



Research Article

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Antifungal activity of *Streptomyces* sp. against environmental and clinical *Cryptococcus* spp. isolates

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ABSTRACT

This study aimed to analyze the antifungal susceptibility profile of environmental *Cryptococcus* spp. and to determine the antifungal activity of bioactive molecules extracted from an actinomycete, strain E96, against these *Cryptococcus* spp. *Cryptococcus* strains were investigated from pigeon droppings, atmospheric air and living materials collected on Eucalyptus trees. The isolates were identified and compared for their sensitivity to antifungal agents to clinical strains of *Cryptococcus neoformans*. Actinomycetes were isolated from Mellah Lake water and screened for antimicrobial activity. Antimicrobial assays were performed on ISP2 agar. The taxonomic position of the strain E96 was determined regarding phenotypic and 16S DNA sequences features. It was tested for its antifungal activity against the *Cryptococcus* spp. isolates using both double layers and agar disc diffusion methods. Time course of E96 antifungal metabolites production was determined on ISP1, ISP2, GYEA and Bennett culture media. The actinomycete E96 strain showed a very promising activity against the *Cryptococcus* spp. strains, especially interesting when they are resistant to 5-fluorocytosine, azole or echinocandins.

Keywords : *Cryptococcus albidus*. *Cryptococcus diffluens*. Environment. *Streptomyces*. Antimicrobial activity.

INTRODUCTION

The genus *Cryptococcus* includes 100 basidiomycetous fungal species characterized as variously encapsulated budding yeasts. *C. neoformans* and *C. gattii* are the major human and animal pathogens. However, other species such as, *C. laurentii*, *C. uniguttualus*, *C. gastricus*, *C. albidus*, *C. loteolus*, *C. adeliensis*, *C. humicola*, *C. magnus*, *C. diffluens*, *C. curvatus*, and others have been occasionally involved in moderate-to-severe disease [1]. Cryptococcosis can result in serious meningitis, encephalitis, or meningoencephalitis and is acquired by inhalation of infective yeasts present in wood, fruits, rotting vegetables, soil, dairy products, and urban pigeon (*Columba livia*) droppings [2]. The drug of choice in the treatment of cryptococcosis is amphotericin B (AMB), which may or may not be combined with other drugs, such as fluconazole (FCZ) or 5-fluorocytosine [3, 4]. But it is responsible for many side effects and recent studies indicate that some patients remained resistant to new antifungal drugs [5]. Actinomycetes have been recognized as the potential producers of metabolites such as antibiotics, growth promoting substances for plants and animals, immunomodifiers, enzyme inhibitors and many other compounds of use to man [6].

In this study, we reported for the first time the isolation of *C. albidus* and *C. diffluens* from pigeon droppings and atmospheric air sampled near bird guano in Eastern Algeria. The antifungal susceptibility profile of these isolates was compared to the one of *C. neoformans* clinical strains. The relatively high antifungal resistance of these strains prompted us to search for other antifungal agents. Therefore, we attempted to identify *Actinomycetales* strains

isolated from Lake El Mellah that presented antifungal activity against these clinical and environmental strains of *Cryptococcus* spp.

EXPERIMENTAL SECTION

Collection and identification of yeast strains

Three clinical strains were used in this study: CN104 (isolated from the blood of a 46 year-old male in 2003), CN721 (isolated from the blood of a 19 year-old male in 2007), CN954 (isolated from the CSF of a 22 year-old male in 2003). They were provided by the Parasitology-Mycology laboratory of the Marseille Timone Hospital, France.

The environmental samples were collected from public hospitals (inside and outside the buildings), private clinic, places of public affluence, as well as downtown streets in Algeria. We collected a total of 117 samples, 91 obtained from pigeon droppings, 16 from atmospheric air sampled near bird droppings and 10 from living materials collected on Eucalyptus trees, including flowers, fruits, leaves, bark, debris and soil [7] in the Annaba area (Figure 1). Air was sampled using the SAS AIR SAMPLER Duo 360 (Bioscience International, Rockville, USA). The device was placed in the sampling sites and programmed to aspirate 180l of air per minute for 2 min. Pigeon droppings and living materials from the Eucalyptus trees were collected in sterile bags and transferred immediately to the laboratory.

Pigeon dropping samples and Eucalyptus samples were suspended in sterile saline solution (NaCl 0.9%) at a ratio of 1:5 by vortexing and allowed to sediment for 20min. Supernatant aliquots of 0.5 ml were inoculated on Sabouraud Dextrose Agar (SDA) medium supplemented with (0.1 g/l) chloramphenicol. The plates were incubated at 26°C for 8 days and were daily examined to observe the appearance of the colonies and to select those corresponding to *Cryptococcus* species [8]. They were subcultured on malt extract agar at 30°C and were examined under the microscope, after India ink staining, to analyze their morphology and to visualize the presence of a capsule [9]. The phenoloxydase activity was tested using Niger (*Guizotia abyssinica*) agar. The biochemical identification relied on urease testing and ID32C auxanogram profile [8]. After morphological and biochemical identification, yeasts were conserved using the Protect Microorganism Preservation System (Technical Service Consultants Ltd, Heywood, Lancashire, UK) and maintained at -80°C until further subculture.

Fungal DNA was extracted using the EZ1 DNA Tissue Kit with the EZ1 Advanced XL instrument. Colonies were incubated in 200 µl Buffer G2 and 10 µl proteinase K at 56°C until total lysis. DNA was then purified as described by the manufacturer and eluted in 100 µl of buffer. Primers D1 (AAC TTA AGC ATA TCA ATA AGC GGA GGA) and D2 (GGT CCG TGT TTC AAG ACG G), and primers ITS3 (GCA TCG ATG AAG AAC GCA GC) and ITS4 (TCC TCC GCT TAT TGA TAT GC) were used to amplify the D1-D2 variable region of the 26S and the internal transcribed spacer 2 of the ribosomal RNA gene, respectively, as described in Cassagne *et al.* [10]. The obtained sequences were queried against nucleotide sequences deposited in GenBank using the program BLAST through the NCBI server and the International Society for Human and Animal Mycology (ISHAM)-ITS reference DNA barcoding database [11].



Figure 1: Sample location and *Cryptococcus* spp. isolated in (A) Annaba area and (B) Annaba city (Algeria)

☆ : Environmental samples; △ : *Cryptococcus salbidus* ; ○ : *Cryptococcus diffluens*.

Isolation, characterization and antifungal activity of the Actinomycete strain

Water samples of the Mellah Lake were collected in sterile bottles kept at 4°C and transferred immediately to the laboratory. They were then inoculated onto seven different media: ISP1, ISP2, ISP4, ISP5, Gauss, Cross and Casein starch agar. Plates were incubated for 7, 14 and 21 days at 28°C. Characteristic colonies were subcultured on ISP2 agar and kept at 4°C [12]. Among these strains, we were interested in the E96 strain that displayed an antifungal activity against *Cryptococcus* spp. A taxonomic study based on morphological, biochemical and physiological characters was performed. The morphological characteristics and the production of melanoid pigment of the organism were determined by visual examination of 14-day-old cultures grown using the International Streptomyces Project (ISP) media (ISP1, ISP2, ISP3, ISP4, ISP5, ISP6, and ISP7) recommended by Shirling and Gottlieb [13] and casein starch medium. Advocated by Wink [14], the biochemical properties were detected using the available commercially API ZYM, API 50CH and API 20NE according to the manufacturer's instructions. A total of 55 tests have been performed to study the physiological characteristics, including the utilization of 12 carbohydrate compounds evaluated on ISP9 basal medium with a final concentration of 1% [13], degradation of valine, asparagine, proline, arginine, serine, paraffin and chitin according to Goodfellow's methods [15], hydrolysis of casein, urea, indole and starch [16] and hydrolysis of gelatin [17]. The strain was also tested for its ability to grow in the presence of 18 antibiotics, using the paper disc method of Goodfellow and Orchard [18] and inhibitory compounds including (w/v): sodium azide 0.001%, and phenol 0.1% on YEA medium as described by Goodfellow [15]. Growth at 15°C, 29°C, 37°C and 43°C, and at pH4, pH5, pH6, pH7, pH8, pH9 and pH10 was also tested.

Genomic DNA of the E96 strain was extracted from colonies grown after 48h at 30°C in COS medium by the method described by Roux *et al.* [19]. PCR amplification of 16S rDNA of Actinomycete strain was carried out using the universal primers Fd1 (5'-AGAGTTTGATCCTGGCTCAG-3') and rp2 (5'-ACGGCTACCTTGTTACGACTT-3') [20]. The 3130 Genetic Analyzer automated sequencer was used for nucleotide sequence determination. The eight resulting sequences were assembled into a unique contig with ChromasPro version 1.5. The resulting nucleotide sequences were compared using the program BLAST via the NCBI server, with those included in a bank we constituted. The sequences included in the bank were those of the type strain of each species.

The antifungal activity was detected using two different methods: the double layers method described by Zitouni & *al.* [21] and the agar disc diffusion method described by Saadoun and Muhana [22]. The E96 strain production kinetics of metabolites with antifungal activity was determined using various media (ISP2, ISP1, Bennett and GYEA (glucose yeast extract agar)) for 14 days [23].

The extraction of the bioactive compounds was performed according to the technique detailed by Badji & *al.* [24]. The E96 strain was inoculated in very tight streaks onto the surface of the medium in a 90 mm diameter Petri dish. After 1 day of incubation at 30°C, the agar of one Petri dish was cut into small cubes which were then transferred into an Erlenmeyer flask containing 40 ml of one of the following four solvents: n-hexane, ethyl acetate, n-butanol or dichloromethane. The extraction was carried out with stirring for 2 h at room temperature. The organic extract obtained was filtered and then desiccated under vacuum at 45°C using a rotary evaporator. The dry residue was then suspended in 5 ml of methanol.

The in vitro antifungal susceptibility was determined using the SENSITITRE™ YEASTONE™ YO10 containing a serial dilutions of eight antifungal agents: anidulafungin (AND), micafungin (MF), caspofungin (CAS), 5-Flucytosine (FC), posaconazole (PZ), voriconazole (VOR), itraconazole (IZ), fluconazole (FZ) and amphotericin B (AB). This broth microdilution assay provides quantitative results of Minimum Inhibitory Concentration (MIC) for each tested antifungal. Yeast cells collected from a 48 hours culture were suspended in sterile water, turbidity was adjusted to 0.5 McFarland, and the suspension was inoculated into each well of the plate. The plates were incubated at 30°C and visually read after 48 h. Colorimetric MIC result was read as the first blue (no growth) well that showed a distinct color change as compared to the positive growth (red or purple) well [25, 26]. Because no clinical MIC breakpoints have been determined for *Cryptococcus* spp., the susceptibility of the isolates was not categorized. *Candida parapsilosis* ATCC 22019 and *Candida krusei* ATCC 6258 were used for quality control [27].

The antifungal activity of the bioactive compound was tested using 6 mm diameter paper discs. The discs are soaked with 50 µl and 100 µl of methanol extract and placed onto Sabouraud agar previously inoculated with the yeast strain. The diameter of the inhibition zones was measured for each strain after 48h of incubation at 30°C.

RESULTS AND DISCUSSION

Collection and identification of yeast strains

Identification of clinical yeast strains showed that all isolates belonged to the species *C. neoformans* with 99-100 % homology with the sequences deposited in the Genbank database and the ISHAM-ITS reference DNA barcoding database.

We collected a total of 117 environmental samples, 91 pigeon droppings, 16 air samples and 10 from living materials collected on plant material or soil [7] in the Annaba area. Species belonging to the *Cryptococcus* genus were isolated from 5.5% of the pigeon dropping and 12.5% of the air samples (Table1). Many diverse fungal colonies were observed on the agar plate. The genera *Cryptococcus*, *Mucor*, *Cladosporium*, *Penicillium*, *Alternaria*, *Fusarium*, *Abisidia*, *Rhodotorula* and *Aspergillus* were identified according to morphological criteria. Thereafter, only the strains which morphology was compatible with *Cryptococcus* species, i.e. smooth colonies (creamy or mucous) with white beige or ocher color and a capsule observed at the microscopic examination of an India ink stain preparation, were kept. These isolates were further identified using biochemical and DNA-sequence comparison. All isolates belonged to the species *C. albidus* except one (F9) that was identified as *C. diffluens*. The 26S rRNA gene and ITS2 sequences of the *C. albidus* isolates were identical and showed 99-100 % homology with the sequences deposited in the Genbank database. The F9 isolate sequences showed 99-100 % homology with the *C. diffluens* sequences deposited in the GenBank database.

Table 1: Distribution of *Cryptococcus spp* isolated from environmental samples

Location	Samples (117)								
	Pigeon droppings (91)			Atmospheric air (16)			Eucalyptus trees (10)		
	Sample number	Positive samples	Isolates	Sample number	Positive samples	Isolates	Sample number	Positive samples	Isolates
Hospital	19	5	F1: <i>C. albidus</i> F2: <i>C. albidus</i> F6.5: <i>C. albidus</i> F8: <i>C. albidus</i> F9: <i>C. diffluens</i>	16	2	AF2: <i>C. albidus</i> AF10: <i>C. albidus</i>		0	
Clinic	31	0							
Mosque	12	0							
Annaba downtown	8	0							
Villages around Annaba	18	0					5		
El Kala's region	3	0					5		

In this study, we reported for the first time, the isolation of *Cryptococcus sp.* from pigeon droppings and atmospheric air sampled in Algeria. Only three studies have reported *Cryptococcus spp.* isolation in Algeria. Mounira & al. [28] isolated *Cryptococcus sp.* in patients hospitalized with neurological disorders. Akdouche & al. isolated *Cryptococcus sp.* in the mammary glands of cows, and Bendjama & al. isolated *C. terreus*, *C. albidus* and *C. neoformans* from irrigation water samples from the Ouargla area (south of Algeria). One limitation of these studies was that their identification strains relied on morphological and biochemical criteria and not on nucleotide sequence analysis [29, 30]. In our study, *Cryptococcus albidus* was isolated from pigeon droppings and atmospheric air, whereas *Cryptococcus diffluens* was found only in pigeon droppings. It has been shown that the presence of birds in the environment was the main factor associated with *C. neoformans* infection and thus would be a source of human infection [29, 8, 7]. Our findings suggested that pigeon droppings offered suitable conditions for the survival, and might act as reservoir of various *Cryptococcus* species in the spore form which might infect humans via air borne transmission.

Identification and antifungal activity of the Actinomycete strain

The cultural characteristics of the Actinomycete strain were observed on various media after 14 days incubation at 30°C. The strain E96 showed good growth on ISP1, ISP2, ISP3, ISP4 and casein starch media and moderate on ISP5, ISP6 and ISP7. No soluble pigment was produced. Light microscopy observation indicated that the strain was Gram positive with a filamentous branched and no fragmented aerial mycelium. The spores of the strain were spirale chain (S), Rectus-Flexibilis (RF) and Retinaculum- Apertum (RA).

The physiological and biochemical proprieties of E96 strain are detailed in the Table 2. The E96 strain assimilates the following carbon sources: glucose, galactose, D-fructose, D-mannitol, sorbitol, mannose. It showed a moderate growth for L-rhamnose, but no growth on raffinose, saccharose, L-arabinose, D-xylose, D-melibiose. It showed a good growth with valine, asparagine, proline, arginine, and no growth with serine. Good growth was observed at pH 5, 6, 7, 8, 9, 10 and at 15°C, 29°C, 37°C, but reduced growth was observed at 43°C and pH 4. E96 could growth in

the presence of several antibiotics such as ampicillin, nalidixic acid, amoxicillin, cefotaxime), pefloxacin, cefixime, cefazolin, pipemidic acid, and cephalixin. E96 produced chitinase, caseinase, urease, amylase and gelatinase.

Table 2: Physiological and biochemical characteristics of the strain E96

Tests	Result
<i>Utilization of carbohydrates</i>	
Glucose	+
Galactose	+
Raffinose	-
Saccharose	-
L-Arabinose	-
D-Fructose	+
D-Mannitol	+
D-Xylose	-
Sorbitol	+
D-Melibiose	-
Mannose	+
L-Rhamnose	+/-
Simmons citrate	+
<i>Utilization of nitrogen sources</i>	
Valine	+
Asparagine	+
Proline	+
Arginine	+
Serine	-
<i>Degradation of</i>	
Chitin	+
Casein	-
Urea	-
Indole	+
Starch	+
Gelatin	+
<i>Growth at</i>	
15°C	+
29°C	+
37°C	+
43°C	+/-
pH 4	+/-
pH5	+
pH6	+
pH7	+
pH8	+
pH9	+
pH10	+
<i>Growth in presence of</i>	
Phenol (0.1%)	-
Sodium azide (0.001%)	-
Paraffin	-
<i>Growth in presence of antibiotics</i>	
Ampicillin (25µg)	+
Nalidixic acid (30µg)	+
Kanamycin (30µg)	-
Erythromycin (15µg)	-
Amoxicillin (30µg)	+
Clindamycin (2µg)	-
Tobramycin (10µg)	-
Imipenem (10µg)	-
Cefotaxime (30µg)	+
Pefloxacin (5µg)	+
Nitroxoline (30µg)	-
Pristinamycin (15µg)	-
Spiramycin (100µg)	-
Cefixime (10µg)	+
Cefazolin (30µg)	+
Doxycycline (30µg)	-
Pipemidic acid	+
Cefalexin (30µg)	+

According to the Bergey's Manual of Systematic Bacteriology, the physiological and biochemical proprieties strongly suggested that the strain E96 belonged to the genus *Streptomyces* [31]. Molecular identification was based on the analysis of the 16S rDNA gene. A 1508pb fragment was obtained and compared with 16S rDNA genes sequences included in the bank we constituted. *Streptomyces celluloflavus* and *S. kasugaensis* sequences were

reported as the most similar with 98.8% identity. The position of E96 in the neighbor joining phylogenetic tree is shown in Figure 2.

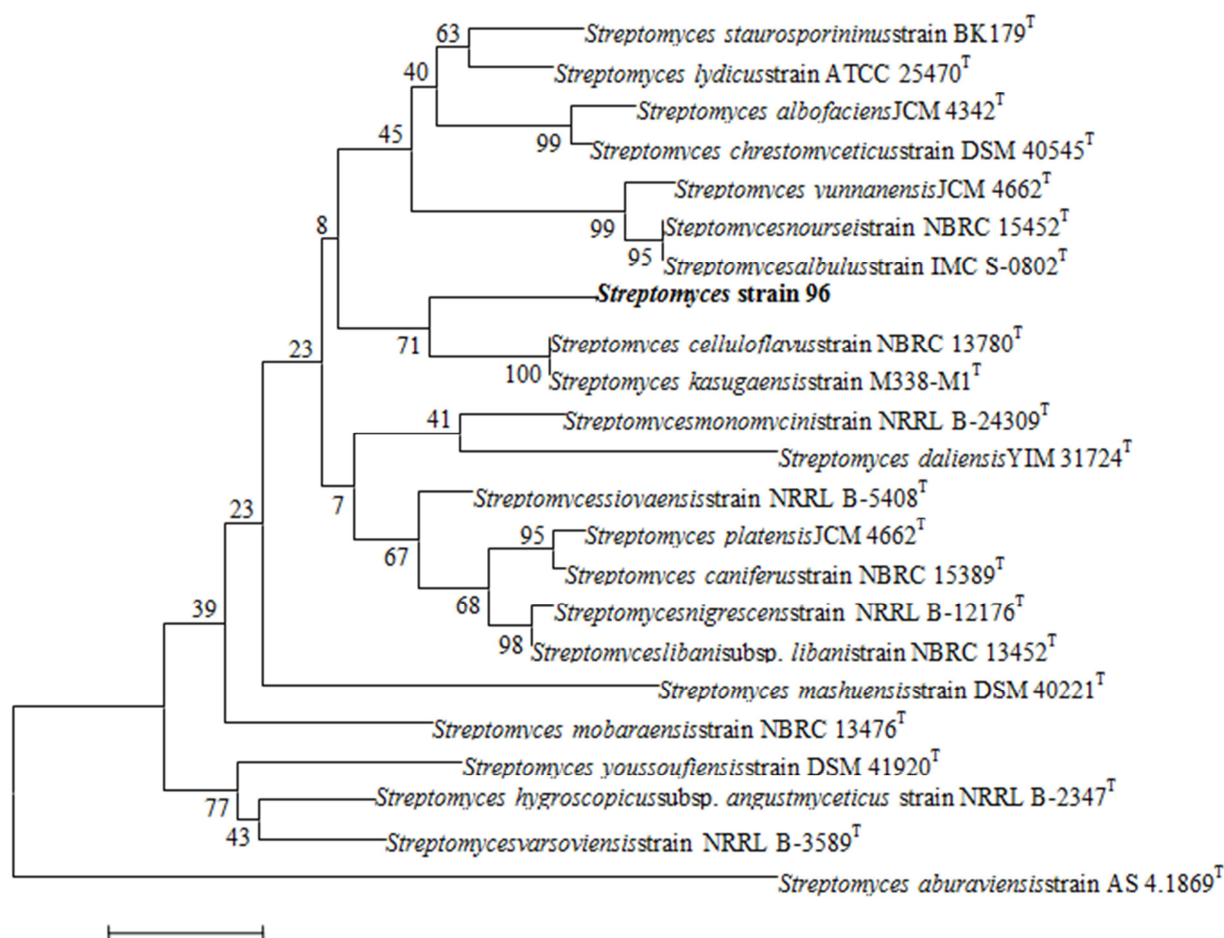


Figure 2: Neighbor-joining phylogenetic tree based on 16S rRNA sequence gene comparison showing relationships between *Streptomyces* E96 and related species
The scale bar indicates 0.005 substitutions

To evaluate the antifungal activity and to determine the best antifungal-active production medium, two techniques and four media respectively, were used. Antifungal activity was observed on all media tested (Figure 3). The ISP2 medium yielded the best production and was thus selected for antifungal compounds production. The antifungal-active metabolite production by the E96 strain on ISP1, ISP2, Bennett and GYEA started at the first day of culture on all media tested and reached a maximum inhibition zone (27 mm) on ISP2 medium (Figure 4). Moreover, n-hexane solvent yielded the best secondary metabolites extraction (data not shown). The optimal antifungal extraction conditions were thus: at the first day of culture on ISP2 medium using n-hexane organic solvent.

Regarding the *in vitro* susceptibility to the antifungal commercial agents (Table 3), ANI, MIC and CAS displayed elevated MIC against 100% of the tested isolates. 5FC displayed low MIC against 100% of the clinical *C. neoformans* isolates but in contrast it displayed elevated MIC against 67% of the environmental isolates. The azole antifungal agents (POS, VORI, ITRA, FLU) displayed low MIC against 71% of all isolates. The antifungal active extract (EXT96) of the E96 strain showed a wide inhibition zone (23 mm) at 0.06mg/l. It was impossible to subculture isolate F2 and thus its susceptibility to antifungal agents could not be determined. In our study, only the strains isolated from droppings pigeon displayed a multi-antifungal resistance profile. Therefore azole resistance could be not intrinsic to the species *C. albidus* but to some isolates of this species. Clinical strains displayed a lower azole resistance level than environmental strains. Like for the other non-*C. neoformans* infections, the treatment of *C. albidus* infections is not well defined, which may explain the 50% mortality in *C. albidus* encephalitis despite antifungal therapy [32]. Increasing drug resistance in microbes, including fungi, prompted to search for new and enhanced therapeutic compounds from different sources, including the metabolites excreted by *Actinomycetes* [33]. At screening, our strain showed an important antifungal activity against both environmental and clinical strains of *Cryptococcus spp.* and displayed a higher inhibition zone than the tested commercially available antifungal agents.

Secondary metabolites production usually occurs during the stationary growth phase. However, we found that the antifungal compound produced by the E96 strain was highly correlated with the growth of strain (J1, Figure 4). Albeit, most of the antifungal antibiotics were extracted from liquid media and using ethyl acetate [6], those produced by E96 could not be extracted under these conditions but they were extracted from solid media using n-hexane solvent.

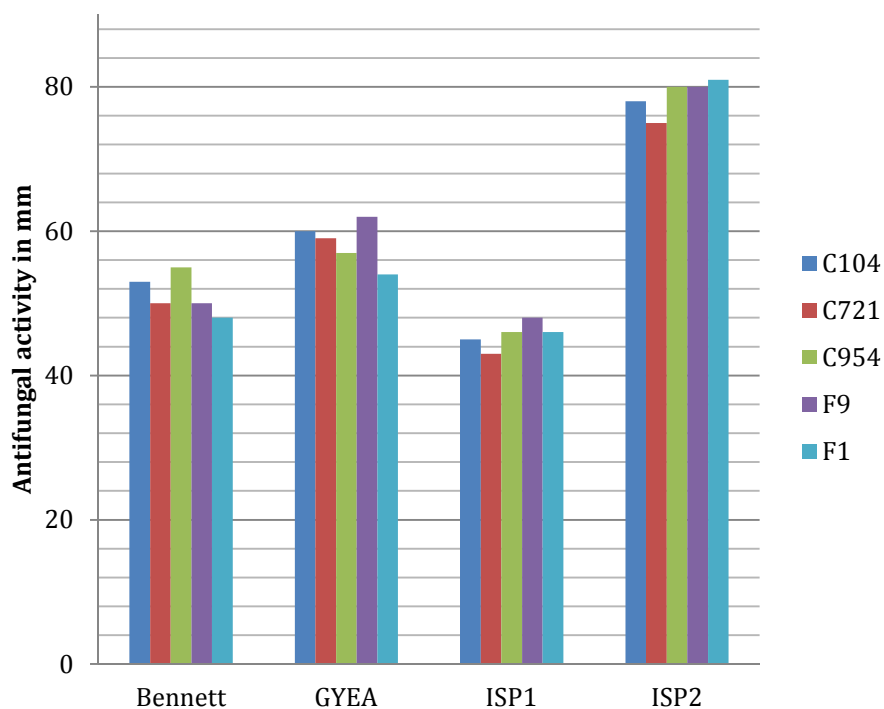


Figure 3: Antifungal activity determination of 5 *Cryptococcus* isolates on 4 different media by the double layer method

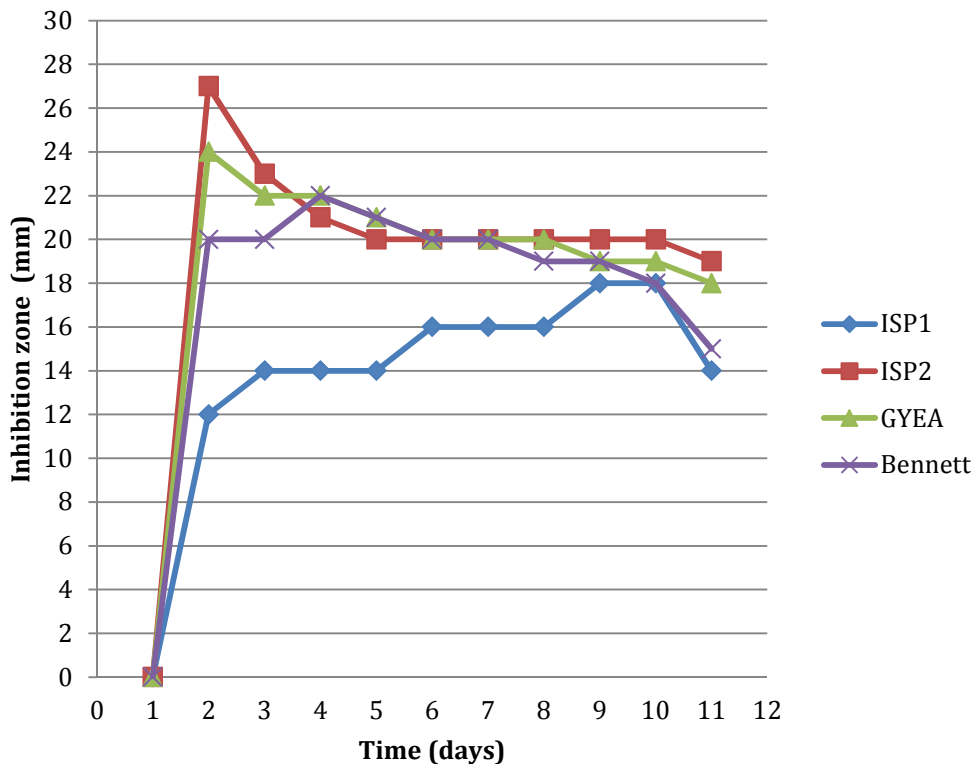


Figure 4: Time course of antifungal metabolites production by *Streptomyces* E96 in four culture media (ISP1, ISP2, GYEA, Bennett)

Table 3: Determination of the minimum inhibitory concentration (MIC, mg/l) of environmental and clinical isolates
 ANI: Anudulafungin, MICA: Micafungin, CAS: Caspofungin, 5FC: 5-fluorocytosin, POS: Posaconazole, VORI: Voriconazole, ITRA: Itraconazole, FLU: Fluconazole, AMB: Amphotericin B. ND: not determined

Drugs	Range MIC(mg/l)	Samples (MIC/Susceptibility)									
		C. albidus F1	C. albidus F2	C. albidus F6.5	C. albidus F8	C. diffluens F9	C. albidus AF2	C. albidus AF10	C. neoformans CN104	C. neoformans CN721	C. neoformans CN954
ANI	8	>8	ND	>8	>8	>8	>8	>8	>8	>8	>8
MICA	8	>8	ND	>8	>8	>8	>8	>8	>8	>8	>8
CAS	8	>8	ND	>8	>8	>8	>8	>8	>8	>8	>8
5FC	4 – 64	>64	ND	>64	>64	>64	8	4	2	2	2
POS	0.25 – 1	1	ND	0.5	0.25	0.25	0.5	0.25	0.06	0.25	0.06
VORI	0.12 – 0.25	0.5	ND	0.25	0.12	0.12	0.25	0.12	0.03	0.25	0.03
ITRA	0.06 – 0.25	0.5	ND	0.25	0.5	0.06	0.12	0.12	0.06	0.12	0.06
FLU	8– 64	64	ND	16	16	32	16	8	8	8	4
AMB	0.12 – 0.25	0.25	ND	1	0.25	<0.12	<0.12	0.12	0.12	0.12	0.12
EXT96	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06

CONCLUSION

Our findings showed that *Cryptococcus* spp., which are potential human pathogens, are present in environments located in the close vicinity of human activities. We found that the E96 *Actinomyces* strain produces molecules that are highly active against *Cryptococcus* spp. clinical and environmental isolates and could be especially interesting when *Cryptococcus* spp. are resistant to 5-fluorocytosine, azole or echinocandins. The identification and the characterization of this molecule are ongoing.

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