Isolation and Molecular Identification of *Streptomyces griseorubens* from Al Saman Region Cave as a Producer of Antibacterial Agent

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ABSTRACT

Isolation and exploitation of actinomycetes from extreme habitats were increased leading to the discovering of potent bioactive natural compounds and avoid re-isolation of known strains. The unique characteristics and the microbiome residing inside including actinobacteria have made the cave ecosystems the interest of the research community. In this study, a total of 12 actinomycete isolates were isolated from two cave ecosystems (Hotel and Reda caves), situated at Al Saman region, 200 km northeast of Riyadh, Saudi Arabia. All these isolates were characterized and screened for antibacterial activity. They were able to abundantly grow on starch nitrate agar, ISP-4, and ISP-7 media and grew optimally at 25-30℃, pH 6.0-7.0. Only, five isolates had antibacterial activity against at least one of the tested bacteria (Staphylococcus aureus, methicillin-resistant Staphylococcus aureus (MRSA), Enterococcus faecalis, Acinetobacter baumannii, Klebsiella oxytoca, and Escherichia coli). Among them, the most potent isolate was CL2 which showed antibacterial activity against three pathogenic bacteria (S. aureus, MRSA, and E. faecalis). The selected isolate CL2 was screened for some enzyme production, and it was positive for amylase, protease, keratinase, gelatinase, chitinase, and lipase. Using morphological, physiological, and 16S rRNA patterns, the isolate CL2 was belonging to the genus Streptomyces and identified as *Streptomyces griseorubens* CL2 with a 99.24% similarity level to S. griseorubens strain NBRC 12780. The results obtained indicate that actinobacteria isolated from cave soils could be sources of industrial enzymes and antibacterial substances.

Key Words: *Streptomyces griseorubens,* Al Saman region cave, Antibacterial agent, 16S rRNA.

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INTRODUCTION

Recently, there is an urgent need for new drugs due to the emergence of multidrug-resistant pathogenic microorganisms and new infectious diseases. Also, the demand for industrial enzymes with novel properties was urgently needed [1, 2]. For many years, natural products have been used as medicinal agents all over the world [3].

Actinomycetes are regarded as the most prolific source of bioactive compounds commercially available antibiotics. They produce approximately two- thirds of all know antibiotics in the market and most of these are from members of genus *Streptomyces* [4]. Actinomycetes form active substances (antibiotics) that inhibit the development of pathogens on the roots of tree plantations [5]. Also, various genera of actinomycetes reported a wide array of enzymes and their products that have been applied in biotechnological industries and biomedical fields. Enzymes from other sources are less active than the enzymes of actinomycetes because enzymes of actinomycetes have high stability and unusual substrate specificity [6].

However, isolating and exploitating actinomycetes for novel compounds from conventional environments accounts for the rediscovery of known compounds [7]. So, a rare genera of actinomycetes switched from normal habitats or to the discovery of strains/species because of the

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search for novel products found in unusual habitats. The idea of these approaches is that novel bioactive compounds can be produced by such strains [8]. Rare-actinobacteria (non-*Streptomyces*) are mostly categorized as strains other than *Streptomyces* [9] or actinobacteria strains with less frequency of isolation under normal parameters [10]. Soil is a separate natural unit in which protection is a top priority issue when it is used by industry or agriculture [11]. Rare actinobacteria were discovered in different kinds of soil [12, 13], but caves, volcanic caves included, are still underexploited environment that can be screened for industrial compounds that are important [14].

During the past decade, studying microbes in caves was done in the area of many kinds of research. The interest of many scientists with microbial diversity in caves is increased due to unusual conditions like high humidity, extreme temperatures, and poor nutrients. These regions may contain new microbes with biotechnological importance [15, 16] Actinomycete populations were dominant among the other microorganisms in many cave habitats [17-19]. Some reviews provide evidence that actinobacteria from caves are might be a good source that drugs can be discovered from [17, 19]. Hence, the present investigation aimed at the isolation and characterization of actinomycetes from poorly studied cave habitat as a source of antimicrobial agents.

MATERIALS AND METHODS

Chemicals and media

Cultivation media components were obtained from different companies: BDH Limited Poole (England), Biomatic Corporation (Canada), Difco Laboratories (USA), FlukaChemie (Switzerland), Himedia (India), Holyland (Saudi Arabia), LobaChemie (India), and Techno Pharmchem Haryana (India). Glycerol was obtained from Merck (Germany) and commercial antibiotics disks were obtained from Mast Group Ltd (UK).

Sample collection

Six soil samples were obtained from two caves (Hotel and Reda caves) of Saudi Arabia situated at Al Saman region 200 km northeast of Riyadh (Fig. 1 and Fig. 2). The samples were collected aseptically from different parts of the caves (entrance, ground, and wall) using sterile bottles along with a sterile spatula, and the soils were taken at 5-40 cm depth. All samples were transported at 4℃ and processed directly.

Isolation and purification of actinomycetes

The soil samples were air-dried at room temperature for about 7 days before inoculation onto isolation plates. Then, the soil suspension of each sample was made separately by mixing 1g with 10 ml distilled water and vortexed for 2-5 min, then the mixtures could settle [20]. For each soil dilution, about 0.5 ml was taken [21] and spread evenly with a sterile glass rod onto starch nitrate agar [22]. The inoculated plates were incubated at 30℃ for 7 days [23]. After the incubation period, differentiation of actinomycete isolates from other microbial colonies by characteristics such as tough, leathery colonies which are not fully submerged into the agar [24]. Morphologically distinct colonies were chosen and sub-cultured to obtain pure isolates. The pure cultures were maintained on starch nitrate agar slants at 4℃; for long-term storage, cultures were maintained in glycerol broth (50%, v/v) at -20℃ [25]. Cultural characteristics and having antibacterial activity against pathogenic microorganisms were studied for all isolates. The best antagonistic actinomycete isolate was analyzed for producing some important enzymes and studied its taxonomic characterization.

Figure 1. Location of sampling site, Al Saman region, northeast of Riyadh, Saudi Arabia

Figure 2. The two studied caves. (A) Hotel cave and (B) Reda cave.

Cultural characteristics of the isolates

Growth capability (heavy, moderate, scanty, or no growth), the color of substrate and aerial mycelia, and the presence of soluble pigment were examined using various media [22]. These media were International *Streptomyces* Project (ISP) including yeast extract-malt extract agar (ISP-2), inorganic salts-starch agar (ISP-4), tyrosine agar (ISP-7),

and starch nitrate agar. The production of melanoid pigment was examined using tyrosine agar (ISP-7) and dark brown to black color pigment means positive result [26]. In all previous media, the plates were incubated at 30℃ for 7 days. The color of the substrate mycelium was described by the colors of the RAL code.

Screening of the isolates for their antibacterial activity

All actinomycete isolates were tested for antibacterial activity by agar plug method [27] against test pathogens bacteria obtained from King Fahad Armed Forces Hospital, Jeddah. The test bacteria were *Staphylococcus aureus* (ATCC 29213), methicillin-resistant *Staphylococcus aureus* (MRSA) (ATCC 52923), *Enterococcus faecalis* (ATCC 29212), *Acinetobacter baumannii* (ATCC BAA-747), *Klebsiella oxytoca* (ATCC 49131) and *Escherichia coli* (ATCC 25922).

The isolates were spread over the starch nitrate agar medium and incubated at 30℃ for 10 days for better growth and antibiotic production. Isolates of actinomycetes were removed from their agar using a sterile cork bore (5 mm in diameter) and placed onto Muller-Hinton agar (Himedia, India) plates seeded separately with cell suspensions of the test bacteria (12-24 hours old) [28]. The plates were kept 2 hrs at room temperature for the diffusion of antibiotic compounds from actinomycetes discs and the plates were incubated at 37℃ for 24-72 hours [29]. Following incubation, antibacterial activity was indicated by the formation of a clear zone surrounding the agar plug. The diameter of the inhibition zone was measured in millimeter (mm) and the absence of an inhibition zone indicated a negative result.

Screening of the selected isolate CL2 for its extracellular enzymes production

Production of extracellular enzymes by a potential isolate CL2 was studied by inoculated on different agar media at 30℃ for 7-14 days. The isolate CL2 was subjected to screen for amylase, protease, keratinase, gelatinase, chitinase, and lipase activity.

For screening amylolytic activity, the selected isolate was grown on ISP-2 containing 1% soluble starch [30]. After growth, the plates were flooded with an iodine solution. Indicator of hydrolysis of casein was assessed by growing the isolate on ISP-2 containing 1% skimmed milk [30]. Regarding the screening of keratinase, feather meal agar plates were inoculated with the bacterial isolate. The medium contained the following constituents (g/l): NH_4Cl, 0.5; NaCl, 0.5; K_2HPO_4, 0.3; KH_2PO_4, 0.4; $MgCl_2.6H_2O$, 0.1; yeast extract, 0.1; feather meal powder, 10; and agar, 20 and pH was maintained at 7.5 [31]. Gelatinase production for the isolate CL2 was tested using a gelatin medium containing 12% gelatin in test tubes [32]. The actinomycete culture was further examined for its chitinase activity on plates containing chitin agar medium [33]. The composition of the previous medium was (g/l): colloidal chitin, 1; KH_2PO_4, 0.7; K_2HPO_4, 0.3; NaCl, 4; $MgSO_4.7H_2O$, 0.5; and agar, 20). To determine lipase activity, the isolated bacterium CL2 was streaked on solid agar medium with Tween 80 as substrate [34]. Medium was prepared with (g/l): peptone, 10; NaCl, 5; $CaCl_2.2H_2O$, 0.1; Tween 80, 10; and agar, 20.

Taxonomical studies of the selected isolate CL2

Micromorphological characteristics

Light and scanning electron microscope was used to observe the morphology of the selected isolate. Chitin agar plate was inoculated by CL2 isolate and incubated at 30℃ for 7 days to examine by direct microscopic observation at 400X magnification of the selected filamentous bacterium [35].

Physiological characteristics

All physiological tests were performed on starch nitrate agar medium and incubated for 7-14 days at an appropriate temperature. The growth was assayed at different temperatures (15, 20, 25, 30, 35, 40, and 45℃) and at various pH (5, 6, 7, 8, and 9) to determine the optimal temperature and pH [21].

Biochemical characteristics

Gram staining was carried out by using the standard Gram reaction. Antibiotic susceptibility was examined on Muller-Hinton agar (Himedia, India) plates at 30℃ for 24-72 hrs with disks containing different antibiotics such as amikacin, ceftazidime, aztreonam, piperacillin, imipenem, and ciprofloxacin. After incubation, the plates were examined for the presence of inhibition zones around the disc of antibiotic [23].

Molecular identification

DNA extraction

Genomic DNA extraction was performed at King Fahd Medical Research Centre, KAU according to Kumar *et al.* [36] with some modifications. The purified actinobacteria isolate CL2 was grown for approximately 5 days on a starch nitrate agar plate. In a clean tube, bacterial colonies were mixed with 500 µl of TE buffer. This was followed by 3 cycles of heating in a water bath at 99℃ and treating by liquid nitrogen (3 min in each per cycle). The crushed mycelium was treated with lysozyme (20 mg/ml) and incubated in a water bath at 37℃ for 30 min. After cell lysis with 10% SDS (w/v) and proteinase K at 55℃ for 30 min, the lysate was cooled, extracted for 5 min with a mixture

of phenol: chloroform (1:1 v/v) at 10,000 rpm. In the aqueous phase, DNA was precipitated by adding 70-90% ethanol at -20℃ for 30 min. After centrifugation, the formed pellet was washed twice with 90% ethanol and dissolved in TE buffer. To obtain RNA free DNA, 20 µl of RNAase solution (20 µg/ml) was added and then incubated at 37℃ for 1 hr. The sample was once again extracted with an equal volume of phenol: chloroform and precipitated as above. The purity and concentration of DNA were checked using NanoDrop 2000 spectrophotometer (Thermo Scientific).

PCR amplification

The 16S rRNA was amplified using a thermal cycler (Applied Biosystems Veriti™ 96-Well Thermal Cycler). Amplification was performed using primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 519R (5'-GWATTACCGCGGCKGCTG -3') [37]. PCR conditions consisted of an initial denaturation at 95℃ for 5 min; followed by 35 cycles of denaturation (94℃ for 40 sec), annealing (58℃ for 40 sec), and extension (72℃ for 1 min), and a final extension at 72℃ for 10 min. The amplification product was examined by 1% agarose gel electrophoresis and the gel was visualized by Ultra-Violet Product (UVP BioSpectrum ® Imaging System).

Phylogenetic analysis of 16S rRNA sequence

The sequencing of the PCR amplicon of the selected isolate CL2 was done by Macrogen, Inc (Seoul, Korea). The 16S rRNA sequence was compared with available sequence data in the GenBank at the National Center of Biotechnology Information (NCBI) using the BLAST program [38].

RESULTS

Isolation of actinomycetes

A total of 12 morphologically distinct colonies were obtained from six cave soil samples using starch nitrate agar medium and named CL1 to CL12.

Cultural characterization of all isolates

From the result, it is indicated that the cultural characteristics of isolates on the isolation medium showed various colonies appearance with soluble pigments were observed in the media for isolates CL1, CL2, CL4, and CL8. Also, it was noticed that most colonies (about 83.33%) had grey color in different degrees and only two isolates were white (Table 1 and Fig. 3). Colonies' characteristics of the isolates on the isolation medium can be transformed into different appearances when the organism is sub-cultured on another growth medium. All isolates showed heavy growth on starch nitrate agar, ISP-4, and ISP-7 plates, however; the growth on ISP-2 medium was heavy for all colonies except five isolates. The three isolates CL1, CL6, and CL10 had moderate growth and the isolates CL2 and CL4 were poorly grown on ISP-2. Melanoid pigment was showed on IPS-7 (tyrosine agar) of the isolates CL5, CL6, CL9, CL10, CL11, and CL12.

Antibacterial activity screening of the isolates

Bioactive compounds from isolates were produced by agar surface fermentation and their activity was tested by agar plug method against *S. aureus*, MRSA, *E. faecalis*, *A. baumannii*, *K. oxytoca*, and *E. coli*. About 41.7% of actinobacterial cultures had antibacterial activity against at least one of the test bacteria (Table 2 and Fig. 4). The strong inhibitory activity of these actinomycetes was the isolate CL2 because it inhibited the growth of *S. aureus*, MRSA, and *E. faecalis* with inhibition zones 10, 11, and10 mm respectively (Fig. 5). However, the isolates CL11 and CL12 were active against two pathogenic bacteria, *S. aureus* and MRSA, whereas; the isolates CL1 and CL4 could inhibit *E. faecalis* only. No inhibition zone was observed against *A. baumannii*, *K. oxytoca*, or *E. coli*.

Table 1. Cultural characteristics of the actinomycetes on starch nitrate agar medium at 30℃ for 7 days.

Isolate no.	Growth	Source of isolation	Color of aerial mycelium	Color of substrate mycelium	Presence of soluble pigment
CL1	Heavy	Hotel cave	Grey	Brown beige	+
CL2	Heavy	Hotel cave	Grey	Tomato red	+
CL3	Heavy	Reda cave	Pale grey	Ivory	-
CL4	Heavy	Reda cave	Grey	Tomato red	+
CL5	Heavy	Reda cave	Pale grey	Beige brown	-
CL6	Heavy	Reda cave	Pale grey	Pearl gold	-
CL7	Heavy	Reda cave	White	Cream	-
CL8	Heavy	Hotel cave	White	Ochre yellow	+
CL9	Heavy	Hotel cave	Grey	Pearl gold	-
CL10	Heavy	Hotel cave	Grey	Terra brown	-
CL11	Heavy	Hotel cave	Grey	Ochre yellow	-
CL12	Heavy	Reda cave	Grey	Pearl gold	-

+: Soluble pigment presents; -: No soluble pigment presents.

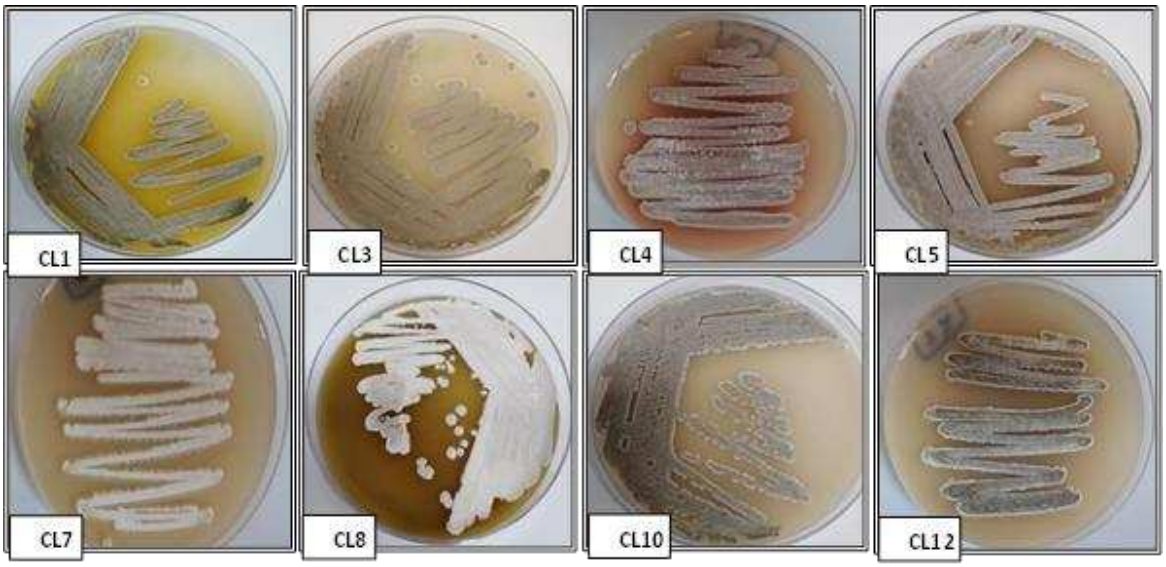

Figure 3. Colonies morphology and color of cave actinomycetes on starch nitrate agar medium at 30℃ for 7 days.

Table 2. Actinobacterial cultures showing antibacterial activity against Gram-positive bacterial pathogens.

Isolate no.	Zone of inhibition (mm)		
	The tested Gram-positive bacteria		
	S. aureus	MRSA	*E. faecalis*
CL1	-	-	12
CL2	10	11	10
CL3	-	-	-
CL4	-	-	10.5
CL5	-	-	-
CL6	-	-	-
CL7	-	-	-
CL8	-	-	-
CL9	-	-	-
CL10	-	-	-
CL11	9	6	-
CL12	7	8	-

-: No inhibition zone detected under the study.

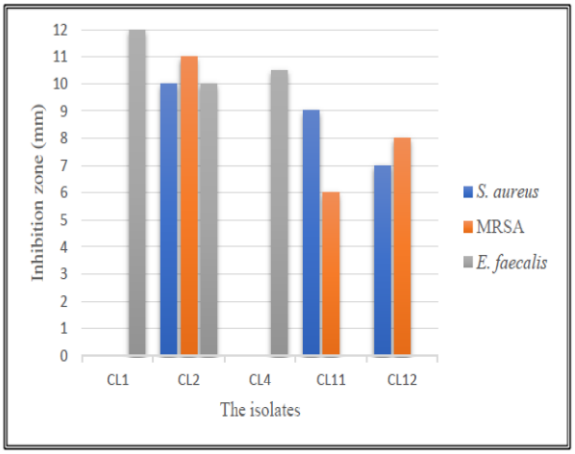

Figure 4. Antibacterial activity of some active bacterial isolates from caves.

Enzymatic screening of the selected isolate CL2

It is evident from the results that the isolate CL2 could produce amylase, protease, keratinase, gelatinase, chitinase, and lipase. The positive result of enzymes production for this isolate was illustrated in Figure 6.

On adding iodine to starch agar plates with the growth of the isolate, a zone of clearance was seen around the colony. Iodine forms a bluish-black complex with starch but not with hydrolyzed starch, thereby the isolate was amylase producing. The isolate CL2 showed positive proteolysis because of appearing a clear zone around the colony in the medium. The isolate also exhibited a clearing zone around the growth in the feather meal agar medium indicating positive keratinolytic production. Partial or total gelatin liquefaction of the inoculated tubes even after exposure to cold temperature (4℃) showed that the isolate CL2 produced gelatinase. To determine chitinase activity, a clearing zone surrounding the actinobacterial colony on a creamish background revealed that its growth depends, at least partially, on its ability to solubilize chitin. The lipolysis was detected due to the occurrence of a zone of clearance around the growth and subsequent formation of a white precipitate of calcium monolaurate because of the combination of released fatty acids and Ca^{2+} ions [39].

Taxonomical studies of the selected isolate CL2

Morphological, physiological, and biochemical characteristics

The selected isolate CL2 showed heavy growth on starch nitrate agar with soluble pigment that was observed in the medium. The growth on ISP-4 and ISP-7 plates were heavy, however; the growth on ISP-2 medium was poorly grown (Table 3 and Fig. 7). No melanoid pigment was

showed on the ISP-7 plate. Using a light microscope (at 400X magnification), direct observation of the isolate CL2 growing on chitin agar showed a filamentous bacterium (Fig. 8A). The results of physiological and biochemical properties revealed that the selected isolate grew well at a temperature between 15 and 45℃, and the optimal growth temperature was 25-30℃. The isolate could grow at a pH between 5 and 9 with a normal pH of 6.0-7.0. The CL2 isolate was Gram-positive and the microscopic characterization (at 1000X magnification) illustrated the substrate and aerial mycelia (Fig. 8B). A spore chain with a smooth surface was noticed (Fig. 8C). The growth ability of the selected isolate in the presence of 6 standard antibiotics was evaluated by the disc diffusion method. Isolate CL2 recorded a clear sensitivity against the following antibiotics: amikacin, imipenem, and ciprofloxacin with different degrees. Some morphological, physiological, and biochemical characteristics of the isolate CL2 were listed in Table 4.

Figure 5. The antagonistic actinomycete isolates CL2 against (A) *S. aureus*, (B) MRSA and (C) *E. faecalis*.

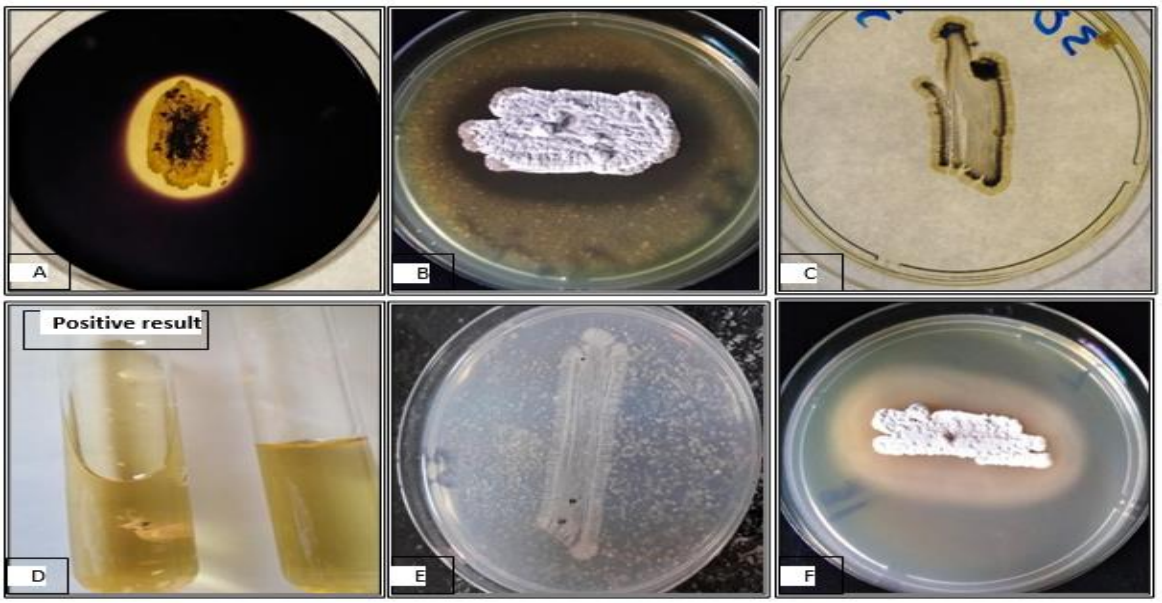

Figure 6. Enzyme production by the selected isolate CL2 on different enzymatic agar media. (A) A clear zone of starch hydrolysis, (B) A zone of skimmed milk clearance, (C) Keratinase degradation activity, (D) Gelatin liquefaction (positive result), (E) Chitinase producing and (F) Lipolytic activity detected by a precipitate around the growth.

Table 3. Cultural characters of the selected isolate CL2 grown on different agar media at 30℃ for 7 days.

Media	Growth	Aerial mycelium color	Substrate mycelium color	Presence of pigment
Starch nitrate agar	Heavy	Grey	Tomato red	+
ISP-2	Scanty	Pale yellow	Signal yellow	-
ISP-4	Heavy	Gray	Clay brown	-
ISP-7	Heavy	Pale gray	Saffron yellow	-

+: Soluble pigment presents; -: No soluble pigment presents.

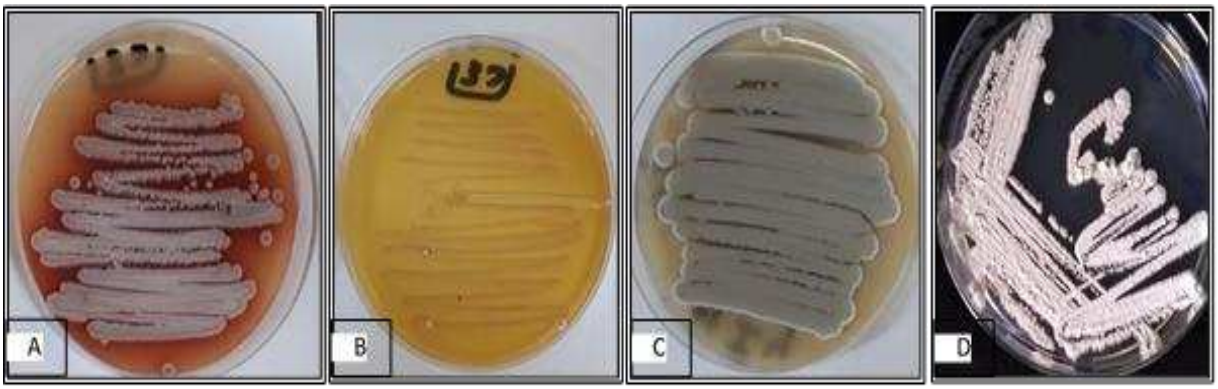

Figure 7. Different media used for cultural characteristics of the selected isolate CL2. (A) Starch nitrate agar, (B) IPS-2, (C) IPS-4 and (D) IPS-7.

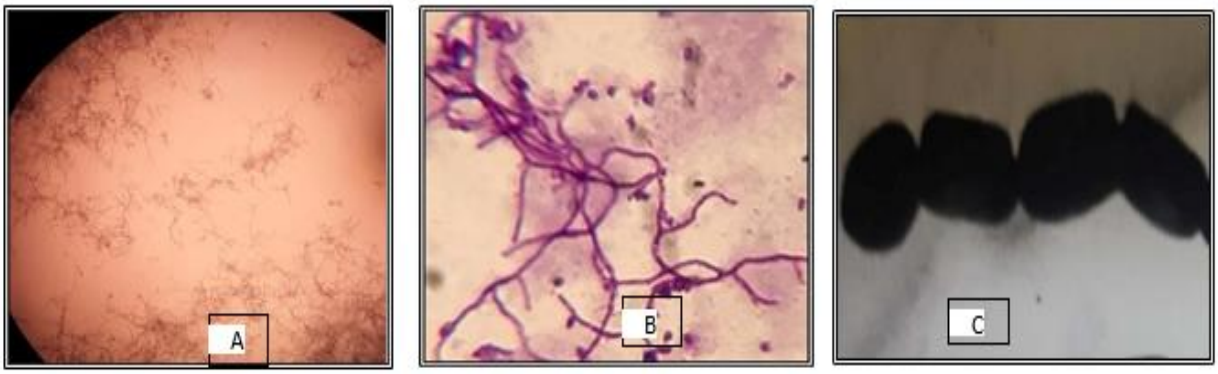

Figure 8. The isolate CL2 showed filamentous bacterium under a light microscope (A) On chitin agar plate (400X magnification) and (B) Gram staining (1000X magnification). (C) The isolate CL2 under an electron microscope.

Table 4. Some morphological, physiological, and biochemical characters of the tested isolate, CL2.

Tested Character	Result
Melanin pigment on tyrosine agar	-ve
Motility of spore	Absent
Shape of spore	Cylindrical
Spore chain	Straight chain
Number of spores/chains	40-60
Aerial and substrate mycelia	Well developed
Zoospore, sporangium, sclerotia, and fragmented mycelia	Absent
Growth temperature range	15-45℃
pH range	5-9
Gram stain	Gram-positive
Resistance to antibiotics:	
Amikacin	S
Ceftazidime	R
Piperacillin	R
Imipenem	S
Ciprofloxacin	S

-ve: Negative result, R: Resistance, S: Sensitive.

Molecular identification

Identification of actinomycetes using molecular tools proved to be faster and least tedious compared to classical biochemical methods. The 16S rRNA sequence of the

isolate CL2 was compared to the GenBank database in the National Center for Biotechnology Information (NCBI) using the BLAST program.

The phylogenetic analysis revealed that the CL2 isolate belongs to the genus *Streptomyces* sp. MBRC-120. The rooted phylogenetic tree for MBRC-120 and related representative type strains of the genus *Streptomyces* demonstrated that *Streptomyces* CL2 isolate was most closely related to *Streptomyces griseorubens* strain NBRC 12780 (99.24%), as shown in Figure 9.

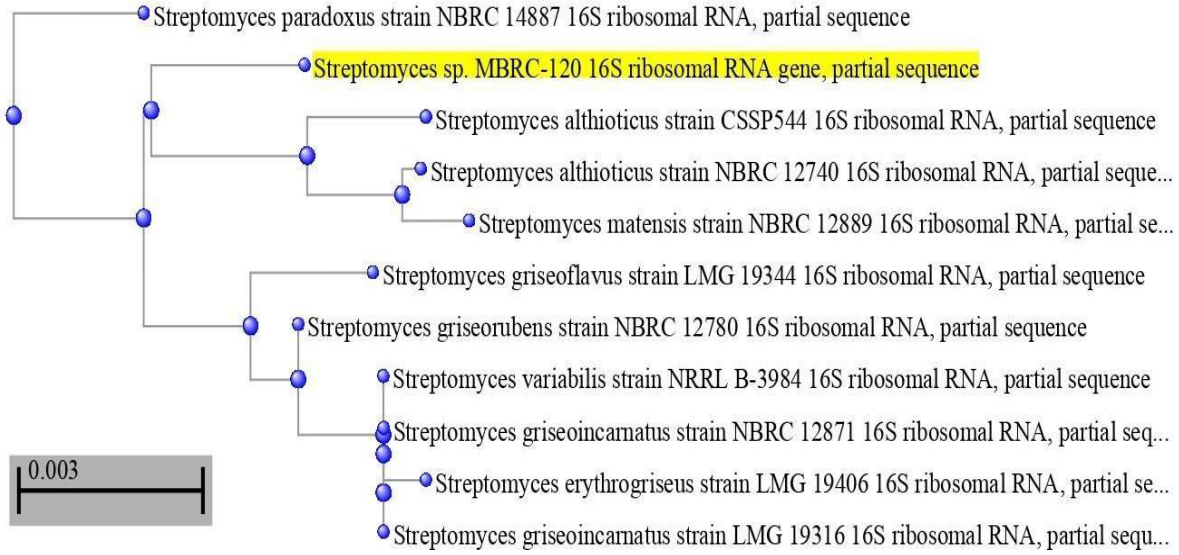

Figure 9. Phylogenetic tree based on 16S rRNA sequence comparisons of *Streptomyces* CL2, using neighbor-joining tree method, maximum sequence difference =0.003.

DISCUSSION

The extreme habitats are characterized by chemical or physical conditions that differ significantly from those found in environments that support more abundant and varied life forms [40]. Caves provide a quasi-extreme environment for living organisms which is due to the relatively low organic nutrient input and lack of light [41]. The extreme conditions in the caves might create stress for the inhabitant microorganisms at the genetic level, which leads to the evolution of new species and their novel metabolites [42]. Therefore, caves are considered as an attractive source for the isolation of novel actinomycete taxa [2].

In this study, about 12 actinomycete colonies were obtained from different parts of Hotel and Reda's caves located at Al Saman region 200 km northeast of Riyadh in Saudi Arabia. Several factors were considered for the isolation of actinomycetes from cave samples. Before diluting soils, the soils were air-dried at room temperature for about a week to decrease the population of Gram-negative bacteria [43] because actinomycetes are slow growers relative to that of other soil bacteria [44]. The samples were grown on selective medium (starch nitrate agar) at 30°C for 7 days. This medium was recommended for improving the isolation of actinomycetes [23, 45]. All isolates were screened for antimicrobial activities using different resistant bacteria as test organisms. The most active isolate was CL2 isolate, thus it was selected for more detail studies. Using morphological, physiological, and 16S rRNA sequencing analysis, the isolate CL2 was belonging to the genus *Streptomyces*. It was identified as *S. griseorubens*CL2 with a 99.24% similarity level to *S. griseorubens* strain NBRC 12780. Similar results were obtained by other authors who reported that actinobacteria are abundant in caves [46], and the highest number of novel species was from genus *Streptomyces* [2]. Further, the predominant actinomycetes isolated from different caves were members of the genus *Streptomyces* [47, 48].

The genus *Streptomyces* was classified in the family Streptomycetaceae and received particular attention for three main reasons. First, streptomycetes are abundant and important in the soil. They played major roles in the recycling the carbon that is trapped in insoluble organic debris, especially the ones from fungi and plants. This action is enabled by the production of diverse hydrolytic exoenzymes [4]. Second, the genus exhibits a wide phylogenetic spread [49]. Thirdly, streptomycetes are among nature's most competent chemists and produce a stunning diversity of bioactive secondary metabolites; consequently, they are of great interest in medicine and industry [50]. This genus serves as a model system for bacterial antibiotic production [4].

During the screening of antibacterial activity in this study, five (42%) isolated actinomycetes out of 12 isolates

showing activity against Gram-positive bacteria. Gram-negative strains were highly resistant and were consistent with the known susceptibility differences among the target organisms [51]. The reason for the different sensitivity between Gram-positive and Gram-negative bacteria could be explained to the morphological differences between these microorganisms. Gram-negative bacteria have an outer polysaccharide membrane carrying the structural lipopolysaccharide components which makes the cell wall impermeable to lipophilic solutes. The Gram-positive should be more susceptible to having only an outer peptidoglycan layer which is not an effective permeability barrier [52].

These results confirmed the observation of some studies which reported that actinobacterial isolates have antibacterial activity against Gram-positive microorganisms. New derivatives Cervimycin were obtained from *S. tendae* which was isolated from a rock wall of the Grottadei Cervi, Italy. The previous antibiotics inhibited both Gram-positive (*Bacillus subtilis*, *S. aureus*, MRSA and vancomycin-resistant *E. faecalis* (VRE) [53]. Xiakemycin A is a new pyranonaphthoquinone obtained from *Streptomyces* sp., isolated from remote karst soil, China and this material strongly inhibited Gram-positive bacteria [54]. Sixteen isolates of *Streptomyces* spp. from Chaabe cave in Algeria showed strong antibacterial activities against Gram-positive bacteria [55].

Unlike the present study, antimicrobial activity was found in the isolated actinomycetes against Gram-positive and Gram-negative bacteria. In a study of cultivable actinobacteria from Azores volcanic caves that was carried out in Portugal, 18.1% of 148 actinobacterial isolates had antibacterial activity against at least one of the following bacteria: *Salmonella typhimurium*, *E. coli*, *Pseudomonas aeruginosa*, *Proteus* sp., *Listeria monocytogenes*, *L. innocua*, and *S. aureus*. Most of the active isolates belong to the genus *Streptomyces* (*S. nojiriensis, S. spiroverticillatus, S. avidinii,* and *S. mauvecolor*) [56]. A total of 40 taxa, belonging to the different genera as *Streptomyces*, were recovered from moon milk deposits, Belgium. Antimicrobial activities of these isolates were 87% and 59% against Gram-positive and Gram-negative bacteria, respectively [57]. A report revealed that the Turkish karstic caves harbor actinobacteria, in which 62% of the isolates, were active against several microbial pathogens (Gram-positive and Gram-negative bacteria, yeast, and filamentous fungi). Yücel and Yamac [58] reported that *Streptomyces* sp. had strong activity against clinical strains of MRSA, VRE, and *A. baumanii*.

Studies on the microbial potential of extreme environments can be utilized to produce novel enzymes that can become future harbingers of green biotechnology. Actinomycetes have great importance because they can make and then secrete different kinds of extracellular hydrolytic enzymes that are safe for the environment. A wide array of these enzymes can be used in different industrial applications such as food, medicine, pulp and paper, detergent, textile, agriculture, and biorefineries [59]. In the present investigation, the selected actinomycete CL2, obtained from the soil sample of Hotel cave, was tested for its capacity to produce some important enzymes. The results represented that the identified isolate CL2 produced different enzymes such as amylase, protease, keratinase, gelatinase, chitinase, and lipase which are of biotechnological and industrial importance. Similarly, Narasaiah *et al*. [60] reported that *Streptomyces* are being recognized as the best-known producers of array enzymes like amylase, lipase, protease, keratinase, and chitinases.

In conclusion, cave and karst environments have not been explored microbiologically and should be considered as one of the sites for searching and discovering novel actinobacteria and their chemical diversity of compounds that are quite useful. The results of this investigation revealed that the caves of the Al- Saman region were a potent source of actinomycetes with useful bioactive compounds. Hence, these isolates can be further evaluated and studied in detail for commercial-scale production of enzymes and antibacterial substances. Currently, the isolation strategy specifically for cave actinobacteria is lacking. There is still an urgent need for improved selective isolation to target specific actinobacterial taxa of interest. Modification of growth conditions and the use of new culturing methods were proposed for the cultivation of previously uncultivable microorganisms. A combination of enrichment techniques which included adjusting media pH, heat pretreatments of samples, and calcium salts supplements were effectively used to separate rare actinobacteria from karstic caves [48]. The study on cave actinobacteria and the bioactive compounds that are found there is still at an early stage. There remain rare bacteria to be discovered and further studied as producers of new bioactive compounds. These bacteria will benefit the human and can be a magic solution [2].

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