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Full Length Research Paper

Isolation and molecular identification of antibiotic-producing actinomycetes from an old house in the medina of Fez, Morocco

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Six actinomycetes strains were isolated from soil and deteriorated wood taken from an old house in the medina of Fez, Morocco. The antimicrobial activity of these strains was tested against Gram-positive bacteria (Bacillus strains and Staphylococcus strains) and Gram-negative bacteria (Pseudomonas strain and Escherichia strain) and *Candida albicans*. Among the six isolates, five showed activity against at least one of the test microorganisms. The activity was also found in organic extracts of the isolates. Furthermore, the organic extract from one strain was sensitive to temperature and proteinase K indicating that the active substance would be of protein nature. Molecular identification of the isolates was performed on the basis of 16S rDNA sequence analysis. Three isolates were attributed to Streptomyces genus, namely: Sj32 which could be identified as *Streptomyces flavolimosus*, Sj33 (*Streptomyces spiroverticillatus)* and Sj38 (*Streptomyces parvus)* whereas Sj68 and Sj69 could be indicated as *Saccharothrix violacea.*

Key words: Actinomycetes, Streptomyces, Saccharotrix, antimicrobial activity, molecular identification, 16S rDNA.

INTRODUCTION

The increasing emergence of resistant and multi-resistant bacteria worldwide and the lack of appropriate antibiotics to fight them continue to be the main concern of the medical community (Kuti et al., 2002). Obtaining new antibiotics (antibacterial and antifungal agents) is now a necessity. To achieve this goal, much research has been directed towards the screening of new production strains. Actinomycetes are very promising because of their ability to produce a large number of natural molecules such as antibiotics. Indeed, about 75% of the molecules used in medicine and approximately 60% of antibiotics developed for agricultural purposes are produced by the filamentous bacteria belonging to the genus Streptomyces (Miyadoh, 1993; Tanaka and Mura, 1993). However, the rate of discovery of new antibiotics has decreased since the ubiquitous species have been widely investigated (Suzuki et al., 1999). The search for new molecules having unique therapeutic properties continues to be an active field of research and many studies are oriented towards the isolation of actinomycetes species from new habitats.

Thus, we were interested in this study to carry out isolation and molecular identification of new strains of actinomycetes from an old house in the medina of Fez, Morocco and their screening for the production of

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antimicrobial substances.

MATERIALS AND METHODS

Sample selection and used strains

In this study, an old house in the Medina of Fez, Morocco was used to isolate actinomycetes strains endowed with antimicrobial activities. Different samples were taken from soil and deteriorated wood (cedar wood) of this house. Soil samples were collected by a sterile spatula and wood samples were cut and fragmented by a sterile scalpel. All samples were placed in sterile vials and returned promptly to the laboratory.

The target strains used for screening antimicrobial activity are: Gram-positive bacteria (*Bacillus subtilis* CIP 5262, *Bacillus cereus* CIP 14579, *Staphylococcus aureus* 7625, *Staphylococcus epidermidis* 6821), Gram-negative bacteria (*Pseudomonas aeruginosa* 76110, *Escerichia coli* CIP 7624) and the yeast *Candida albicans*.

Isolation of actinomycetes

Six gram of each samples were diluted in 100 ml of physiological water and incubated at room temperature for one hour with agitation. After decantation, the supernatant was diluted (10^{-1} to 10^{-5}) and 100 µl of each dilution was spread on the surface of three different actinomycetes isolation medium: Arginine-glycerol-salts (AGS) agar medium (g/l: arginine, 1; glycerol, 12.5; K_2HPO_4, 1; NaCl, 1; $MgSO_4.7H_2O$, 0.5; $Fe_2(SO_4)_3.6H_2O$, 0.010; $CuSO_4.5H_2O$, 0.001; $ZnSO_4.7H_2O$, 0.001; $MnSO_4.H_2O$, 0.001 and agar, 15 with pH 7.2) (El-Nakeeb and Lechevalier, 1963), glucose-yeast extract-malt (GLM) agar medium (g/l: yeast extract, 3; malt extract, 3; peptone, 5; glucose, 10 and agar, 20 with pH 7.2) (Kitouni et al., 2005) and starch casein (SCA) agar medium (g/l: starch, 10; casein, 0.3; KNO_3, 2; NaCl, 2; K_2HPO_4, 2; $MgSO_4.7H_2O$, 0.05; $CaCO_3$, 0.02; $FeSO_4.7H_2O$, 0.01 and agar, 18 with pH 7.2) (Kuster and Williams, 1964); All media were supplemented with 50 mg/l of cycloheximide. The plates were then incubated at 30°C for three weeks. Colonies of actinomycetes were recognized by their macroscopic and microscopic characteristics (optical microscopy and Gram stain). The isolates were then purified and conserved at 4°C for shorts periods and at -20°C in glycerol stock (20%, v/v) for a longer period.

Detection of antimicrobial activity

Primary screening for antibiotic production was undertaken on International Streptomyces Project 2 (ISP2) medium (g/l: yeast extract, 4; malt extract, 10; glucose, 4 and agar, 16 with pH 7.2) using the cross streaking method (Zitouni et al., 2005). The isolate of actinomycete was inoculated in straight line on a Petri plate containing the ISP2 medium and incubated at 30°C for seven days. The test strains were seeded in cross streaks to actinomycete culture. The antibacterial inhibition was evaluated after incubation at 30°C for 24 to 48 h.

The results were confirmed using the agar piece method (Petrosyan et al., 2003; Saadoun and Al moumani, 1997; Shomura et al., 1979). The actinomycetes isolates were grown in scratches tightened in SCA medium and incubated at 28°C for 7 to 10 days. Agar cylinders (3 mm in diameter) were cut and placed on the surface of Muller Hinton medium (beef infusion solids, 2 g/l; starch, 1.5 g/l; casein hydrolysate 17.5 g/l with pH 7.4) already sown by the test microorganisms. The plates were kept at 4°C for 2 h for a better distribution of the bioactive substance and then incubated 24 to 48 h at 30°C for *C. albicans* and 37°C for the test bacteria.

Organic crude extract

Crude extract of actinomycetes isolates was performed from solid and liquid culture using ethyl acetate solvent. In liquid medium, the actinomycetes isolates were inoculated in a 500 ml Erlenmeyer flask containing 100 ml of ISP2 medium and incubated at 30°C under constant agitation of 250 rpm for 5-7 days. The cultures were then centrifuged at 8000 g for 20 min to remove the mycelium. Ethyl acetate was added to the supernatant in the ration 1:1 (v/v) and shaken vigorously for 2 h at room temperature. The organic solvent was then evaporated to dryness in a vacuum evaporator and the dry residue dissolved in 1 ml of methanol (Zitouni et al., 2005).

The extraction from solid medium was done as follows: A dish of 9 cm in diameter containing ISP2 medium was streaked by actinomycete isolate and incubated at 30°C for 3 weeks. The agar medium was then cut into small cubes which were placed in an Erlenmeyer flask containing 40 ml of ethyl acetate. After stirring for 2 h at room temperature, the organic extract obtained was filtered and then evaporated at 45°C using a rotary evaporator. The dry residue obtained was taken up in 1 ml of methanol (Badji et al., 2006).

Antimicrobial activity of the organic extract was evaluated using the following method: 10 ml of Mueller Hinton soft agar (0.7%, w/v) was inoculated by 200 µl of an overnight culture of target strain. After solidification, 10 µl of the organic extract was deposed on the wells or on the surface of the plates and allowed to dry under the hood. Plates were then incubated at 30°C for *C. albicans* and 37°C for test bacteria.

Kinetics of antibiotic production

The kinetic of antibiotic production was determined for the isolates Sj32, Sj33, Sj38 and Sj68. Pre-cultures of actinomycetes isolates were carried out in 250 ml Erlenmeyer flasks containing 50 ml of the ISP2 medium. After incubation for 48 h at 30°C under constant agitation at 250 rpm, the flasks were homogenized and 5 ml of each pre-culture was used to inoculate a 500 ml flask containing 100 ml of ISP2 medium. The flasks were then incubated under the same conditions as above. Immediately after inoculation and at 24 h intervals, samples of 5 ml were taken and used for antimicrobial activity testing by the agar well diffusion method (Boudjella et al., 2006).

Partial characterization of the antimicrobial products

The organic extract recovered in water was subjected to heat treatment at different temperatures to test the thermal stability of the active substance. Hundred microliter of organic extract was incubated for 60 min at 60°C, 45 min at 80°C and 30 min at 100°C (Badji et al., 2006) and tested for antimicrobial activity using the protocol described previously above. In addition, the sensitivity of organic extract to proteinase K was tested as follow: 40 µl of a solution of 1 mg/ml of this enzyme was mixed with 100 µl of the extract and incubated for 2 h at 37°C (Shimei et al., 2005). Then, the antimicrobial activity was tested using the protocol described previously paragraph above.

Amplification and sequencing of 16S rDNA

The extraction of actinomycetes DNA was performed with the “Genomic DNA from Tissue kit according to manufacturer instruction (NucleoSpin Tissue (Macherey-Nagel). The 16S rDNA gene of the isolates was amplified by polymerase chain reaction (PCR) from chromosomal DNA using primers fD1

Table 1. Actinomycetes isolates in relation to their origin and isolation medium.

Strain	Biotope of isolation	Medium of isolation
Sj32	Soil	Arginine-glycerol-salts agar
Sj33	Soil	Starch casein agar
Sj38	Soil	Starch casein agar
Sj49	Wood	Starch casein agar
Sj68	Soil	Starch casein agar
Sj69	Soil	Starch casein agar

(5'-AGAGTTTGATCCTGGCTCAG-3') and rD1 (5'_TAAGGAGGTGATCCAGCC_3') as described by Weisburg et al. (1991). PCR mixture (50 µl) contained 1 µl of genomic DNA extract, 1.2 µl $MgCl_2$ (25 mM), 4 µl Taq buffer (5 ×), 4 µl dNTPs (1 mM), 1 µl of each primer (20 µM) and 2.5 U of Taq. PCR amplification was performed using the following protocol: initial denaturation (94°C for 5 min), followed by 35 cycles of denaturation (94°C for 1 min), primer annealing (56°C for 1 min 15 s) and extension (72°C for 1 min 15 s). A final extension at 72°C for 10 min was performed. PCR products were separated in a 1% agarose gel containing Sybr® Safe DNA gel stain and were subsequently visualized by ultraviolet (UV) illumination. The PCR product length was evaluated with a DNA ladder. Sequencing of PCR products was performed with a set of 5 internal primers: SP1 (5'_ACTCCTACGGGAGGCAGCA_3'), SP2 (5'_ACCGCGGCTGCTGGCACG_3'), SP3 (5'_GATACCCTGGTAGTCCACG_3'), SP4 (5'_CTCGTTGCGGGACTTAAC_3') and SP5 (5'_GGTACCTTGTTACGACTT_3') of the 16S rDNA gene. The five resulting sequences were assembled into a unique contig with BioEdit sequence alignment software (Hall, 1999). The Basic Local Alignment Search Tool 2 program (BLAST) was used for representation of sequence and similarity searches in the GenBank database. The 16S rRNA gene sequences of the five active isolates and those of strains of the most closely related species from GenBank database were aligned using CLUSTALW (Thompson et al., 1994), using MEGA version 5.0 neighbor joining phylogenetic tree was created (Tamura et al., 2011) with Kimura's two-parameter model (Gascuel, 1997; Kimura, 1980) (Figure 3).

RESULTS AND DISCUSSION

Isolation of actinomycetes

The biotope explored in this study is a house located in the oldest of Morocco's imperial cities, Fez. This house, built 450 years ago, is located in the former Derbllamté in the Medina of Fez and is currently in an advanced state of deterioration. Six colonies that exhibited typical macroscopic and microscopic characteristics of actinomycetes were selected. Five isolates (Sj33, Sj38, Sj49, Sj68 and Sj69) were extracted from starch casein agar medium and one (Sj32) from arginine-glycerol-salts agar medium (Table 1). These culture media promote the growth of actinomycetes because they contain starch and glycerol as main carbon sources, respectively. Indeed, utilization of some sources of carbon and nitrogen (starch, chitin, glycerol, casein, arginine and asparagine) makes the culture medium less favorable to the development of other bacteria (Cavalla and Eberlin, 1994). Five different colonies (Figure 1) were isolated from soil and one from deteriorate wood; this is consistent with literature showing that soil is the main reservoir of actinomycetes (Lemriss et al., 2003).

Detection of antimicrobial activity

The antimicrobial activity of the six isolates was determined against Gram-positive bacteria (*Bacillus subtilis* CIP 5262, *B. cereus* CIP 14579, *S. aureus* 7625 and *S. epidermidis* 6821), Gram-negative bacteria (*P. aeruginosa* 76110, *E. coli* CIP 7624) and the yeast *C. albicans* (Table 2). One isolate (Sj69) was active only on *Bacillus* strains. The isolate Sj33 had a broader spectrum of action because it inhibited the growth of the Gram-positive bacteria and *E. coli*. The morphological differences between Gram-positive and Gram negative bacteria could be responsible for their difference of sensitivity. Indeed, Gram negative bacteria have an outer polysaccharide membrane, making the cell wall impermeable to lipophylic compounds; in contrast to Gram-positive bacteria, which have only an outer peptidoglycan layer that is not an effective permeability barrier (Scherrer et al., 1971).

One isolate (Sj49) showed no antimicrobial activity against the microorganisms tested. The antimicrobial activity of this isolate would be tested against other microorganisms and on different culture media. In fact, it is now well established that the composition of the culture medium can affect the production of antibiotics by actinomycetes (Valan et al., 2008; Singh et al., 2009; Kuster and Williams, 1964; Tesic and Lukic, 1966; Waksman, 1967). For example, Lounès et al., (1995) showed that slowly assimilated carbon sources allow higher production of antibiotics whereas other studies showed that glucose causes repression of the formation of several aminoglycosides antibiotics produced by actinomycetes (streptomycin, kanamycin, neomycin and istamycine) by a repression of biosynthetic enzymes (Demain, 1989; Piepersberg and Distler, 1997).

Results of the kinetics of antibiotics production assayed against *S. aureus* 7625 are shown in Figure 2. The antimicrobial activity was detected after two and three days of incubation for Sj32, Sj33 and Sj38 and Sj68, respectively. Furthermore, the maximum of activity was obtained after the 7th day of incubation, mainly for Sj32 and Sj68 isolates.

Partial characterization of the antimicrobial products

The activity of ethyl acetate extracts of the five isolates showing antimicrobial activity and their sensitivity to temperature and proteinase K are shown in Table 3.

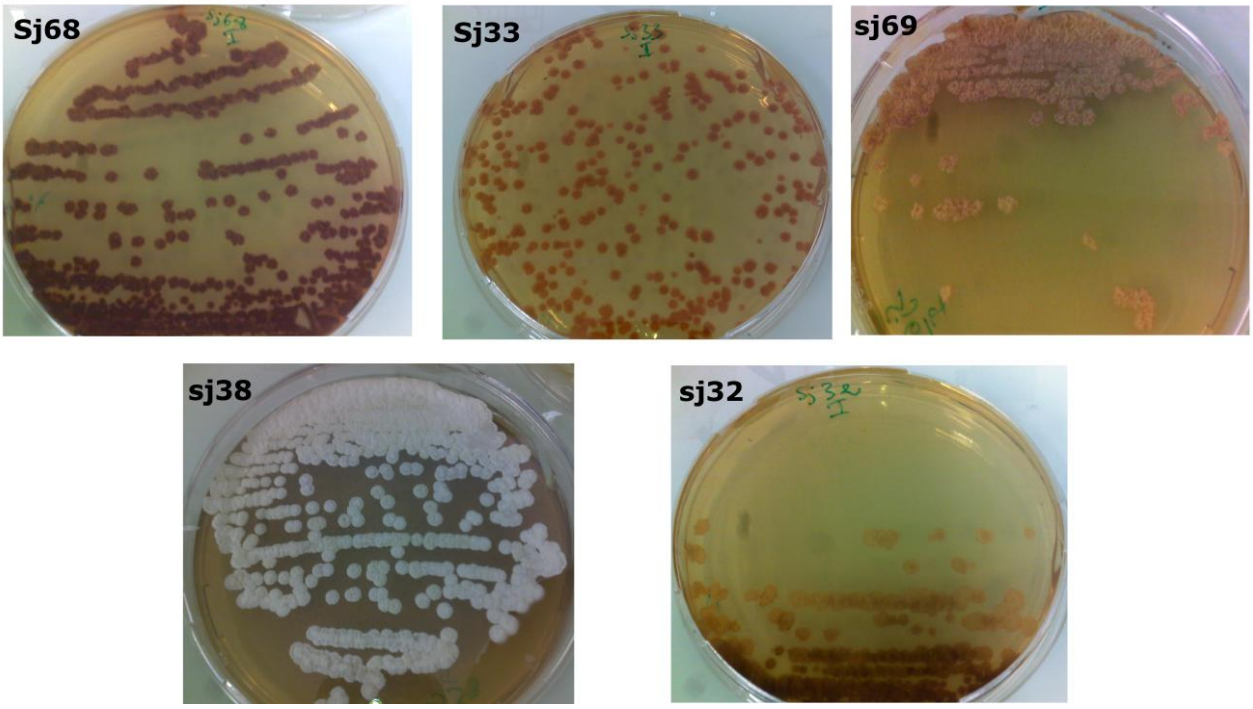

Figure 1. Colonies of active actinomycetes isolates on ISP2 agar medium

Table 2. Antibacterial activity of isolated actinomycetes.

Strain	*S. epidermidis 6821*	*S. aureus 7625*	*B. subtilis 52.62*	*B. cereus 14579*	*P. aeruginosa 76110*	*E. coli 7624*	*C. albicans*
Sj32	+	+	+	+	-	-	-
Sj33	+	+	+	+	-	+	-
Sj38	+	+	+	+	-	-	-
Sj49	-	-	-	-	-	-	-
Sj68	+	+	+	+	-	-	-
Sj69	-	-	+	+	-	-	-

+, inhibition; -, no inhibition. These tests were conducted in triplicates.

Extract of the isolate Sj38 showed sensitivity to temperature and proteinase K suggesting that the bioactive(s) substance(s) would be of protein nature. However, extracts of isolates Sj32, Sj33, Sj68 and Sj69 resisted to high temperature and proteinase K treatment; thus the bioactive substances would be of non protein nature. Research is currently underway to determine the precise nature of the bioactive substance(s). It is worth nothing that antibacterial activity was found in crude extract of actinomycetes isolates, performed from both solid and liquid culture. Indeed, it is recognized that antibiotic production by actinomycetes is more effective from solid than liquid medium and many strains are unable to produce antibiotics from liquid medium (Iwai and Omura, 1982; Shomura et al., 1979).

Analysis of the 16S rDNA sequences

Unlike conventional methods of identification of actinomycetes which are long, difficult and very expensive, molecular approach is often used for its speed and efficiency. Among molecular techniques, PCR remains the most widely used. The 16S rDNA gene is mainly used for molecular identification of bacteria. Indeed, some regions of this gene are highly conserved and common to all bacteria, while others are specific to species. Thus, 16S rDNA analysis offers a great advantage for the identification of actinomycetes (Provost et al., 1997).

Comparison of the resulting nearly complete sequences of 16S rDNA genes of the five active actinomycetes

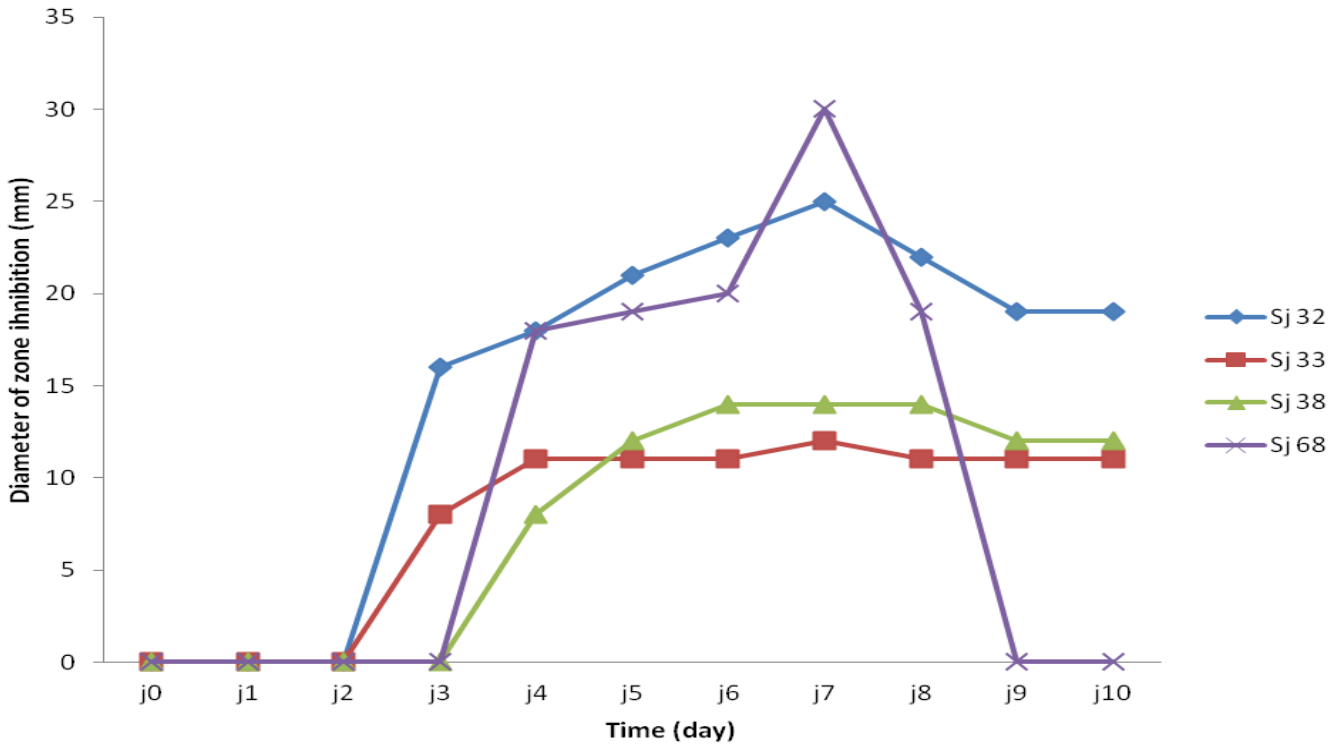

Figure 2. Time course of antibiotics production assayed against *S. aureus* 7625.

Table 3. Organic extracts activities of the five isolates showing antimicrobial activity and their sensitivity to temperature and proteinase K.

Strain	Organic extract (not treated)	Organic extract treatment			
		60°C	80°C	100°C	Proteinase K
Sj32	+	+	+	+	+
Sj33	+	+	+	+	+
Sj38	+	-	-	-	-
Sj68	+	+	+	+	+
Sj69	+	+	+	+	+

+, Activity; -, no activity. These tests were conducted in triplicates.

isolates with those of public databases allowed the affiliation of three isolates to Streptomyces genus and two to Saccharotrix. The 16S rDNA sequences of strains Sj32, Sj33, Sj38, Sj68 and Sj69 have been deposited in NCBI GenBank under the accession number JX013966, JX013967, JX013965, JX013968 and JX013969, respectively. In a neighbour-joining dendrogram based on the sequences from this study and sequences from the GenBank database, the phylogenetic position of the five active actinomycetes isolates was determined (Figure 3). According to their percentage of identity, Sj32, Sj33 and Sj38 could be identified as *Streptomyces flavolimosus* (99.9%), *Streptomyces spiroverticillatus* (99.7%), and *Streptomyces parvus* (100 %), respectively.

For Sj68 and Sj69, the highest sequence similarity (99.7 %) was obtained with the 16S rRNA gene of *Saccharothrix violacea*. However, morphological characteristics (Figure 1) and spectrum of activity (Table 2) of these two isolates show that they are different. In the literature, it is known that rare actinomycetes like Saccharotrix are found in small amounts in different ecosystems and cannot be isolated by usual techniques used in microbiology. Their selection must pass through the elimination of other organisms that may embarrass

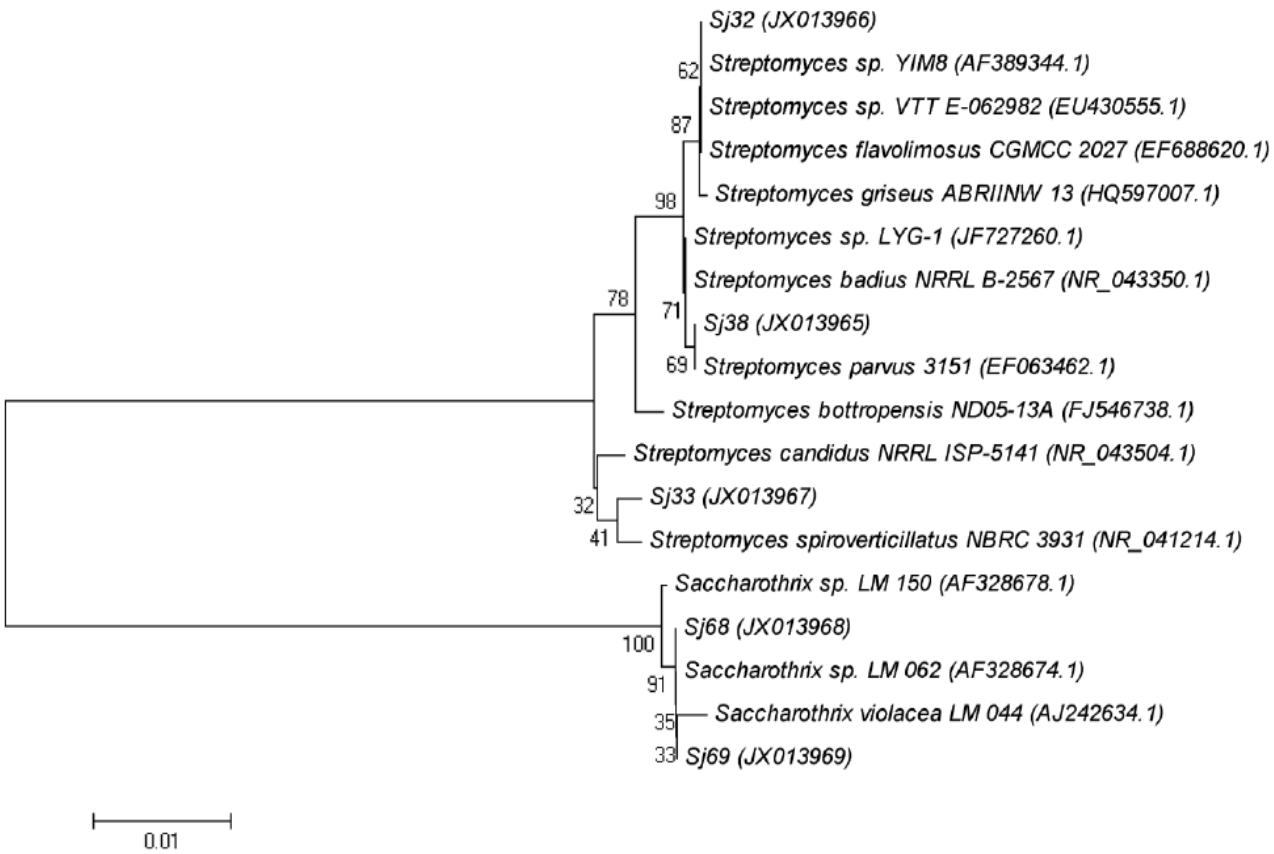

Figure 3. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequence (1460 nt) analysis, showing the relationships of actives actinomycetes isolates (Sj32, Sj33, Sj38, Sj68, Sj69) with some strains of *Streptomyces* and *Saccharothrix* species. Bootstrap values based on 1000 replicates are shown at branch nodes. Bar, 0.01 substitutions per nucleotide position.

the growth of actinomycetes. Several techniques can be used to increase the number of actinomycetes isolated from a given sample such as chemical or physical pretreatments (use of phenol or calcium carbonate), the use of certain carbon and nitrogen sources (Cavalla and Eberlin, 1994) or by the addition of antibiotics that inhibit the growth of other bacteria and fungi (Larpent and Larpent, 1990). In this study, we used the starch casein agar medium supplemented with the antifungal cycloheximide (50 mg/l) as selective medium of actinomycetes. It is important to point out that rare actinomycetes may be an important source of new antibiotics (Lazzarini et al., 2001).

Sj68 and Sj69 have a narrow spectrum of action since they act only on the Gram- positive strains tested. In particular, Sj69 acts only on *Bacillus* strains. These rare actinomycetes strains would be good candidates for the isolation of new antimicrobial molecules. Indeed, actinomycetes genera other than *Streptomyces* appear as a promising source of new antibacterial and antifungal compounds (Wang et al., 2010). Furthermore, organisms classified as Saccharothrix have been shown to be producers of novel secondary metabolites (Bush et al., 1987; Takeuchi et al., 1992).

In summary, results of this work permit the isolation and characterization of five actinomycetes strains producing antimicrobial substances. Some of these substances would be important in particular those produced by rare actinomycetes like Sj68 and Sj69. Further investigations are in progress to characterize these substances and to demonstrate their importance in combating microbial infections.

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