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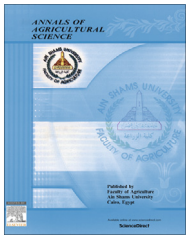

Molecular Identification of *Streptomyces* producing antibiotics and their antimicrobial activities

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Abstract Five strains of *Streptomyces*, namely S, N, W, E and C (designations should be mentioned in detail here) isolated from the rhizosphere soil cultivated with palm Alajua (date, pressed dates), AlMedina city, Saudi Arabia, were induced to produce antibiotics. Antimicrobial activities were determined on solid medium supplemented with starch. The detection was based on the formation of transparent zones around colonies. The results indicated that isolates had antibacterial activities against *Staphylococcus aureus*, *Bacillus cereus*, *B. subtilis*, *Pseudomonas aeruginosa* and also showed antifungal activity against *Candida albicans* and *Aspergillus niger*. DNA extracted from five isolates was used as template for 16s rDNA gene amplification. The expected PCR size was 1.5 kbp;1.6 kbp; 1.25 kbp; 1.25kbp and 1.0 k bp for S, N, W, E and C isolates respectively using universal 16s rDNA gene primers using direct PCR. The isolates varied morphologically on the basis of spore color, aerial and substrate mycelium formation, and production of diffusible pigment. Isolates were tested under a microscope by using slide culture technique. The results indicate that the soil of this region is source of *Streptomyces* having antibacterial and antifungal activity and thus better utilization of these microorganisms as biological control agents.

Introduction

Actinomycetes play a relevant role in soil ecology and are also of important biotechnological interest as they produce several bioactive metabolites. Within the filamentous actinomycetes, it would be desirable to identify and characterize environmental samples containing unusual genera (Alharbi et al., 2012). They provided many important bioactive compounds of high commercial value and continue to be routinely screened for new bioactive compounds (Khanna et al., 2011).

Therefore, the exploration of new habitats with unusual environment and poorly explored areas of the world has become important and useful for the discovery of novel compounds produced by actinobacteria (Learn-Han et al., 2012). *Actinomycetes* play a relevant role in soil ecology. The soil is the main hub for microbial population, especially the actinomycetes. Actinomycetes constitute a formidable group of industrially important microorganisms that have been explored for the production of Antibiotics which are known

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to be derived from several fungi, bacteria and actinomycetes (Pandey et al., 2000; Asgher et al., 2007). The ecological importance of antibiotic production by soil actinomycetes has been debated, but only limited evidence for production in natural environments was obtained (Williams and Vickers, 1986). The production of antibiotics by *Streptomyces* was demonstrated and included chloramphenicol (Gottlieb et al., 1954) gentamycin, (Rothrock and Gottleib, 1984), and thiostrepton (Wellington et al., 1993). Evidence for production under more natural conditions was difficult to obtain, possibly due to problems associated with adsorption of the antibiotics to clays and the very small amounts likely to be produced in oligotrophic environments, such as soil (Williams, 1982). *Streptomyces* have been recommended for the biocontrol of fungal root and seed pathogens (Tahoven et al., 1995) but are better known as prolific producers of commercially, important clinical antibiotics, Bizuye et al. (2013).

Streptomyces species was considered as major producers of bioactive compounds for the biotechnology industry. They are the source of most clinically used antibiotics, as well as of several widely used drugs against common diseases. Actinomycetes are the dominant group of soil population together with bacteria and fungi and are originally considered as an intermediate group between them. They are free living saprophytic bacteria and a major source for production of antibiotics (Unaogu et al., 1994). They play a major role in recycling of organic matter, production of novel pharmaceuticals, nutritional materials, enzymes, antitumor agents, enzyme inhibitors, immune modifiers and vitamins (Wellington et al., 1992), *Streptomyces*, a gram-positive, antibiotics producer (Unaogu et al., 1994), play a major role in recycling of organic matter. *Streptomyces*, the gram-positive, filamentous bacteria capable for secondary metabolite production such as antibiotics and antifungal compounds (Ref). The study aimed for isolation and characterization of *Streptomyces* collected from soil samples of agricultural soil (rhizosphere of plant) and its ability for antibiotic production. This study was performed to isolate actinomycetes having antibacterial and antifungal activities from soil samples collected from 5 different agricultural soil (S, N, W, E, and C), the soil of palm Alajua (date, pressed dates) Al-Medina city, Al-Medina region, located at the west of Saudi Arabia.

Materials and methods

*Sample collection and **Actinomycetes** isolation*

Rhizosphere soil samples were randomly collected from soil cultivated with palm Alajua (date, pressed dates) in different locations from AlMedina city, Saudi Arabia. The collected samples were air-dried, ground, then mixed with $CaCO_3$ and followed by sieving in 4 mm mesh screen. One gram of each prepared soil was dissolved in 100 ml sterile water and serial dilution was prepared until 10^{-6}. One ml of each dilution was spread plated on Petri-dish containing starch nitrate agar medium according to Tadashi (1975). The dishes were incubated for 7 days at 28 °C till *Streptomyces* colonies appear.

The purification was achieved by picking up unique single identical morphological streptomycetal colonies. Twenty purified *Streptomyces* isolates were sub-cultured on specific medium and stored at 4 °C.

Culture media

Peptone-Glycerol-Yeast extract agar medium (Oskay et al., 2004) was used for isolation and purification of *Streptomyces*. Glycerol asparagines agar medium (Williams and Cross, 1971) was used for isolation and purification of *Streptomyces*. Starch mineral salt agar medium (Williams et al., 1983) was used to detect colors of substrate and aerial agar mycelium. Additional media such as Nutrient agar medium (Oxoid), Potato dextrose, Tyrosine agar, and Czapek-Dox Agar Medium (CAM) were implemented in this study.

Antimicrobial activities

The antibacterial and antifungal of five isolates were assessed by the cork-borer method as following: Gram positive bacteria (*Staphylococcus aureus* ATCC6538 *Bacillus subtilis* ATCC6633 and *B. cereus* ATCC6354) and Gram negative bacteria (*E. coli* ATCC7839; *Pseudomonas aeruginosa* ATCC9027) cultures were inoculated in the form of a loopful of each bacteria in 50 ml of nutrient agar medium and poured in sterilized plates and left to solidify. *Candida albicans*, ATCC10231 culture was inoculated in the form of loopful in 50 ml of sabaroud agar medium and poured in sterilized plates and left to solidify. *Aspergillus niger*, TCC16404 (spore suspension) were inoculated in 50 ml of Dox agar medium and poured in sterilized plates and left to solidify. Plugs of each five *Streptomyces* test were cut out by the cork-borer and placed on the surface of the agenized medium seeded with test organisms. The plates were incubated at 37 °C for 24 h (bacteria), 30 °C for 48 h (yeast) and 25 °C for 72 h (Fungi). This method based on the observation of the microbial growth inhibition zone is an indication of the existence of a biologically active substance.

Morphological characterization

Spore chain morphology of five *Streptomyces* isolates was determined by light microscopy and described according to Pridham et al. (1958).

Genomic DNA isolation

DNA of five *Streptomyces* isolates was extracted using DNeasy Blood & Tissue Kit according to the manufacturer instructions. Antagonistic isolates were grown on agar medium containing (g/L): malt extract agar 0.08 mg, distilled water 1 L, pH 7.0. For DNA isolation the organism was grown with shaking for 24 h at 37 °C in baffled flasks containing 50 ml Nutrient broth medium.

PCR primers

In order to identify five *Streptomyces* isolates and classify them according to their phylogenetic criteria, the advantage of the sequence diversity of the 16s–23s intragenic spacer regions (SRs) of *Streptomyces* was considered. The SR primer was paired with a specific primer from within the 16s rDNA gene. The primer set 24 f and 1492r are illustrated in Table 1 and were used in the amplification 1.5 kbp product of 16s rDNA

Table 1 Sequence specificity of PCR primers used for amplification.

	Primers	Sequence
Set 1	24 F	(5′-AGAGTTTGATCCTGGCTCAG-3′)
	1492 R	(5′-TACGGTTACCTTGTTACGACTT-3′)
Set 2	ACT235F	(5′-CGCGGCCTATCAGCTTGTTG-3′)
	ACT878R	(5′-CCGTACTCCCCAGGCGGGG-3′)

gene. To increase the sensitivity of PCR, the primer sets ACT235f and ACT878r designed to amplify a portion of 16s rDNA gene were used in a nested-PCR reaction.

PCR amplifications

All amplification reactions were performed in a final volume of 50 µl, containing 50 mM KCl, 10 mM Tris HCl, pH 8.3, each primer at 500 nM, each dNTP at 0.2 mM, 1.5–3.5 mM $MgCl_2$, 1.5–2.5 U of Ampli Taq Gold DNA Polymerase (Applied Biosystems) and 3 µl of 10U Denhardt s reagent (per liter: 2 g Ficoll 400, 2 g polyvinylpyrrolidone, 2 g bovine serum albumin) (Volossiouk et al., 1995). Optimized cycling parameters, enzyme and $MgCl_2$ concentration for each primer pair are shown in Table 1.

Each amplification program was initiated by denaturation for 5 min at 95 °C (10 min when using AmpliTaq Gold) and finished with an extension step of 10 min at 72 °C. Reactions were performed either in a PE 9600 in a PCRExpress thermal cycler (Hybaid Ltd.) equipped with a gradient block for optimization of the annealing temperature. As a positive control, all DNAs yielded the expected amplification fragment of about 1.5 kb when tested with the degenerated primers (F24 and R1492, Table 2), specific for all bacteria (Heuer et al., 1997).

Electrophoretic analysis

Aliquots of 10 µl each of the amplification products were loaded onto 1.2% agarose slabs and run in TBA (40 mM Tris-acetate, 1 mM EDTA) buffer at 80 V for two hours. Slabs were stained with 0.4 µg of ethidium bromide/ml. After electrophoresis, the PCR products were visualized with UV transilluminator and documented with a Gel Doc 2000 gel system (Bio-Rad, USA). In addition, gels were photographed using a Polaroid camera. Molecular weight analysis of the resulted patterns was performed with the Quantity One software version 4.2.1 (Bio-Rad), as compared to the 1-kb DNA ladder (Invitrogen).

Statistical analysis

The obtained data were statistically analyzed using the Analysis of Variance (ANOVA) one way with the MSTAT-C statistical package The Least Significant Difference procedure (LSD) was used at 0.05 level of probability.

Results and discussion

Single colony of *Streptomyces* was isolated in pure culture using Nutrient agar medium. The isolates were grouped in five color series based on their aerial mycelia color and screened for their antibacterial and antifungal activities. Five isolates were found to have high activity against gram-positive and gram-negative bacteria besides pathogenic fungi.

Morphological characters

Morphological analysis was performed using light microscopy. The strains chosen for this study were gram stained, examined for shape, size and the mycelium structure. The arrangement of spore on the mycelium was examined using the light microscope as performed by Ara et al. (2012).

Table 2 Growth characteristics of five *Streptomces* isolated from Rhizosphere soil palm Alajua (date, pressed dates).

	Medium *Streptomyces*	Growth	Aerial mycelium	Substrate mycelium	Diffusible pigment
Glycerol asparagine agar	Isolate (STG)	Good	Gray	White	None
	Isolate (STL)	Moderate	White	Brown	Brown
	Isolate (STF)	Good	White	Brown	Brown
	Isolate (STC)	Moderate	White	White	None
	Isolate (STA)	Good	White	Brown	Brown
Starch mineral salt agar	Isolate (STG)	Good	Green	None	None
	Isolate (STF)	Good	Red	Brown	Brown
	Isolate (STC)	Moderate	Gray	None	None
	Isolate (STA)	Good	Gray	White	None
Peptone-glycerol-yeast extract agar	Isolate (STG)	Moderate	Red	None	Brown
	Isolate (STL)	Good	White	Brown	None
	Isolate (STF)	Moderate	White	None	None
	Isolate (STC)	Good	Red	Brown	Brown
	Isolate (STA)	Good	Gray	White	None
Tyrosine agar	Isolate (STG)	Moderate	Gray	Brown	Brown
	Isolate (STL)	Moderate	White	None	Brown
	Isolate (STF)	Good	Gray	White	None
	Isolate (STC)	Good	Red	None	Brown
	Isolate (STA)	Moderate	Red	Brown	Brown

Table 3 Antimicrobial activity of five *Streptomces* isolated from Rhizosphere soil palm Alajua (date, pressed dates).

Isolates	Microorganism					
	Diameter of zone of inhibition (mm)					
	S. aureus	*B. cereus*	*B. subtilus*	*P. aeruginosa*	*A. niger*	*C. albicans*
St. cyaneus (N)	14	14	12	12	15	14
St. antibiotics (S)	12	14	12	*ND*	17	*ND*
St. lavendulae (C)	11	*ND*	13	13	15	15
*St. griseorube*r (E)	14	15	*ND*	*ND*	*ND*	15
*St. fulvissimu*s (W)	14	15	13	13	14	14

ND = not detected.

Table 4 DNA concentration of five *Streptomyces* isolated from Rhizosphere soil palm Alajua (date and pressed dates).

Streptomyces isototes	DNA conc. (ng/µl)
C-*Streptomyces fulvissimus*	24.9
E-*Streptomyces cyaneus*	25.5
W-*Streptomyces antibiaticus*	41
S-*Streptomyces griseoruber*	23.4
N-*Streptomyces lovendulae*	13.2

The study included the isolation of five isolates belonging to the genus *Streptomyces* from five different rhizosphere soil of palm Alajua (date, pressed dates) AlMedina city, Saudi Arabia. *Streptomyces* isolates were identified on the basis of chalky appearance of colonies and production of moist earthy odor then confirmed by using slide culture technique (Table 2). The isolates were morphologically distinct on the basis of spore color, aerial and substrate mycelium formation, and production of diffusible pigment (Table 2). Morphological examination of these isolates, indicates that they belong to the genus *Streptomyces* (Waksman, 1961). The results showed that *Streptomyces* produced branched substrate and aerial mycelium which upon maturation, they were differentiated into spiral spore chains. The mycelial growth as well as development of spiral spore chains was studied microscopically under a light microscope. Cultural characteristics (Table 2) were determined in Peptone-Glycerol-Yeast extract agar medium, Starch mineral salt agar medium, Glycerol asparagine agar medium and Tyrosine agar. The addition of $CaCO_3$ and heat treatment led to raising the value of hydrogen power which limits the growth of most fungi and increase in growth of Actinomycetes.

Table 5 Expected PCR product (kb).

Target	Primers	Amplified fragment (kb)
N-*St. lovendulae*	24 f/ 1492 r ACT235f/ACT878r	1.5
S-*St. griseoruber*	24 f/1492 r ACT235f/ACT878r	1.25
C-*St. fulvissimus*	24 f/1492 r ACT235f/ACT878r	0.6
E-*St. cyaneus*	24 f/1492 r ACT235f/ACT878r	1.0
W-*St. antibiaticus*	24 f/1492 r ACT235f/ ACT878r	0.5

Antimicrobial activity

In this study, five *Streptomyces* isolates showed antimicrobial activity against the fulvissimus test organisms (Table 3). The isolated *St. cyaneus* (N) and *St. Fulvissimu*s (W) showed effect against gram positive and gram negative tested bacteria (*Staph. aureus*; *B. cereus*; *B. subtilis* and *P. aeruginosa*) and pathogenic fungi (*A. niger* and *C. albicans*), while the isolated *St. antibiotics* (S) had no effect against *Ps aeruginosa* and *C. albicans*. Also *St. Lavendulae* (C) isolate no effect against *B. cereus*. On the other hand *St. griseorube*r (E) showed low antimicrobial activity against *S. aureus*; *B. cereus* and *C. albicans*. These results are similar to those obtained by Sahin and Ugur (2003).

Bacterial DNA extraction

The concentrations of DNA extracted were, 24.9 ng/µl; 25.5 ng/µl; 41 ng/µl and 23.4 ng/µl for *St. fulvissimus*; *St. cyaneus*; *St. antibiaticus*; *St. griseoruber* and *St. lovendulae* respectively Table 4. DNA extraction was done using the kit (DNeasy Blood & Tissue Kit).

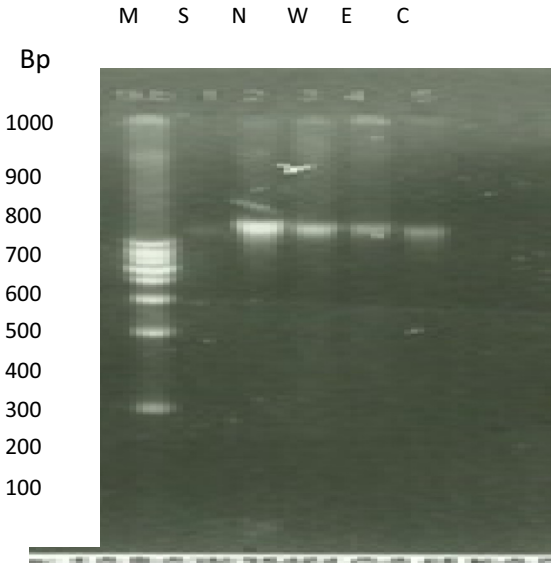

Fig. 1 Agarose gel electrophoresis (0.8%) of the extracted DNA (~12 kb). N: negative control (sterile water), lines 1–5: PCR product corresponding to the different actinomycetes (S, N, W, E, C) respectively. M: molecular weight marker (1kbp ladder).

Molecular screening

For the amplification of the 16S rRNA gene, two different PCR reactions were prepared with 2 couples of primers (24f/1492r and ACT235f/ACT878r). The PCR products sizes are summarized in Table 5 and visualized using agarose gel electrophoresis (Fig. 1) These results support that *Streptomyces* have been investigated predominantly as biocontrol agents, since they are frequently and easily isolated, and their antibiotics production arouses significant commercial interest. Since the isolates showed inhibitory activity against indicator bacteria and fungi, it is suggestive that Saudi Arabian soil could be an interesting source to explore for antibacterial, antifungal and antitumor secondary metabolites.

Antibiotics are the best known products of *Actinomycetes*. Over 5000 antibiotics have been identified from the cultures of Gram positive and Gram-negative organisms, and filamentous fungi, but only about 100 antibiotics have been commercially used to treat human, animal and plant diseases (Demain and Davies, 1999). The genus, *Streptomyces*, is responsible for the formation of most known antibiotics while a further 15% are made by a number of related genera such as *Actinomyces Micromonospora*, *Actinomadura*, *Streptoverticillium* and *Thermoactinomycetes* (Waksman, 1954).

Conclusion

The isolated microorganisms from rhizosphere soil of fertile areas were identified as a member of *Streptomyces* genus and have antibacterial and antifungal activity. This indicates that the soils of this region may be an interesting source for new antibiotics. Extensive study will be carried out in the future to explore more bioactive compounds from this region. Antibiotics production by these strains could easily be detected on solid medium. The present data suggest that the number and diversity of actinobacteria in deserted soils which is the case of Saudi Arabia representing a vast unexplored resource for the biotechnology of bioactive production. Studies are currently being conducted to produce bioactive compounds from actinobacteria through fermentations of different substrates.

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