

Original Article

ISOLATION AND CHARACTERIZATION OF ACTINOMYCETES FROM SOIL OF AD-DAWADMI, SAUDI ARABIA AND SCREENING THEIR ANTIBACTERIAL ACTIVITIES

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ABSTRACT

Objective: To isolate and characterize novel actinomycetes and to evaluate their antibacterial activity against drug-resistant pathogenic bacteria

Methods: In the present study, 19 soil samples were collected from different localities of Ad-Dawadmi, Saudi Arabia. Actinomycetes were isolated from these samples using serial dilution and plating method on Actinomycetes isolation agar supplemented with nalidixic acid and actidione to inhibit bacteria and fungi. Crude extracts of potential actinomycetes were produced by submerged fermentation. The antimicrobial activity of crude extracts of actinomycetes was tested against different bacteria using the agar well diffusion method. Characterization of the isolates was done by morphological, physiological and biochemical methods.

Results: A total of 9 (47%) isolates of actinomycetes were isolated from 19 different soil samples tested. Among them, 4 (44%) isolates confirmed as *Streptomyces* sp. showed potential antimicrobial activity against one or more test organisms. Crude extracts were made from these 4 actinomycetes isolates (DOM1, DOM3, DP3, DP4) and tested for their antibacterial activities against 4 different clinical bacterial strains (*Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Enterococcus*). Crude extract from DP3 isolate showed highest antibacterial activity against all the four test organisms (28 mm, 21 mm, 20 mm and 18 mm) respectively and DP4 showed lowest antibacterial activity against all the four test organisms (14 mm, 12 mm, 0 mm, 6 mm) respectively. The highest zone of inhibition was shown by DP3 against *Staphylococcus aureus* (28 mm) and *Escherichia coli* was resistant for DP4. Most of the inhibition zones produced by crude extracts showed significant differences when compared with control, tested against test organisms (P<0.05). Inhibition zones produced by DP3 and DOM1 against *Staphylococcus aureus* were 28 mm and 23 mm, respectively which were strong active when compared with control Ciprofloxacin (18 mm).

Conclusion: Further studies for purification of bioactive metabolites and molecular characterization analysis of isolated *Streptomyces* sp. are in progress which would be helpful in discovering novel compounds of commercial value.

Keywords: Actinomycetes, Drug resistance, Antimicrobial activity, *Streptomyces*

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INTRODUCTION

Actinomycetes are a group of prokaryotic microorganisms which are gram-positive bacteria with high guanine+cytosine in their DNA [1]. They are considered as biotechnologically important organisms since they are responsible for producing about half of the bioactive secondary metabolites including antibiotics [2, 3]. They are filamentous bacteria which produce two types of branching mycelium, namely aerial and substrate mycelium. Factors influencing the number and types of actinomycetes present in a particular soil are a geographical location, such as soil type, temperature, organic matter content, moisture content, cultivation and aeration. Actinomycetes act as a major component of the microbial population in most of the soil. About 90% of the total actinomycetes population consists of *Streptomyces* species [4]. Soil actinomycetes can produce a wide range of secondary metabolites and about 70% of the naturally derived antibiotics that are currently in clinical use are produced by them [5]. Almost 80% of the world's antibiotics are known to come from actinomycetes, mostly from the genera *Streptomyces* and *Micromonospora* [6]. A major global healthcare problem has occurred in the 21st century because of increasing resistance of microorganisms against commonly used antibiotics. A renewed search for new antimicrobial agents from *Streptomyces* has occurred because of this rapid emergence of antimicrobial resistance among pathogenic microorganisms [7]. The most multiple drug-resistant bacteria causing important community-acquired infections include methicillin/oxacillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Staphylococcus aureus* (VRSA), vancomycin-resistant *Enterococcus* (VRE), extended-spectrum beta-lactamase (ESBL) producing

bacteria such as *E. coli* and *Klebsiella* spp and penicillin-resistant *Streptococcus pneumoniae* (PRSP). Therefore, it is important to search for new, efficient and safe antibiotics from actinomycetes sources to overcome the menace of drug-resistant infections. The aim of this present study was to isolate, identify and characterize the actinomycetes isolates from different soil samples from Ad-Dawadmi, Saudi Arabia and to determine their antagonistic activity against selective human pathogenic micro-organisms.

MATERIALS AND METHODS

Chemicals and media

Culture media and chemicals used in this study such as Starch Casein agar (SCA), Actinomycetes isolation agar (AIA), Mueller Hilton agar, nutrient broth, starch casein broth, glucose phosphate broth, peptone water, simmons citrate agar, triple sugar iron agar, dimethylsulfoxide, nalidixic acid, actidione, ciprofloxacin were purchased from Himedia Laboratories, Mumbai, India.

Sample collection

19 Soil samples were collected from different areas of Ad-Dawadmi, Saudi Arabia such as Applied Medical Science College campus, Ad-Dawadmi Park and Ad-Dawadmi old market. Soil samples were collected from 5-25 cm depth in sterile plastic bags and transported aseptically to the Microbiology laboratory, Applied Medical Science College, Ad-Dawadmi. After tacking, soil sample is directly transferred into polyethylene bags to minimize moisture losses during transportation. Clinical strains of bacteria such as *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Enterococcus* were obtained from the Ad-Dawadmi General Hospital, Saudi Arabia.

Isolation of actinomycetes

The samples were air dried for one week at room temperature. Isolation and enumeration of Actinomycetes were done by serial dilution and spread plate technique [8]. One gram of soil was suspended in 9 ml of sterile double distilled water and then the dilution was carried out up to 10⁻⁵ dilutions. Aliquots (0.1 ml) of 10⁻², 10⁻³, 10⁻⁴ and 10⁻⁵ were spread on the actinomycetes isolation agar. To minimize the bacterial and fungal growth, nalidixic acid 100 mg/land actidione 20 mg/l were added respectively. Then the plates were incubated at 30 °C for 10 d [9]. The plates were observed intermittently for the actinomycetes growth during incubation. After incubation, actinomycetes colonies which are morphologically distinct were picked from the actinomycetes isolation agar plates and further purified by repeated streak plate method [10]. Once the pure colonies were obtained, each colony was further identified based on its characteristics such as earthy like the smell, colony morphology, the color of hyphae and the presence or absence of aerial and substrate mycelium. Then, selected and identified colonies of actinomycetes were transferred to starch casein agar slant and incubated at 27 °C for their growth. After incubation, the slants containing pure actinomycetes isolates were stored at-4 °C for further studies [11].

Screening of actinomycetes and extraction of antibacterial compound

Clinical strains of bacteria such as *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Enterococcus* were obtained from Ad-Dawadmi General Hospital, Saudi Arabia. The isolates of actinomycete were screened for antibacterial activity against these four clinical isolates using nutrient agar by cross streak method [12]. Each nutrient agar plate was streaked with each actinomycete isolate at the center of the plate and incubated at 37 °C for 6-7 d. Then, 24-hour subcultures of bacteria were streaked perpendicular to the actinomycete isolates and the plates were incubated at 37 °C for 1 d. After incubation, the zone of inhibition of the test organisms indicates that the actinomycete isolate has antibacterial activity, whereas the full growth of the test organism indicates that the actinomycete isolate has no antibacterial activity. The actinomycete isolates that show potent antibacterial activity (designated as DOM1, DOM3, DP3 and DP4) were selected for extraction of the crude antibacterial compound by submerged culture technique [13]. They were inoculated in 250 ml flasks containing 75 ml of starch casein broth and incubated on a rotary shaker (200 rpm) at 30 °C for 6 d. A 20liter total volume was filtered through Whatmanno.1 filter paper and then centrifuged at 5000 rpm for 20 min and the clear supernatant broths obtained was stored at 4 °C in screw-capped bottles for further study. The supernatant broths were tested for their antimicrobial activity against the selected pathogens by agar well diffusion method [14].

Characterization and Identification of actinomycetes

Characterization of potent actinomycete isolates was carried out by morphological and biochemical and physiological studies as described in the International *Streptomyces* Project (ISP) [15]

Morphological Identification

Gram staining and lactophenol blue staining were done to study the morphology of the actinomycetes cells and spore chain morphology

was studied coverslip culture technique with a light microscope (Accuscope 3002 series) [16].

Physiological and cultural characterization

Colony morphology of the isolates of actinomycete was studied under a high power magnifying lens by observing color of the colony, nature of the mycelium, spore surface and felling the consistency with a sterile loop.

Biochemical characterization

Actinomycete isolates were characterized by various biochemical tests such as indole test, methyl red test, vogus-proskauer test, citrate utilization test, triple sugar iron test, nitrate reduction test, starch hydrolysis test, catalase test, mannitol and sucrose utilization tests.

Antagonistic activity by agar well diffusion method

Agar well diffusion method was used to determine the antimicrobial activity of isolates. The supernatant broth containing compounds of actinomycetes was tested for their antibacterial activity against four different clinical isolates namely, *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Enterococcus*. The bacterial test isolates were inoculated in test tubes containing nutrient broths separately and labelled. Then they are incubated at 37 °C for overnight for obtaining broth cultures. After overnight incubation, bacterial cultures were swabbed in solidified Muller Hinton agar plates and 6 mm diameter wells were punched in the plate. Then 100 µl of supernatant broths containing actinomycetes compounds were dispensed in separate wells. The plates were incubated at 37 °C for 24 h. After incubation, the diameter of the zone of inhibition around the wells was measured and recorded. The antibiotic ciprofloxacin is used as a control and dimethylsulfoxide without antibacterial compound is used as a blank. Inhibition zones produced by crude extracts showed significant differences when compared with control ciprofloxacin tested against different test organisms (P<0.05).

Statistical analysis

The experiment is repeated three times. The mean and standard deviation were calculated. The significance of the differences between the mean values of inhibition zones produced by crude extracts and the control antibiotic tested against test organisms was determined using students *t*-test (using Primer of statistics) and the results were recorded.

RESULTS AND DISCUSSION

Isolation of actinomycetes

After processing of the soil samples collected from different areas of Ad-Dawadmi, Saudi Arabia in Microbiology laboratory, Applied Medical Science College, Ad-Dawadmi, a total of 9 (47%) isolates of actinomycetes were isolated from 19 different soil samples. Out of 9 isolates, 2 (22.2%) were isolated from Applied Medical Science College campus, 03(33.3%) from Ad-Dawadmi old market and 4(44.4%) from Ad-Dawadmi park (table 1). Isolation and enumeration of actinomycetes were done by serial dilution and spread plate technique using actinomycetes isolation agar media. All the 9 isolates grown on actinomycetes isolation agar showed morphology typical of actinomycetes. The colonies were slow growing, aerobic, folded and with aerial and substrate mycelia of different colors.

Table 1: Actinomycetes isolates from soil samples collected from different sites of dawadmi

Collection sites	No. of soil samples	No. of isolates	Isolate number
Applied Medical College campus	6	2	AMC1, AMC2
Dawadmi old market	6	3	DOM1,DOM2, DOM3
Dawadmi park	7	4	DP1, DP2,DP3,DP4

Screening of actinomycetes and extraction of antibacterial compound

All the 9 Actinomycetes isolates were subjected antagonistic activity by cross streak method against 4 types of bacteria, namely,

Escherichia coli, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Enterococcus*. As a result of the cross streak method, all actinomycete isolates showed antimicrobial activity against one or more bacterial strains. 4(44.4%) out of 9 isolates which exhibited high activity against all the pathogens were designated as DOM1,

DOM3, DP3 and DP4 respectively. For studying antagonistic activity, potential actinomycetes isolates were obtained in pure form by submerged culture technique and preserved at 4 °C for further studies. In a similar study conducted in soil samples taken from the Garden of Sathyabama University, Chennai, India 13 out of 22 (59%) actinomycete isolates showed potential antimicrobial activity against one or more test bacteria and/or fungus [17]. In a study conducted in soil samples of different sites of Chambal territory and other parts of Madhya Pradesh, India only 5 out of 85 actinomycete isolates showed antimicrobial activity [18]. In another study conducted in soil samples from Konark and western terrestrial sea, India only 3 out of 20 actinomycetes isolates significant antimicrobial activity [19]. Several other studies have shown that actinomycetes can produce antagonistic molecules that can inhibit or kill the pathogenic microorganisms [20-22].

Characterization and identification of actinomycetes

The potent actinomycetes capable of high antibacterial activity were characterized by morphological and biochemical characteristics

(table 2 and table 3). The observed structure was compared with Bergey's manual of determinative bacteriology [23]. Indole production was strictly negative, methyl red was positive for all except DOM1, vogus-proskauer was negative for all the isolates, citrate positive was for all except DP3, TSI was negative for all the isolates, nitrate reduction was positive for all the isolates except DOM3, starch hydrolysis was positive for all the isolates and catalase was also positive for all the isolates. Mannitol utilization was positive for DP3 and DP4. Sucrose utilization was positive for all the isolates except DP3. After observation of cultural, morphological, physiological and biochemical characteristics it was confirmed that these isolates belong to the species of the genus, *Streptomyces*. Previous studies conducted by [13, 17-22] showed that *Streptomyces sp.* being producers of useful bioactive metabolite have an antibacterial effect with a broad spectrum of activities. Another study showed that among *Streptomyces* species produced about 7,600 compounds which have antimicrobial properties which are highest among actinomycetes producing antibiotics in the soil [24].

Table 2: Morphological characteristics of potential isolate

Organism identified	Nature of mycelium	Color of the colony	Spore surface	Gram stain
<i>Streptomyces species (DOM1)</i>	extensively branched, aerial and substrate mycelium	white yellow	wrinkled	+
<i>Streptomyces species (DOM3)</i>	branched, aerial and substrate mycelium	white pink	wrinkled	+
<i>Streptomyces species (DP3)</i>	granular, aerial and substrate mycelium	yellowish brown	smooth	+
<i>Streptomyces species (DP4)</i>	branched, aerial and substrate mycelium	creamy	wrinkled	+

+= Positive

Table 3: Biochemical characteristics of potential isolates of actinomycetes

Biochemical test	DOM1	DOM3	DP3	DP4
Indole	-	-	-	-
MR	-	+	+	+
VP	-	-	-	-
Citrate	+	+	-	+
TSI	-	-	-	-
Nitrate reduction	+	-	+	+
Starch hydrolysis	+	+	+	+
Catalase test	+	+	+	+
Mannitol utilization	-	-	+	+
Sucrose utilization	+	+	-	+

+= Positive; -= Negative

Antagonistic activity by agar well diffusion method

The agar well diffusion method is used for the antimicrobial evaluations. In this work, 4 potential actinomycetes isolates were tested for their antagonistic activities against 4 different bacterial strains (*Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Enterococcus*. 24 hour broth culture of the bacterial strains were used to test the antagonistic activity of crude extracts of potential actinomycetes isolates and mean diameters of the developing inhibition zones (mm) were calculated (table 4). The crude extracts showed antibacterial activities against 4 different bacterial strains with zones of inhibition ranging between 6-28 mm. A total of 9 (47%) isolates of actinomycetes were isolated from 19 different soil samples tested. Crude extract from DP3 isolate showed highest antibacterial activity against all the four test organisms (28 mm, 21 mm, 20 mm and 18 mm) respectively and DP4 showed lowest antibacterial activity against all the four test organisms (14 mm, 12 mm, 0 mm, 6 mm) respectively. The highest zone of inhibition was shown by DP3 against *Staphylococcus aureus* (28 mm) and *Escherichia coli* was resistant for DP4. Most of the Inhibition zones produced by crude extracts showed significant differences when compared with control, tested against test organisms (P<0.05). Inhibition zones produced by DP3 and DOM1 against *Staphylococcus aureus* were 28 mm and 23 mm, respectively, which were strong active when compared with control Ciprofloxacin (18 mm). The results also showed that

the inhibition zones maximum against gram-positive bacteria (*Staphylococcus aureus* and *Enterococcus*) when compared to gram-negative bacteria *Escherichia coli*, *Pseudomonas aeruginosa*). The reason for lower sensitivity of gram-negative organisms towards antibiotics is due to the difference in cell wall components between these microorganisms, gram-negative bacteria having an outer polysaccharide membrane carrying the structural lipopolysaccharide components which are absent in gram-positive organisms. Several studies on isolation, characterization and antibacterial activity of actinomycetes have been conducted in Saudi Arabia previously. In a study, actinomycetes isolated from soil samples of Jeddah and Al-Madina Al-Munawarah, Saudi Arabia showed antibacterial activity against *Staphylococcus aureus*, *Escherichia coli*, and *Salmonella typhimurium* [25].

Another study shows that actinomycetes isolated from soil samples of Al-Kharj, Saudi Arabia showed antibacterial activity against *Staphylococcus aureus*, *Bacillus subtilis*, *Streptococcus epidermidis* and *Escherichia coli* [26]. Several other studies on isolation and characterization of actinomycetes from soil and evaluation of antibacterial activities of actinomycetes against human pathogens also prove that the actinomycetes are able to produce a generation of new antimicrobial agents [17-22]. The results of the present study also support previous studies that the soil actinomycetes have the potential to produce novel antibiotics and were found to be antagonistic against test organisms.

Table 4: Antagonistic activity of crude extracts of actinomycetes against human pathogens by agar well diffusion method (mm)

Test organisms	Zone of inhibition (mm)					
	DOM1	DOM3	DP3	DP4	Control	Blank
<i>Staphylococcus aureus</i>	23±2*	18±1.39 ^{NS}	28±1.9***	14±2 ^{NS}	18±2	0±0
<i>Enterococcus sp.</i>	15±2*	19±1 ^{NS}	21±1 ^{NS}	12±2**	21±3	0±0
<i>E. coli</i>	8±1**	15±2 ^{NS}	20±2**	0	13±2	0±0
<i>Pseudomonas aeruginosa</i>	16±1 ^{NS}	9±1***	18±2*	6±2***	15±1	0±0

Values are mean±SD of three replications; (0) denotes no zone of inhibition, NS = non-significant, * = P<0.05, ** = P<0.01, *** = P<0.001 when crude extracts compared with control, DOM1, DOM3, DP3, DP4 = crude extracts from different isolates; Blank = 1% Dimethyl sulfoxide; Control = Ciprofloxacin (30µg/ml)

CONCLUSION

These findings indicated that the soil samples of the Ad-Dawadmi region have actinomycetes with metabolites that control bacterial pathogens. The crude metabolites obtained from four isolates of *Streptomyces* in our study showed very good antimicrobial activity against four different clinical pathogens. Bioactive metabolites from these *Streptomyces* sp. have to be purified and molecular characterization of the isolates has to be done for discovering novel compounds which have commercial value. Further studies on these are underway.

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AUTHORS CONTRIBUTION

1. Study conception and design including acquisition of data: Gouse Basha Sheik
2. Analysis and interpretation of data: Muazzam Sheriff Maqbul
3. Drafting of manuscript: Gokul Shankar S
4. Critical revision: Ranjith MS

CONFLICT OF INTERESTS

Declared none

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