Evaluation of antifungal activity of novel marine actinomycete, *Streptomyces* sp. AA13 isolated from sediments of Lake Oubeira (Algeria) against *Candida albicans*

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A new actinomycete strain, designated AA13 was isolated from a marine sediment sample obtained from Lake Oubeira, in the North-East of Algeria and selected for its antifungal activities against *Candida albicans*. Morphological, physiological and biochemical properties and 16S rRNA gene sequencing strongly suggested that this strain was a new species, which belonged to the genus *Streptomyces*. Study of the influence of different nutritional compounds and culture conditions on growth and production of compounds with antifungal activity by the *Streptomyces* sp. strain AA13 indicated that the highest biomass and biological activities were obtained by utilizing the glycerol and peptone as carbon and nitrogen sources, respectively, with pH 7.0 and incubation temperature of 30°C. Two bioactive spots were detected by analysis of the ethyl acetate extract by thin-layer chromatography (TLC) and bioautography analyses. Among these bioactive compounds (antibiotics), a complex AA13-B that showed the interesting antifungal activity, was selected and purified by high performance liquid chromatography (HPLC), which indicated the presence of three peaks. Interestingly, the infrared spectroscopy (IR) studies showed that the molecule AA13-B2 contain an aromatic ring substituted by aliphatic chains. However, the investigations which determine the structure of the antifungal molecule are in progress.

Key words: *Streptomyces*, antifungal activity, *Candida albicans*, Lake Oubeira, sediments.

INTRODUCTION

The incidence of invasive fungal disease (IFDs), have been widely studied in recent years, largely because of the increasing population at risk. The FDs, are a significant cause of morbidity and mortality in

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immunocompromised patients, and are associated with increased healthcare costs. In fact, FDs, which is nevertheless a serious international health problem, has dramatically increased over the past few decades in both the hospital and community settings paralleling the rising number of immunocompromised patients (Joshua, 2006), and immunodeficiency diseases (Kwon-Chung and Bennett, 1992; Zaehner and Fiedler, 1995; McGinnis et al., 1999; Van Burik and Magee, 2001; Wavre et al., 2001; Bennett and Klich, 2003), as well as the changing spectrum of pathogens and antibiotic resistance (Tanaka and Mura, 1993; Culotta, 1994; Cassell, 1997).

*Candida albicans* is an opportunistic human fungal pathogen that causes candidiasis. It is found in the oral and gut mucosae in approximately 50-60% of healthy humans. Nevertheless, *C. albicans* can also be the agent of different types of infections, reaching from relatively harmless superficial infections like vaginal candidiasis or oral thrush of newborns to life-threatening blood stream infections (Glick and Siegel, 1999).

With the exponential emergence of *C. albicans* becoming resistant to antifungal antibiotics, the problems of drug resistance, patient sensitivity and inability to control infectious diseases have given real impetus for continuous search for new antibiotics all over the world (Chopra et al., 1997). This situation highlights the need for advent of safe, novel, and effective antifungal compounds. Microbial natural products still appear as the most promising source of the future antibiotics that the society is expecting. It is well known that the actinobacteria are the potential products of antibiotics, which could profitably be developed in the pharmaceutical industries. Among the so far reported actinobacteria organisms, is the genus *Streptomyces*, which are widely recognized as industrially important microorganisms because of their ability to produce many kinds of novel secondary metabolites including antibiotics, seem to offer a wide range of advantages, including accelerated accumulation of biomass (Williams et al., 1983; 1989; Crandall and Hamil, 1986; Korn-Wendisch and Kutzner, 1992; Ozgur et al., 2008). Furthermore, the importance of streptomycetes to medicine results from their production of over two-thirds of naturally derived antibiotics in current use (and many other pharmaceuticals such as anti-tumor agents and immunosuppressants), by means of complex secondary metabolic pathways (Miyadoh, 1993; Tanaka and Mura, 1993).

In the course of screening for new antibiotics, several studies are oriented towards isolation of streptomycetes from different habitats. Marine sediments constitute a large carbon reservoir and an untapped source for many useful drugs and an assessment of this potential is imperative. It is an environment with numerous microorganisms (Whitman et al., 1998; Biddle et al., 2005). Recent investigations indicate the tremendous potential of marine actinomycetes, particularly *Streptomyces* sp. as a useful and sustainable source of new bioactive natural products (Benouagueni et al., 2015).

Of particular relevance to the present study, the Lake Oubeira located in the North-East of Algeria, being an unexplored area in this field, with unique ecological niches and rich in biodiversity. The microbiology of sediment has to be further explored in order to get benefit out of the precious bio-wealth, because any new antibiotics and its producing organisms have been a great demand from the health care point of view to combat against the existing and emerging drug resistant pathogens. Accordingly, the present study reports, for the first time, on the effects of various nutritional and environmental factors on cell growth and antimicrobial metabolite production from *Streptomyces* sp. strain AA13, newly isolated from the Lake Oubeira, against *C. albicans*. It also provides basic information on the partial characterization of the bioactive molecules from the strain AA13.

**MATERIALS AND METHODS**

**Substrates and chemicals**

Unless specified, all substrates, chemicals, and reagents were of the analytical grade or highest available purity and purchased from Sigma Chemical Co. (St. Louis, MO, USA).

**Sample collection**

Sediment samples were collected from the sediments of the Lake Oubeira in the North East of Algeria, using a sediment grab sampler and transferred to one liter sterile plastic containers. Next, the container volume was filled with 60% sediment and 40% seawater from the sampling site. This was done in order to ensure aerobic conditions under storage upon processing. All the samples were kept at 4°C until use.

**Strain isolation**

One gram of sediments was taken in 9 ml of distilled water and shaken vigorously for 1 min. Different aqueous serial dilutions (10-1 to 10-5) of the suspension were carried out, and were decanted for 30 min. The supernatant (100 µl) was spread on medium casein-starch 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 30°C for 21 days and colonies were purified by streaking on medium called International *Streptomyces* Project (ISP)-2 agar.

**Cultural and microscopic characteristics**

Cultural features of strain AA13 were characterized following the directions given by the ISP media Viz., ISP-1, ISP-2, ISP-3, ISP-4, and ISP-5. The production of melanoides pigments was carried out on ISP-6 and ISP-7 at 30°C for 7-14 days (Shirling and Gottlieb, 1966), and the Bergey’s Manual of Systematic Bacteriology (Cross, 1989).
Morphology of spore bearing hyphae with the entire spore chain was observed with a light microscope (Model SE; Nikon) using cover-slip method in ISP-2 media after Gram staining (You et al., 2005).

Biochemical and physiological characteristics

These characteristics included the ability of the isolate to utilize different carbon. It was determined on plates containing ISP basal medium 9 to which carbon sources were added to a final concentration of 1% (Pröhid and Gottlieb, 1948), using glucose as positive control and using carbon source free medium as negative control (for comparison more suitable). The plates were incubated at 30°C for 7 to 21 days.

Hydrolysis of gelatin, starch and nitrate reduction was examined as described by Williams et al. (1983), Gordon et al. (1974) and Boudjella et al. (2006), respectively. The degradation of casein was given according to method of Gordon et al. (1974).

Analytical profiling index (API) strip tests were carried out to identify the genus to which the AA13 strain belonged. The nature of Gram staining, motility in hanging drop preparations, and physiological and biochemical characteristics of the strain were investigated using API 50 CH and API ZYM strips in accordance with the manufacturer's instructions (bioMérieux, SA, Marcy-l'-Etoile, France). The reading was carried out after incubation at 30°C for 24 h and then 48 h (Humble et al., 1977). The API LAB software (bioMérieux) was then used to obtain the percentage admitted, the identity of the bacteria sought by comparison with standard profiles. The other physiological and biochemical characteristics were determined using the method described by Williams et al. (1983). All tests were performed at 30°C.

Antifungal bioassay

*C. albicans* ATCC 10231 strain resistant to antibiotics like Ficonazole, Nystatin and Amphterocin B was grown at 30°C on Sabouraud dextrose medium. The culture was stored at 4°C. The antifungal activities of the isolates were determined by using the double layer agar method as described by You et al. (2005). The actinomycetes were inoculated on Petri dishes containing 15 ml ISP-2 agar and incubated at 30°C for 5 days, then Sabouraud dextrose medium was poured onto the basal layer containing strain AA13 colonies, and the *C. albicans* were plated onto the top layer. The inhibition zones were measured after incubation at 30°C for 48 h.

Extraction of the genomic DNA

The total preparation of DNA from the strain AA13 was carried out according to Higdon et al. (1985). Preparations of small size of plasmids from *Escherichia coli* were given according to Sambrook et al. (1989) and Fourati-Ben Fguira et al. (2005). Digestion with ribonucleases of restriction, the separation of the fragments of DNA by the electrophoresis on agarose gel, the dephosphorylation with the alkaline phosphatase of calf intestine, the ligation of the fragments of DNA and the transformation of *E. coli* were all performed in accordance with the method of Sambrook et al. (1989).

Amplification by polymerase chain reaction (PCR)

The 16S rRNA gene of the strain AA13 was amplified by PCR using two universal primers: forward primer, called pA: AGA GGT TGA TCC TGG CTC AG (8-28), and reverse primer, called pB: AAG GAG GTG ATC CAG CCG CA (1542-1522), designed from base positions 8 to 27 and 1541 to 1525, respectively, which were the conserved zones within the rRNA operon of *E. coli* (Edwards et al., 1989; Mellouli et al., 2003). Roughly, 50 ng of the DNA matrix is employed with 30 pmol each primer by a final volume of the reaction mixture of 50 µl. To improve the denaturation of the DNA, 50% (v/v) DMSO was added with the mixture to the reaction. Amplification was carried out with a thermocycler automated (Perkin-Elmer) by using one unit of DNA Tag polymerase (Stratagene, Agilent Technologies, Santa Clara, CA, USA) according to the following amplification profile: a stage of denaturation of the matrix: 94°C (3 min), followed by 40 cycles of which each one include: a stage of denaturation: 94°C (30 s), a stage of hybridization of the oligonucleotides to the matrix: 50°C (1 min), a stage of elongation: 72°C (10 min). The product of PCR was analyzed by electrophoresis on agarose gel 0.8%.

Sequencing of the 16S rRNA gene

The nucleotide sequences of both strands of the cloned 16S rRNA gene sequence were determined using BigDye Terminator Cycle Sequencing Ready Reaction kits and the automated DNA sequencer ABI PRISM® 3100-Avant Genetic Analyser (Applied Biosystems, Foster City, CA, USA). Multiple nucleotide sequence alignment was performed using the BioEdit version 7.0.2 software program and CLUSTALW program at the European Bioinformatics Institute server (http://www.ebi.ac.uk/clustalw). Phylogenetic and molecular evolutionary genetic analyses were performed using the molecular evolutionary genetics analysis (MEGA) software version 4.1. Distances and clustering were calculated using the neighbor-joining method. The tree topology of the neighbor-joining data was evaluated by Bootstrap analysis with 100 re-samplings.

Optimization of nutritional and cultural conditions

Culture medium on growth and bioactive metabolite production of the strain were optimized by using different parameters such as carbon and nitrogen sources, incubation temperature and pH as follows:

**Effect of carbon and nitrogen sources**

Different carbon and nitrogen sources were used to replace the carbon and nitrogen sources in basal medium, starch nitrate broth and all other components were kept constant. The sources were sterilized separately and added just prior to inoculation. Glucose, maltose, lactose, sucrose and glycerol were added separately as carbon sources into the basal medium at 1% concentration. Different nitrogen sources such as KNO₃, tryptone, peptone and meat extract were provided separately into the basal medium at 1% concentrations. The respective biomass and antifungal metabolites production were also recorded.

**Effect of incubation temperature**

The optimum temperature was assayed by incubating the production medium at temperature ranges varying from 25 to 37°C, and maintaining all other conditions at optimum levels at original concentration.

**Effect of initial pH of the culture medium**

To determine the influence of initial pH value of culture medium on growth and bioactive metabolite production, the strain AA13 was
cultivated in basal medium with different initial pH values (5-11). The pH was adjusted using hydrochloric acid or sodium hydroxide at 0.1 M.

Biomass determination
Samples (10 ml) were centrifuged at 5000 rpm for 5 min. The supernatants were discarded and the cell pellet was washed twice with distilled water and then dried at 70°C. Dry weight was measured and recorded.

Disk diffusion method
A paper disk, impregnated with the supernatant and then placed on the surface of Muller Hinton agar pre-inoculated with the pathogenic strain test and incubated at 30°C after 48 h. Plates were examined for evidence of antimicrobial activities represented by a zone of inhibition of growth around the paper disk.

Extraction and purification of active compounds
The cultivation medium ISP-2 from 1000 ml of shake flask culture of the Streptomyces sp. strain AA13 at 30°C for 7 days was harvested to remove the biomass. The cell-free supernatant was extracted with an equal volume of organic solvent. Four extraction solvents were tested for effectiveness, including n-hexane, ethyl acetate, acetic acid 5% and n-butanol. Each organic extract was evaporated to dryness using a Rotavapor. The resulting dry extract was recuperated in 1 ml of methanol (Zitouni et al., 2005; Boudjella et al., 2006).

For the TLC analysis, the crude extract was used loading a capillary tube on silica-coated plate (Merck, Darmstadt, Germany) on the line drawn around 1.5 cm from one of the plate. The plate was immersed in the solvent just below the line where samples were loaded. The solvents used were n-butanol-acetic acid-water (3:1:1). After the solvent front reached about half of the plate, the plate was removed and dried. Pigment spots were detected by bioautography (Betina, 1973) on silica gel plates seeded with C. albicans. The active spots were visualized using UV irradiation at 254 (absorbance) and 365 nm (fluorescence) and Rf values were calculated (Palanichamy et al., 2011). The fraction that showed antifungal activity was purified by HPLC. The TLC analysis was performed using the R package Version 3.1.1 (Vanderbilt University, USA).

Statistical analysis
All determinations were performed in three independent replicates, and the control experiment without xylanase was carried out under the same conditions. The experimental results were expressed as the mean of the replicate determinations and standard deviation (mean ± SD). Statistical significance was evaluated using t-tests for two-sample comparison and one-way analysis of variance (ANOVA) followed by t-test. The results were considered statistically significant for P values of less than or equal to 0.05. Statistical analysis was performed using the R package Version 3.1.1 (Vanderbilt University, USA).

Nucleotide sequence accession number
The 16S rRNA gene sequence (1507 bp) of strain AA13 has been deposited into publicly available databases (DDBJ/EMBL/GenBank) under the accession number JQ965757.

RESULTS

Cultural and microscopic characteristics
Actinomycetes have been intensively studied in several underexplored environments; niche and extreme habitats in various parts of the world in the last few years. However, their presence in marine sediments has not been extensively investigated, although their ubiquitous presence in the marine sediments has been well-documented (Jensen et al., 1991; Takizawa et al., 1993; Moran et al., 1995). The characterization of Streptomyces sp. was studied by following methods recommended by ISP.

The morphology of strain AA13 in different ISP media showed filamentous bacterium with extensively branched aerial mycelia and grew well on ISP medium, which include both synthetic and organic media described and the colonies were spreading. The aerial mycelium of strain appeared grayish with white outline on casein starch (Figure 1A) and ISP-2 media, greyish white on ISP-1 and ISP-5, grayish on ISP-3, 4, 6, and 7 media, and the substrate mycelium was light and dark brown, light and dark yellow (Table 1). The strain produced brown diffusible pigments in ISP-6 and ISP-7.

The observation with light microscopy (100× magnification), on the ISP-2 to ISP-5, showed that the spore-bearing hyphae were Spiral chain (S), Rectus-Flexibilis (RF), and Retinaculum-Apertura (RA) (Figure 1B). The number of the spores was higher than ten, which made them to be referred to as the long chains of spores. The vegetative hyphae was branched but not fragmented.

The characteristics of AA13 strains were compared with those of the known species of actinomycetes described in Bergey's manual of systematic bacteriology (Whitman et al., 1998), and obtained morphological properties suggested strongly that strain AA13 belonged to the genus Streptomyces.

Physiological and biochemical characteristics
The physiological and biochemical tests were performed according to standard methods described for actinomycetes. The strain showed an ability to assimilate 21 carbon sources but it could not utilize 11 other sugar (Table 2). It produces seven enzymes such as phosphatases, lipase, proteases, urease and osidases (Table 3). This strain also peptonized milk, liquefied gelatin and reduced nitrate, but it did not produce H2S.

Molecular identification of the strain AA13
A rapid method for the identification of filamentous
Table 1. Culture characteristics of isolate AA13 in different media after 7 days of incubation at 30°C.

| Medium      | Growth | Aerial mycelium           | Substrate mycelium | Soluble pigment |
|-------------|--------|---------------------------|--------------------|-----------------|
| Casein-starch | Good   | Grey with white outline   | Light brown        | -               |
| ISP-1       | Moderate | White-Grey              | Darkbrown          | -               |
| ISP-2       | Good    | Grey with white outline  | Light brown        | Brown           |
| ISP-3       | Good    | Grey                      | Light brown        | -               |
| ISP-4       | Good    | Grey                      | Light brown        | -               |
| ISP-5       | Good    | White-Grey                | Darkyellow         | -               |
| ISP-6       | Good    | Grey                      | Light yellow       | Brown           |
| ISP-7       | Moderate | Grey                     | Light yellow       | Brown           |

Table 2. Biochemical characteristics of isolate AA13.

| Carbon source | Growth characteristic | Carbon source | Growth characteristic |
|---------------|-----------------------|---------------|-----------------------|
| Esculin       | +                     | L-Arabinose   | +                     |
| Cellobiose    | +                     | Ribose        | +                     |
| Maltose       | +                     | D-Xylose      | +                     |
| Lactose       | +                     | L-Xylose      | +                     |
| Sucrose       | +                     | Adonitol      | -                     |
| Trehalose     | +                     | Galactose     | +                     |
| Melibiose     | -                     | Glucose       | +                     |
| Raffinose     | -                     | Fructose      | +                     |
| Melezitose    | +                     | Mannose       | +                     |
| Starch        | +                     | L-Sorbose     | -                     |
| Glycogen      | -                     | Rhamnose      | -                     |
| Inulin        | -                     | Dulcitol      | -                     |
| Xylitol       | +                     | Inositol      | -                     |
| Glycerol      | +                     | Sorbitol      | +                     |
| Erythritol    | -                     | Mannitol      | -                     |
| D-Arabinose   | +                     | N-Acetylglucosamine | +                     |

actinomycete was based on the analysis of the 16S rRNA gene restriction fragment patterns. This method begins
by isolating DNA and amplifying the gene coding for 16S rRNA using the polymerase chain reaction. The total nucleotide sequence of 1507 pb was determined in both strands (accession No. JQ965757). The alignment of this sequence through matching with the 16S rRNA reported genes sequences in GeneBank indicated that it belonged to the genus *Streptomyces* and represented a novel species that was readily distinguished from all recognized *Streptomyces* species (Figure 2).

**Antifungal bioassay**

According the tests of antifungal activity on ISP-2 medium by using the technique of double-layer, the *Streptomyces* sp. AA13 showed a significant antifungal activity against *C. albicans* with zone of inhibition of 42 mm.

**Optimization of nutritional and cultural conditions**

A number of carbohydrates were investigated for their effect on growth of AA13 and on its antibiotic production. The strain AA13 is able to grow and produce antifungal activities with the five kinds of carbon sources. Kinetics of growth and active molecules production studies, showed that the secretion of biological activities was closely correlated with the biomass production. Maximum biological activities and biomass production were obtained after 5 days of incubation for all tested carbon sources. Glycerol, lactose, maltose and sucrose were propitious to growth and antifungal antibiotic production by the strain AA13. The medium including glycerol gave the highest biomass (0.173 g/l) and antifungal activity (DIZ = 59 mm) (Figure 3). Other carbon sources such lactose, maltose, sucrose and glucose also favored growth but their intensity was less when compared with glycerol.

The findings of utilization of nitrogen source indicated that the medium including peptone gave the highest biomass (0.121 g/l) and anti fungal activity (DIZ = 35 mm), followed by cultures containing meat extract, tryptone, and KNO$_3$ but did not show significant effect (Figure 4).

In order to investigate the effect of incubating temperature on growth and antifungal production, the strain AA13 was cultivated at 25-37°C. The results indicated in the Figure 5 showed maximum antifungal activity at 30°C with the highest biomass of 0.116 g/l and the maximum diameter of inhibition zone of 48 mm. The strain AA13 was cultivated in the above improved medium with different initial pH values (5-11). The results showed that the best pH for antifungal antibiotic production was 7.0, and the corresponding maximum diameter of inhibition zones was 57 mm with the highest biomass of 0.118 g/l (Figure 6).
Figure 2. Phylogenetic tree based on 16S rRNA gene sequences showing relationships among *Streptomyces* sp. AA13 and the most close type strain species of *Streptomyces*.

**Extraction and purification of active compounds**

Antifungal compounds were extraction from the supernatant with equal volumes of four organic solvents, including n-hexane, ethyl acetate, acetic acid and n-butanol. The crude extract obtained by ethyl acetate showed maximal zonation of 31 mm against *C. albicans*. The residues were analyzed by TLC and bioautography. Two bioactive regions were detected and the Rf values are 0.31 to 0.48. HPLC was used in an attempt to purify the antimicrobial compounds from the crude solvent extract of isolate AA13. Among, two pure molecules, AA13-B complex that made the strongest antifungal activity were purified by HPLC. The chromatogram showed three peaks. Only, AA13-B2 had the highest antifungal activity against *C. albicans*.

The partial characterization by infrared spectrum of AA13-B2 (Figure 7) showed hydroxyl group (bands at 3540 and 3460 cm\(^{-1}\)), alkyl groups (bands between 3000 and 1800 cm\(^{-1}\)) and carbonyl group (band at 1645 cm\(^{-1}\)). The band 1560 cm\(^{-1}\) strongly indicates the presence of aromatic ring, and the bands at 1500 to 1300 cm\(^{-1}\) showed presence of C-H and C-O bonds as secondary bands. The aromatic ring is supported by secondary bands at 760 and 610 cm\(^{-1}\).

**DISCUSSION**

The emergence of fungal resistance threatens to return us to the era before the development of antifungal antibiotics (Smith et al., 1999; Shantikumar et al., 2006). The need for the investigation of new, safe and effective antimicrobials for replacement with invalidated antimicrobials or use in antibiotic rotation programs is necessary (Gerding et al., 1991; Quale et al., 1996; Niedreman, 1997).

As already mentioned, *C. albicans* is the agent of different types of infections, reaching from relatively harmless superficial infections like vaginal candidiasis or oral thrush of newborns to life-threatening blood stream infections. The human immune system is normally able to limit the abundance of *C. albicans*, keeping a healthy equilibrium in the commensally flora of the human mucosa (Glick and Siegel, 1999). Intriguingly, when the immune system is seriously weakened, *C. albicans* is able to become dominant in the mucosa, colonize different zones of the human body and cause severe infections. From a natural products perspective, marine bacteria remain a relatively unexplored resource for novel secondary metabolites. However, recent data suggest that actinomycetes, in particular genus *Streptomyces* are
widely distributed in marine environments (Sergey, 2012).

Of particular interest of this study, the Lake Oubeira is rich in biodiversity of flora, fauna, and microbial diversity (Morakchi et al., 2009; Benouagueni et al., 2015). The microbiology of its sediments has to be further explored in order to get benefit out of the precious bio-wealth. In our screening program for bioactive compounds, an antifungal activity of *Streptomyces* sp. strain AA13 isolated from the sediments of Lake Oubeira highlights its importance as candidate for further investigation in biological control of *C. albicans*. There is little published information describing the ecology of actinomycetes in marine habitats, because actinomycetes represent a small component of the total bacterial population in marine sediments, and their role in the marine environment is difficult to assess.

The actinomycetes have been isolated from Neuston sediments, as well as from marine sponges and sea
weeds (Bull and Stach, 2007; Goodfellow and Fiedler, 2010; Sergey, 2012). Jensen et al. (2005) isolated five actinomycetes phylotypes from marine sediments collected around the island of Guam. Marine actinomycetes *Kocuria erythryxa*, *Rhodococcus erythropolis* and *Dietzia maris* were isolated from a sub-seafloor sediment core collected at a depth of 1225 m of Hokkaido (Inagaki et al., 2003). However, recent studies have shown that the distribution of actinomycetes in marine sediments and the requirements of seawater for growth give conclusive evidence that actinomycetes adapted to the marine environment represent a physiologically unique class of microorganisms (Morakchi et al., 2009). On the other hand, the presence of indigenous marine actinomycetes indicate the wide distribution in different marine environments and habitats.
Figure 5. Kinetics of incubation temperature, growth and antifungal activities in starch nitrate medium, against Candida albicans.

(Küster, 1976; Al-Diwany and Cross, 1978; Collins and Jones, 1980; Goodfellow and Williams, 1983; Lam, 2006). Whereas other studies indicate that the Streptomycetes are not part of the indigenous microflora, as the possibility of wash-in from surrounding terrestrial habitats must always be considered (Goodfellow and Williams, 1983).

This latter view was supported by the observation that the number of actinomycetes in marine habitats decrease with increasing distance from land (Collins and Jones, 1980; Goodfellow and Williams, 1983). In this study, the sediment samples were collected from distance of 4 m from land by inserting a grab sampler 20 cm into the sediments.

Several reviews describing biologically active molecules isolated from marine actinomycetes have recently been published (Fenical and Jensen, 2006; Bull and Stach, 2007; Goodfellow and Fiedler, 2010; Mayer et al., 2011; Sergey, 2012), and this article provides a rather brief and general overview of this subject.

A recent publication describes the isolation of four macrodiolide antibiotics, marinomycins A-D from Marinispora strain CNQ-140 that exhibit impressive cancer cell toxicities against eight melanoma lines in the
Based on its morphological properties, the isolate AA13 was classified in the genus *Streptomyces*. Methods described by Shirling and Gottlieb (1966) have been used in the ISP. Those characteristics were considered important and are now commonly used in the key for classification of *Streptomyces* species. The taxonomy of *Streptomyces* species was mainly based on, the color of aerial and substrate mycelia and of soluble pigment, the shape and ornamentation of spore surface because of its stability (Forar Laidi et al., 2007). The aerial mycelium, substrate mycelium growth and pigmentation showed distinct variation based on the culture media in which the isolates were grown. Among the culture media used, the isolate growth was excellent in starch casein agar and this may be due to sufficient amount of nutrient included in this media (Ghanem et al., 2000; Gebreselema et al., 2013). Furthermore, some additional physiological characteristics (such as degradation of starch, gelatin, casein and reduction of nitrates) were carried out for adequate identification. Some additional tests relative to the use of some carbon source are also considered as certain species classification of new isolates strains are recommended by Shirling and Gottlieb (1972) and Holt et
The *Streptomyces* sp. strain AA13 contained various baggage of enzymes. This important producing actinomycetes have been reported (Ramesh and Mathivanan, 2009; Ayari et al., 2012; Ramesh and Aalbersberg, 2012). Such cellulolytic activity of marine actinomycetes was described by Chandramohan et al. (1972), and chitinolytic activity was reported by Pisano et al. (1992). Actinomycetes are also reported to contribute to recycling of organic compounds (Goodfellow and Haynes, 1984). In addition, they play a significant role in mineralization of organic matter and fixation of nitrogen (Valli et al., 2012).

Boudemagh et al. (2005) mentioned that molecular approaches for identification were often used due to their speed and efficiency. For this, the 16S rRNA gene sequence of the strain AA13 (1507 pb) was PCR-amplified, sequenced and submitted to GenBank (accession number is JQ965757). A neighbour-joining tree based on 16S rRNA sequences showed that the isolate occupies a distinct phylogenetic position within the radiation including representatives of the *Streptomyces* family.

In the current study, strain AA13 that seem to be strong fungal inhibitor, showed antibiosis against *C. albicans*. Although, the exact mechanisms by which this actinomycete isolate operate to reduce disease incidence is not elucidated, one possibility is that these biocontrol agents exert a direct inhibitory effect on structure of yeast pathogens (Zakalyukina and Zenova, 2007; Logman et al., 2009; Oskay, 2009). Several hypotheses were formulated on the possible natural role of these molecules. Most probably, they were produced under stress conditions, such as nutrient starvation, to protect and preserve the producer from the other competitors present in the same environment (Marinelli, 2009). Antibiotic production usually occurs late in growth, during late stages of the development of the aerial mycelium on solid medium and just before entry into stationary phase in liquid cultures (Chouayekh and Virolle, 2002). The genes responsible for the biosynthesis of an antibiotic were usually gathered on the chromosome, and their growth phase-dependent coordinated expression was under the control of one or several specific pathways as well as pleiotropic regulators (Hopwood et al., 1995).

In *Streptomyces*, several physiological studies have demonstrated that antibiotic biosynthesis, was elicited by phosphate limitation and conversely, strongly repressed by exogenous phosphate (Chouayekh and Virolle, 2002). Nevertheless, 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 (Bode et al., 2002), pH, temperature (Sujatha et al., 2005), carbon and nitrogen source, which vary from organism to organism (Stanbury et al., 1997; Dahiya et al., 2006; Asha Devi et al., 2008; Yu et al., 2008; Oskay, 2009).

From the results it was evident that maximum growth and antifungal metabolite production was obtained at 30°C, which clearly indicates the mesophilic nature of the isolate. Previous reports, illustrate that optimal temperature range was between 26 and 35°C for antibacterial metabolites by *Streptomyces* strains (Macedo et al., 2007; Madan and SingaraCharya, 2013). Maximum zone of inhibition was observed when the glycerol was used as carbon source. This indicated the presence of an active uptake system for these compounds in the isolate. Similar results have been reported by many investigators (Mansour et al., 1996; Vahidi et al., 2004; Madan and SingaraCharya, 2013). Maltose, sucrose and glucose are poor carbon source for antibiotic production. It is possible that these carbon

**Figure 7.** Infrared spectrum of antibiotic AA13-B2.
sources were utilized rapidly for the synthesis of cellular material so that little would be available as carbon and energy source for antibiotic synthesis. Therefore, carbon source plays a critical role as sources of precursors and energies for synthesis of biomass building blocks and secondary metabolite production (Jia et al., 2009; Oskay, 2011). However, Tanaka et al. (1986) observed that in certain cases, some excessive nutritional components such as glucose, amino acids and other carbon and nitrogen sources affected antibiotic production in fermentation broth.

However, controversially, there was no production of antimicrobial compound by Nocardiosis sp. strain MAD08 when the medium was supplemented with different carbon sources at a concentration of 1% (w/v) (Selvin et al., 2009). The present study results also indicated that peptone served as ideal nitrogen source. The change in initial pH of the culture medium that affected maximum growth and antifungal production was obtained at 7.0.

Augustine et al. (2005) showed that cultural conditions affected antifungal metabolite production by Streptomyces rochei strain AK 39. The change in pH of the culture medium induces production of new substances that affect antibiotic production. However, El-Mehalawy et al. (2005) reported the effects of factors on fungal production Streptomyces lydicus, Streptomyces ederensis, Streptomyces erumpens and Streptomyces antimycoticus. Glycerol had positive effects on antifungal production followed by starch. In addition, it was observed that the optimum temperature for antifungal production by S. lydicus and S. ederensis was 24°C, while for S. erumpens and S. antimycoticus, it was 28°C. The optimum pH value for antifungal production by these species was 7.0.

The crude extracts showed wide range of inhibition zone against tested C. albicans. Similar findings were reported earlier by Gebreselema et al. (2013). In general, the antifungal activity of crude extracts fluctuated widely. Therefore, crude extracts could be a potent source for antibiotic production, which leads to the development of novel drugs for the treatment of infectious diseases. It has been reported that the antimicrobial activity of the compounds from different strains of actinomycetes vary depending on the strains, from which the compound was obtained, the solvent used for the extraction and the nature of the pathogens tested against such compound (Pridham and Gottlieb, 1948; Saadoun and Al-Momani, 2000; Sahin and Ugur, 2003; Narayana et al., 2005). It is evident that the antimicrobial efficacy of the bioactive compound is the expression of the genetic potentiality of the organisms, and the sensitivity of the test organisms is one of the genetic properties of the organism. Hence, studies on the genetic relationship between the organisms involved in the microbial interaction could throw more light on the underlying mechanisms. In addition, some studies were done in relation to the industrial enzymes production by microorganisms, eg: Sharma and Pant (2001) isolated aquatic actinomycetes in Bengal gulf and their results showed that the aquatic actinomycetes could be a source for production of bioactive compounds (Ward and Bora, 2006). Isamycins, Apasmomycins and Altemicidin, isolated by researchers at the Institute of Microbial Chemistry in Tokyo, were produced by various actinomycetes isolated from marine sediment samples collected from Sagami Bay, Japan (Okami et al., 1979; Fenical and Jensen, 1993).

The Rf values from TLC showed the presence of two active compounds from Streptomyces sp. strain AA13 in the finding of Hongjuan et al. (2006). The sizes of the inhibition zones varied with the most antagonistic isolate producing bigger zones in the bioautograms. This corroborates reports by Asha Devi et al. (2008) that the size of the inhibition zone positively correlates with the amount of antibiotics produced. The various separation and purification steps led to the isolation of two pure bioactive molecules. The infrared spectrum indicated that the antifungal AA13-B2 belongs to the group, which contain aromatic ring substituted by aliphatic chains. Boudjella et al. (2006) mentioned that the antifungal B extracted from Streptosporangium Sg 10, purified via HPLC and characterized by infrared spectroscopy, belonged to the group of glycosylated aromatics. Badji et al. (2006) indicated that four antibiotics produced by Actinomadura sp. strain AC104 isolated from Algerian Saharian soil, belonged to the same chemical family containing a benzenic ring di-substituted by aliphatic chains. Narayana et al. (2008) mentioned that the bioactive compounds isolated from Streptomyces sp. strain ANU 6277 were identified as benzyl alcohol, phenylethyl alcohol and 2H-1, 4-benzoxazin-3 (4H)-one. Although, the two active molecules were characterized from the new isolated Streptomyces sp. strain AA13 strain, we think that our strain is very interesting because it produces simultaneously two active molecules, which can be used in human therapy.

Study on the influence of different nutritional compounds and culture conditions on growth and production of compounds with antifungal activity by the Streptomyces sp. strain AA13 strain isolated from the sediments of Lake Oubeira, showed that the high biomass and biological activities were obtained when glycerol or peptone were added in basal medium with pH 7.0 and incubation temperature at 30°C.

The antimicrobial substance produced by Streptomyces sp. strain AA13 was purified and its antifungal characteristics were investigated in this study. The crude culture supernatant showed antifungal activity against C. albicans. The extraction and purification steps led to isolation of two pure molecules having biological activities. The partial characterization of the compound AA13-B2, indicated that the presence of aromatic ring was substituted by aliphatic chains.

These findings indicated that the produced substance
might be the alternative antimicrobial substance which is a tool for controlling fungi diseases.

Conflict of Interests

The authors have not declared any conflict of interests.

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