Kaohsiung Journal of Medical Sciences (2014) 30, 435–446

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ORIGINAL ARTICLE

Isolation, screening and partial purification of antimicrobial antibiotics from soil *Streptomyces* sp. SCA 7

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Received 12 November 2013; accepted 6 February 2014
Available online 9 July 2014

KEYWORDS
Antimicrobial activity;
Actinomycetes;
Methanolic extract;
Minimum inhibitory concentration;
Streptomyces sp. SCA 7

Abstract Thirty-seven actinomycetes strains were isolated from soil samples collected from an agriculture field in Vengodu, Thiruvannamalai District, Tamil Nadu, India (latitude: 12° 54′ 0033″, North; longitude: 79° 78′ 5216″, East; elevation: 228.6/70.0 ft/m). The isolates were assessed for antagonistic activity against five Gram-positive bacteria, seven Gram-negative bacteria, and two pathogenic fungi. During the initial screening, 43% of the strains showed weak activity, 16% showed moderate activity, 5% showed good activity, and 35% showed no antagonistic activity. Among the strains tested, SCA 7 showed strong antimicrobial activity. Maximum biological activity was obtained on modified nutrient glucose agar (MNGA) medium. The mycelia of SCA 7 were extracted with methanol and tested against microbial pathogens using the disc diffusion method. The crude extract was purified partially using column chromatography and assessed for antimicrobial activity. Fraction 10 showed good activity against *Staphylococcus epidermidis* (31.25 μg/mL) and *Malassezia pachydermatis* (500 μg/mL) and the active principle (fraction 10) was identified as 2,4-bis (1,1-dimethylethyl) phenol. Based on morphological, physiological, biochemical, cultural, and molecular characteristics (16S rDNA sequencing), this strain was identified as *Streptomyces* sp. SCA 7. It could be used in the development of new substances for pharmaceutical or agricultural purposes.

Conflicts of interest: All authors declare no conflicts of interest.

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http://dx.doi.org/10.1016/j.kjms.2014.05.006

Introduction

The development of new antimicrobial agents, preferably naturally occurring ones with novel mechanisms of action, is an urgent medical need. Soil in particular is an intensively exploited ecological niche, the inhabitants of which produce many useful biologically active natural products, including clinically important antibiotics. However, the emergence of drug and multidrug-resistant pathogens [1] necessitates a continuing search for new antimicrobial compounds with potent antipathogenic activity. Searching for previously unknown microbial strains is an effective approach for obtaining new biologically active substances [2]. There is an urgent need to find new drugs, especially antibiotics, to control the spread of antibiotic resistant pathogens [3—5] and to treat life-threatening diseases such as cancer [6]. Microbial metabolites are rich sources for new potential therapeutic drugs [7]. Over 5000 antibiotics have been identified from the cultures of Gram-positive, Gram-negative and filamentous fungi, but only about 100 antibiotics alone have been used commercially to treat human, animal, and plant diseases [8]. The need for less toxic, more potent antibiotics from noninfective organisms, which overcome the resistance exhibited against the existing antibiotics, is felt acutely. During the past decades, the actinomycetes have provided many important bioactive compounds of high commercial value. Consequently, they are continuing to be routinely screened for new bioactive substances. These searches have been remarkably successful, and approximately two-thirds of naturally occurring antibiotics have been isolated from actinomycetes [9,10]. Actinomycetes, which are prolific producers of antibiotics and important suppliers to the pharmaceutical industry, can produce a wide variety of secondary metabolites [11]. Actinomycetes are widely distributed in nature, and are typically useful in the pharmaceutical industry for their seemingly unlimited capacity to produce secondary metabolites with diverse chemical structures and biological activities [12]. Although thousands of antibiotics have been isolated from *Streptomyces*, these represent only a small fraction of the repertoire of bioactive compounds produced [13,14]. Therefore, isolation of new *Streptomyces* from natural resources and characterization of their secondary metabolites is a valuable endeavor. *Streptomyces* species are Gram-positive, aerobic microorganisms with high DNA G + C contents and produce about half of all known antibiotics from microorganisms [15]. The species belonging to the genus *Streptomyces* constitutes 50% of the total population of soil actinomycetes and 75—80% of the commercially and medicinally useful antibiotics [16]. The aim of the present study was to isolate actinomycetes from soil samples collected from Vengodu, Thiruvannamalai District, Tamil Nadu, India. This area is poorly studied and represents the diverse and largely unscreened ecosystem.

Materials and methods

Chemicals

Media, standard antibiotics and all other chemicals were purchased from Himedia, Mumbai, India. Freshly prepared double distilled water was used throughout the experimental work.

Isolation of actinomycetes from soil samples

Soil samples were collected from an agriculture field in Vengodu, Thiruvannamalai District, Tamil Nadu, India (latitude: 12° 54′0033″, North; longitude: 79° 78′ 5216″, East; elevation: 228.6/70.0 ft/m). The samples were collected from 5—25 cm depth in sterile plastic bags and transported aseptically to the laboratory. The soil samples were air-dried for 1 week at room temperature. Isolation and enumeration of actinomycetes were performed by serial dilution and spread plate technique [17]. One gram of soil was suspended in 9 mL of sterile double distilled water. 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 starch casein agar (SCA, Himedia). To minimize the fungal and bacterial growth, actidione 20 mg/L, and nalidixic acid 100 mg/L were added. The plates were incubated at 30°C for 10 days. Based on the colony morphology, the actinomycetes cultures were selected and purified on International *Streptomyces* Project 2 (ISP-2) medium. In our pilot scale screening, a total of 37 actinomycetes were isolated and designated as SCA 1—37. They were used for the screening against microbial pathogens; active isolates were further characterized by the 16S rRNA sequencing technique.

Morphological, physiological, and biochemical observations

Cultural and morphological features of SCA 7 were characterized following the methods [18]. Cultural characteristics of pure isolates in various media including, Actinomycetes isolation agar (AIA), Mueller Hinton Agar (MHA), SCA, Sabouraud dextrose agar (SDA), *Streptomyces* agar (STP), yeast peptone glucose (YPG) agar, Zobell marine agar (ZMA), and ISP-2 were recorded after incubation at 30°C for 7—14 days. Morphology of spore bearing hyphae with entire spore chains was observed with a light microscope (Model SE; Nikon, West Patel Nagar, Delhi, India) using the cover-slip method in ISP medium (ISP-3—ISP-6). The shape of cell, Gram-stain, color determination, presence of spores, and colony morphology were assessed on solid ISP agar medium. Biochemical reactions, different temperatures, NaCl concentration, pH level, pigment production, and acid or gas production were done following standard methods [19,20].

Determination of antibiotic sensitivity and resistance pattern of SCA 7

Antibiotic sensitivity and resistance of *Streptomyces* sp. SCA 7 were assayed by the disc diffusion method [21]. A *Streptomyces* inoculum was prepared by growing cells in SCA for 48 hours at 30°C. Petri plates were prepared with 25 mL of sterile SCA medium. The test culture was swabbed on the top of the solidified media and allowed to dry for 10 minutes. Different antibiotic loaded discs were placed on the surface of the medium and left for 30 minutes at room temperature for diffusion of the antibiotics. The

plates were incubated for 48 hours at 30°C. After incubation, the organisms were classified as sensitive or resistant to an antibiotic according to the diameter of inhibition zone given in a standard antibiotic disc chart.

Cross streak method

The antimicrobial activity of actinomycetes isolates was performed by using the cross streak method [22]. YPG plates were prepared and inoculated with isolates by a single streak in the center of the Petri plate and incubated at 30°C for 7 days. The plates were then inoculated with the test organisms by a single streak at 90° angles to the actinomycetes strains and incubated at 37°C overnight. Antagonism was observed by the inhibition of the test organism. The results are presented in the following manner: +++ good activity (100% activity); ++ moderate activity (50% activity); + weak activity (25% activity) and − no activity.

Microbial organisms

The following Gram-positive and Gram-negative bacteria and some fungi were used for the experiment. Gram-positive: *Staphylococcus aureus* MTCC 96, *Micrococcus luteus* MTCC 106, *S. epidermidis* MTCC 3615, *S. aureus* ATCC 25923, and methicillin-resistant *S. aureus* (MRSA). Gram-negative: *Klebsiella pneumoniae* MTCC 109, *Enterobacter aerogenes* MTCC 111, *Salmonella typhimurium* MTCC 1251, *Shigella flexneri* MTCC 1457, *Proteus vulgaris* MTCC 1771, *Salmonella typhi-B*, and *S. typhi*. Fungi: *Candida parapsilosis* and *Malassezia pachydermatis*. The reference bacterial cultures were obtained from the Institute of Microbial Technology, Chandigarh, India-160 036 and all the fungal cultures were obtained from the Department of Microbiology, Christian Medical College, Vellore, Tamil Nadu, India. Bacterial and fungal inocula were prepared by growing cells in Mueller Hinton broth (MHB) (Himedia) for 24 hours at 37°C.

Optimization of media, mass production and intracellular extraction

Streptomyces sp. SCA 7 was grown on the following media for the production of bioactive compounds in an orbital shaker (150 rpm at laboratory temperature 30°C). Antibiotic production medium, fermentation medium, glucose yeast extract malt medium, M3 medium, modified nutrient glucose (MNGA) medium, M6 medium, and YPG medium were used. The pH of the medium was adjusted to 7.0 using 1M HCl and 1M NaOH. The culture was grown with continuous shaking on a rotary shaker (150 rpm) at 30°C for 14 days. After the fermentation of the culture, biomass was harvested by centrifugation (5000 rpm) at 20°C for 20 minutes, and then the mycelia were washed three times with sterile distilled water under aseptic conditions. The cells were then resuspended in a little amount of methanol and ground with the help of a pestle and mortar. Methanol was added to the ground cells in the ratio of 1:1 (w/v) and the mixture was shaken vigorously overnight; the extracts were then filtered through a blotting paper. The filtrates were then evaporated using a rotary evaporator at 50°C. The concentrated extract was then transferred into glass screw cap tubes and stored at 4°C for further use.

Antimicrobial assay

The antibacterial activity of the crude extract was assayed using the standard Kirby-Bauer disc diffusion method. Petri plates were prepared with 20 mL of sterile MHA (Himedia). The test cultures were swabbed on the top of the solidified media and allowed to dry for 10 minutes. The tests were conducted at 5.0 mg/disc concentrations of each crude extract. The loaded discs were placed on the surface of the medium and left for 30 minutes at room temperature for compound diffusion. A negative control was prepared using the respective solvent [dimethylsulfoxide (DMSO)]. Streptomycin (10 μg/disc) for bacteria and ketoconazole (30 μg/disc) for fungi were used as positive controls. The plates were incubated over night at 37°C and zones of inhibition were recorded. Diameters of the zones of inhibition were measured using a zone scale from Himedia and expressed in millimeters. All of the experiments were done in triplicate.

Fractions of methanol extract and their antimicrobial activity

The *Streptomyces* sp. SCA 7 was cultured, isolated, identified, and extracted for secondary metabolites according to the procedure described earlier [23]. The crude methanol extract was chromatographed over a silica gel chromatography column (60—120 mesh Ramken Si gel) and eluted with mixtures of $CHCl_3$:MeOH (chloroform:methanol). Finally, 20 fractions were obtained and all were screened against microbes.

In vitro antimicrobial assay

Based on biological activity, fraction 10 (8 mg) was dissolved in 0.2 mL of DMSO and used for the antimicrobial study using the standard broth microdilution method [24]. MHB was prepared and sterilized by autoclaving at 121°C, 15 lbs for 15 minutes. The required concentrations of the extract (1000 μg/mL, 500 μg/mL, 250 μg/mL, 125 μg/mL, 62.5 μg/mL, 31.25 μg/mL, and 15.625 μg/mL) were added to the 96 well microtiter plate containing 0.1 mL broth. An amount of 3 μL of log phase culture was introduced into the respective well and the final inoculum size was 1×10^5 cfu/mL. The plates were incubated at 37°C for 18 hours. Negative and solvent controls (DMSO) were also included. Streptomycin for bacteria and fluconazole for fungi were included as positive controls. An amount of 5 μL of the test broth was introduced on plain MHA plates to observe the viability of the organism. Minimum inhibitory concentration (MIC) was determined as the complete growth inhibition at a low concentration of the extract. All the experiments were done in triplicate.

Gas chromatography—mass spectrometry analysis

The active fraction 10 was subjected to gas chromatography—mass spectrometry (GC-MS) analysis on GC-MS-5975 (Agilent Palo Alto, CA, United States), column DB 5 ms Agilent, dimension length = 30.0 m, Internal Diameter

(ID) = 0.2 mm, film thickness = 0.25 μm, with a temperature program of 70–300°C, 10°C/minute, injection temperature = 240°C, carrier gas = helium, flow rate = 1.51 mL/minute, equipped with GC-MS NIST-II library.

16S rRNA regions based characterization

Genomic DNA isolation

The genomic DNA was isolated using the Hipura *Streptomyces* DNA spin kit-MB 527-20 pr (Himedia), according to the manufacturer's protocol. Briefly, the freshly cultured cells were pelleted by centrifuging for 2 minutes at 10,000*g* to obtain 10–15 mg (wet weight). The cells were resuspended thoroughly in 300 μL of lysis solution and 20 μL of RNase A solution was added, mixed, and incubated for 2 minutes at room temperature. About 20 μL of the proteinase K solution (20 mg/mL) was added to the sample and mixed; the resuspended cells were transferred to a Hibead Tube and incubated for 30 minutes at 55°C. The mixture was vortexed for 5–7 minutes and incubated for 10 minutes at 95°C followed by pulse vortexing. Supernatant was collected by centrifuging the tube at 8000*g* for 1 minute at room

Table 1 Preliminary screening of actinomycetes isolates using cross streak method.

	Gram-positive[a]					Gram-negative[b]								
	96	106	3615	25923	MRSA	109	111	1251	1457	1771	SPB	SPT	CP	MP
SCA-1	−	−	+++	−	++	+	++	−	+	+++	++	−	+++	+
SCA-2	+++	+	+++	+	+	+	+	−	−	+	−	−	+++	+
SCA-3	−	+	+++	−	+	+	+	−	−	−	−	−	+	+
SCA-4	−	++	+++	−	+	−	++	+++	−	−	−	−	+++	++
SCA-5	+	+	+++	+++	−	−	+	+	−	+	+	+++	+	−
SCA-6	+	++	++	−	+	+	++	+++	++	−	−	−	+++	+++
SCA-7	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	−
SCA-8	−	+++	++	−	++	+	+	++	++	++	−	−	−	−
SCA-9	−	+	+++	−	+++	−	+	−	−	+++	−	−	+++	+
SCA-10	−	−	−	−	−	−	−	−	−	−	−	−	−	−
SCA-11	−	−	−	−	−	−	−	−	−	−	−	−	−	−
SCA-12	−	+	+	−	+	−	+	+	+	+	−	−	++	+
SCA-13	−	+	+++	+	++	+	+	−	−	+++	−	−	+	+
SCA-14	−	−	−	−	−	−	−	−	−	−	−	−	−	−
SCA-15	+	+++	+++	+++	+++	+++	+++	+++	+++	+++	−	++	+++	+++
SCA-16	−	−	−	−	−	−	−	−	−	−	−	−	−	−
SCA-17	−	−	+	−	−	−	−	−	−	−	−	−	−	−
SCA-18	−	+	−	−	+++	+++	++	−	−	−	−	−	−	−
SCA-19	−	−	−	−	−	−	−	−	−	−	−	−	−	−
SCA-20	−	−	+++	−	−	−	−	−	−	+++	−	−	+	−
SCA-21	−	−	−	−	−	−	−	−	−	−	−	−	−	−
SCA-22	−	−	−	−	−	−	−	−	−	−	−	−	−	−
SCA-23	++	+++	−	−	+++	+++	−	−	−	−	−	−	−	−
SCA-24	−	−	−	−	−	−	−	−	−	−	−	−	−	−
SCA-25	−	+	+++	−	+++	++	++	−	−	+++	−	−	+++	+
SCA-26	+	+	−	−	+	−	−	−	−	−	−	−	−	−
SCA-27	−	−	+++	−	−	−	−	−	−	+++	−	−	−	−
SCA-28	−	−	−	−	−	−	−	−	−	−	−	−	−	−
SCA-29	−	−	−	−	−	−	−	−	−	−	−	−	−	−
SCA-30	−	−	+++	−	++	+	+	−	−	+++	−	−	+++	+
SCA-31	++	+++	+++	−	++	++	++	++	++	+++	−	−	+++	+++
SCA-32	−	−	−	−	−	−	−	−	−	−	−	−	−	−
SCA-33	−	−	−	−	−	−	−	−	−	−	−	−	−	−
SCA-34	−	−	−	−	−	−	−	−	−	−	−	−	−	−
SCA-35	−	−	−	−	−	−	++	++	−	−	−	−	−	−
SCA-36	++	+++	+++	−	+++	++	+++	+++	+++	+++	−	−	+++	+++
SCA-37	−	−	−	−	−	−	−	−	−	−	−	−	−	−

− = no activity (0% inhibition); + = weak activity (25% inhibition); ++ = moderate activity (50% inhibition); +++ = good activity (100% inhibition).

[a] Gram-positive: *Staphylococcus aureus* MTCC 96, *Micrococcus luteus* MTCC 106, *Staphylococcus epidermidis* MTCC 3615, *S. aureus* ATCC 25923 and Methicillin resistant *S. aureus* (MRSA).

[b] Gram-negative: *Klebsiella pneumoniae* MTCC 109, *Enterobacter aerogenes* MTCC 111, *Salmonella typhimurium* MTCC 1251, *Shigella flexneri* MTCC 1457, *Proteus vulgaris* MTCC 1771, *Salmonella typhi-B* (SPB), *Salmonella typhi*, and fungi such as *Candida parapsilosis* (CP) and *Malassezia pachydermatis* (MP).

Table 2 Morphological features of *Streptomyces* sp. (SCA 7) on different media.

Media	Aerial mycelium	Substrate mycelium	Soluble pigment	Reverse side	Growth
AIA	Whitish gray	Gray	—	Whitish	+++
MHA	—	Slimy yellow	—	Slimy yellow	+
SCA	Whitish gray	Gray	—	Yellow	+++
SDA	—	Yellow	—	Whitish yellow	++
STP	Dark gray	Dark gray	—	Yellowish gray	+++
YPG	Grayish white	Yellow	—	Yellow	+++
ZMA	—	Slimy yellow	—	Whitish yellow	+
ISP-2	White	White	—	Brownish yellow	+++

— = absent; + = weak growth; ++ = moderate growth; +++ = good growth; AIA = Actinomycetes isolation agar; ISP-2 = International *Streptomyces* Project 2 medium; MHA = Mueller Hinton Agar; SCA = starch casein agar; SDA = Sabouraud dextrose agar; STP = *Streptomyces* agar; YPG = yeast peptone glucose; ZMA = Zobell marine agar.

temperature. About 200 μL of lysis solution was added, mixed thoroughly by vortexing, and incubated at 55°C for 10 minutes. An amount of 200 μL of ethanol (96—100%) was added to the lysate and mixed thoroughly by vortexing for 15 seconds. The lysate was transferred to a new spin column and 500 μL of prewash solution was added to the spin column and centrifuged at 8000*g* for 1 minute; the supernatant was discarded. The lysate was then washed in 500 μL of wash solution and centrifuged at 8000*g* for 3 minutes. The elution buffer (200 μL) was pipetted out and added directly into the column without spilling and incubated for 1 minute at room temperature. Finally, the DNA was eluted by centrifuging the column at 8000*g* for 1 minute.

Preparation and analysis of 16S rRNA

The primers 27F (51 AGT TTG ATC CTG GCT CAG 31) and 1492R (51 ACG GCT ACC TTG TTA CGA CTT 31) were used to amplify the 16S ribosomal sequence from genomic DNA in a thermal cycler (ep gradient, Eppendorf, Kilpauk, Chennai, India). The cyclic conditions were as follows: initial denaturation at 94°C for 3 minutes, 35 cycles of 94°C for 1 minute, 54°C for 1 minute, and 72°C for 2 minutes, and final extension of 10 minutes at 10 minutes and hold at 4°C. The polymerase chain reaction products were confirmed by 1% agrose gel electrophoresis [25].

Table 3 Physiological and biochemical characteristics of *Streptomyces* sp. (SCA 7).

Characteristics	Results
Gram staining	Positive
Shape and growth	Filamentous aerial growth
Production of diffusible pigment	—
Range of temperature for growth	25—37°C
Optimum temperature	30°C
Range of pH for growth	3—11
Optimum pH	7
Growth in the presence of NaCl	1—11%
H_2S production	—

— = absent.

DNA sequence determination

Automated sequencing was carried out according to the dideoxy chain termination method using the Applied Biosystems automated sequencer (Synergy Scientific Services, Chennai, Tamilnadu, India) [26].

Database searching and nucleotide sequence accession number

The sequence was compared for similarity with the reference species of bacteria contained in genomic database banks, using the nucleotide blast (blastn) tool (http://www.ncbi.nlm.nih.gov/BLAST). The partial 16S rRNA gene sequences of isolate SCA 7 were deposited in the GenBank with accession number (KC 315781). A phylogenetic tree was constructed using the neighbor-joining DNA distance algorithm using software MEGA (version 4.0) [27].

Table 4 Antibiotic sensitivity tests for *Streptomyces* sp. (SCA 7).

Standard antibiotics	Resistant	Sensitivity
Amikacin 30 mg/disc	—	S
Ampicillin 10 mg/disc	—	S
Chloramphenicol 30 mg/disc	R	—
Cephalothin 30 mg/disc	R	—
Cephoxitin 30 mg/disc	R	—
Ciprofloxacin 10 mg/disc	—	S
Co-trimoxazole 25 mg/disc	—	R
Erythromycin 15 mg/disc	—	S
Gentamicin 10 mg/disc	—	S
Imipenem 1 mg/disc	—	S
Norfloxacin 10 mg/disc	—	S
Oxacillin	R	—
Penicillin-G 100 mg/disc	R	—
Piperacillin	R	—
Rifamycin 30 mg/disc	—	R
Tetracycline 30 mg/disc	—	S
Vancomycin 30 mg/disc	—	S

Results and discussion

Isolation of actinomycetes

Actinomycetes have been intensively studied in several underexplored environments, niche and extreme habitats in various parts of the world in the past few years. The soil samples were collected from different parts of Vengodu with a view to isolate actinomycetes strains. Based on the colony morphology and stability in subculturing, 37 suspected actinomycetes cultures were purified on ISP-2 slants. Among 37 isolates, 13, 9, 7, 3, 3, and 2 were gray, white, green, blue, orange, and black pigmented, respectively. Interestingly, gray and white mycelial pigmented actinomycetes were prominent in the soil. For long time storage, the strains were grown in ISP-2 broth for 7 days; then glycerol was added to make the final concentration 15% and storage was at −20°C [28].

Antagonistic potential of the isolates

It has been well established that most actinomycetes exhibit antimicrobial activity. The actinomycetes were initially screened to determine their ability to produce

Figure 1. Phylogenetic tree of *Streptomyces* sp. SCA 7 (KC 315781) constructed using the neighbor-joining method with the aid of MEGA 4.0 program.

antimicrobial compounds by the cross streak method [29]. In the initial screening, 16 (43%) showed weak activity, 6 (16%) showed moderate activity, 2 (5%) showed good activity and 13 (35%) showed no antagonistic activity. Among the strains tested, SCA 7 showed strong antimicrobial activity against the tested pathogens (Table 1). So, the SCA 7 strain was identified by biochemical and physiological characteristics and 16s rDNA amplification and sequencing. It was further cultivated in large scale and the bioactive metabolite was extracted.

Morphological, physiological, and biochemical characteristics of *Streptomyces* sp. (SCA 7)

Table 2 shows the cultural characteristics of the SCA 7 isolate. It was found that SCA 7 was a Gram-positive filamentous bacterium forming long spore chains in the aerial mycelia. Cultural characteristics of SCA 7 were observed after 7—14 days of growth, on eight different types of media. Strain SCA 7 grew well on AIA, SCA, STP, YPG agar, and ISP-2. Moderate growth was observed on SDA medium and weak growth was observed in MHA. The colors of colonies were whitish gray or grayish white and grayish brown and spore chains were whitish gray in color. The spores grew better on AIA, SCA, STP, YPG agar, ISP-2, and SDA than on MHA. The pigment did not diffuse into the surrounding medium on any medium used. The isolate was found to be a *Streptomyces* sp. based on the above mentioned cultural, morphological, and biochemical characteristics. The physiological characters are depicted in Table 3. The strain SCA 7 exhibited relatively good growth between the temperatures of 25°C and 30°C; the optimal temperature was 30°C. The SCA 7 isolate had tolerance limits of 11% to NaCl; test for H_2S production and diffusible pigments showed negative results.

Antibiotic sensitivity and resistance pattern

An antibiotic sensitivity test was conducted against the most commonly used antibiotics for bacterial infections by means of the disc diffusion method. SCA 7 exhibited high sensitivity towards amikacin, ampicillin, ciprofloxacin, erythromycin, gentamicin, imipenem, norfloxacin, tetracycline, and vancomycin. However, SCA 7 isolate showed resistance towards chloramphenicol, cephalothin, cephoxitin, co-trimoxazole, oxacillin, penicillin-G, piperacillin, and rifamycin (Table 4).

16S rDNA sequencing and phylogenetic analysis

16S rDNA sequence was determined and Blast analysis was performed, which confirmed that the isolate belonged to the *Streptomyces* species (bases 1—1470 linear DNA). The strain SCA 7 had 98% similarity with *Streptomyces sp.* (GU 045548) and *Streptomyces katrae* (HQ 607777). SCA 7 also shared a maximum of 94%, 93%, 92%, and 91% similarity with *Streptomyces sp.* (JF 820336), *Streptomyces lavendulae* (EF 371426), *Streptomyces herbaricolor* (DQ 442502), and

Table 5 Antimicrobial activity of extracts using the disc diffusion method produced from different media in which *Streptomyces* sp. (SCA 7) were grown.

	APM	FEM	GLM	M3 medium 5 mg/disc	M6 medium	MNGA	YPG	Streptomycin 10 μg/disc
Zone of inhibition (mm)								
Gram-positive[a]								
96	—	—	—	—	—	19	—	27
106	—	23	16	21	—	26	—	30
3615	—	—	—	—	—	22	—	23
25923	—	—	—	—	—	20	—	25
MRSA	—	—	—	—	—	15	—	24
Gram-negative[b]								
109	—	—	—	—	—	13	—	28
111	—	—	—	—	—	23	—	24
1251	—	—	—	—	—	13	—	18
1457	—	—	—	—	—	21	—	20
1771	—	—	—	—	—	20	—	21
SPB	—	—	—	—	—	10	—	20
SPT	—	—	—	—	—	10	—	18
Fungal								Ketoconazole (30 μg/disc)
CP	—	—	—	—	—	15	—	35
MP	—	—	—	—	—	12	—	24

— = no activity; AMP = antibiotic production media; FEM = fermentation media; GLM = glucose yeast extract malt media; MNGA = modified nutrient glucose media; SPT = *Salmonella paratyphi*; YPG = yeast peptone glucose media.

[a] Gram-positive: *Staphylococcus aureus* MTCC 96, *Micrococcus luteus* MTCC 106, *Staphylococcus epidermidis* MTCC 3615, *S. aureus* ATCC 25923, and Methicillin resistant *S. aureus* (MRSA).

[b] Gram-negative: *Klebsiella pneumoniae* MTCC 109, *Enterobacter aerogenes* MTCC 111, *Salmonella typhimurium* MTCC 1251, *Shigella flexneri* MTCC 1457, *Proteus vulgaris* MTCC 1771, *Salmonella typhi-B* (SPB), *Salmonella typhi*, and fungi such as *Candida parapsilosis* (CP) and *Malassezia pachydermatis* (MP).

Streptomyces sp. (GU 04559), respectively. A phylogenetic tree was constructed with bootstrap values. A neighbor joining tree based on the 16S rDNA gene sequences showed that the isolate occupied a new phylogenetic position within a subcluster, which was composed of *Streptomyces polychromogenes* (EU 570719) and *Streptomyces roseus* (EF 017710) (Fig. 1). Therefore, SCA 7 was determined to belong to the species *Streptomyces*. The sequences were deposited in GenBank (NCBI) with accession number (KC 315781) *Streptomyces* sp. SCA 7.

Media optimization

The antimicrobial activity of an organism is generally influenced by the nature of the habitat and differences in the composition of the substrate. In addition, there can be variations in terms of different strains and the test organisms. For example, we found that the isolate *Streptomyces* sp. SCA 7 was comparatively more active than the other antagonistic isolates with a higher antibacterial activity against Gram-positive and Gram-negative bacteria and fungal pathogens. This type of variation has also been reported with reference to activity against microbial pathogens in a study performed by Thumar et al., (2010) [30]. These authors isolated halotolerant alkaliphilic *Streptomyces* strains from the saline desert of Kutch, Western India, and determined the preliminary antimicrobial activity of these isolates by the cross streak and well-diffusion methods. One of the isolates exhibited potential activity against *Bacillus subtilis*. Preliminary screening revealed that MNGA medium was a very good base for the production of antimicrobial compounds among the tested media. The diameters of inhibition zones produced by intracellular extracts from of SCA 7 are as follows: *M. luteus* (26 mm), *E. aerogenes* (23 mm), *S. epidermidis* (22 mm), *S. flexneri* (21 mm), *P. vulgaris* (20 mm), MRSA (15 mm), *S. typhimurium* (13 mm), *K. pneumoniae* (13 mm), *S. typhi-B* (10 mm), *C. parapsilosis* (12), and *M. pachydermatis* (15 mm) (Table 5). Similar results were reported earlier [31].

Mass production, extraction, and antagonistic activity of intracellular extract

The isolate SCA 7 exhibited good antimicrobial activity. Hence it was chosen for mass production using MNGA medium; the fully grown mycelia were extracted with methanol. The concentrated crude extract was used for further studies. The antimicrobial activity of the intracellular methanol extract of strain SCA 7 was tested against five Gram-positive and seven Gram-negative bacteria and two pathogenic fungi at a 2.5 mg/disc concentration. Methanol extract showed good activity against *S. flexneri* (16 mm), *S. aureus* MTCC strain (14 mm), *P. vulgaris* (14 mm), *M. luteus* (13 mm), *S. aureus* ATCC strain (13 mm), MRSA (12 mm), *E. aerogenes* (12 mm), *K. pneumoniae* (11 mm), *S. typhimurium* (10 mm), *C. parapsilosis* (11 mm), and *M. pachydermatis* (12 mm). No inhibition was observed for *S. typhi-B* (Table 6). In a previous study by Vijayakumar et al., (2012) [32], the ethyl acetate extract of *Streptomyces* sp. was highly active against *Vibrio cholerae* (26 mm), *S. typhi* (24 mm), *P. vulgaris* (23 mm), *S. epidermidis* (18 mm), *Cryptococcus neoformans* (18 mm), *B. subtilis* (17 mm), *S. typhimurium* (17 mm), *Salmonella paratyphi B* (17 mm), *Candida albicans* (17 mm), *K. pneumonia* (16 mm), *Proteus mirabilis* (15 mm), *S. aureus* (15 mm) and *E. coli* (14 mm).

Table 6 Antimicrobial activity of crude methanolic extract of *Streptomyces* sp. (SCA 7) using the disc diffusion method.

	Methanolic extract 2.5 mg/disc	Streptomycin 10 μg/disc
Zone of inhibition (mm)		
Gram-positive[a]		
96	14	27
106	13	30
3615	11	23
25923	13	25
MRSA	12	24
Gram-negative[b]		
109	11	28
111	12	24
1251	10	18
1457	16	20
1771	14	21
SPB	—	20
SPT	—	18
Fungal		Ketoconazole (30 μg/disc)
CP	11	35
MP	12	24

— = no activity; SPT = *Salmonella paratyphi*.
[a] Gram-positive: *Staphylococcus aureus* MTCC 96, *Micrococcus luteus* MTCC 106, *Staphylococcus epidermidis* MTCC 3615, *S. aureus* ATCC 25923, and Methicillin resistant *S. aureus* (MRSA).
[b] Gram-negative: *Klebsiella pneumoniae* MTCC 109, *Enterobacter aerogenes* MTCC 111, *Salmonella typhimurium* MTCC 1251, *Shigella flexneri* MTCC 1457, *Proteus vulgaris* MTCC 1771, *Salmonella typhi-B* (SPB), *Salmonella typhi*, and fungi such as *Candida parapsilosis* (CP) and *Malassezia pachydermatis* (MP).

Fractions of methanol extract

Streptomyces sp. SCA 7 produced antibiotics during fermentation. They were extracted from the biomass using methanol and subsequently purified by silica gel column chromatography (60—120 mesh Ramken Si gel). About 72 fractions were collected and pooled into 20 fractions based upon their Thin Layer Chromatography (TLC) profile as follows: fractions 1—3 (CHCl3 100%), 4—8 (Chloroform — Methanol, 19:1), 9—11 (Chloroform — Methanol, 9:1), 12—14 (Chloroform — Methanol, 9:1), 15—19 (Chloroform — Methanol, 9:2), 20—25 (Chloroform — Methanol, 8.5:1.5), 26—31 (Chloroform — Methanol, 8.5:1.5), 32—38(Chloroform — Methanol, 4:1), 39—43 (Chloroform — Methanol, 4:1), 44—46 (Chloroform — Methanol, 7.5:1.5), 47—49 (Chloroform — Methanol, 3:1), 50—53 (Chloroform — Methanol, 3:1), 54—56 (Chloroform — Methanol, 3:1), 57—59 (Chloroform — Methanol, 3:1), 60—63 (Chloroform — Methanol, 3:1), 64—65 (Chloroform — Methanol, 1:1), 66—68 (Chloroform — Methanol, 1:1), 69—71 (Chloroform — Methanol, 1:1), and 72 (MeOH, 100%). The fractions were tested against different

Table 7 Antimicrobial activity of fractions of *Streptomyces* sp. (SCA 7) using the disc diffusion method.

Gram-positive[a]									Gram-negative[b]					
	96	106	3615	25923	MRSA	109 1000 μg/disc	111 disc	1251	1457	1771	SPB	SPT	CP	MP
Zone of inhibition (mm)														
Fr-1	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Fr-2	–	–	10	–	10	–	–	–	–	10	–	–	–	–
Fr-3	10	13	18	10	16	10	15	–	12	10	–	–	–	–
Fr-4	10	10	11	–	10		10	–	10	–	–	–	–	–
Fr-5	10	14	18	–	17	10	19	–	11	10	–	–	–	–
Fr-6	14	14	22	–	20	–	22	–	17	–	–	10	10	–
Fr-7	12	16	16	–	18	–	22	–	15	10	–	10	10	–
Fr-8	12	14	17	–	19	–	20	–	15	10	–	10	–	–
Fr-9	16	16	28	–	13	–	18	–	15	–	–	–	–	–
Fr-10	19	16	30	13	17	11	19	–	20	10	–	–	–	12
Fr-11	16	18	27	12	11	–	20	–	19	10	–	–	–	11
Fr-12	17	11	30	10	14	–	21	–	20	–	–	–	–	–
Fr-13	17	11	28	11	14	–	19	–	19	–	–	–	–	–
Fr-14	15	17	26	–	15	–	18	–	20	–	–	–	–	–
Fr-15	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Fr-16	13	15	19	10	15	–	17	–	15	–	–	–	–	–
Fr-17	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Fr-18	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Fr-19	12	10	17	–	11	–	12	–	14	–	–	–	–	–
Fr-20	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Streptomycin 10 μg/disc													Ketoconazole (30 μg/disc)	
	27	30	23	25	24	28	24	18	20	21	20	18	35	24

– = no activity; Fr = fraction; SPT = *Salmonella paratyphi*.

[a] Gram-positive: *Micrococcus luteus* MTCC 106, *Bacillus subtilis* MTCC 441, *Staphylococcus epidermidis* MTCC 3615, and Methicillin resistant S. *aureus* (MRSA).

[b] Gram-negative: *Klebsiella pneumoniae* MTCC 109, *Enterobacter aerogenes* MTCC 111, *Salmonella typhimurium* MTCC 1251, *Shigella flexneri* MTCC1457, *Proteus vulgaris* MTCC 1771, and *Salmonella typhi-B* (SPB), and fungi such as *Candida parapsilosis* (CP) and *Malassezia pachydermatis* (MP).

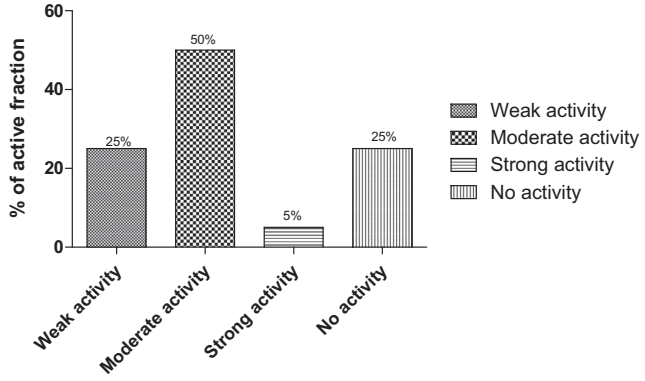

Figure 2. Percentage activity of different fractions against microbial pathogens.

test organisms by the disc diffusion method. An amount of 10 mg of each fraction was dissolved in 500 μL DMSO and loaded into 1000 μg/disc test for antimicrobial activity. Fraction 10 showed good activity; fractions 3, 5—9, and 11—14 showed moderate activity; fractions 2, 4, 16, and 19 showed weak activity, and fractions 1, 15, 17, 18, and 20 did not exhibit any activity against tested bacteria (Table 7, Fig. 2). Fraction 10 alone was assayed for MIC. Fraction 10 showed MIC values of 31.25 μg/mL against *S. epidermidis*, 250 μg/mL against MRSA, *E. aerogenes*, and *S. aureus*, 500 μg/mL against *M. luteus*, *S. aureus*, *S. flexneri*, and *M. pachydermatis*, and 1000 μg/mL against *K. pneumoniae* and *P. vulgaris* (Table 8). Similar results were reported by Valan Arasu et al., (2008) [12]. Further studies on the purification and characterization of antimicrobial principles of *Streptomyces* sp. SCA 7 are needed.

Table 8 Minimum inhibitory concentration of fraction 10 of methanol extract of *Streptomyces* sp. (SCA 7).

	Fr-10 (μg/mL)	Streptomycin (μg/mL)
Zone of inhibition (mm)		
Gram-positive[a]		
96	>250	>6.25
106	>500	>6.25
3615	>31.25	>6.25
25923	>500	>6.25
MRSA	>250	>25
Gram-negative[b]		
109	>1000	>25
111	>250	>25
1457	>500	>6.25
1771	>1000	>30
		Fluconazole
MP	>500	>30

[a] Gram-positive: *Staphylococcus aureus* MTCC 96, *Micrococcus luteus* MTCC 106, *Staphylococcus epidermidis* MTCC 3615, *S. aureus* ATCC 25923, and Methicillin resistant *S. aureus* (MRSA).
[b] Gram-negative: *Klebsiella pneumoniae* MTCC 109, *Enterobacter aerogenes* MTCC 111, *Shigella flexneri* MTCC 1457, *Proteus vulgaris* MTCC 1771, and fungi *Malassezia pachydermatis* (MP).

GC-MS

The GC-MS profiles of the active fraction 10 and its constituents are shown in Fig. 3 and Table 9. The structure of the major constituent is provided in Fig. 4. The chromatograph showed seven constituents. The phenolic constituent identified as 2,4-bis(1,1-dimethyethyl) constituted 16.51%, S.I 96% and this may the active principle. The other constituents in Table 9 are straight chain hydrocarbons or alcohols, which are known to be inactive. The highest antimicrobial activity was in the fractions containing a high amount of 2,4-bis(1,1-dimethyethyl) [33]. In the study conducted by Rangel-Sánchez et al. (2014) [34], the phenolic compound isolated from the Avocado roots showed marked antifungal activity against a wide range of

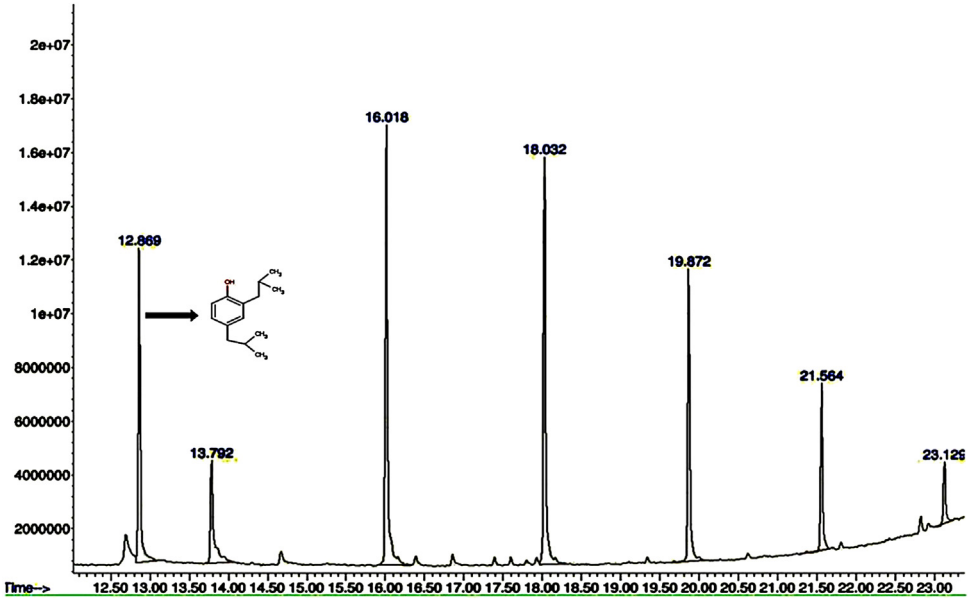

Figure 3. GC—MS chromatogram of the active fraction 10 from soil *Streptomyces* sp. SCA 7.

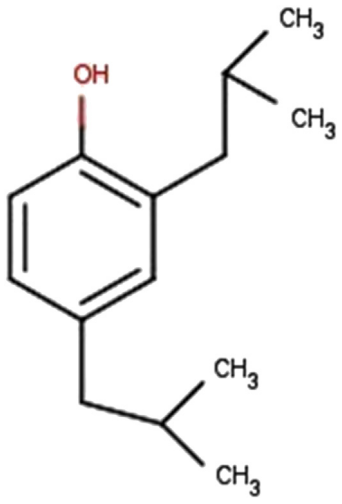

Figure 4. Structure of 2, 4-bis (1, 1-dimethylethyl) phenol.

Table 9 Gas chromatography—mass spectrometry (GC—MS) profile of the active fraction 10 from soil *Streptomyces* sp. SCA 7.

Sample no.	Retention time	Name of the compound	Peak (%)
1	12.869	Phenol, 2,4-bis (1,1-dimethylethyl)	16.51
2	13.792	3-Octadecene, (E)	8.74
3	16.018	1-Nonadecene	23.49
4	18.032	3-Eicosene (E)	23.93
5	19.872	Behenic alcohol	15.88
6	21.564	1-Heneicosanol	8.05
7	23.129	1-Nonadecene	3.40

fungi. Kubo et al. (1995) [35] proposed that the antimicrobial activity may be due to a balance between the polar hydrophilic and nonpolar hydrophobic portions of the molecule. Voda et al. (2004) [36] and Kim et al. (2007) [37] suggested that the hydroxyl (OH) group of the aromatic compounds and fatty acids could enter the membrane, orient into the aqueous phase by hydrogen bonding and nonpolar carbon chain aligned to the lipid phase by dispersion forces. The dispersion forces cause the disturbance of the fluidity of the cell membranes and the growth of the bacteria gets affected [36,37]. The compound has also been reported to have immunomodulatory effects [38]. The compound is sold commercially by Jinan JuHeng Chemical Co., Ltd., Tianqiao District, Jinan City, Shandong Province, P. R. China as an antioxidant principle.

Conclusion

The strain *Streptomyces* sp. SCA 7 isolated from an agricultural field showed a broad range of antimicrobial activity. The methanol extract and fractions of *Streptomyces* sp. SCA 7 were examined for antimicrobial activity. Results showed that the methanolic fractions possessed activity; 25% showed good activity, 50% showed moderate activity, 20% showed weak activity, and 5% showed no activity against the tested microbes. More specifically, fraction 10 exhibited the best antimicrobial activity; this was subjected to GC-MS analysis. It showed compound 2,4-bis(1,1-dimethylethyl) phenol to be the active principle. The other constituents identified by the GC-MS analysis were inactive straight chain hydrocarbons and alcohols.

Acknowledgments

The authors would like to extend their sincere appreciation to the Deanship of Scientific Research at King Saud University for its funding of this research through the Research Group project No. RGP-VPP-213.

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