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INFLUENCE OF SOME ANTIBIOTIC-PRODUCING ACTINOMYCETES ISOLATED FROM ECOLOGICALLY DIFFERENT AREAS IN SAUDI ARABIA ON THE HUMAN PATHOGENIC *STAPHYLOCOCCUS AUREUS*

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Short Profile

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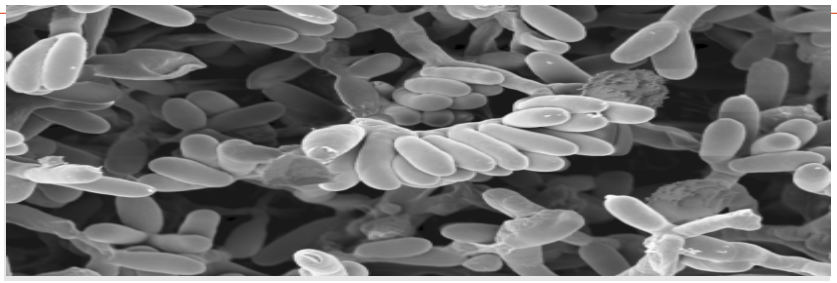

ABSTRACT:
This research was implemented to investigate the ecological distribution of antibiotic-producing actinomycetes in Saudi Arabia and their effect on the human pathogenic *Staphylococcus aureus*. A total of 124 actinomycetes isolates was isolated from the rhizosphere soil of some economic crops, i.e. date palm tree, tomato plants, beans plants, mango trees, squash plants and clover plants cultivated in ecologically different regionsin KSA (Northern, Southern, Central, Eastern and Western regions). The highest temperature was recorded in the West region at Makkah and Jeddah in the summer season that ranged from 35-50 oC and 39-47 oC consecutively. On the other hand, the lowest temperature was recorded in Tabuk at the North region that ranged from 0-15 oC in the winter season.Out of the 124 actinomycetes isolates, 15 isolates revealed antagonistic effect against the human pathogenic *Staphylococcus aureus* ATCC 25923. The 15 actinomycetes-producing antibiotics were identified using molecular technique of 16srRNA.The ethyl acetate extract of the actinomycetes cultures supernatant recorded inhibition growth zones of *Staphylococcus aureus* (11.5-13.6 mm) more that those obtained with the cultures supernatantas such (8.0-11.7 mm). It was detected that, except Abhagovernorate in the South region, each region was inhabited by one species of actinomycetes despite the difference in the plant rhizosphere from which these species were isolated. Two strains of Streptomyces enissocaesilis were isolated for the rhizosphere of a date palm tree cultivated in Tabuk at the North region; while for the Middle Region (Al-Riyadh), five strains were isolated from the rhizosphere of three different plant (date palm tree, clover and tomato) and identified as Streptomyces sp. Two strains of Streptomyces fungicidicus were isolated from the rhizosphere of date palm tree and clover cultivated in Al-Dammam at the East region; while the three strains that isolated from the rhizosphere of a mango tree and beans plants were identified as Streptomyces ambofaciens. Regarding the South region (Abha), it was the only exception that found outside the context; where three different strains identified as *Streptomyces maritimus, Streptomyces rochei* and *Actinobacterium* sp. were isolated from the rhizosphere region date palm tree and beans plants. In general, this study reveals that the predominant ecological conditions in a region play an important role in the distribution of the antibiotic-producing actinomycetes.

KEYWORDS
Antibiotic-producing actinomycetes, ecological different regions in KSA, Staphylococcus aureus.

1.INTRODUCTION:

Actinomycetes are the most economically and biotechnologically valuable prokaryotes. They are responsible for the production of about half of the discovered bioactive secondary metabolites such as antibiotics, herbicides, pesticides, anti-parasitic and enzymes (Berdy, 2005). Of these compounds, antibiotics predominate in therapeutic and commercial importance (Waksman, 1961; McCarthy and Williams, 1990 and Ouhdouche*t al*., 2001).Among Actinomycetes, *Streptomycetes* species are very potent producers of secondary metabolites. Out of the approximately 10000 known antibiotics, 45-55 % is produced by *Streptomycetes* (Demain, 1999 and Lazzarini*et al*., 2000). The secondary metabolites produced by them have a broad spectrum of biological activities,e.g. antibacterial, antiviral, antiparasitic, immunosuppressive, antitumor, enzyme inhibitory).The antimicrobial spectrum of antagonistic properties of *Streptomyces* species againstGram-positive and Gram-negative bacteria, unicellular and filamentous fungi, as one of the important criteria in species differentiation, were early reported by some investigators (Krassilinikov, 1960 and 1970; Hottaet al., 1983; Williams *et al*., 1983; Schael, 1986 and Hara *et al*., 1991).Many investigators are still looking for new bioactive compounds from Streptomyces sp. and have recently found that particular Streptomyces sp. can produce antibacterial activities against S. aureus, Methicillin-resistant *Staphylococcus aureus* (MRSA), *Vancomycin-resistant Staphylococcus aureus(VRSA),B. subtilis, S. epidermidis, Enterococcus faecalis, Micrococcusluteus, E. coli, P. aeruginosa,Klebsiellasp. (Arasuet al., 2008; Selvameenalet al., 2009; Yadavet al., 2009 and Duraipandiyanet al., 2010).*

In view of the foregoing, the objective of the present investigation was to screen soilsamples collected throughout the Kingdom of Saudi Arabia, which are large, diverse and largely unscreened ecosystems, for the isolation of potent antibiotic-producing Actinomycetes against *Staphylococcus aureus.*

MATERIALS AND METHODS

Samples Collection:

Rhizosphere soil samples were collected randomly from different soil areasin Saudi Arabia (Northern,Southern, Central, Easternand Western). Each sample was taken from 5-15 cm depth of the soil by using sterile degraded metal tube (30 cm in length). Soil samples were mixed and sieved to remove stones, leaf, stem and roots. Then, samples were packed in cleaned and sterile plastic bags, received and stored until analysis (Parungao*et al*., 2007).

Isolation of Actinomycetes:

After purification of selected soil samples, 10gm from sample was added to 90 ml sterile distilled water to make a suspension, a thorough mixing was done and serial dilutions of the suspension were done to 10^{-1} to 10^{-6}. Actinomycetes selective isolation medium i.e. Starch nitrate agar ISP2 (Shirling and Gottlieb, 1966), was prepared and inoculated in duplicate by 1 ml from the above mentioned dilutions from each soil sample using pour plate method. The inoculated plates were gently rotated several times for the purpose of even distribution of the isolates, solidified andincubated at 30°C for 8 days. Streak plate method was used to recover isolated pure colonies of actinomycetes which were then transferred

to slant media as stock isolates.The optimum temperature30°C and the initial pH 7 weremaintained for the production of antimicrobial metabolites by the isolates.

Screening for antimicrobial activities:

The actinomycetes isolates were inoculated into 50 ml ISP- 2 broth medium in 250ml conical flask and incubated at 30°C into a rotary shaker at 180 rpm for 8 days. For testing the antibacterial activity, the disc diffusion method for antimicrobial susceptibility testing was carried out according to the standard method by (Bauer *et al*., 1966). Filter paper discs of 7 mm diameters were impregnated with the liquid culture of the respective actinomycetes then three filter paper discs placed on sterile Petri dishes containing Mueller Hinton agar media(Mueller and Hinton, 1941)inoculated with Staphylococcus aureusATCC 25923 cultures.The Petri plates then incubatedfor 24 hours at 37°C; the inhibition zoneswere measured in mm.

Antibacterial Solvent extraction:

Ethyl acetate solvent was used for extraction of antimicrobial metabolites. A quantity of 500 µl of the crude extracellular extract was taken in Eppendorf tubes and 500µl of the respective solvent was added. Gentle mixing was done for 1 hour and the tubes were spun at 10000 rpm for 10 minutes, ethyl acetate phase (upper) containing dissolved metabolites was collected in weighed tube and it was kept in a hot air oven (50◯C) for drying of ethyl acetate. The tube once again weighed and amount of metabolite extracted was calculated by subtracting the weight of empty tube from the weight of tube after drying. The metabolite was dissolved in 500 ml of sterile distilled water (Alam khan et al., 2011).

Identification of Actinomycetes:

The actinomycetes isolates active in the production of antibiotics were identified by application ofthe molecular technique 16s rRNA sequencing (the analysis of 16srRNA sequences done by Macrogen Korea 10 F, 254 Seoul Company).

For the DNA extraction, actinomycetes were grown on an ISP-2 plate at RT for 3 to 4 weeks. The DNA of each isolate was extracted by suspending some colonies of actinomycetes in 400 µ of TE buffer, and 8 µ of lysozyme (50 mg/ml) in a micro-tube. The mixture was agitated and incubated at 37°C for 30 min. Then 4 µ of proteinase K (20 mg/ml),20 µof 10%SDS and 4 µ of RNase A (100 mg/ml) were added. The mixture was mixed together and incubated at 37°C for 30 min. After that, 70 µ of 5M NaCl, 55 µ of 10%CTAB/0.7M NaCl were added and incubated at 65 °C for 10 min. Then an equal volume of chloroform was added and centrifuged at 15,000 rpm, RT for 5 min. This step was repeated twice. The supernatant was transferred to a new micro-tube, added an equal volume of phenol/chloroform and centrifuged at 15,000 rpm, RT for 5 min. The supernatant was transferred to a new micro-tube, and then isopropanol was added and centrifuged at 8,000 rpm, RT for 2 min. DNA pellet was washed twice with 1 ml of 70% ethanol and centrifuged at 8,000 rpm for 1 min. After drying DNA pellet, it was re-suspended in 20 µ of water or TE buffer for PCR amplification.

RESULTS

Table (1) indicates the environmentally different regionsin Saudi Arabia (North, South, Middle,

East and West)and their dominant weather from which the actinomycetes were isolated. This table reveals the great variations between the different regions for temperature, humidity and annual rain levels. The highest temperature recorded in the West region at Makkah and Jeddahin the summer season that ranged from 35-50 °C and 39-47 °Cconsecutively. On the other hand, the lowest temperature was recorded in Tabuk at the North region that ranged from 0-15°C in the winter season.

Table 1: The weather parameters dominant in various regions of KSAselected for antibiotic-producing actinomycetes isolation.

Weather / Regions	Temperature (°C)		Humidity (%)	Annual rain level (mm)	Weather type
	Summer	Winter			
North					
Tabuk	23-34	0-15	35	39	Temperate in summer, cold in winter
South					
Abha	20-28	9-18	60	200-500	Temperate in summer, cold in winter
Al-Baha	23-30	11-15	52-67	229-580	Temperate in summer cold in winter
Middle					
Al-Riyadh	25-48	3-14	70	10-20	Hot in summer, cold in winter
East					
Al-Dammam	30-44	10-15	35-45	30	Hot in summer, cold in winter
West					
Makkah	35-50	20-25	35-45	10-33	Hot in summer, temperate in winter
Al-Medina	36-46	15-20	22-35	35	Hot in summer, cold in winter
Jeddah	39-47	21-30	70-80	15-25	Hot in summer,temperate in winter
Al-Taif	23-28	8-17	45	200-381	Temperate in summer, cold in winter

One hundred and twenty four (124) isolates were isolated form the different regions in KSA representing the environmentally different regions (North, South, Middle, East and West). Out of the 124 actinomycetes isolates, 15 isolates revealed adverse effects against the human pathogenic S*taphylococcus aureus*(Table 2). Both effects of the culture supernatant of the actinomycetes or the ethyl acetate extract of theircultures supernatanton the growth of the human pathogenic *Staphylococcusaureus* were presented in Table (2).Generally, the ethyl acetate extracts of the actinomycetes supernatant produced inhibition zones wider than their respective cultures supernatant. The inhibition zones diameters of the cultures supernatant ranged from 8.0 to 11.7 mm being the highest with the isolate no. 5 (Plate 1) that isolated from the Middle region. The ethyl acetate extract of the actinomycetes cultures induced inhibition zone diameters ranged from 11.5 to 13.6 mm being the highest with the isolate no. 9 (Plate 2) also isolated from the Middle region.

Table 2: Antagonistic effect of different actinomycetes isolates against pathogenic *S. aureus.*

Isolates code no.	Inhibition Zones (mm)		Region
	Cultures supernatant	Ethyl acetate extract	
7	10.3	11.7	North
12	9.0	12.8	North
3	11.0	12.8	South
11	9.8	11.5	South
13	8.2	12.4	South
5	11.7	12.9	Middle
8	10.8	13.0	Middle
9	11.6	13.6	Middle
14	8.5	13.0	Middle
15	8.7	11.9	Middle
2	9.0	12.3	East
6	8.5	12.1	East
1	11.5	13.2	West
4	8.0	11.5	West
10	10.2	12.9	West

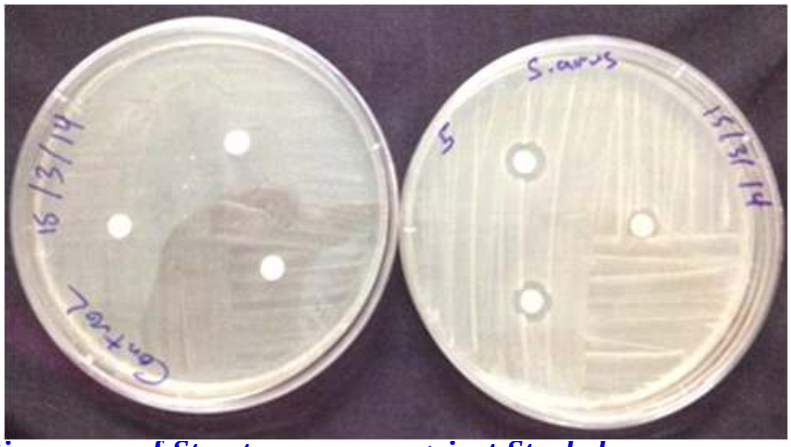

Plate 1: The inhibition zones of *Streptomyces* sp. against *Staphylococcus aureus* ATCC 25923 pathogen in comparison with the control.

Plate 2:The inhibition zones of Streptomyces sp. against *Staphylococcus aureus* ATCC 25923

pathogen in comparison with the control (Ethyl acetate solvent).

The actinomycetes' isolates active in the production of antibiotics were identified by the molecular technique (the analysis of 16srRNA sequences done by Macrogen Korea 10 F, 254 Seoul Company). All morphological characters were observed on ISP-2 agar according to Taddei*et al*. (2006).

Table (3) illustrates the identification of the 15 actinomycetes isolates showing antagonistic effect against the human pathogenic *Staphylococcus aureus* in addition to the different regions and the standing crops from their rhizosphere the actinomycetes were isolated. It was found that, except Abhagovernorate in the South, each region was inhabited by one species of actinomycetes despite the difference in the plant rhizosphere from which these species were isolated. The data recorded in Table (3) reveal that two strains of *Streptomyces enissocaesilis* were isolated for the rhizosphere of a date palm tree cultivated in Tabuk at the North region. For the Middle Region (Al-Riyadh) five strains that isolated from the rhizosphere of three different plant (date palm tree, clover and tomato), were identified as *Streptomyces* sp. Two strains of *Streptomyces fungicidicus* were isolated from the rhizosphere of date palm tree and clover cultivated in Al-Dammam at the East region; while the three strains that isolated from the rhizosphere of a mango tree and beans plants were identified as *Streptomyces ambofaciens*. Regarding the South region (Abha), it was the only exception that found outside the context; where three different strains identified as *Streptomyces maritimus*, *Streptomyces rochei* and *Actinobacterium* sp.were isolated from the rhizosphere region of date palm tree and beans plants.

Table 3: The antibiotic-producing actinomycetes and their ecological distribution in different KSA regions.

Regions	Standing crop	Isolates code no.	Identification
North			
Tabuk	Date palm tree	7&12	*Streptomyces enissocaesilis*
South			
Abha	Date palm tree	3	*Streptomyces maritimus*
		13	*Actinobacterium*sp.
	Beans	11	*Streptomyces rochei*
Middle			
Al-Riyadh	Date palm tree	5.8&14	*Streptomyces* sp.
	Clover	9	*Streptomyces* sp.
	Tomato	15	*Streptomyces* sp.
East			
Al-Dammam	Date palm tree	6	*Streptomyces fungicidicus*
	Clover	2	*Streptomyces fungicidicus*
West			
Makkah	Mango	1&10	*Streptomyces ambofaciens*
Al-Taif	Beans	4	*Streptomyces ambofaciens*

DISCUSSION

In the present study, the dominate antibiotic producing soil Actinomycetes recovered is found to belong to genus *Streptomyces* which agree with study result by Atta *et al*.(2009),Thangapandian(2007) and Berdy(2005)who found that the most dominant genus of Actinomycetes producing antibiotics in soil

are *Streptomyces* and produced about 7.600 compounds.Furthermore, Almost 80% of the world's antibiotics are known to come from Actinomycetes, mostly from the genus *Streptomyces*(Pandeyet al., 2004).

In the present study the strains of *Streptomyces* sp. (code numbers 5, 8, 9, 14 and 15) that isolated from Al-Riyadh at the Middle region proved to produce antibiotic when tested against Staphylococcus aureus, they produce inhibition zones ranged from 8.5 to 11.7 mm for the whole culture and from 11.9 to 13.6 mm for the ethyl acetate extract.These results are similar to that obtained by El-Shobaky(2010)who stated that nine of his isolated actinomyceteswere active against *Staphylococcus aureus*.Furthermore, two strains of *Streptomyces enissocaesilis isolated* from the rhizosphere of date palm tree (code numbers 7 and 12) producedinhibition zones for *Staphylococcus aureusreached* 10.3 and 9.0mm for the whole culture and 11.7 and 12.8 mm for the ethyl acetate extract.These results are in agree with those obtained by Sirishaet al.(2014)who mentioned that *Streptomyces enissocaesilis* was active in inhibiting the growth of *Staphylococcus aureus.*

The strain of Streptomyces fungicidicus(code numbers 2 and 6) induced inhibition zoneson growth ofthe pathogenic Staphylococcus aureus reached 9.0 and 8.5mm for the whole culture and 12.3 and 12.1 mm for the ethyl acetate extract.Thesefindings are on the same line with those obtained by Hoang-Chuong Nguyen et al. (2013) and Xihou Yin et al.(2011)whofound that *Streptomyces fungicidicus* inhibited the growth Staphylococcus aureus. The strain of *Streptomyces rochei*(code numbers11) was proved to produce antibiotic when tested against *Staphylococcus aureus* and produce an inhibition zone of 9.8 mm.The obtained results are in agreement with the study by Reddy et al.(2014) and Kavitha et al.(2007).

In the present study the isolate *Streptomyces martimus* sample number (3) was proved to produce Antibiotic when tested against *Staphylococcus aureus* pathogen and produce an inhibition zone of (11 mm),this result similar to that mentioned by (Hoang-Chuong Nguyen*et al*., 2013) which proved that Streptomyces martimuswas active against Staphylococcus aureus.In the present study the *isolate Actinobacterium* sp. (code number13) was proved to produce Antibiotic when tested against *Staphylococcus aureusand* produce an inhibition zone of (8 mm) this result agree with the study by (Kumar and Bhaskara, 2012). In the present study the crude antibiotic produced by all the isolates are concentrated using Ethyl acetate method and found to produce a wider diameter of inhibition zones against *Staphylococcus aureus* than the crude antibiotic filtrate,this agree with result of several studies (Selvameenal*et al*., 2009 and El-Shobaky, 2010).

In conclusion, 15 out of 124 isolates of soil actinomycete shadthe capability of inhibiting growth of the human pathogenic *Staphylococcus aureas* ATCC 25923. The favorable conditions under which these antibiotics produced were pH (7),temperature (30 ^{o}C), agitation speed (180 rpm), working volume (50 ml medium/250 ml conical flask) and incubation period of 8 days.The present study reveals that the predominant ecological conditions in a region play an important role in the distribution of the antibiotic-producing soil actinomycetes.

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