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Research Article
Isolation and Characterization of Antagonistic Activity of Some Streptomycetes and their Specific Actinophages from Soil

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Abstract

Background and Objective: Phages specific to actinomycetes are common, active in the soil and gladly detected. Soil streptomycetes are having antibiosis activities against numerous bacteria, fungi and plant viruses. Thus, this study was designed to isolate, purify and characterize some streptomycetes active against some microorganisms from soil followed by isolation of their specific phages. **Materials and Methods:** Antagonistic activities of these streptomycetes isolates were tested against *Bacillus subtilis*, *Pseudomonas* sp., *Serratia* sp. and *Aspergillus niger*. To confirm their biological characterization of the streptomycetes isolates under investigation, the 16SrRNA gene was also used. The presence of specific lysate actinophages in the soil samples were tested by spot test technique and then propagated and purified for further characterization. The morphology of the purified actinophages was determined by electron microscopy. **Results:** The five selected *Streptomyces* isolates having effective antagonistic activity were biologically and molecularly identified as *Streptomyces sclerogranulatus* (QQ06), *Streptomyces mutabilis* (QQ07), *Streptomyces heilongjiangensis* (QQ08), *Streptomyces sparsus* (QQ09) and *Streptomyces purpurascens* (QQ10) strains. Electron micrographs showed the presence of filamentous virus-like particles with lengths of 21.4×928.57, 25×750, 21.4×857.14, 21.4×885.7 and 21.4×857.14 nm specific to *Streptomyces* strains QQ06, QQ07, QQ08, QQ09 and QQ10, respectively and belong to the family Inoviridae. **Conclusion:** Phage of Inoviridae was considered as the first time against streptomycetes isolates, therefore, additional and advanced studies should be carried out at the level of molecular characterization.

Key words: Actinophages, streptomycetes, isolation, antibiosis, characterization, 16SrRNA gene, inoviridae

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Streptomycetes used to take place abundantly in soil rich in organic matter, marine and represented in nature by a large number of species or varieties[1-3]. One of the strictest aerobes microorganisms is streptomycetes which can grow in sterile soil under low oxygen concentration or dried-soil[4]. Several streptomycetes were recently isolated from soil and biologically and/or molecularly identified[5,6]. The possibility of use actinomycetes in the biological control field as a safe alternative to chemical pesticides has been studied. Mohamed *et al.*[7] isolated several actinomycetes from the rhizosphere soil collected from different plants and able to inhibit or reduce the growth of some plant pathogens.

Bacteriophages are considered the most broadly abundant viruses on planet Earth[8,9]. Some soil bacteria could be controlled by bacteriophages that accessible in the soil at an estimated level of 1.5×10^8 phages g^{-1} of soil[10-12]. The composition and variety of the actinomycetes population could be decreased by actinophages as biological control agents. These phages were belonging to three families (Myoviridae, Siphoviridae and Podoviridae) and suggested to control plant-pathogenic bacteria[13,14]. Two phages (ØS and ØL) from soil were active against *Streptomyces scabies* MR13[8] while Donadio *et al.*[15] isolated three phages that were specific to *Streptomyces erythreus*. Phages attacking *Streptomyces* spp. were pervasive in the soil environment due to phage endurance and replication in soil[11-14]. Luis *et al.*[16] isolated from soil samples nine dissimilar phages specific to *S. antibioticus*. The phages had polyhedral heads and long, non-contractile tails. In Western Australia, El-Tarabily *et al.*[17] collected some soil samples from Jarrah forest for isolation of three phages belonging to the family Siphoviridae and apply *S. diastaticus, S. griseus* and *S. hygroscopicus* as propagative hosts.

In Egypt, Marei and Elbaz[18] isolated three different lytic actinophages from the soil and classified them as Sf1, Sf2 and Sf3 and were specific to *S. flavovirens*. These phages were characterized by the shape and size of the head and tail with a size of 67.2 and 114 nm, respectively. Filamentous bacteriophages from the Inoviridae family were reported to infect both Gram-negative and positive bacteria and construct an important contribution to host physiology, ecology and virulence[19,20-22].

This study aimed at isolation and purification of some streptomycetes from some soil samples having antagonistic activities against some microorganisms. Isolation and partial characterization of actinophages specific to the isolated streptomycetes also aimed.

MATERIALS AND METHODS

Study area: The study was carried out at the Department of Agricultural Microbiology, Faculty of Agriculture, Ain Shams University, Egypt from June, 2018-2020.

Soil samples collection: Four fresh heterogeneous mixture soil samples were collected from the farm of Faculty of Agriculture, Ain Shams University, El-Qalyubia Governorate and kept at 4°C till used as described by Mohamed *et al.*[7]. The soil samples were analyzed for their chemical, physical and biological properties at arid land Labs Faculty of Agriculture, Ain Shams University, Egypt.

Isolation of streptomycetes: Isolation of streptomycetes was approved by poured plate technique and purification by single colony technique as described by Mohamed *et al.*[7] using starch nitrate agar medium[23].

Antagonistic activities: Antimicrobial activities of the selected streptomycetes against four test microorganisms (*Bacillus subtilis*, *Pseudomonas* sp., *Serratia* sp. and *Aspergillus niger*) were determined based on the methods of Mohamed *et al.*[7]. These microorganisms were obtained from the Microbiology Laboratory, Department of Agricultural Microbiology, Faculty of Agriculture, Ain Shams University, Egypt.

Identification of selected streptomycetes

Biological identification: Up to genus the anticipated keys of Bergey's Manual of Determinative Bacteriology[24] were used for partial identification of all purified streptomycetes-like isolates. Each growth culture and morphology of spore chain characters by a direct microscopic method as well as spore surface of the five selected *Streptomyces* isolates using Transmission Electron Microscopy (TEM) was studied based on the method of Tresner *et al.*[25]. The physiological properties of the same *Streptomyces* isolates including the ability to produce melanoid pigments and formation of diffusible pigments were determined[26]. The ability to use different sugar sources as exclusive of carbon sources was also studied as reported by Mohamed *et al.*[7]. Medium with D-glucose and medium without carbon source were served as positive control and negative controls, respectively. The isolates were also tested for their abilities to grow on Czapek-Dox agar medium[27].

Molecular identification: The *Streptomyces* isolates showed highly antagonistic activities were sent as slants (Pure

cultures) to Macrogen Laboratory, Seoul, South Korea for molecular identification by determining the nucleotide sequences of the 16SrRNA gene. For PCR-isolation of 16SrRNA gene two universal primer pairs (785F: 5'GGA TTA GAT ACC CTG GTA3' and 27F: 5'AGA GTT TGA TCM TGG CTC AG3' and 907R: 5'CCG TCA ATT CMT TTR AGT TT3' and 1492R: 5'TAC GGY TAC CTT GTT ACG ACT T3' were used. The protocol of Mohamed *et al*.[7] for Polymerase Chain Reaction (PCR) mixture and PCR program was applied for isolation of the 16SrRNA genes of the five selected *Streptomyces* isolates. The DNA sequences of the PCR products of the five selected isolates were blasted with the linked nucleotide sequences of the universal isolates collected from HTTP:// www.ncbi.nlm.nih.gov/, using the DNA Star Software Package-Lasergene.

Isolation and purification of actinophages: The actinophages specific to the five identified *Streptomyces* strains were isolated from soil samples following the protocol of Stenholm *et al*.[28]. The presence of actinophages in the soil suspensions was detected qualitatively by the spot test technique. The phages were propagated as showed by Carey-Smith *et al*.[29]. The actinophages were purified by the modified method of Marei and Elbaz[18]. The morphology of purified actinophages particle was examined using negative staining with 2% uranyl acetate by TEM as applied by Nugent and Cole[30]. Electron micrographs were obtained by using a Model Beckman 1010 TEM at Al-Azhar University, the Regional Center for Mycology and Biotechnology, Cairo, Egypt.

RESULTS

Isolation, purification of Streptomycetes and their antagonistic activities: Data in Table 1 showed that a total number of 15 out of 30 Streptomycetes-like Colonies (SLC) were isolated, purified and kept on slants of starch nitrate agar medium. These isolates were introduced to the determination of their antagonistic activities against one fungal isolate (*Aspergillus niger*) (Fig. 1a-f) and three bacterial isolates (*Pseudomonas* sp., *Serratia* sp., *Bacillus subtilis*) (Fig. 2a-c). The 15 SLC isolates were diverse in their antagonistic activities

Table 1: Total number of SLC isolated from Plant-cultivated soils

Soil samples	Number of SLC	Number of purified isolates on selective medium
KB-01	6	3
KB-02	7	4
KB-03	9	4
KB-04	8	3
Total	30	15

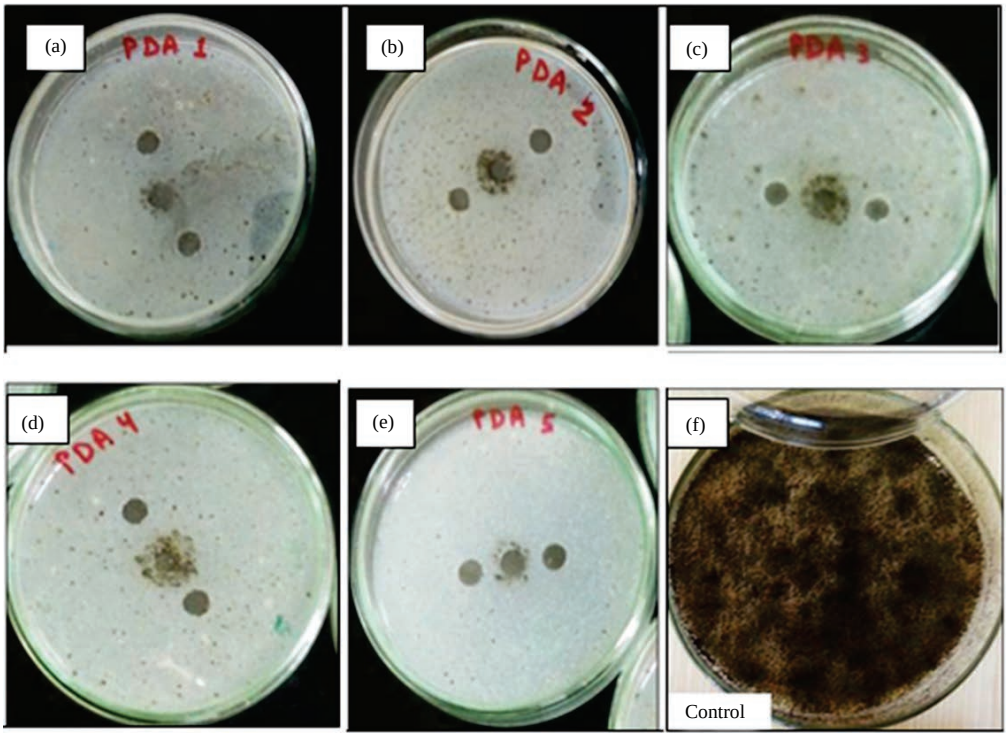

Fig. 1(a-f): Antifungal activities of the five selected streptomycetes isolates against *Aspergillus niger*
1: QQ06, 2: QQ07, 3: QQ08, 4: QQ09 and 5: QQ10

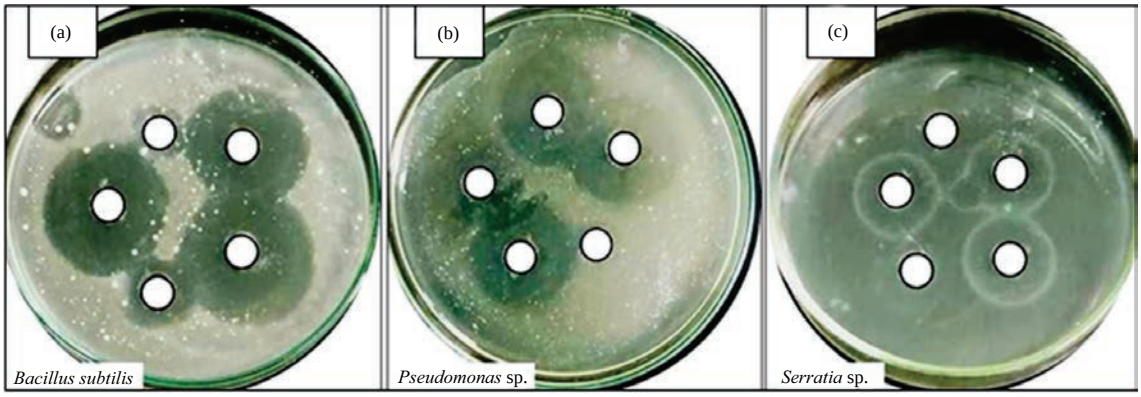

Fig. 2(a-c): Antibacterial activities of the five selected streptomycetes isolates 10 days post-incubation compared to untreated (with no streptomycete-filtrate as a control)

Table 2: Antagonistic activities of the fifteen purified *Streptomyces* isolates

	Antimicrobial activities			
	Antibacterial		Antifungal	
Streptomyces isolates	*Bacillus subtilis*	*Pseudomonas* sp.	*Serratia* sp.	*Aspergillus niger*
1	+	+	+	-
2	+++	+++	+++	+++
3	++	-	-	-
4	+++	++	+++	++
5	++	++	++	++
6	++	-	++	-
7	++	-	+	-
8	-	+	++	-
9	++	++	++	++
10	-	++	-	+
11	++	++	-	+
12	++	++	++	+
13	-	+	-	-
14	+++	+++	+++	+++
15	++	+	-	+

-: No antimicrobial activity, +: Weak activity, ++: Moderate activity, +++: High activity

against the tested microbial strains (Table 2). The highest active *Streptomyces* isolates, i.e., isolates No. 2, 4, 5, 9 and 14 were selected for their partial biological and molecular identification.

Biological identification of the selected streptomycete isolates: The experimental results showed that the streptomycete isolate-02 (QQ06) had a white to pale yellow aerial mycelium, RF spore-chain and smooth spore surface (Table 3 and Fig. 3a). The isolate was not able to produce melanoid pigments on the four media used. Moderate growth on Cazpek's agar medium was recorded. its substrate mycelium was reddish and produces yellow diffusible pigments. The isolate was not vigorously utilized 30% of added sugars (L-Arabinose, L-Rhamnose and Sucrose) as a sole carbon source for growth. QQ06 isolate had antimicrobial (Bacterial and Fungal) activities.

The isolate-04 (QQ07) was having a grey aerial mycelium and spiral spore chain with a smooth surface (Table 3 and Fig. 3b). Neither melanoid nor diffusible pigments were produced by isolate-04 (QQ07) and poor growth on Cazpek's agar medium was recorded. The isolate was able to use 80% of the sugars as sole carbon sources but not able to utilize both raffinose and sucrose. Antimicrobial activities of the isolate under investigation were reported. Therefore, the isolate was classified as a strain of *S. mutabilis*.

A grey and pale-yellow mycelium carrying RF spore chain with a smooth surface was recorded for isolate-5 (QQ08) (Table 3 and Fig. 3c). Moderate growth on Cazpek's agar medium, brownish diffusible pigments and producing

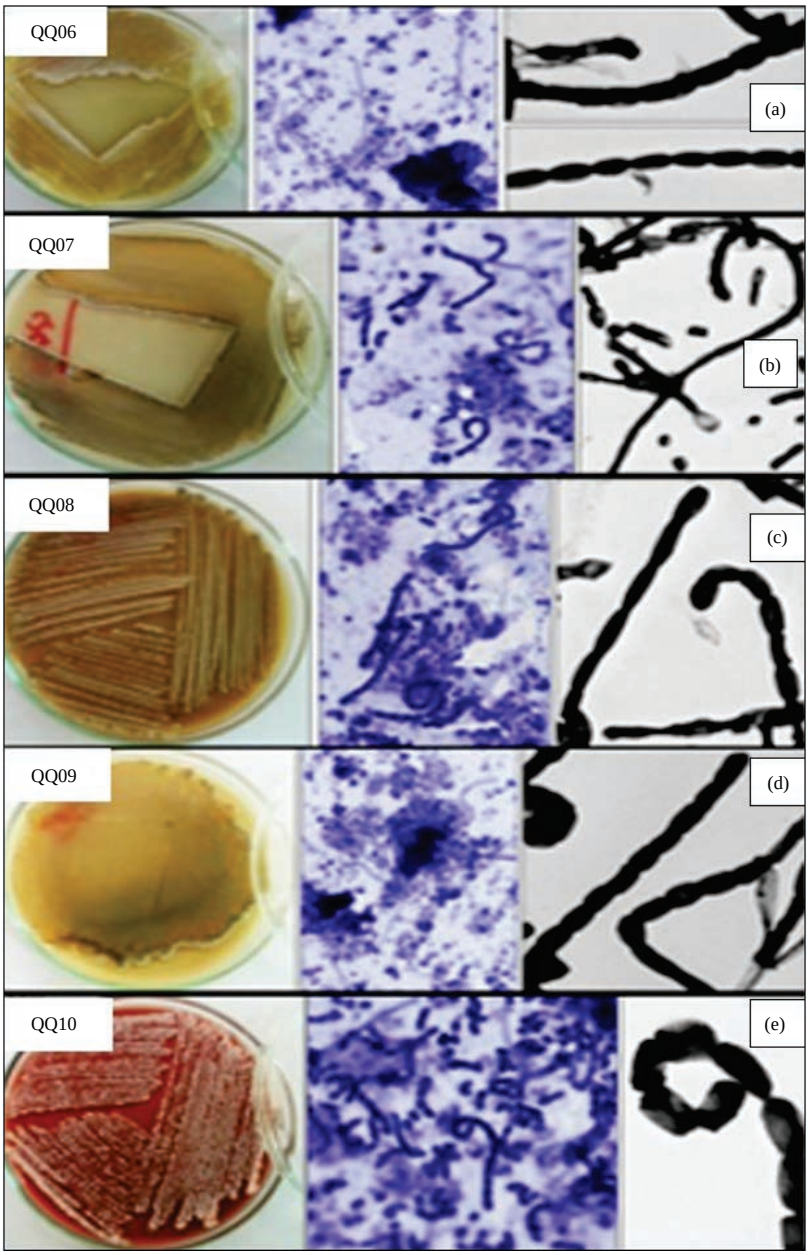

Fig. 3(a-e): Color of aerial mycelia, the shape of spore chains and spore surfaces of the five streptomycetes

(a) Streptomycete isolates-02 (QQ06): White to pale yellow aerial mycelium, rectus flexible spore chain (1000-X) and smooth spore surface (20,000-X). (b) Streptomycete isolate-04 (QQ07): Grey aerial mycelium, Spiralspore chain (1000-X) and smooth spore surface (10,000-X), (c) Streptomycete isolate-05 (QQ08): Grey aerial mycelium, rectus flexible spore chain (1000-X) and smooth spore surface (15,000-X), (d) Streptomycete isolate-09 (QQ09): Grey aerial mycelium, rectus flexible spore chain (1000-X) and smooth spore surface (20,000-X) and (e) Streptomycete isolate-14 (QQ10): Red aerial mycelium, Spiralspore chain (1000-X) and spiny spore surface (20,000-X)

melanoid pigments were noted. About 90% of the sugars as sole carbon sources was utilized as carbon sources by this streptomycete, while it was not able to utilize the D-Galactose sugar. The growth of the three tested microorganisms was inhibited by the filtrate of QQ08 streptomycete isolate.

Results revealed the characters of the streptomycete isolate-09 (QQ09) which is characterized by grey aerial mycelium, reddish substrate mycelium, RF spore chain with a smooth surface (Table 3 and Fig. 3d), positive-melanoid pigment, moderate growth on Cazpek's agar medium and not produce diffusible pigments and

Table 3: Characters of the five streptomycete isolates (QQ06, QQ07, QQ08, QQ09 and QQ10) under investigation

Characters	Streptomycete isolate-02 (QQ06)	Streptomycete isolate-04 (QQ07)	Streptomycete isolate-05 (QQ08)	Streptomycete isolate-09 (QQ09)	Streptomycete isolate-14 (QQ10)
Colour of aerial mycelium	White to pale yellow	Grey	Grey	Grey	Red
Spore-chain	RF	Spiral	RF	RF	Spiral
Melanoid pigment	C-	C-	C+	C+	C+
Spore surface	Smooth	Smooth	Smooth	Smooth	Spiny
Growth on Cazpek's medium	Moderate	Poor	NR	Moderate	Good
Colour of substrate mycelium	Reddish	Yellow	Pale yellow	Reddish	Reddish
Diffusible pigments	Yellow			-	Red
Utilization of carbon					-
No carbon	NR	-	-	NR	
D-Glucose	+	+	+	+	+
D-Xylose	+	+	+	+	+
L-Arabinose	-	+	+	+	+
L-Rhamnose	-	+	+	-	+
D-Fructose	+	+	+	+	+
D-Galactose	+	+	-	-	+
Raffinose	+	-	+	+	+
D-Mannitol	+	+	+	+	+
i-Inositol	+	+	+	+	+
Sucrose	-	-	+	+	+
Antagonistic activity	Antibacterial and antifungal		Antifungal	Antifungal	Antifungal and antiviral

RF: Rectus flexible, C-: No Melanoid pigment, C+: Produces melanoid pigments, NR: Not recorded, +: Positive, -: Negative

Table 4: Identities percentage of nucleotide sequences of the 16SrRNA gene of the five *Streptomyces* isolates and the most similar Streptomycetes recorded in GenBank

Description	Query cover (%)	Identities (%)	Accessions
QQ06 (LC42888.1)			
Streptomyces sclerogranulatus strain IHBB 4104	100	99.71	JX912955.1
Streptomyces alboniger strain ATCC 12461	100	97.27	CP023695.1
Streptomyces aureocirculatus strain CSSP728	096	97.92	NR_043371.1
QQ07 (LC42889.1)			
Streptomyces rochei strain NBRC 12908	097	99.43	NR_041091.1
Streptomyces mutabilis strain SW103	097	99.43	LM644088.1
Streptomyces nodosus strain ATCC 14899	097	97.99	NR_041730.2
QQ08 (LC42890.1)			
Streptomyces heilongjiangensis strain FREP14 16S	100	98.71	MK226482.1
Streptomyces cacaoi strain NBRC 12748	097	96.39	NR_041061.1
Streptomyces griseorubiginosus strain LMG 19941	097	95.28	AJ781339.1
QQ09 (LC42891.1)			
Streptomyces sparsus strain RHRM30	098	99.43	MH209254.1
Streptomyces noboritoensis strain NBRC 13065	097	95.25	NR_041107.1
QQ10 (LC42892.1)			
Streptomyces purpurascens strain JCM 4509	099	99.52	NR_104281.1
Streptomyces violaceus strain NBRC 13103	099	98.92	NR_041115.1
Streptomyces yokosukanensis strain NRRL B-3353	099	95.96	NR_043496.1

Five *Streptomyces* isolates (LC42888.1, LC42889.1, LC42890.1, LC42891.1 and LC42892.1)

antifungal activity. The isolate can't utilize L-Rhamnose and D-Galactose as sole carbon sources.

Regarding the streptomycete isolate-14 (QQ10) were found to be belonged to the red colour series, while the substrate mycelium was pigmented with reddish. This isolate had a spiral spore chain with a spiny surface (Table 3 and Fig. 3e). Producing melanoid pigments on tyrosine agar medium, peptone-yeast extract iron agar medium and tryptone-yeast extract broth medium was reported. Good growth on Cazpek's agar medium and actively utilizing the 100% of added sugars as carbon sources for growth were also found. This isolate has antimicrobial (Bacterial and fungal) activities.

The nucleotide sequences of 16SrRNA genes of the five selected *Streptomyces* isolates were partially determined. Lengths of 694, 711, 704, 708 and 841 nts were recorded for the *Streptomyces* isolates 02 (QQ06), 04 (QQ07), 05 (QQ08), 09 (QQ09) and 14 (QQ10), respectively. These sequences were submitted in GenBank as *Streptomyces* sp. QQ06 (LC42888.1), *Streptomyces* sp. QQ07 (LC42889.1),

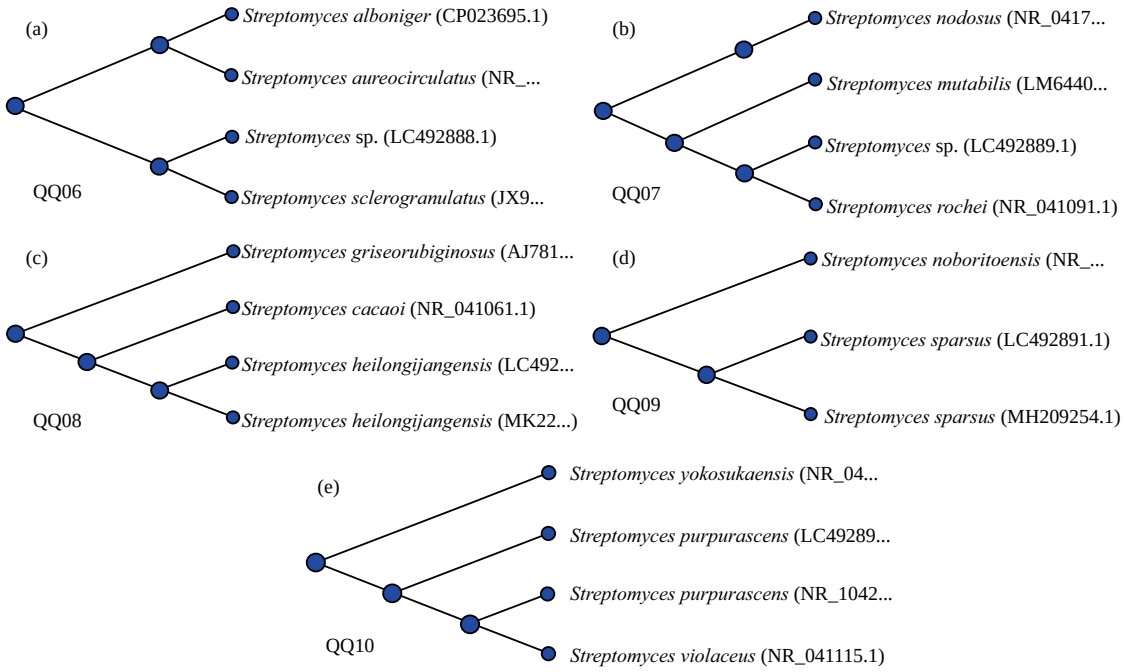

Fig. 4(a-e): Phylogenetic trees of the partial nucleotide sequences of 16SrRNA gene of the five *Streptomyces* isolates and the most similar streptomycetes recorded in GenBank
Five Streptomyces isolates (LC42888.1, LC42889.1, LC42890.1, LC42891.1, and LC42892.1)

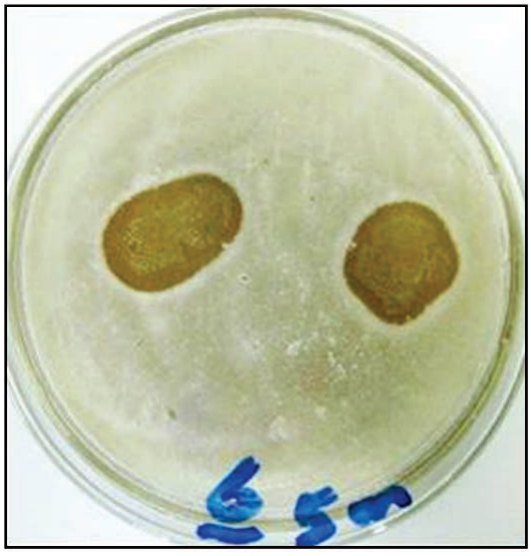

Fig. 5: Spot test technique used for detection of the presence of actinophages in virus suspensions of streptomycete isolates QQ06

Streptomyces heilongjiangensis QQ08 (LC42890.1), *Streptomyces sparsus* QQ09 (LC42891.1), *Streptomyces purpurascens* QQ10 (LC42892.1). Highly similar sequences of partial nucleotide sequences of 16S rRNA gene of soil-*Streptomyces* strains compared to the most overseas strains recorded in GenBank with E-value (0.0) showed variation in the percentage of query cover of 96-100, 97, 97-100, 97-98 and 99% and percentage identities of 97.27-99.71, 97.99-99.43, 95.28-98.71, 95.25-99.43, 95.96-99.52% (Table 4) for the five selected *Streptomyces* strains under investigation, respectively. The experimental results of sequences producing significant alignments of partial nucleotide sequences of 16SrRNA gene of the five selected *Streptomyces* isolates (QQ06, QQ07, QQ08, QQ09 and QQ10) approved the taxonomy of these isolates as strains of *Streptomyces sclerogranulatus*, *Streptomyces mutabilis*, *Streptomyces heilongjiangensis*, *Streptomyces sparsus* and *Streptomyces purpurascens*, respectively. This taxonomy was highly supported by the phylogenetic trees of partial nucleotide sequences of the 16SrRNA gene of *Streptomyces* strains compared to overseas strains recorded in GenBank (Fig. 4a-e).

Characterization of actinophages isolate: In this study presence of actinophages specific to the five streptomycete isolates in the soil suspensions prepared from four soils was done. Results of the spot test technique represented by Fig. 5 showed the presence of Streptomycetes lysis on starch nitrate

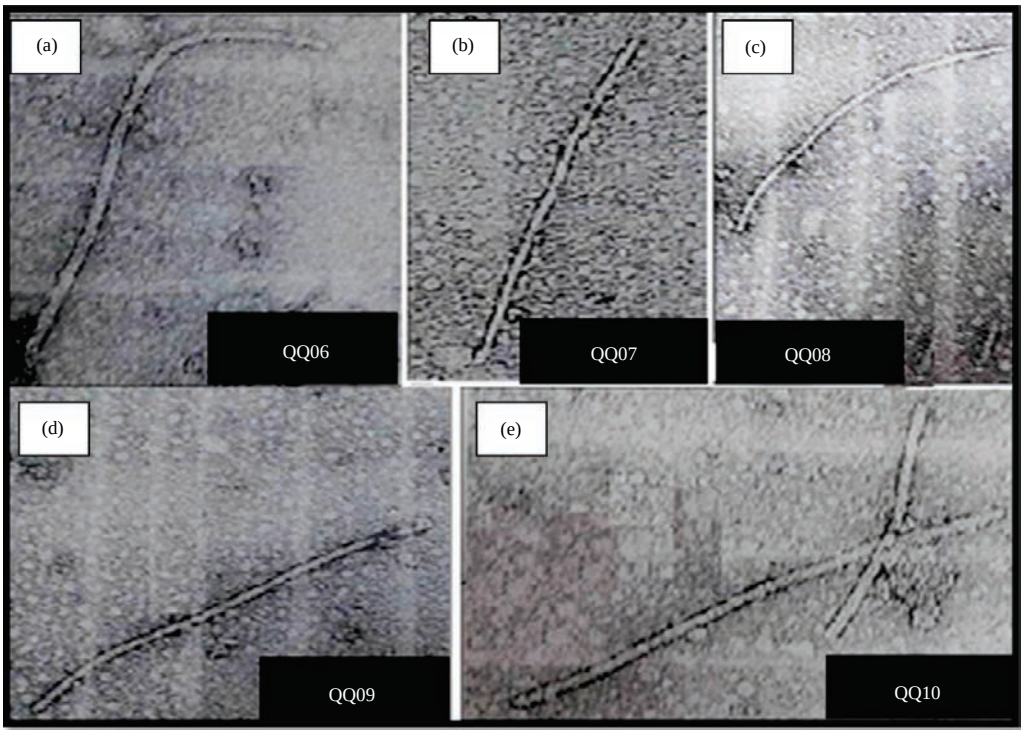

Fig. 6(a-e): Electron micrographs show the morphology of filamentous virus-like particles specific to the five identified *Streptomyces* strains

Table 5: Size distances of filamentous virus-like particles of actinophages specific to *Streptomyces* strains

Streptomycetes isolates	Actinophages isolates	Size distances
QQ06 (LC42888.1)	QQ06	21.4×928.57
QQ07 (LC42889.1)	QQ07	25×750
QQ08 (LC42890.1)	QQ08	21.4×857.14
QQ09 (LC42891.1)	QQ09	21.4×885.7
QQ10 (LC42892.1)	QQ010	21.4×857.14

agar medium inoculated with the Streptomycetes strain QQ06 as indicator host. The morphology of the purified actinophages was determined by negative staining technique and electron microscopy. Filamentous virus-like particles with lengths of 21.4×928.57, 25×750, 21.4×857.14, 21.4×885.7 and 21.4×857.14 (Table 5 and Fig. 6a-e) nm specific to Streptomyces strains QQ06, QQ07, QQ08, QQ09 and QQ10, respectively and belonging to family Inoviridae were electron micrographed.

DISCUSSION

In this study four clay loam soil were collected from under plant-cultivated soils used for isolation of both streptomycetes and their specific actinophages. The fifteen purified streptomycete isolates were varied in their antimicrobial activities against the four microbial strains. Actinomycetes including streptomycetes were isolated from different soils[31,32]. The variation of antimicrobial activities of actinomycetes epically streptomycetes were also reported in some previous studies[5,32-34].

The highest active streptomycetes isolates were belonging to white (one isolate, QQ06), grey (3 isolates, QQ07, QQ08 and QQ09) and red (1 isolate, QQ10) colour series. On comparing the cultural, morphological and physiological characters of the five selected streptomycetes to the most similar strains identified by several studies[35,36-39], the QQ06, QQ07, QQ08, QQ09 and QQ10 isolates could be classified as strains of *S. sclerogranulatus*, *S. mutabilis*, *Streptomyces heilongjiangensis*, *Streptomyces sparsus* and *Streptomyces purpurascens*, respectively, despite minor differences in between were recorded. Biological identification based on the cultural and morphological and physiological characters of streptomycetes was previously applied[3,6-8,24,35-37,40].

The classification of the five selected streptomycetes strains was confirmed by determining the nucleotide sequences of the 16SrRNA genes (LC492888.1, LC492889.1, LC492890.1, LC492891.1 and LC492892.1). These results showed the importance of the 16S rRNA gene as one of the molecular tools that necessary to be used for a taxonomy of bacteria[1,7,23]. It is well known Streptomycetes viruses or

actinophages exist wherever hosts are present, whether in the soil[17]. These viruses may play an important role in reducing the damage caused by economically harmful microbes[12,13].

Filamentous virus-like particles specific to different bacterial strains were isolated and characterized[19,20,41-43] and their applications were also reseacrhed[22]. Bacteriophages from the Inoviridae family were characterized by their unique morphology and exhibit unique genetic features[23]. Results of this study considered as the first record for isolation and characterization of filamentous virus-like particles specific to some *Streptomyces* strains and belonging to the Inoviridae family. These bacteriophages are characterized by dsDNA genomes encapsulated into icosahedral capsids. Inoviruses were characterized by rod-shaped or filamentous virions and circular single-stranded DNA genomes of ~5-15 kb[22,43].

One can recommend the completion of further studies on extracting and identifying active substances that inhibit the tested microorganisms and then producing them as broad-spectrum antibiotics. Also, the phages of actinomycetes should be subjected to more investigations in a trial to be available for use in the field of biological control.

CONCLUSION

In this study, several five *Streptomyces* strains having antagonistic activities against some test microorganisms were isolated from soil, purified and completely identified depending on either biological or molecular characterization. Their specialized lysate actinophages were isolated and purified and classified as members of the family Inoviridae based on their filamentous morphology shapes. Phage of Inoviridae was considered as the first time against streptomycetes isolates, therefore, further studies should be carried out at the level of molecular characterization.

SIGNIFICANCE STATEMENT

This study paid attention to the importance of Soil-streptomycetes as an important source of antibiotics effective against several microorganisms and could be successfully used as biological control agents. The presence of phages specific to streptomycetes in the soil was proved and further studies at the level of molecular biology should be done for its characterization.

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