and Citrate Utilization (+). There was no color change after the Kovac's reagent was added for the Indole test. Upon addition of Methyl red, a red ring was formed in the test tube, and the test was negative for VP as no color change was observed. Simon's citrate agar slants turned from green to blue upon incubation with the isolated bacteria, and hence it was positive for citrate utilization. TSI agar slants showed a red slant/yellow bottom and there was no growth on Macconkey agar, indicating that the microorganism was not a lactose fermenter. The results of the biochemical tests were cross-checked with the Bergey's Manual of Systematic Bacteriology and the *probable identification* of the isolated bacteria was *Proteus mirabilis.* The antibiotic production media was made and the microorganism was inoculated, and the optical density of the medium was measured every day for seven days using a UV-vis spectrophotometer. The values were plot against the time (in hours) and the antibiotic production curve was plotted (Fig8). It was observed that there was high production of secondary metabolites on the 6th day, indicating that this stage was the antibiotic producing stage of the bacteria's life cycle.

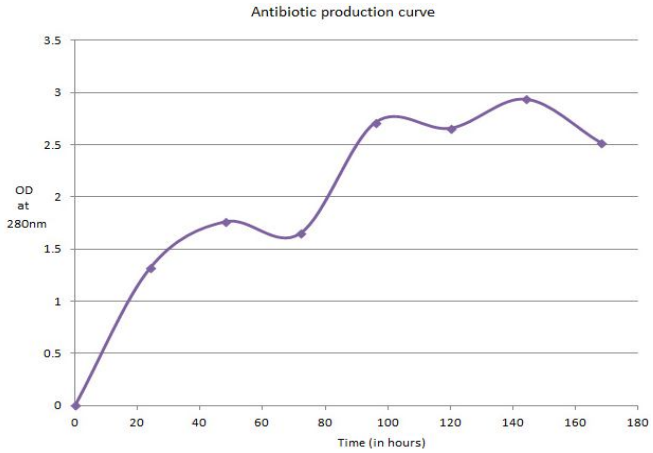

Fig. 8: Graph of antibiotic production plotted against time.

CONCLUSION

New antibiotics extracted from potential microorganisms are the key to fighting the multi-drug resistant bacteria that are emerging as of today's scenario (Bizuye *et al.,* 2013). There are already many existing *Actinomycetes* derived antibiotics available (Gebreyohannes *et al.,* 2013), and hence new soil microbiota that has the ability to produce antibiotics should be screened and isolated. The soil environment harbors a variety of microorganisms, and they need to me explored to find such potential novel antibiotic producers (D'Costa *et al.,* 2007).

From the results, the colonies that were associated with the clear zones of inhibition observed in the crowded plate technique were taken and sub-cultured repeatedly to obtain a pure culture. This was because the clear zone of inhibition indicated that the colony exude substances or compounds that inhibited the growth of the other microorganism in the crowded plate. This colony was then subject to morphology and biochemical tests to determine the genus of the bacteria. The pure culture was then estimated for antibiotic productivity. Further purification, structural elucidation and characterization are recommended to know the quality, novelty and commercial value of these antibiotics.

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