Isolation and Identification of some halotolerant Actinomycetes having antagonistic activities against some plant pathogens (i.e., Tobacco mosaic virus, Aspergillus Sp., Fusarium Sp.) from soil of Taif Governorate KSA

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ABSTRACT

Actinomycetes are considered one of the important bacteria due to their ability to produce several substances as secondary metabolites, found to be effective in the control of some plant pathogens. In this study, a number of soil samples were collected from different locations of Taif as well as Jeddah and Makah. This was followed by determination of the microbial total counts, i.e., bacteria, fungi and actinomycetes. *Actinomycetes* were isolated and purified and their color groups were determined. The actinomycete isolates were identified based on their cultural and morphological properties as *Streptomycetes.* The salt tolerance range of the purified *Streptomyces* isolates were determined by growing them on starch nitrate agar medium supplemented with different NaCl concentrations ranged from 3.5 to 10.5%. The highly tolerant streptomycete isolates were grown on a starch nitrate broth medium for 6 days under shaking conditions at 28-30^{o}C. Then, their antiviral and antifungal activities against TMV, *Aspergillus* sp. and *Fusarium* sp. were determined. The halotolerant *Streptomycete* isolates having antifungal and/or antiviral activities were completely identified based on their cultural, morphological and physiological properties. The DNA of the identified isolates was extracted and used for determination of DNA fingerprinting of these isolates using the RAPD-PCR molecular tool.

Key Words: Actinomycete, Streptomycetes, Taif region, Antiviral and antifungal activity, TMV, *Aspergillus*, *Fusarium*, RAPD-PCR.

INTRODUCTION

Actinomycetes are microscopic soil microorganisms are known to play a very supporting role in the degradation of organic matter in coffee habitats (Mythili and Ayyappa Das, 2011). Atta *et al.,* (2011b) isolated twenty eight actinomycete strains from soil samples collected from Farm Jabbar districted, Al-Khurmah Governorate, KSA. One of the actinomycete cultures, symbol 143 from two cultures was found to produce a wide spectrum antifungal agent (unicellular and filamentous fungi). The nucleotide sequence of the 16s RNA gene (1.5 Kb) of the most potent strain evidenced a 77% similarity with *S. albidoflavus*.

Actinomycetes particularly *Streptomyces* spp. has antagonistic activity against a wide range of plant pathogens (Dhanasekaran *et al*., 2005; Dormanns-Simon, 2007; Singh *et al*., 2009; Oliveira *et al*., 2010). In the recent decades, they have attracted high interests as biological control agents (Qiu *et al*., 2009; Zarandi *et al*., 2009). The genus of *Streptomyces*, a saprophytic Gram-positive bacterium, has properties, which make them useful as pharmaceutical and bio-control agents. A *Streptomyces* strain MY02 from soil samples showed significant antagonism against 14 plant pathogenic fungi including *Fusarium oxysporum* f. sp. *cucumerinum* (Qiu *et al*., 2009).

Mohamed and Galal (2005) reported that four halotolerant *Streptomyces* isolates (QS01, QS02, QS03 & QS04) were obtained from Qaroon lake, which had the ability to grow on 7% NaCl concentration in the starch nitrate agar medium. They were identified with a numerical method using two, three and four marker species belonging to red, gray and yellow series color groups. Their antiviral activities against *Tobacco mosaic tobamovirus* (TMV) and *Potato Y potyvirus* (PVY) were also determined. The isolates QS03 and QS04 identified as strains of *S. naganishii* and *S. michigansis* with similarities of 99.0 and 92.3%, respectively. QS01 and QS02 isolates were identified as duplicate strains of *S. erythraeus* with similarities of 93.8 and 90.2%, respectively. As interesting, both of filtrates and pellets (Cells of streptomycetes) were found to contain substances with antiviral activities, as the number of necrotic local lesions (NLL) produced by TMV and PVY on *Nicotiana glutinosa* and *Chenopodium quinoa, respectively* were decreased.

MaLi *et al*., (1997) found that a strain of soil actinomycetes isolated in Taiwan produced a series of metabolites with antimicrobial activity. The compounds exhibited broad spectrum of antimicrobial activity *in vitro* against fungi (*Candida albicans, C. krusei, C. tropicalis, Cryptococcus neoformans, Penicillium chrysogenum, Aspergillus fumigatus, Rhizoctonia solani, Fusarium oxysporum* f.sp. *cubense*). The strain was identified as *S. bacillaris*. One of the antifungal metabolites was identified as a cyclic depsipeptide, valinomycin by spectroscopic analysis. At the level of molecular studies on *Streptomyces* species, some investigators carried out trials to identify some *Streptomyces* isolates (strains) using the random amplified polymorphic DNA (RAPD) polymerase chain reaction (PCR) (Mehling *et al*., 1995; Mohamed, 1998; Mohamed *et al*., 2001; Mahfouz and Mohamed, 2002; El-Domyati and Mohamed, 2004; Abdel-Fattah, 2005).

This study aimed to isolate, purify and identify halotolerant actinomycete having antiviral and/or antifungal activities against TMV, *Aspergillus* sp. and *Fusarium* sp. as some plant pathogens.

MATERIALS AND METHODS

Collection of some soil samples from different locations of Taif, KSA: Rhizosphere and non-rhizosphere soils were collected from different locations of Taif, Jeddah, and Makah area. At each location, soil samples were

randomly collected from five sites in sterile bottles and thoroughly mixed together to form one representative sample. All samples were subjected to microbiological analyses.

Determination of microbial (bacteria, fungi and actinomycetes) total counts in soil samples: Microbiological analyses include the determinations of total bacterial (Jacobs and Gerstein, 1960), fungal (Mohamed 1998) and actinomycetes (Waksman and Lechevalier, 1961) counts were carried out.

Isolation and purification of actionmycete isolates: Isolation and purification of actinomycete isolates was done as described by Mohamed (1998) using starch nitrate agar medium (Waksman and Lechevalier, 1961).

Determination of salt tolerance range of the purified streptomycete isolates: The purified *Streptomyces* isolates were tested for their abilities to grow at increasing salt concentrations of 0.02 (normal salt concentration of the medium), 3.5, 7.0, and 10.5% salt, NaCl) using starch nitrate agar medium (Waksman and Lechevalier, 1961) as reported by Mohamed *et al*., (2000).

Antifungal and antiviral activities: Regarding the antifungal activities, the streptomycete filtrates were tested against two fungi (*Aspergillus* sp. and *Fusarium* sp.) as described by Mohamed (1998). On the other hand their antiviral activities were done against TMV using *Datura metel* as a necrotic local lesion indicator host based on the method of Mohamed and Galal (2005).

Identification of selected actionmycete isolates: The halotolerant streptomycete isolates having antibiosis activities were selected and further identified up to species according to keys proposed by Bergey's Manual of Determinative Bacteriology (1974). Media as well as methods used in these keys were described by Shirling and Gottlieb (1966). Identification was based on cultural, morphological and physiological characteristics. Micromorphology of spore surface was determined using transmission electron microscope (Jeol JEM-1008 electron microscope) by the technique described by Tresner *et al*., (1961). The spore chains of the selected streptomycete isolates were determined according the morphological groups as recorded by Shirling and Gottlieb (1966).

DNA extraction and purification: The DNA was extracted from the selected streptomycete isolates as described by El-Domyati and Mohamed (2004).

RAPD-PCR analysis: Ten RAPD-PCR primers (OPB09,OPB16,OPB17, OPE04, OPE05,OPF06, OPG07, OPO03, OPW18 and OPZ16 were used for determination of DNA fingerprinting of DNA-streptomycete strains as described by El-Domyati and Mohamed (2004).

RAPD-PCR program and electrophoresis: PCR amplification was performed in a GeneAmp 2400 PCR machine using the following program: Denaturation (5 min at 94°C, 1 cycle; 35 cycles, each of denaturation for 1 min at 94°C, annealing for 1 min at 36°C and extension for 2 min at 72°C. The primer extension segment was extended to 5 min at 72^{o}C in the final

cycle. Amplified products were visually examined under UV transilluminator and the presence or absence of each sizes class was scored as 1 (present) or – 0 (absent), respectively. Bands of the same mobility were scored as identical. The similarity coefficient (F) between isolates was defined by the formula of Nei and Li (1979). A phylogenetic tree was derived from the distance by un-weighted paired-group method (Sneath and Sokal, 1973).

The DNA of the selected *Streptomyces* isolate was used as a template for PCR-isolation of 16S rRNA gene using two universal primers (F: AGA GTT TGA TCC TGG CTC AG; R: ACG GCT ACC TGT TAC GAC TT, Cook and Meyers, 2003). PCR amplification was performed in a GeneAmp 2400 PCR machine using the following program: Denaturation (5 min at 95°C, 1 cycle; 35 cycles, each of denaturation at 95°C for 1 min, Annealing for 1 min at 56°C and extension for 2 min at 72°C. The primer extension segment was extended to 5 min at 72^{o}C in the final cycle. The PCR products were resolved by electrophoresis in a 1.2% agarose gel at 80 volts for 1 hr with 1X TAE buffer and then stained with ethidium bromide solution for around 10-15 minutes. Amplified fragments were visually examined under UV trans-illuminator.

RESULTS AND DISCUSSION

Genus *Streptomyces* comprises, by far, the largest number of species of actinomycetes now known to occur in nature (Williams *et al*., 1989). Many investigators throughout the world are isolating cultures of streptomycetes from soils (Atta *et al*., 2011a & b; Mythili and Ayyappa Das, 2011; Oliveira *et al*., 2010) as well as coastal soils (Gulve and Desgmukh, 2011).

In this study, actinomycetes were isolated and purified (Figure 1) from different soil samples were collected from Taif, Jeddah and Makah. The total counts of bacteria were the highest followed by actionmycetes and fungi. A number of 20 actinomycete isolates were obtained from the rhizosphere soil and 10 from the non-rhizosphere soil samples. These isolates were divided based on their serial color groups to 12, 8, 5, 3 and 2 isolates belonging to violet, gray, white, red and yellow color groups, respectively (Table 1). This study agrees with that of Ping *et al*., (2006).

Data in Table -2 show that all streptomycete isolates were able to grow in the presence of 3.5% NaCl in the starch nitrate agar medium. At concentration of 7% NaCl, only 6 out of 30 isolates showed moderate growth (++) (Figure 2), while 50% showed weak growth and 30% were not able to grow on this concentration. The streptomycetes isolates showed no growth (-, 20 isolates) or in-doubt (±, 10 isolates) when the NaCl was added to the growth medium with 10.5%. This result agrees with that found by Mohamed *et al*., (2000), Mohamed and Chaudrhy (2005) and Singh *et al*., (2009). In Egypt, a number of studies were achieved corresponding to the halotolerant streptomycetes isolated from soils (Mohamed 1998; Mohamed *et al.,* 2000; Abdel-Fattah, 2005).

Attempts have been made to develop *Streptomyces* species as fungal root disease control agents, since *Streptomyces* spp. are capable of producing a remarkably wide spectrum of antibiotics as secondary metabolites (Lechevalier, 1988, Franklin *et al*., 1989 and El-Sherbiny, 2006). In the present study,the antagonism activities of the three soil *Streptomyces* isolates under investigation and obtained from soil were determined against two different fungi (*Fusarium* sp. and *Aspergillus* sp.) (Figure-3) and a tobamovirus (TMV) (Figure- 4). Similar results obtained by Mohamed and Galal (2005) who isolated halotolerant streptomycetes having antiviral activity against TMV and PVY. Only three isolates were appeared highly antiviral activities (Isolates 3, 8 and 15) (Table 3). Two out of them showed antiviral and antifungal activities against one fungus. Zarandi *et al*., (2009) reported that the soil actinomycetes were having antagonistic activity against a wide range of plant pathogens. Our results in agree with that found by Qiu *et al*., (2009), who showed that the *Streptomyces* from soil could be used as biocontrol agent as it had antifungal activity against *Fusarium oxysporum* f.sp. *cucumerinum*. Also, Dormanns-Simon (2007) pointed to the ability of use the *Streptomyces* for controlling the damping-off and Fusarium wilt. The result of this study was supported by that of MaLi *et al*., (1997) showed that soil actinomycetes produced antimicrobial activity against *Aspergillus fumigates* and *Fusarium oxysporum* f.sp. cubense. Also, Dhanasekaran *et al*., (2005) supported our results, as they found antagonistic activity of Streptomyces against *Rhizoctonia solani*.

The results paid an attention to the possibilities of extraction, purifycation and identification of such substances. Mahfouz and Mohamed (2002) also pointed to the same idea. The results also encourage the idea for the use of such active streptomycetes as a biopesticide for controlling such plant pathogens, i.e, viruses.

Based on the proposed key of Pridham and Tresner (1974) the three selected antagonistic *Streptomyces* isolates were found to be strains of *S. fulvoviolaceus* (Isolates 8 & 15) and *S. antibioticus* (Isolate 3) based on their cultural, morphological and physiological characteristics (Table 4 and Figure 5).

At the molecular level,the DNA finger printing of the three *Streptomyces* isolates (3,8 and 15) were determined using RAPD-PCR technique (Figure 6). As a total number of 111 amplified fragments were amplified using the ten RAPD-PCR primers, and the three isolates showed 62, 58 and 46 fragments were amplified from the DNA extracts of the isolates 8, 15 and 3, respectively. The dendrogram showed that the first cluster included isolates 8 and 15 with similarity of 26.3%, while, isolate 3 lied in a separate subcluster (Figure 7). This results in the same tend with that of Mohamed (1998); Mohamed *et al*., (2001); Mahfouz and Mohamed (2002); El-Domyati and Mohamed (2004); Abdel-Fattah (2005).

Table-1: Color serial groups of streptomycetes isolates obtained from collected soil samples.

Soil samples	Number of streptomycete isolates among color serial groups					
	Violet	Gray	White	Red	Yellow	Total
Rhizosphere	7	5	4	3	1	20
Non-rhizosphere	5	3	1	0	1	10
Total	12	8	5	3	2	30

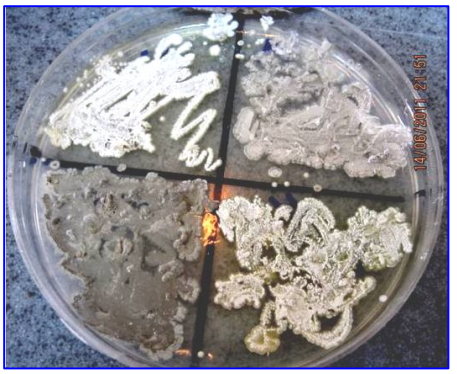

Figure-1: Purified streptomycete isolates (Upper) and model of serial color groups (Lower) of some streptomycete isolates obtained from soil.

Table-2: Salt tolerance range of streptomycete isolates.

NaCl concentrations (%)				Total
Actinomycete isolates	3.5	7.0	10.5	
1, 3, 8, 15, 23, 29	+++	++	±	6
2, 4, 5, 6, 9, 10, 13, 17, 19, 20, 22, 24, 26, 27, 28	+++	+	-	15
7, 11, 12, 14, 16, 18, 21, 25, 30	+++	±	-	9
Total				30

+++: Good growth, ++: Moderate growth, +: weak growth, **±**: In doubt, - : No growth.

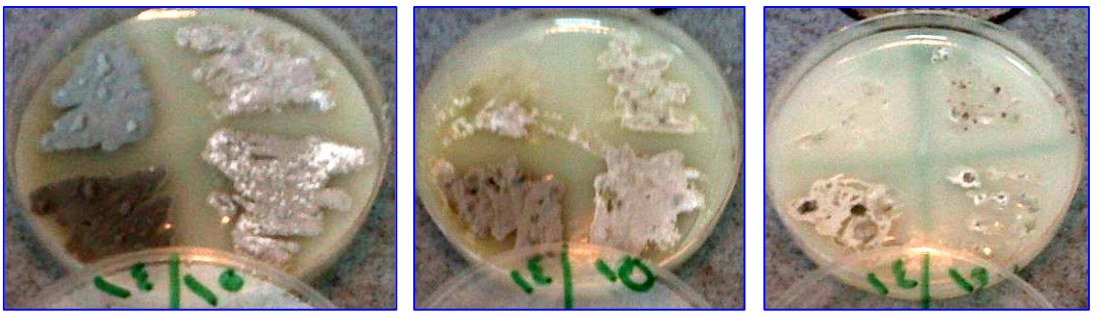

Figure-2: Salt tolerance range of some streptomycete isolates. Right: control. Middle: 3.5% NaCl. Left: 7% NaCl.

Table -3: Antifungal and antiviral activities of halotolerant streptomycete isolates.

Actinomycete isolates	Antifungal		Antiviral (TMV)	
	Aspergillus sp.	*Fusarium* sp.	No. NLL	Level of antiviral (%)
1	--	+	400	14.9
3	+	--	079	83.2
8	--	+	055	88.3
15	--	--	048	89.8
23	--	--	208	55.7
29	--	--	216	45.9
Control			470	000

Table-4: Cultural, morphological and physiological characteristics of streptomycete isolates 3, 8 and 15 compared with those of similar species reported in the key proposed by Pridham and Tresner (1974).

Characters	**Isolate** 8	Isolate **15**	*S. fulvoviolaceus*	**Isolate** 3	*S. antibioticus*
Color of aerial mycelium	GY	GY	GY	GY	GY
Spore-chain	RA	RA	RF	RA	RF
Melanoid pigment	C+	C+	C+	C+	C+
Spore surface	SM	SM	SM	SM	SM
Growth on Czapek's medium	Excellent	Very good	Excellent	Excellent	Poor
Color of substrate mycelium	Dark brown	Light brown	Brown to gray brown	Dark brown	ND
Diffusable pigments	Red	Grayish	Different on some media	Red	ND
Utilization of Carbon:					
No carbon	-	-	-	-	-
D-Glucose	+	+	+	+	+
D-Xylose	+	+	+	+	+
L-Arabinose	+	+	+	+	+
L-Rhamnose	+	+	+	+	+
Raffinose	+	+	+	+	+
D-Mannitol	+	+	+	+	+
i-Inositol	+	±	+	+	+
Sucrose	+	+	+	-	-
Antagonistic activity	Antifungal + antiviral	Antiviral and antifungal	Antibacterial and antiviral	Antifungal+ antiviral	ND
Sensitivity to streptomycin	NS	NS	S	NS	S
NaCl tolerance	7%	7%	ND	7%	≥7%

+: Growth, - : No growth, ND: No data.

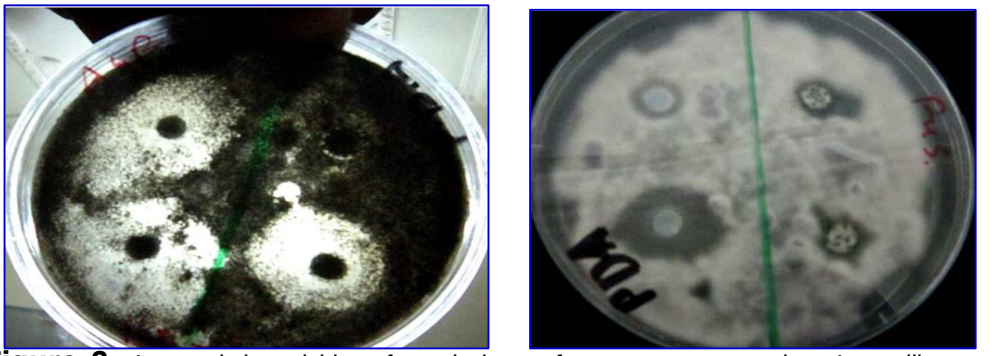

Figure-3: Antagonistic activities of two isolates of streptomycetes against *Aspergillus* sp. (Left) and *Fusarium* sp. (Right).

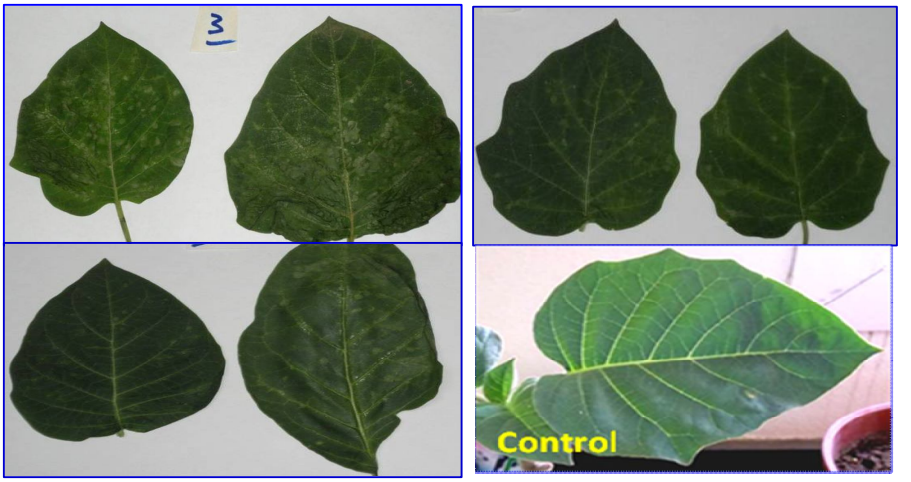

Figure-4: Determination of antiviral activities of the selected *Streptomyces* isolates (3, 8 and 15) on *D. metel* plant as a Necrotic local lesion diagnosis host.

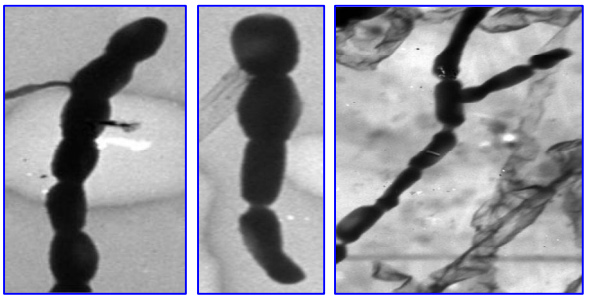

Figure-5: Electron micrograph of streptomycete isolate No. 3, 8 and 15, from left to right, shows smooth spore surphace. (X-10000).

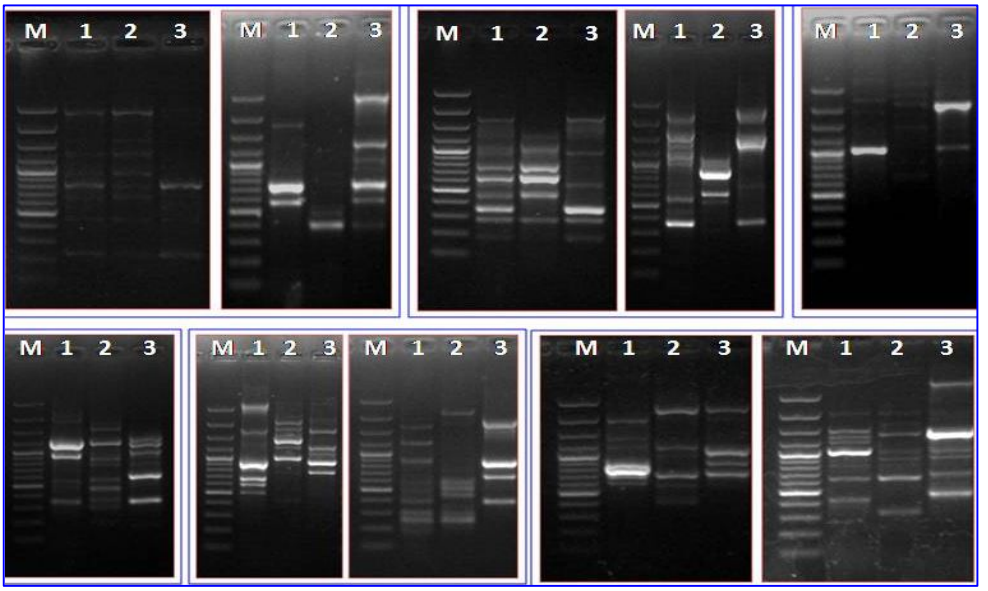

Figure-6: Agarose gel (1.5%) stained with ethidium bromide shows the DNA polymorphims amplified from the DNA of the three *Streptomyces* isolates (3, 8 and 15, Lanes 1-3 after the DNA marker) using OPB09, OPB16, OPB17, OPE04, OPE05, OPF06, OPG07, OPO03, OPW18 and OPZ16 RAPD-PCR primers, respectively, from left to right.

Streptomyces isolates	Percent identities between the three *Streptomyces* isolates		
	03	15	08
03	100		
15	22.6	100	
08	23	26.3	100

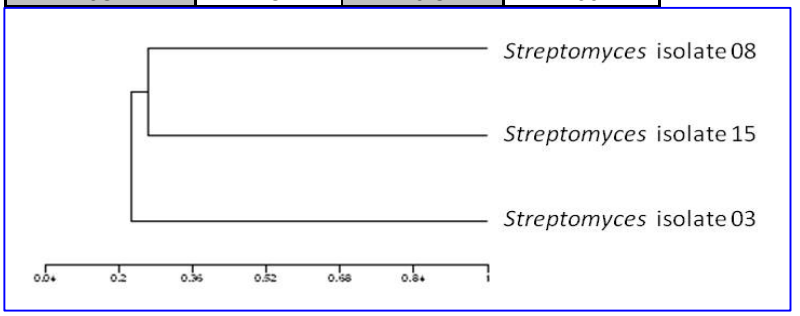

Figure-7: Percent identities and phylogenetic tree of DNA polymorphisms of the three *Streptomyces* isolates (3, 8 and 15) amplified by RAPD-PCR using 10 RAPD-PCR primers.

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