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Full Length Research Paper

Identification and antifungal activity of *Streptomyces* sp. S72 isolated from Lake Oubeira sediments in North-East of Algeria

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The aim of this study was to identify a new actinomycete isolate *Streptomyces* sp. S72 with antifungal activity from sediments of Oubeira's Lake located at the North-East of Algeria. This isolate was identified based on a great variety of morphological, cultural, physiological and molecular characteristics analysis of 16S rDNA sequence. The test of antifungal activity for several pathogens fungi causing invasive aspergillosis and systemic candidiasis revealed that the *Streptomyces* sp. S72 was a good moderate antifungal compound producer against *Aspergillus fumigatus* and *Candida albicans*, and had no activity against *Aspergillus flavus, Aspergillus niger*, *Candida pseudotropicalis* and *Candida tropicalis*.

Key words: *Streptomyces*, Oubeira's Lake, antifungal activity, *Candida albicans*, *Aspergillus fumigatus*.

INTRODUCTION

Fungal infections have been gaining prime importance because of the morbidity of hospitalized patients (Beck-Sague and Jarvis, 1993). In particular, candidiasis and aspergillosis have remained the opportunist fungal infections that occur most frequently. Presently, they represent a major area of concern in the medical field; however, the occurrences of invasive fungal diseases, particularly in AIDS and other immunocompromised patients, are life-threatening and they increase economic burden (Beck-Sague and Jarvis, 1993). To limit this occurrence, considerable progresses were accomplished in the fields of the biosynthesis of the antimicrobial compounds. The interest in searching for new molecules has been enhanced with the development of new approaches and ideas, such as the use of genetically engineered microbes and cells as screening organisms, and newer sources of bioactive materials, like marine organisms, coral reefs and sponges from ecological niches (Monghan and Tkacz, 1990; Jacob and Zasloff, 1994; Persidis, 1998; Fudou et al., 2001a; Phoebe et al., 2001; Gupte et al., 2002).

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Actinomycetes are best known for their ability to produce antibiotics; moreover, the pioneering work of Waksman showed that actinomycetes are capable of producing medically useful antibiotics (Nolan and Cross, 1998; Baltz and Roundtable, 2006). Actinomycetes are diverse group of heterotrophic prokaryotes forming hyphae at some stage of their growth; hence, they are referred to as filamentous prokaryotes (Gottlieb, 1973). They are the prolific producers of antibiotics and other industrially useful secondary metabolites (Okami and Hotta, 1988; Koehn and Carter, 2005; Nermeen and Gehan, 2006).

Approximately 70% of all antibiotics known were isolated in bacteria from actinomycetes, in which 75% were employed in medicine and 60% in agriculture (Miyadoh, 1993; Tanaka and Mura, 1993; Ben Ameur-Mehdi et al., 2006). Among the most known genus, are Streptomyces. These are used in the manufacturing of fermentation of the active pharmaceutical compounds, such as the antifungal ones, antiviral, anti-cancer, agents of immuno-suppressor, insecticides, weed killers, etc (Yoshiteru, 2007). The marine environment could be a source of the rare bacterial groups and promising sources of the bioactive molecules (Munro et al., 1999; Pomponi, 1999; Asha devi et al., 2006; Pelaez, 2006). Members of the actinomycetes which live in marine environment, are poorly

understood and only few reports are available (Siva Kumar, 2001; Vikineswary et al., 1997; Rathana Kala and Chandrika, 1993; Lakshmanaperumalsamy, 1978; Bredholt et al., 2008).

Among these natural sources which remain unexploited, Lake Oubeira, situated at the extreme North-East of Algeria, constitutes a good example type representative of the natural wet area in the Mediterranean region, after those from the Delta region of Ebre, in Spain and the Camargue in France. It is registered in the Ramsar list of the wet lands of international importance. It is a permanent fresh water lake, and has a maximum depth of 4 m, and a minimum of 1.30 m. The lake constitutes a tank of deposit of sediments coming from the catchment area (Direction of the protection of the flora and fauna, 2002; Head office of the forests, 2003).

The objective of this study was to describe the isolation of *Streptomyces* sp. S72 from sediments of Oubeira's Lake and its identification by studying the morphological, cultural, physiological and molecular characteristics as well as the production of its antifungal activity.

MATERIALS AND METHODS

Sample collection

Sediment samples were collected from the sediments of Oubeira's Lake in the North-East of Algeria using a sediment grab sampler and transferred to 1 L sterile plastic containers. The container volume was filled with 60% sediment and 40% sea water from the sampling site. This was done in order to ensure aerobic conditions under storage upon processing. All of the samples were kept at 4°C until they were used.

Strain isolation

One gram of sediments was taken in 9 ml of distilled water, agitated vigorously for 1 min. Different aqueous dilutions (10^{-1} to 10^{-3}) of the suspension were carried out, and were decanted for 30 min. The supernatant (100 µl) was spread on medium casein-starch (starch, 10 g; casein, 0.3 g; K_2HPO_4, 2 g; KNO_3, 2 g; $CaCO_3$, 0.02 g; $FeSO_4$, $7H_2O$, 0.01 g; $MgSO_4$, $7H_2O$, 0.05 g; agar, 20 g; H_2O, 1000 ml, pH 7.2), recommended by Shirling and Gottlieb (1966). The medium was supplemented with 10 µg/ml of gentamicin and 25 µg/ml of nystatin. Plates were incubated at 28°C for three weeks and colonies were purified by streaking on medium ISP2-agar (International Streptomyces Project 2-agar).

Cultural and micro-morphological characteristics

Cultural features of strain S72 were characterized following the directions given by the International Streptomyces Project (ISP) media namely: ISP1 Agar (tryptone, 5 g; yeast extract, 3 g; agar, 20 g; H_2O, 1000 ml, pH 7.2), ISP2 agar (yeast extract, 4 g; malt extract, 10 g; glucose, 4 g; agar, 20 g; H_2O, 1000 ml, pH 7.2), ISP3 agar (meals, 20 g; $MnCl_2$, $4H_2O$, 0.1 g; $FeSO_4$, $7H_2O$, 0.1 g; $ZnSO_4$, $7H_2O$, 0.1 g; Agar, 18 g, H_20, 1000 ml, pH 7.2), ISP4 Agar (starch soluble, 10 g; K_2HPO_4, 1 g; $MgSO_4$, $7H_2O$, 1 g; NaCl, 1 g; $(NH_4)_2SO_4$, 2 g; $CaCO_3$, 2 g; agar, 20 g; H_20, 1000 ml, pH 7.2), ISP5 agar (L-asparagine, 1 g; glycerol, 10 g; K_2HPO_4, 1 g; $MnCl_2$, $4H_2O$, 0.1 g; $FeSO_4$, $7H_2O$, 0.1 g; $ZnSO_4$, $7H_2O$, 0.1 g; agar, 20 g, H_2O, 1000 ml, pH 7.2), peptone yeast extract iron agar (ISP6) and tyrosine agar (ISP7) at 28°C for seven to 14 days (Shirling and Gottlieb, 1966), and the Bergey's Manual of Systematic Bacteriology (Williams et al., 1983a; Cross, 1989).

Morphology of the spore bearing hyphae with the entire spore chain was observed with a light microscope (Model SE; Nikon) using cover-slip method in ISP2 media after stain Gram (You et al., 2005).

Physiological and biochemical characteristics

Physiological criteria include the ability of the isolate to utilize different carbons that were determined on plates containing ISP basal medium 9 ($(NH_4)_2SO_4$, 2.64g; KH_2PO_4, 2.38g; K_2HPO_4, 5.65g; $MgSO_4$, $7H_2O$, 1g; standard saline solution, 1ml; agar, 15g; H_20, 1000ml, pH 7.2) to which carbon sources were added to a final concentration of 1% (Pridham and Gottlieb, 1948). The plates were incubated at 28°C and the growth was read after 7, 14 and 21 days using glucose as positive control and carbon source free medium as negative control (more suitable for comparison).

Hydrolysis of gelatin, starch and nitrate reduction was examined as described by Williams et al. (1983a), Gordon et al. (1974) and Boudjella et al. (2006). Degradation of casein was given according to the method of Gordon, growth at different temperatures, other physiological and biochemical characteristics were determined using the method described by Williams et al. (1983a). All tests were performed at 28°C. The production of melanoïdes pigments was carried out on ISP6 Agar (peptone, 20 g; ferric citrate ammoniacal, 0.5 g; sodium thiosulfate, 0.08 g; yeast extract, 1 g; K_2HPO_4, 1 g; Agar 15 g; H_2O, 1000 ml, pH 7.2) and ISP7 agar (glycerol, 15 g; L-tyrosin, 0.5 g; L-asparagine, 1 g; K_2HPO_4, 0.5 g; $MgSO_4$, $7H_2O$, 0.5 g; NaCl, 0.5 g; $FeSO_4$, $7H_2O$, 0.01 g; standard saline solution, 1 ml; agar, 18 g; H_20, 1000 ml, pH 7.2) (Shirling and Gottlieb, 1966).

Determination of pH range and temperature for growth

The actinomycete isolate was tested for its ability to grow at pH 5 to 10 and a temperature range of 25 to 50°C. Cultures were spot inoculated onto plates of ISP2 media to pH 5, 5.5, 6, 6.5, 7, 8, 8.5, 9 and 10. Cultures were checked for growth after seven days of incubation at 28°C. Plates were evaluated visually for little or no observable growth (+), some growth (+ or ++), or excellent growth (+++).

Antifungal bioassay

Antifungal activity was evaluated on ISP2 medium, by the double layer method against pathogenic fungi, *Aspergillus niger* (ATCC 16404), *Aspergillus fumigatus* (ATCC 9197), *Aspergillus flavus* (ATCC 10124) and yeast, *Candida albicans* (ATCC 10231), *Candida pseudotropicalis* (ATCC 46764), *Candida tropicalis* (ATCC 13803). The strain S72 was sown by touch, in the center of Petri dish and incubated at 28°C during seven days. The culture was covered by 8 ml of ISP2 medium containing 10 g of agar, already sowed by the test germs. It was then incubated at 28°C, after which the diameters of inhibition were determined after 48 h (Boughachiche et al., 2005).

Extraction of active compounds and kinetics of antifungal products

Strain S72 was sown in very tight scratches on ISP2 medium. After three weeks of culture at 28°C, the medium was cut out in small cubes which were placed in Erlenmeyer flasks containing 40 ml of

organic solvent (methanol). The extraction was carried out under agitation for two hours at room temperature. The organic extract obtained was filtered and then the vacuum was evaporated at 45°C using a rotary evaporator. The dry residue was then taken again in 5 ml of methanol and the antifungal activity was evaluated by using paper disks (6 mm in diameter). The soaked disks were deposited on the surface of ISP2 medium, already sown by the test germs. The plates were incubated at 4°C for two hours and later at 28°C. The diameter of the aureoles of inhibition was measured after 24 and 48 h.

Kinetics of production of *Streptomyces* sp. S72 was followed for 28 days on ISP2 medium. Thus, the medium contained in a Petri dish was taken daily to be extracted by organic solvent. The activities were measured by the method of the disks.

Extraction of the genomic DNA

The total preparation of DNA from strain S72 was carried out according to Hopwood et al. (1985) (Fourati-Ben Fguira et al., 2005). Preparations of small size of plasmids from *Escherichia coli* were given according to Sambrook et al. (1989) (Fourati-Ben Fguira et al., 2005). Digestion with ribonucleases of restriction, separation of the fragments of DNA by electrophoresis on agarose gel, dephosphorylation with the alkaline phosphatase of calf intestine, ligation of the fragments of DNA and the transformation of *E. coli* were carried out according to Sambrook et al. (1989) (Fourati-Ben Fguira et al., 2005).

Amplification by PCR

Fragment amplification of gene 16S rRNA of strain S72 was carried out by using two primers, pA: AGA GTT TGA TCC TGG CTC AG (8-28) and pH: AAG GAG GTG ATC CAG CCG CA (1542-1522) described by Edwards et al. (1989) (Mellouli et al., 2003). Roughly, 50 ng of the DNA matrix was employed with 30 pmol of each primer by a final volume of the reactional mixture of 50 µl. To improve the denaturation of the DNA, 50% (v/v) of dimethyl sulfoxide (DMSO) was added to the mixture with the reaction. Amplification was carried out with an automated thermocycler (Perkin-Elmer) by using 1U DNA Tag polymerase (stratagene) according to the following amplification profile: a stage of denaturation of the matrix: 94°C (3 min), followed by 40 cycles each of which include a stage of denaturation: 94°C (30 s), a stage of hybridization of the oligonucléotides to the matrix: 50°C (1 min), and a stage of elongation: 72°C (10 min). The product of PCR was analyzed by electrophoresis on agarose gel.

Sequencing of the 16S rRNA gene

The sequencing was carried out by using an automatic sequencer ABIPrism 3100 (Applied Biosystem) of the Center of Biotechnology of Sfax, with the same primers used. The nucleotidic sequence of the entire gene 16S rRNA (1.5 KB) of the strain of actinomycete was deposited in GenBank (EMBL) under the number of accession GQ140296.

RESULTS AND DISCUSSION

The antifungal potential of Streptomyces strains against some pathogenic fungi was previously reported from different locations of nature. Studies on actinomycetes are very limited and have been mentioned incidentally on the microbial community of marine habitats.

Further, only little information is available on the actinomycetes isolated from the sediments of Oubeira's Lake, as a novel source for the discovery of new bioactive compounds. Morakchi et al. (2009) have shown the ability of strain Streptomyces SLO-105 isolated from sediments of Lake Oubeira to produce antimicrobial compounds against microorganisms, especially the multiple antibiotic resistant Gram positive bacteria MRSA and the fungi *A. niger* and *Rodotorulla mucilaginosa*. Therefore, the study reported here was undertaken to determine the antifungal potential of *Streptomyces* sp. S72 against some pathogenic fungi and yeast.

Identification of the strain

The characteristics of strain S72 were compared with those of the known species of actinomycetes described in Bergey's manual of systematic bacteriology (Shirling and Gottlieb, 1966), and the obtained morphological properties suggested strongly that the strain belonged to the genus Streptomyces. These classical approaches described in the identification key by Nonomura (1974) and Bergey's manual of systematic bacteriology, are very much useful in the identification of streptomycetes. These characteristics have been commonly employed in the taxonomy of streptomycetes for many years.

Among these approaches, morphological, cultural, physiological, biochemical and other characteristics of strain S72 were studied. The observation with light microscopy on the ISP2 media after Gram staining indicated that the strain was a Gram positive, filamentous bacterium, with extensively branched but not fragmented aerial mycelia. The spore-bearing hyphae of the strain were spirale chain (S), Rectus-Flexibilis (RF) and Retinaculum-Apertum (RA) (Figure 1). The number of spores was higher than 10 per chain, which indicated that they referred to the long chains of the spores.

The selected isolate, S72, was properly grown on seven different ISP media after seven days and it produced brown melanoid pigment on ISP6 and ISP7. The aerial mycelium was white-grey in ISP1, ISP4 and ISP5 media, while it was white on ISP2 and ISP3 media (Table 1).

The growth of *Streptomyces* sp. S72 was tested on ISP2 media ranging from pH 5 to 10 at different temperatures of 25, 28, 37, 45 and 50°C. It was grown at pH 6.5 to 8.0. The temperature for its growth ranged from 25 to 37°C with an optimal temperature of 28°C, but did not grow at 45 and 50°C. The ability of strain S72 to assimilate 16 different tested carbon sources are represented in Table 2. However, strain S72 hydrolyses gelatin, starch and reduces nitrates (Table 2).

A rapid method for the identification of filamentous actinomycete was based on the analysis of the 16S rRNA gene restriction fragment patterns. This method begins by

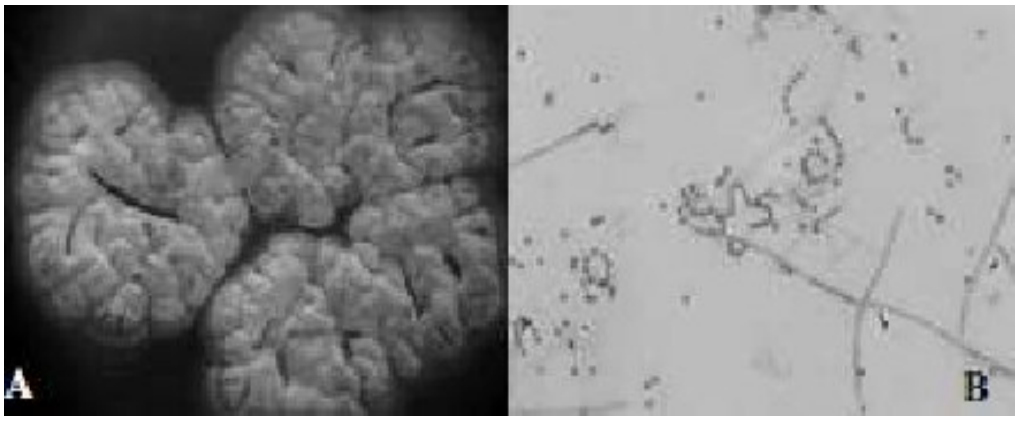

Figure 1. Colonies of *Streptomyces* sp. S72 in ISP2 solid media (A); aerial hyphea and spore chain structure (light microscope 100x) (B).

Table 1. Culture characteristics of *Streptomyces* sp. isolate S72 in different media after 14 days of incubation at 28°C.

Medium	Growth	Aerial mycelium	Substrate mycelium	Soluble pigment
Casein-starch	Good	White	Light yellow	Brown
ISP-1	Moderate	White-grey	Dark yellow	-
ISP-2	Good	White	Light yellow	Brown
ISP-3	Good	White	Light yellow	Brown
ISP-4	Moderate	White-grey	Dark yellow	-
ISP-5	Moderate	White-grey	Dark yellow	-
ISP-6	Good	White-grey	Dark yellow	Brown
ISP-7	Moderate	White-grey	Dark yellow	Brown

isolating DNA (Hopwood, 1985) and amplifying the gene coding for 16S rRNA using the polymerase chain reaction (Siva Kumar, 2001).

The total nucleotide sequence of 1518 pb was determined in both strands (accession N° GQ140296). The alignment of this sequence through matching with the 16S rRNA reported genes sequences in GeneBank showed the highest sequence similarity of 98% with *Streptomyces rochei*.

Antifungal bioassay and kinetics of the antifungal products of *Streptomyces* sp. S72

The results of the test of antifungal activity on ISP2 medium by using the technique of double-layer reveal an important antifungal activity of the strain against *C. albicans* and a moderate activity against *A. fumigatus* (Figure 2), but did not reveal any activity against *A. flavus, A. niger*, *C. pseudotropicalis* and *C. tropicalis* (Table 3).

The kinetics of production was carried out on ISP2 medium. The extraction of the active substances was carried out by methanol and the zones of inhibition were measured by the method of paper discs. The antifungal activities appear only at the fifth day of incubation (Figure 3). They increased gradually to reach their maximum with the 21th day and remain more or less stable thereafter.

The use of microorganisms having an antimicrobial activity to prevent human pathogens offers a supplement for the biological control of human diseases. Currently, actinomycetes and, more specifically, streptomycetes produce various bioactive natural products including antibiotics that are being used as pharmaceuticals and agro-chemical agents (Lazzarini et al., 2001; Watve et al., 2001; Sajid et al., 2008).

A. fumigatus has been reported to be the cause of approximately 85% of all forms of aspergillosis, followed by *A. flavus* (5 to 10%), *A. niger* (2 to 3%) and *A. terreus* (2 to 3%). In addition, some *Aspergillus* sp. are associated with certain clinical forms of aspergillosis, such as *A. niger* with external otitis, *A. flavus* with sinusitis, and *Aspergillus glaucus* with joint infection (Richardson, 1998).

In the current study, strain S72 that seemed to be a great fungal inhibitor showed antibiosis against all highly resistant fungal species (*A. fumigatus* and *C. albicans*). According to different review literature, *Streptomyces* strain SLO-105 showed broad-spectrum of antifungal activity against *A. niger*. However, no activity of the strain was observed against *C. albicans*. While *Streptomyces*

Table 2. Biochemical and physiological characteristics of *Streptomyces* sp. isolate S72.

Culture	Growth characteristic
Range of temperature for growth	25 - 37°C
Optimum temperature for growth	28°C
Range of pH for growth	6.5 - 8
Enzyme production	
Gelatinase	+
Urease	-
Nitrate reductase	+
Milk casein	+
Citrate	+
Esculine	+
Carbon source	
Amidon	+
Amylose	+
Arabinose	+
Celebiose	-
Erythritol	-
Fructose	-
Galactose	-
Glucose	+
Indole	+
Inositol	+
Lactose	-
Maltose	+
Mannose	-
Mannitol	+
Melezitose	+
Melibiose	+
Palatinose	+
Raffinose	+
Rhamnose	-
Ribose	+
Saccharose	+
Sorbitol	+
Sorbose	-
Tréhalose	-
Xylose	+

sp. PM-32 revealed a weak antifungal activity against *A. niger* and *C. albicans* (Manivasagan et al., 2009), certain *Streptomyces* strains showed strong antifungal activity against *A. niger* and *C. albicans* (Laidi et al., 2007; Oskay, 2009). On the other hand, different strains of novel *Streptomyces* identified by Sunanda et al. (2009) were reported to have shown various degrees of antifungal activity against *A. niger* and *C. albicans*. While rare studies have focused generally on sediments actinomycetes, streptomycetes having a widespread distribution are frequently isolated in soil.

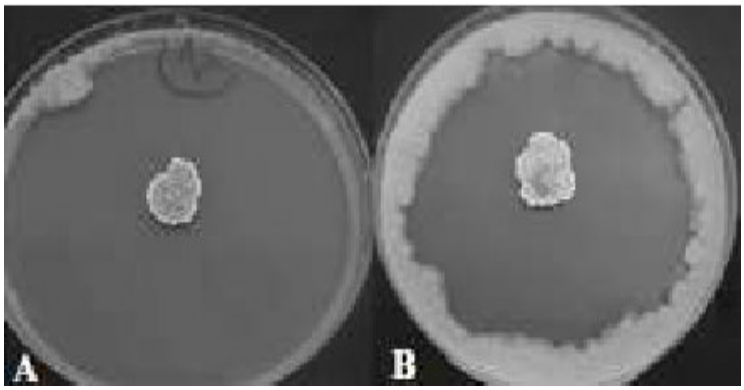

Figure 2. Antifungal activities in ISP2 solid media of *Streptomyces* sp. strain 72 against the two used indicator microorganisms employing double layer method: *Candida albicans* ATCC 10231 (A) and *Aspergillus fumigatus* ATCC 9197 (B).

Table 3. Antifungal activities of *Streptomyces* sp. isolate S72.

Fungi	Diameter of inhibition zone (mm)
Aspergillus fumigatus ATCC 9197	32
Candida albicans ATCC 10231	43
Aspergillus flavus ATCC 10124	00
Candida pseudotropicalis ATCC 46764	00
Candida tropicalis ATCC 13803	00

Many species of actinomycetes have the capacity to inhibit pathogenic fungi (Dahiya et al., 2006). However, the inhibitory effect of this isolate on the growth of human fungal pathogens and disease development was probably derived from more than one mechanism. Although the exact mechanisms by which this actinomycete isolate operated to reduce disease incidence was not elucidated, one possibility was that this strain exerted a direct inhibitory effect on hyphal growth and structure of fungal pathogens (Zaitlin et al., 2004; Zakalyukina et al., 2007; Loqman et al., 2009).

It has been reported that nutritional requirements of Streptomyces play an important role during metabolite synthesis process. Amongst various nutritional requirements, antifungal substance production has been known to be influenced by media components and cultural conditions, such as aeration, agitation, pH, temperature, and carbon source, which vary from organism to organism (Dahiya et al., 2006; Asha Devi et al., 2008; Yu et al., 2008).

Analysis of some morphological and cultural characteristics of strain S72 allowed us to determine its probable taxonomic classification in genus Streptomyces, according to the directions given by Williams et al. (1983a). This is the first study of streptomycetes on their antifungal activities from the sediments of Oubeira's Lake. The results indicate that the strains of *Streptomyces*

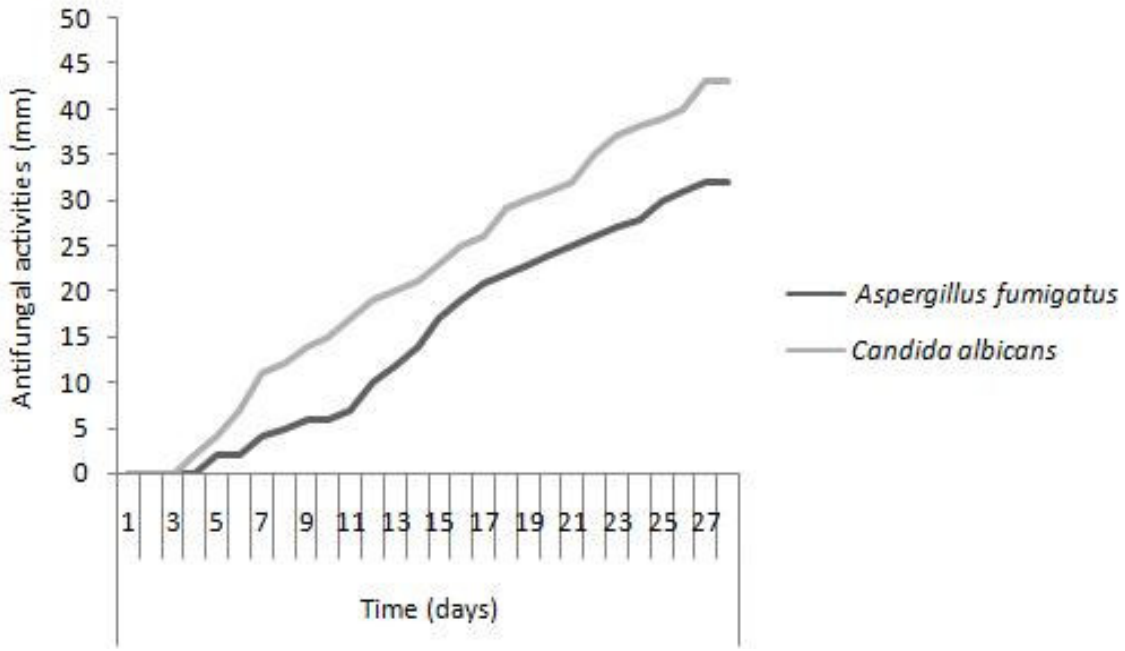

Figure 3. Time course of antifungal production of *Streptomyces* sp. S72 on ISP2 medium.

sp. S72 had a significant inhibitory activity against pathogenic fungi and a potential as biological control agents.

In conclusion, the results of this study show that naturally occurring actinomycetes have a great potential to produce metabolites against dermatophytes enabling the discovery of new antibiotics and hence merit future studies. Current trends suggest that future research in this area should focus on screening molecules from natural sources.

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