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LINCOMYCIN ANTIBIOTIC BIOSYNTHESIS PRODUCED BY *STREPTOMYCES* SP. ISOLATED FROM SAUDI ARABIA SOIL: I-TAXONOMICAL, ANTIMICROBIAL AND INSECTICIDAL STUDIES ON THE PRODUCING ORGANISM

*Ibtisam M Ababutain[1], Zeinab K Abdul Aziz[2] and Nijla A AL-Meshhen[1]
[1]Department of Biology, Faculty of Science, University of Dammam, Kingdom of Saudi Arabia
[2]Department of Botany and Microbiology (Girl's branch), Faculty of Science
Al-Azhar University, Cairo, Egypt

ABSTRACT

The present study began with the isolation of 60 actinomycetes isolates from soil samples collected from different selected locations of Saudi Arabia. The purified actinomycetes isolates were subjected for screening program of antimicrobial and insecticidal activities. Isolate No. 4 isolated from Dammam governorate was found to be the most active actinomycetes isolate in which it produces an active substance belonging to lincomycin antibiotic against Gram positive, Gram negative bacteria and insects. The most active actinomycetes isolates No. 4 was selected for further studies concerning their identification. Morphological, physiological and phylogenetic analysis (16S rRNA); in addition to biochemical studies and culture characteristics as well as the chemical analysis of the cell wall, were carried out for the isolate under study. Based on the phenotypic and genotypic accumulated characteristics of the most active actinomycetes isolate and consulting the recommended International Key's of Bergey's Manual for identification of actinomycetes, it was found that this isolate matched with *Streptomyces* sp. MS-266 and was given the name *Streptomyces* sp. MS-266 Dm4.

Key words: Actinomycetes, diversity, biocontrol, identification, *streptomyces*.

INTRODUCTION

Aerobic actinomycetes are widely distributed in soil. Actinomycetes especially *Streptomyces* sp. are rich sources of bioactive natural products with potential applications as pharmaceuticals and agrochemicals (Atta, 2009; Atta *et al*., 2009; Manteca *et al*., 2008). They are prolific producers of secondary metabolites: antibiotics, herbicides, pesticides and anticancer agents (Atta and Ahmad, 2009; Osada, 1998; Saadoun and Gharaibeh, 2003). Actinomycetes play an important role in the biological control of insects through the production of insecticidal active compounds against the house fly *Musca domestica* (Hussain *et al*., 2002). More than 6000 compounds are produced by *Streptomyces* sp. and many of them have commercial importance as anti-infective (antibiotics, antiparasitic and antifungal agents) anticancer or immunosuppressant agents (Takahashi and Omura, 2003). For example, *S. aureofasies* considered one of important microbes in industrially because of their ability to produce chlortetracycline and tetracycline also; *S. rugosporus* produce pyrroindomycine (Abbanat *et al*., 1999). Antibiotic resistant pathogens have been widely and continuously reported. In consequence, novel antibiotics have been investigated intensively (Holtzel *et al*., 1998; Mukhopadhyay *et al*., 1999; Madigan *et al*., 2000; Aman, 2001; Kim *et al*., 2001; Lewis *et al*., 2003). The aim of this study is the isolation, identification and characterization of the *streptomyces* species isolated from Saudi soil. In addition, the insecticidal and inhibitory effects of these organisms were also investigated.

MATERIALS AND METHODS

Isolation of microorganisms

During the study, 13 soil samples were collected from different locations in Saudi Arabia. Soil samples were taken from a 15-20cm depth, after removing approximately 3cm of the soil surface. The samples were placed in polyethylene bags, closed tightly and stored in a refrigerator until used.

Preparation of Soil Samples

Soil samples were sieved to get rid of any unwanted materials and then dried in the air and mix with calcium carbonate (1%) and incubated at 28°C for several days as the lead to reduce the numbers of vegetative bacterial cells and allowed to increase the numbers of actinomycetes as mentioned by Tsao *et al*. (1960). Dilution method Tsao *et al*. (1960) was used to isolate and purify the actinomycetes by using Starch-nitrate agar as a growth medium.

*Corresponding author email: dr.king2007@hotmail.com

Isolation and purification of the used microorganisms

This was conducted by using soil dilution plate technique according to the method described by Johnson *et al*. (1959).

The antimicrobial activity tests for actinomycetes isolates

Antimicrobial activity for actinomycetes isolates was tested by using the classical diffusion methods described by (Betina, 1983). The following microbial cultures were used for this tests: *Pseudomonas aeruginosa* ATCC 9027, *Escherichia coli* ATCC7839, *Bacillus cereus*, *Bacillus subtilis* ATCC 6633, *Candida albicans* ATCC 10231, *Aspergillus flavus* ATCC 16883, and *Aspergillus niger* ATCC 16404. The Petri dishes were maintained for 2h in a refrigerator at 5°C to allow the diffusion of the bioactive compound. The diameter of complete inhibition zone was measured (in mm) after 24 h for the bacteria and up to 3 days for the fungi incubation at 30°C. The strongest antimicrobial activities of the isolates were selected.

The isolates that gave the strongest antimicrobial activity were grown in submerged culture in 250 ml flasks containing 50ml of the yeast dextrose broth media. The flasks were incubated at 30°C for three days with shaking at 200rpm. One milliliter of the previous culture were transferred to flasks contain 100ml of starch nitrate broth as a production medium which incubated in the same conditions. After growth, Analytical paper disks 740-E/2 diameter that soaked with the cell-free filtrate were placed on the surface of agar plates, which were previously inoculated with the test organisms. The dishes were maintained for 2h in a refrigerator at 5°C to allow the diffusion of the bioactive compound. The diameter of complete inhibition zone was measured (in mm) after 24h for the bacteria and up to 3 days for the fungi incubation at 30°C.

The insecticidal activity tests for actinomycetes isolates:

Origin and rearing of the mosquitoes

Culex pipiens were collected and reared for several generations according to Rady *et al*. (1991) under controlled conditions at temperature of 27 ± 2°C, relative humidity 75 ± 5% and 12- 12 light-dark regime. Adult mosquitoes were kept in (50×50×50cm) wooden cages and daily provided with sponge pieces soaked in 10% sucrose solution for a period of 3-4 days after emergence. After this period, the females were allowed to take a blood meal from a pigeon host, which is necessary for laying eggs (an autogeny). Plastic cup oviposition (15×15cm) containing dechlorinaed tap water was placed in the cage. The obtained egg rafts picked up from the plastic dish and transferred into plastic pans (25×30×15cm) containing 3 liters of tap water left for 24h. The hatching larvae were provided daily with fish food as a diet. This diet was found to be the most preferable food for the larval development and a well female fecundity (Kasap and Demirhan, 1992).

Insect treatment

Ten *C. pipiens* larvae were tested for each 50ml of actinomycetes filtrates in plastic cup. The control tubes were maintained as tap water (50ml) free from actinomycetes filtrate. The experiment was checked daily for recording the biological effects.

Larval mortality percent was estimated by using the Abbott′s formula: % Larval mortality = % A – % B / % A – 100×100, where A = number of controlled larvae and B = number of tested larva (Abbott, 1925).

Methods used for identifying actinomycetes isolates

The identification of genera includes morphological studies, which were done by cover slip culture technique (Kawato and Shinobu, 1959) and total cell hydrolysates analysis of the organisms to detect the meso or the LL-forms of diaminopimelic acid (DAP) according to (Becker *et al*., 1964; Lechevalier and Lechevalier, 1970).

Methods used for classification of the most potent actinomycetes

(a) Studies concerning the morphological characteristics

Determination of the spore-bearing hyphae and spore chains morphology were done by cover slip culture technique (Kawato and Shinobu, 1959) or by direct microscopically examination to the surface of the culture on the growth plates. Electron microscope study was done for spore chain morphology and spore surface.

(b) Studies concerning the cultural characteristics

Diaminopimelic acid (DAP) in the whole cell was analyzed according to Becker *et al*. (1964) and Yamaguchi (1965). Culture characteristics were observed on different kinds of media: Yeast extract-Malt extract agar (ISP2), Oatmeal agar (ISP3), Inorganic salt-starch agar (ISP4), Glycerol-Asparagine agar (ISP5). The color of sporulating aerial mycelium, substrate mycelium and soluble pigments in media were recorded in accordance with the guidelines established by the International Streptomyces Project (Shirling and Gottlieb, 1966) ISP methods. The incubation was carried out at 30°C for 14 days. Colors were assessed on the scale developed by Kenneth (1976).

Growth on Czapek`s agar medium

Ability of the streptomycetes to show good or poor growth on Czapek`s agar medium was carried out by streaking the organism under study on plates of this medium and incubated at 30°C for 14 days.

(c) Studies concerning the physiological properties

Melanoid pigments were observed on the following media: Peptone-Yeast extract- Iron- Agar (ISP 6) (Tresner

and Danga, 1958), Trypton- Yeast extract broth (ISP 1) (Pridham and Gottlieb, 1948) and Tyrosine agar (ISP 7) (Shinobu, 1958). Media were prepared in tubes and inoculated with the isolate No. (4) after sterilization. After incubated for 2- 4 days in 30°C the results have been taken by naked eye the color grayish brown or brown black were considered as a positive result.

Utilization of Carbon Sources was examined by the method of Pridham and Gottlieb (1948). After (10-16 days) incubation, the growth was observed and compared with the positive control (basal medium with glucose) and the negative control (basal medium without carbon source). Sensitivity to streptomycin was carried out according to the method of Bauer *et al.* (1966).

(d) Studies concerning the Phylogenetic characteristics

Genomic DNA extraction was conducted in accordance with the methods described by Sambrook *et al*. (1989). PCR amplification of 16S rDNA gene of the local actinomycete strain was conducted using two primers, F27 with the sequence 5'-AGAGTTTGATCMTGGTCAG-3' and R1492 with the sequence 5'-TACGGYTACCTTGTTACGACTT-3', in accordance with the method described by Edwards *et al.* (1989). Purification of PCR products and sequencing of PCR products for the isolate under study; were preformed in the Genetic analyzer unite of Egyptian company for production of Vaccines, sera and drugs (Vacsera) El-Dokki, Egypt.

Sequence similarities and phylogenetic analysis

The BLAST program (www.ncbi.nlm.gov/blast) was employed in order to assess the degree of DNA similarity. Multiple sequence alignment and molecular phylogeny were evaluated using BioEdit software (Hall, 1999).

RESULTS

Isolation and Purification of Actinomycetes colonies from different habitats

Sixty actinomycetes colony were isolated from thirteen soil samples collected from various locations in Saudi Arabia (Table 1).

Screening for antimicrobial activity of the isolated actinomycetes cultures

Twenty-seven isolates out of sixty showed antimicrobial activity so only 45% of the isolate have antimicrobial activity. The less percentage was the isolates from Khobar (22%), only two out of nine isolates showed antimicrobial activity followed by isolates from Dammam-Riyadh road, three out of twelve (25%) where the isolates from Unaizah one out of two showed antimicrobial activity (50%). The highest percentage was the isolates from Dhahran (60%), three out of five then isolate from Dammam (55%), sixteen out of twenty nine showed antimicrobial activities. However, the isolate from Jubail had no antimicrobial activity (Table 2).

Studies on insecticidal activity of actinomycete isolate No. (4)

The effect of filtrate of isolate No. 4 on the vitality and activity of the larvae of mosquito *C. pipiens* (Culicidae-Diptera) as a vector for some diseases of man and animals, such as "Alfellria" was carried out.

a- Effect on the vitality of mosquito larvae

Table 3 shows the effect of different filtrate concentrations of isolate No. 4 used against mosquito larvae in the third age. It also shows that there is a moral correlation between fatality rates and the different concentrations. When drawing a linear relationship (regression line) between the rates of mortality and

Table 1. Location of the collected samples investigated for isolation of actinomycetes cultures.

Isolate No.	Location	Sample characteristics
1 to 12 (Dm)	Dammam	Sandy soil
13 (Dm)	Dammam	Clay soil
14 to 18 (Dm)	Dammam	Sandy soil
19 to 23 (Dm)	Dammam	Clay soil
24 to 32 (kh)	Khobar	Clay soil
33 to34 (Dm)	Dammam	Clay soil
35 to 39 (Dh)	Dhahran	Clay soil
40 to 42 (Ju)	Jubail	Clay soil
43 (Dm)	Dammam	Clay soil
44 to 46 (Dm)	Dammam	Clay soil
47 to 52 (Dm-Ri)	Dammam-Riyadh	Sandy soil
53 to 58 (Dm-Ri)	Dammam-Riyadh	Sandy soil
59 to 60 (Un)	Unaizah	Clay soil

concentrations, the lethal dose of half the tribe of larvae (LC_{50}) can be calculated ,which was 0.22 ml/L (Fig.1).

b- Effect on the external form of the mosquito larva

After treatment of a group of larva with different doses of isolate No. 4 filtrate, some treated larvae were photographed after 24hours. Figure 2 shows the normal structure of 3rd instars larva of the mosquito *C. pipiens* (Diptera). Figure 3 shows the effect of lower concentration (0.01 ml/L) of isolate No. 4 filtrate on the larvae and the effect will be almost unnoticeable, but from a simple laceration in some areas of the outer layer of the body of the larva (cuticle). Figures 4, 5 and 6 explaining the inhibitory effect of high concentrations of the same isolate on the hardening of cuticle in the body of larvae, where the larvae become transparent, it was possible to observe easily the digestive tract from outside the body.

Table 2**.** Selected actinomycete isolates producing antimicrobial activity.

Isolates No.	Test organisms						
	Ps. aerugina	*E. coli*	*B. cereus*	*B. subtilis*	*Candida albicans*	*Asper. flavus*	*Asper. niger*
1	-	-	-	-	-	-	-
2	-	-	-	- , +	-	-	-
3	-	-	-	+++	-	-	-
4	+++	++	+++++	+++++	+++++	-	-
5	-	-	-	- , +	-	-	-
6	-	-	+	-	-	+	-
8	- , +	-	+	+++	-	+	-
9	-	-	+	- , +	-	-	-
10	-	-	+	-	-	-	-
11	- , +	-	-	- , +	-	-	-
12	-	+	+	++	-	-	-
13	-	+	++	+	-	-	-
14	- , +	-	-	+	-	-	-
15 - 16	-	-	-	-	-	-	-
17	-	+	+	++	-	-	-
18	- , +	+++	++	++	-	- , +	-
19	-	-	-	-	-	-	-
20	-	+	-	+	-	-	-
21	-	+	++	++	-	-	-
22	-	++	-	++	-	+	++
23	-	+	-	+	-	-	-
24	-	-	-	-	-	-	-
25	- , +	- , +	-	- , +	-	-	-
26	-	+	++	+	-	-	-
27	-	-	++	+	-	-	-
28	-	-	-	- , +	-	-	-
29 – 30 -31	-	-	-	-	-	-	-
32	-	-	- , +	- , +	-	-	-
33	-	+	-	-	-	++	++
34	-	-	-	-	-	-	-
35	-	++	-	- , +	-	- , +	-
36	-	-	-	-	-	-	-
37	-,+	-	-	+	-	-	-
38	-	++	-	++	-	-	-
39	-	-	-	-	-	-	-
40	- , +	-	-	- , +	-	-	-
41	-	-	-	-	-	-	-
42	-	-	-	-	-	-	-
43	- , +	-	-	-	-	-	-
44	-	-	-	-	-	-	-
45	-	-	-	+	-	-	-
46-47-48-49	-	-	-	-	-	-	-
50	+	-	-	-	-	-	-
51	-	-	-	-	-	-	-
52	+	-	-	-	-	-	-
53-54-55-56	-	-	-	-	-	-	-
57	++	++	-	-	-	-	-
58	-	-	-	-	-	-	-
59	++	-	++	+	-	-	-
60	-	-	-	-	-	-	-

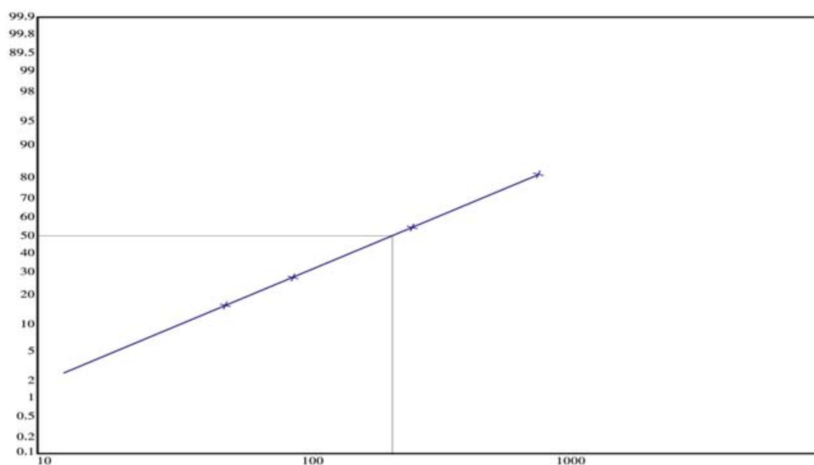

Fig. 1. LC_{50} value of isolate No. (4) filtrate against 3rd larval instars of *Culex pipiens*.

Fig. 2. Normal structure of 3rd instars larva of the mosquito *C. pipiens* (Diptera) (X40).

Fig. 3. 3rd instars larva of *C. pipiens* after treatment with (0.01ml/L) concentration (X40).

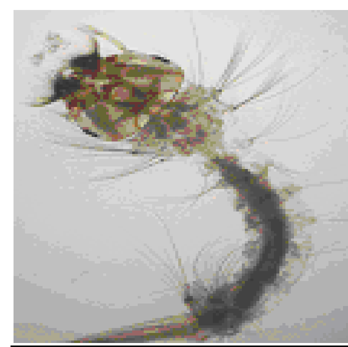

Fig. 4. 3rd instars larva of *C. pipiens* after treatment with (0.1ml/L) concentrations (X40).

Fig. 5. 3rd instars larva of *C. pipiens* after treatment with (0.25ml/L) concentrations (X40).

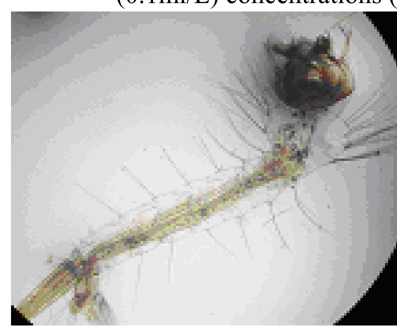

Fig. 6. 3rd instars larva of *C. pipiens* after treatment with (0.5ml/L) concentrations ((X40).

Table 3. The effect of isolate No. 4 filtrates against 3rd larval instars of *C. pipiens*.

Concentration of isolate (ml/L)	% Corrected mortality	LC_{50} value
0.5	90	0.22 ml/L
0.25	56.6	
0.1	26.6	
0.05	16.6	
0.01	13.3	
Control (0.0)	2	

Identification of the most active actinomycetes isolates

The morphological, physiological, cultural and biochemical characteristics for isolate No. 4 see tables 4, 5 and figures 7, 8.

16S rRNA gene sequencing

The 16S rRNA gene sequence was defined to the isolate No. 4 at pb 480 (Fig. 9). Table 6 show the multiple sequence alignment for isolate No. 4 which, showed that isolate No. (4) close to *Streptomyces* sp. MS– 266 by 91

Table 4. Cultural and physiological characteristics of the actinomycetes isolate No. 4.

Types of media	Growth	Aerial mycelium	Substrate mycelium	Diffusible pigments
Starch-nitrate agar	Good	Light Gray (ISCC-NBS 264)	Pale greenish yellow (ISCC-NBS 104)	Light orange yellow (ISCC-NBS 70)
Inorganic-trace salt- starch agar (ISP 4)	Good	Gray (ISCC-NBS 265)	Grayish yellow (ISCC-NBS 90)	Light orange yellow (ISCC-NBS 70)
Glycerol asparagine agar (ISP 5)	Good	Pale yellow (ISCC-NBS 89)	Slightly yellow (ISCC-NBS 84)	Light orange yellow (ISCC-NBS 70)
Yeast extract- malt extract agar (ISP 2)	Good	Gray (ISCC-NBS 265)	Grayish yellow (ISCC-NBS 90)	Light orange yellow (ISCC-NBS 70)
Oat meal agar (ISP 3)	Good	Light Gray (ISCC-NBS 264)	Dark yellow (ISCC-NBS 88)	Light orange yellow (ISCC-NBS 70)
Melanin pigment media 1-Tryptone yeast extract broth (ISP 1)	Weak	White (ISCC-NBS 263)	Slightly yellow (ISCC-NBS 84)	Deep yellowish brown (ISCC-NBS 78)
2- Peptone yeast extract iron agar (ISP 6)	Weak	Pale greenish yellow (ISCC-NBS 104)	Grayish yellow (ISCC-NBS 90)	Strong brown (ISCC-NBS 55)
3- Tyrosine agar (ISP 7)	No Growth	-	-	-

% and it was gave the name *Streptomyces* sp. MS– 266 Dm4.

Table 5. Morphological and biochemical characteristics of actinomycetes isolate No. 4.

Characters	Results
1. Morphological characteristics:	
Spore chain	Spiral
Spore mass	Gray
Spore surface	Smooth
Motility	Non motile
Color of substrate mycelium	Grayish yellow
Diffusible pigment	Light orange yellow
2. Chemotaxonomic analysis: Cell wall hydrolysis for:	
Diaminopimelic acid (DAP)	LL-DAP
Sugar pattern	ND

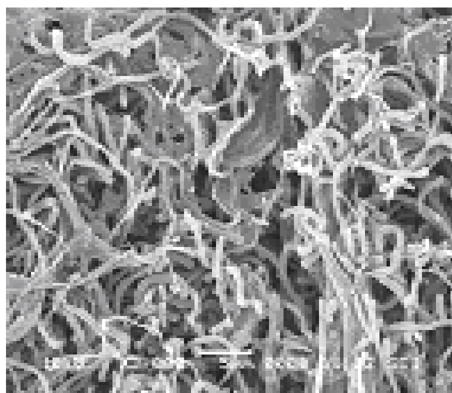

Fig. 7. Scanning electron micrograph of isolates No. 4 showing spiral shaped mycelium (X 3.000).

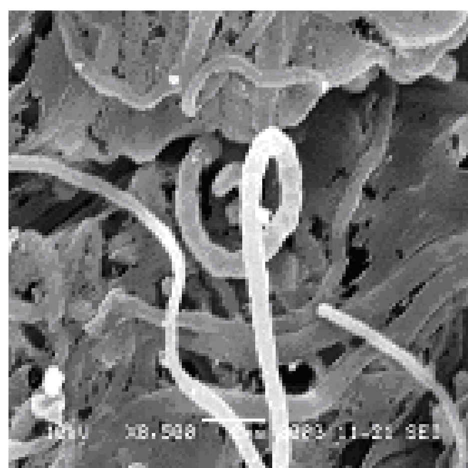

Fig. 8. Scanning electron micrograph of isolates No. 4 showing smooth spore surface (X 8.500).

DISCUSSION

The present study was started by isolation and purification of sixty actinomycetes isolates, which isolated from different regions of Saudi Arabian soil. The 60 isolates were tested for their ability to inhibit the growth of Gram-positive bacteria, Gram-negative bacteria, yeasts, fungi and as insecticide. According to many scientists, which isolate and purify the actinomycetes, Bream *et al*. (2001) isolated different actinomycetes strains from Saudi Arabia and Egypt soil. However, many researchers isolated actinomycetes strains from different places (Sahin and Ugur, 2002; Pandey *et al*., 2004; Ilić *et al*., 2005; Xie *et al*., 2007; Igarashi *et al*., 2008; Malik *et al*., 2008). Recently Dhanasekaran *et al*. (2010) isolated 64

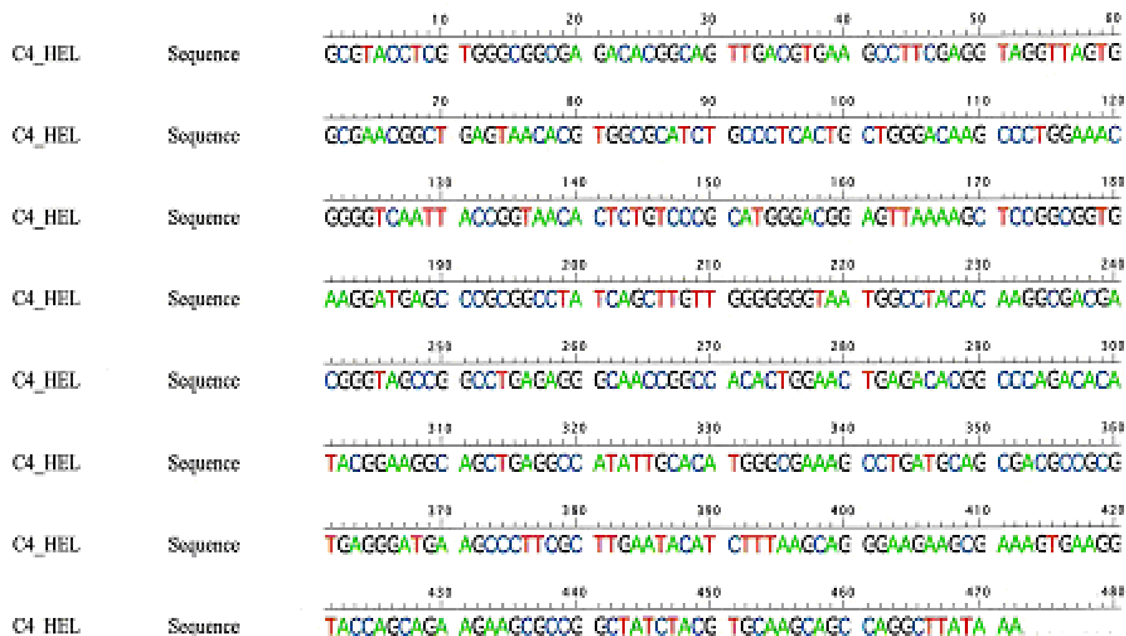

Fig. 9. 16S rRNA gene sequencing for isolate No. 4.

Table 6. Sequences producing significant alignments for isolate No. 4.

Accession	Description	Maximum identities
AY645903.1	*Streptomyces* sp. MS-266	91%
FJ532407.1	*Streptomyces microflavus* strain HBUM174141	91%
FJ481053.1	*Streptomyces cavourensis* strain xsd08096	91%
FJ486354.1	*Streptomyces anulatus* strain HBUM174206	91%
AB184642.1	*Streptomyces cavourensis sub* sp. *washingtonensis* strain: NBRC 15391	91%
AF112162.1	*Streptomyces* sp. EF-41	91%
GU211900.1	*Streptomyces* sp. WZ57	91%
FJ481633.1	*Streptomyces microflavus* strain HBUM174884	91%
FJ486351.1	*Streptomyces microflavus* strain HBUM174178	91%
FJ486435.1	*Streptomyces microflavus* strain HBUM174246	91%

actinomycetes strains from coastal soil. From twenty-seven isolates showed antagonistic activity against test organisms, isolate No. 4 showed the strongest activity against Gram-positive and Gram- negative bacteria. The identification of isolate No. 4 was done according to Bergey`s Manual (Williams, 1989; Holt *et al*., 1994), in which the basis for the definition depends on the morphological characteristics and the pigments produced in addition to the physiological and genetic studies (amplification and sequencing of the 16S rRNA). Kim and Goodfellow (2002) explained that the basis for the definition of *streptomyces* depends on the characteristics of morphology and the production of dyes in addition to the physiological and biochemistry to gain access to the species, and therefore they were differed between the four species of *streptomyces* isolated from British soil. Also, Sahin and Ugur (2002) were identified three isolates belonging to *streptomyces* by following the information in Bergey`s Manual. Whereas, Pandey *et al*. (2004) used the morphology and biochemical methods to describe the actinomycetes isolated from Nepal, and compared the shape with Bergey`s Manual of Determinative Bacteriology Holt *et al*. (1994). Jayasuriye *et al*. (2007) was adopted in its definition of a species on morphological characteristics, as well as analysis of serial 16S rDNA. Ningthoujam *et al*. (2009) identified strain of *streptomyces* sp. LS1-128 as more relevant to *S. sindenensis* based on morphological- physiological–cultural as well as genetic traits. Therefore, it was found that isolate No. 4 matched with *streptomyces* sp. MS-266

and was given the name *Streptomyces* sp. MS- 266 Dm4 in which (Dm) refers to Dammam governorate.

When examining the insecticidal activity of actinomycetes isolates against insect pests, it was noted the existence of effects on the external shape of the larvae of mosquito *C. pipiens* when using filtrate of isolate No. (4), where it have an effect on the cuticle layer which consisting of Arthropod in proteins and "chitin" These compounds gave solidity to the larval body or adult stage (Figs. 2-6). Where the process of Sclerotization had disappeared which responsible for giving the dark color to the cuticle of larva or adult stage. The hardness of cuticle is done by the formation of chemical bonds between cuticle proteins and "Polyphenols" substance secreted from the skin cells layer. The present results are, however, in accordance with several results performed with actinomycetes and other insect species. Dhanasekaran *et al*. (2010) found actinomycetes isolate producing strong larvicidal activity against Anopheles mosquito larvae. Liu *et al*. (2008) obtained on quinomycin A compound isolated from *Streptomyces* sp. which has the effect of inhibiting the growth of many pathogen insects, including mosquito *Culex*.

Since the cuticle of insect species consists largely of chitin, it was postulated that chitinase produced by these isolates could be involved in insect control. Therefore, the production of chitinase was used as the criteria for the selection of potential biocontrol agents of insects. Microbial chitinolytic enzymes have been considered important in the biological control of many insects because of their ability to interfere with chitin deposition (Tripathi *et al*., 2002).

CONCLUSION

Actinomycetes metabolites exhibited its effect against mosquito *C. pipiens*, so based of our results it can be used as an alternative insecticide, because they are free from harmful effects on the environment.

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