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Research article

ISOLATION OF ANTIBIOTIC PRODUCING BACTERIA FROM SOIL

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ABSTRACT: Most antibiotics used today are isolated and extracted from microbial source. The emergence of antibiotic resistance and need for better, broad spectrum antibiotics is always in high demand. In the present study, antibiotic producing bacteria were isolated from a local soil sample. After primary screening, two bacterial strains (strain 1 and 2) were isolated, which showed antimicrobial activity against some common bacteria namely, *E. coli, K. pneumonia, S. aureus, P.aeruginosa and M. smegmatis*. The isolated strains showed prominent zones of inhibition against *E. coli, K. pneumoniae, S. aureus* and *P. Aeruginosa* but not against *M. smegmatis*. To identify the isolated strains, biochemical tests were performed and it was found that both the strains were *Bacillus sp* with some differences in cultural characteristics. The strains were also found to be resistant to some antibiotics such as tetracycline. The screen for the presence of any plasmid which might be influencing antibiotic production by these strains, the cultures were used for extraction of plasmids wherein, both the strains were observed to have a plasmid of ~ 10 kb. Further study in identification of these strains will be helpful in improving these strains for antibiotic production.

Key words: Bacillus, antibiotics, Endospore, primary screening.

INTRODUCTION

Among the bacteria found in soil can be rods, (bacilli) cocci (spherical), and spirilla (spirals) of which, bacillus are more numerous than others. They are one of the major groups of soil bacteria and are widely distributed (Brock *et al*., 1991). While many antibiotics are known to exist, efforts to discover new antibiotics still continue. Therefore, many species such as *Streptomyces*, *Bacillus* and *Penicillium* have been studied continuously for their ability to produce antibiotics. In addition, due to the fact that *Bacillus speci*es have produced antibiotics insoluble form and that these antibiotics have been found to be cheaper and more effective, these microorganisms are preferable for commercial production. Currently, the target is to produce antibiotics such as polymyxin and bacitracin from *Bacillus* (Debavov *et al*., 1982). It was reported that members of the species *Bacillus* generally produced polypeptide type bacteriocins, and that these antibiotics generally affect Gram positive bacteria (Huck *et al*., 1991).The main antibiotic producers of this genus are *B. brevis* (e.g., gramicidin, tyrothricin), *B. cereus* (e.g., cerexin, zwittermicin), *B. circulans* (e.g., circulin), *B. laterosporus* (e.g., laterosporin), *B.licheniformis* (e.g., bacitracin), *B. polymyxa* (e.g., polymyxin, colistin), *B. pumilus* (e.g.,pumulin), *B. subtilis* (e.g., polymyxin, difficidin, subtilin, mycobacillin, bacitracin). As is generally assumed, these antibiotics are mainly polypeptides (Marahiel *et al*., 1993; Berdy *et al*., 1974; D'Ave *et al*.,1997).

Pathogenic bacteria are acquiring resistance to existing antibiotics, most of which are expensive and have been associated with side effects like nephrotoxicity, etc. Bacteria have evolved numerous strategies for resisting the action of antibiotics and antibacterial agents. This is particularly true of those bacteria that are antibiotic producers. Bacteria that produce antibiotics do so to gain a selective advantage over other competing microbes in their natural environment. If they were sensitive to their own metabolic products, such a selective advantage would be lost. Therefore drug sensitivity pattern studies were carried out to other antibiotics.

The aim of this study was to isolate and characterize the microorganisms that have ability to produce antibiotics and to study whether they have property of resistance to other antibiotics along with antibiotic production. To test whether the isolate consists of plasmid, which might be responsible for antibiotic production.

METHODOLOGY

Sample Collection

Soil sample was collected from the Botanical garden at Osmania University, Hyderabad. The soil sample was sieved to extract fine soil particles which were then serially diluted for isolating microorganisms that are potential antibiotic producers.

Collection of Test Strains

Five bacterial strains, namely *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Mycobacterium smegmatis* and *Klebsiella pneumoniae* used in this study were isolated on selective media from urine, pus and wound exudates collected from local hospitals and biochemical tests were performed to confirm the identity of these strains. The purpose for selecting the above bacteria was because all of them are potential opportunistic pathogens commonly associated with bacterial infections.

Crowded plate technique for isolation of microorganisms

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 1:10000 dilution was 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 broth (Hi-Media, India) and their pure cultures were stored in 4^{o}C until further use.

Secondary Screening

The isolated cultures were tested for their anti-bacterial activity against the test strains.The colonies taken were grown overnight in nutrient broth and were further screened for their inhibiting effect on pathogenic microorganisms like *E.coli, P.aeruginosa, S. aureus* and *K.pneumoniae*. Antimicrobial assay was performed using Mueller Hinton agar (by dissolving 3.8g of powdered medium in 1000ml distilled water and then autoclaving it). 0.1 ml of the pathogenic culture was inoculated in each of these plates by spread plate technique. The overnight culture of the colonies isolated was centrifuged and the supernatant was absorbed on the discs. These discs were plated on these plates and then incubated overnight. The isolates that produced the best zones of inhibition were taken and biochemical tests were performed to identify the antibiotic producers.

Characterization of isolated microorganisms

The morphological characteristics of isolated microbes were identified by performing various tests like gram staining, endospore staining, capsular staining and motility test. Biochemical tests like IMViC, sugar fermentation, organic acid production, alkaline production, starch hydrolysis, catalase test and growth on mannitol salt agar were performed for further characterization.

Minimum Inhibitory Concentration

MIC test was performed when the cultures entered stationary phase as evidenced by growth curve. The culture broth was centrifuged at high speed for 15 min. The supernatant was filtered and used for the MIC test. In a series of test tube having test strains (*E. coli and S. aureus*), the centrifuged broth was added with different concentrations and incubated at 37^{o}C for 24 h. The test tubes were then observed for turbidity. The lowest concentration at which turbidity is not seen is taken as MIC.

Plasmid Extraction

1.5 ml of overnight culture was taken and centrifuged at 12000 X g for 5min at 4°C. The supernatant was discarded and pellet was resuspended in 150µl of ice-cold Alkaline Lysis Solution 1(50mM Glucose, 25mMTrisCl (pH 8.0), and 10mM EDTA (ph 8.0)) and vortexed vigorously. 250µl of Alkaline Lysis Solution 2 (10N NaOH, 10% SDS) was added and mixed gently, followed by addition of 250µl of Alkaline Lysis Solution3 (5M potassium acetate, glacial acetic acid) and centrifuged at 13000 X g for 15min at 4°C. Supernatant containing dsDNA was collected in a fresh tube without any traces of pellet. dsDNA was precipitated by adding 2x volumes of absolute ethanol and vortexed.

The mixture was incubated at -20°C for 1h followed by centrifugation at 13000 X g for 10 min at 4°C. Supernatant was discarded and the pellet was rinsed with 70% ethanol and centrifuged at 12000 X g for 5min at 4°C. The pellet was air-dried and resuspended in 50µl of sterile DEPC water and stored at -20°C.

Agarose Gel Electrophoresis

The isolated plasmid DNA was run on 1% agarose (Genei, India) along with 1 kb ladder and the bands were observed in a Gel Documentation systems (Bio-Rad systems). The length of the plasmid DNA was identified by comparing with the 1 kb ladder.

RESULTS

Identification of Bacterial Strains: The identity of bacterial strains *E.coli, P.aeruginosa, S. aureus* and *K.pneuomoniae* collected from a local hospital was confirmed by biochemical tests performed for the individual microorganisms (Table 1). Once the identities of these strains were confirmed, they were stored at 4^{o}C until further use.

Isolation of microorganisms from soil by crowded plate technique: Plates 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. Four different types of colonies from dilutions 1:100 and 1:1000 were found to show clear zone of inhibition around them. These colonies were isolated and their pure cultures were stored at 4^{o}C till further use.

Secondary Screening: The efficacy of the antibiotic agents against a range of gram positive and gram negative microbial strains was assessed through measurement of zones of inhibition (Schmalenberger *et al*., 2005; Bertrand *et al*., 2005; Kay *et al*., 2002; Weller *et al*., 2002).The bacterial isolates were tested for their antibiotic efficiency by disc diffusion assay against the test strains *E. coli, K. pneumonia, S. aureus, P.aeruginosa and M.smegmatis*. The zone of inhibition was measured. Isolate 1 and 2 showed prominent zone of inhibition against *E. coli, K. pneumoniae, S. aureus* and *P. aeruginosa*. Isolates 3 and 4 showed no antimicrobial activity against *K. pneumoniae, S.aureus, P.aeruginosa* and *M.smegmatis*. None of the isolates were effective against *M. smegmatis*. Isolates 1 and 2 were efficient antimicrobial producers among the four colonies isolated. Further biochemical tests were performed on these colonies to confirm the identity of these strains (Table-2 and 3)..

Table 1: Biochemical tests to confirm the identity of the bacterial strains collected from the hospital.

Biochemical tests	*E. coli*	*K. pneumonia*	*P. aeruginosa*	*S. aureus*
Gram staining	-	-	-	+
Indole	+	-	-	-
Methyl red	+	-	-	+
Voges-proskauer	-	+	-	+
Citrate utilization	-	+	+	-

Table 2: Antimicrobial activity of microbes isolated from soil against the pathogenic test strains.

Pathogen	colony 1	colony 2	Colony 3	colony 4
E.coli	+	+	+	+
K. pneumoniae	+	+	-	-
S.aureus	+	+	-	-
P. aeruginosa	+	+	-	-
M.smegmatis	-	-	-	-

+ve = Inhibition, -ve = no inhibition.

Characterization of microbial isolates: Various morphological and biochemical tests were performed to deduce the identity of the isolates 1 and 2. Isolate 1 on Nutrient agar showed creamy, raised and opaque colonies whereas isolate 2 showed creamy, round, small, raised and pin pointed colonies when grown on nutrient agar plates. Biochemical tests indicated that both the colonies belonged to genus *Bacillus sp.*

Plasmid extraction: The plasmids from colonies 1 and 2 were extracted by alkaline lysis method and run on 1% agarose gel electrophoresis along with 1kb DNA ladder. Conspicuous bands were seen on electrophoresis which showed the presence of plasmid of ~10 kb in both the isolates (Fig 1).

Table 3: Biochemical tests for colonies producing effective antimicrobial activity.

Tests/Colonies	Colony 1	Colony 2
Gram staining	+	+
Endospore	+	-
Capsular	-	-
motility	+	+
Indole test	-	-
Methyl red	-	-
VogesProskauer	+	+
Citrate	+	+
Carbohydrate fermentation		
1. Glucose	+	+
2. Fructose	+	+
3. Maltose	+	+
4. Xylulose	+	+
5. Mannose	+	+
Organic acid production	-	-
Base production	+	+
Catalase test	+	+
Starch hydrolysis	-	-
Growth on mannitol salt agar	White big irregular colonies	White irregular colonies

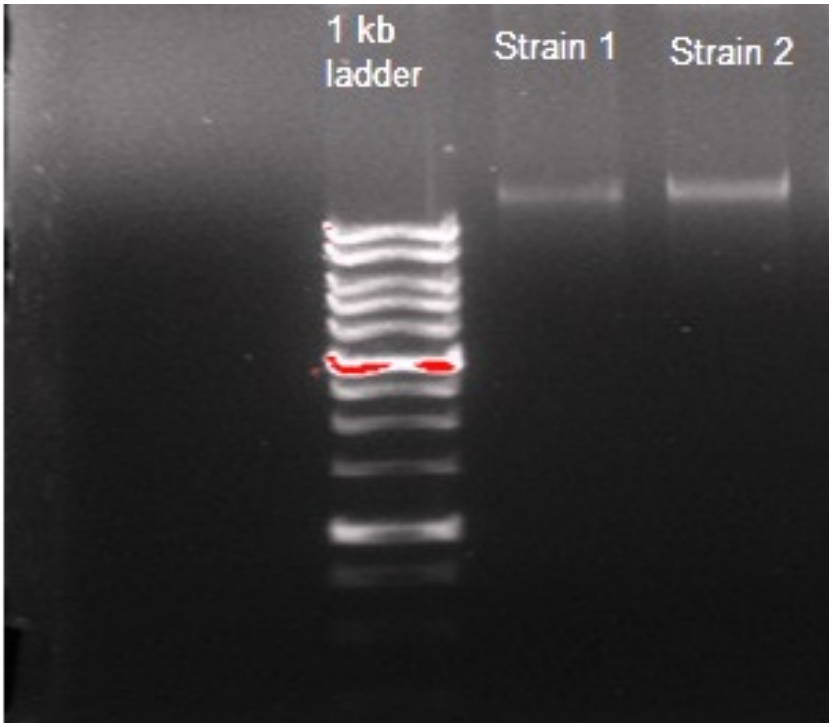

Fig 1: Agarose gel electrophoresis of plasmids extracted by alkaline lysis method. Lane 1,2 and 3 showing DNA ladder and plasmids of isolates 1 and 2 respectively.

DISCUSSION & CONCLUSION

In the present study, two Gram positive *Bacillus* strains were isolated from soil. The culture showed creamy white colonies with irregular margins on nutrient agar medium and pellicle growth in nutrient broth. It is aerobic, endospore forming, non-capsulated and motile. It can ferment sugars like glucose, fructose, xylulose, maltose and mannose and produce gas. It has a characteristic odour in nutrient broth. Both the organisms were positive for Voges-Proskauer and citrate utilization test and exhibited the ability to hydrolyze starch. They were observed to be halophiles as they have the ability to grow at high salt concentration. Based on these properties, the bacterial strains isolated are identified as strains belonging to the genus *Bacillus.*

The *Bacillus* species are known for the synthesis of secondary metabolites with remarkable diversity both in structure and function. (SiloSuh *et al*., 1994). For instance cerecin 7,Tochicin, Thuricin 7, thuricin439 and entomocidus 9 (Cherif *et al*.,2003; Cherif *et al*.,2001) and few may be ribosomal in origin including subtilin (Zhang *et al*.,2004), sublancin (Paik *et al*.,1998), subtilosin A (Babasaki *et al*., 1985), and TasA (Stover *et al*.,1999) polymyxin, difficidin, subtilin, mycobacillin, bacitracin, barnase, etc. Polymyxins are anti-Gram-negative; difficidin is broad spectrum; and mycobacillin and zwittermicin are anti-fungal. The antibiotic produced by the presently isolated strains needs to be further characterized by HPLC, NMR, etc., to pinpoint its nature and properties.

The Bacillus strains were observed to inhibit growth of Gram positive and Gram negative test organisms such as *E.coli, Staphylococcus aureus, Klebsiella & Pseudomonas*, indicating that antibiotic produced by the strains is a broad spectrum antibiotic. Interestingly, the isolated strains showed resistance to tetracycline. This property can be utilized in maintaining pure cultures of the strains. Also, like several known species of the genus Bacillus, the isolated strains showed presence of plasmid. The nature of the plasmid is presently unclear. Whether the plasmid harbors resistance to tetracycline or is responsible for production of the antibiotic, or both, needs to be further characterized.
Hence it can be concluded that since the antibiotics produced by *Bacillus sp* strains observed in the study seem to be broad spectrum, they could be further exploited to know their efficacy compared with currently available antibiotics.

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