



# Effect of *Streptomyces sp* 3400 JX826625 Metabolites on Multidrug Resistant *Candida albicans* Development and Chemical Characterization of Antifungal Metabolites

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**Abstract:** The search of new antimicrobial metabolites remains until now an alternative to mitigate concerns caused by antimicrobial resistance. This work aims to demonstrate the ability of actinomycete strain (*Streptomyces sp* 3400 JX826625) to inhibit pathogen yeast growth (*Candida albicans*), isolated from a woman infected by recidivate candidiasis and to reveal chemical characteristics of the antifungal metabolites produced. Antifungal test using cylinder agar technique showed that the yeast pathogen was resistant to the nystatin 100.000 and the ketoconazole 50 while *Streptomyces sp* 3400 displayed activity with 25mm of inhibition zone diameter. The optimization of antifungal production parameters by the strain recapitulates that its culture on sporulation agar medium at a pH=5,13, incubated at 30°C for 7 days promoted the activity of the actinomycete; the butanol was the best solvent for antifungal metabolites extraction. Chemical investigation showed that liquid-liquid fractionation method of crude extract allowed to obtain four fractions (hexane, dichloromethane, butanol and aqueous fractions) in which butanol fraction exhibited the best antifungal activity (19mm) according to antifungal test by disk method. Separation of active compounds from this active fraction by TLC method revealed 10 bands and its bioautography showed two active compounds against the pathogen yeast of which the diameters of inhibition zone were 19mm and 10mm, respectively. Chemical screening of the butanolic fraction revealed the presence of terpenes, alkaloids, coumarins and anthracene derivatives family with colorimetry by TLC method. The recovering of active compounds by TLC preparative gave two methanolic fractions (MF1 and MF2) of which MIC and MFC were respectively 1,562µg/ml and 3,625µg/ml for MF1; 17µg/ml and 34µg/ml for MF2. The two compounds were stable in a range of temperature from 19°C to 46°C; however, a best antifungal activity was recorded at -20°C. UV- visible spectra of the two active compounds revealed that *Streptomyces sp* 3400 contained non-polyene and heptaene group of polyene molecules.

**Keywords:** *Streptomyces sp* 3400, *Candida albicans*, Antifungals, Polyene, Non-polyene

## 1. Introduction

Resistance to antimicrobials becomes nowadays a main

concern of health sector and the entire world. Moreover, the emergence of new pathogens and the recrudescence of old diseases worsen the situation. Many microbes are actually

known resistant to conventional antibiotics used; multidrug resistant bacteria are represented by methicillin resistant *Staphylococcus aureus*, extended spectrum  $\beta$ -lactamase producing enterobacteria, vancomycin resistant *Enterococcus*, multi-resistant *Pseudomonas aeruginosa* and multi-resistant *Acinetobacter baumannii*. For fungi, antifungal resistance is mostly attributed to the genera *Aspergillus* and *Candida* among which fluconazole resistant *Candida auris*, *C. tropicalis*, *C. parapsilosis*, *C. glabrata* and *C. albicans*. Therefore, the number of patients infected by mycosis increases significantly each year, causing serious problems in public health [1]. For the genus *Candida*, about ten species are revealed potentially pathogen for human but *Candida albicans*, on which the present work is focused, is generally detected. It is a commensal microorganism including in endogenous gastrointestinal, oropharyngeal, female genital organ and skin microbial flora [2]. However, *C. albicans* is also human opportunist pathogen causing infections in case of immune and hormonal imbalances [3] and inducing high mortality rate in immunocompromised patients [4]. The infection is due to the transformation of the saprophyte yeast into filamentous form, responsible of microorganism high virulence, which proliferates and becomes pathogen by releasing toxins causing the candidiasis. Antifungal drugs commonly used to treat *Candida albicans* infections are azoles (synthetic antifungal molecules as fluconazole), echinocandins (semi-synthetic antifungal molecules as caspofungin) and polyenes (natural molecules as nystatin and amphotericin B) but recently, most of them were shown to be inactive against the target yeast [5]. Several factors can be implied in antifungal resistance that can be either intrinsic or acquired, among others, the repetitive use of the antifungal drugs in immunocompromised patients [6] and the interaction with antibacterial medications [7]. Hence, this situation requires urgently the necessity to find out novel antifungal molecules and the use of microorganisms from unexplored or rarely explored site is an alternative. Polyenes such nystatin and amphotericin B are natural antifungal molecules isolated from *Streptomyces* species discovered in the 20<sup>th</sup> century. However, the development of resistance mechanism by the target yeast to these antifungal drugs and their high toxicity limit their use [8]. Work researches in actinomycetes isolated from different ecological niches pay yet researchers' attention in the hope to discover novel secondary metabolites to fight against recurrent diseases due to antimicrobial resistance. This microorganism group is selected in this study for their ability to produce diverse types of secondary metabolites with different biological activities. They are also known as potential sources of different bioactive molecules used in human therapy since actinomycin's discovery in 1940, an antibiotic isolated from an actinomycete of the genus *Streptomyces*. Thus, the present work aims to assess the effect of secondary metabolites produced by *Streptomyces sp 3400 JX826625* isolated from ginger rhizospheric soil [9, 10] on multidrug resistant *Candida albicans*. Optimization of antifungal production parameters and chemical aspects of antifungal metabolites

are reported.

## 2. Materials and Methods

### 2.1. *Streptomyces sp 3400 JX826625*

The actinomycete strain, *Streptomyces sp 3400 JX826625* used for antifungal test in this study was furnished by the LME (Laboratoire de Microbiologie de l'Environnement) of CNRE (Centre National de Recherches sur l'Environnement), Antananarivo-Madagascar. It was isolated from ginger rhizospheric soil, conserved in the microbial strain bank and recorded in the actinomycetes dataset of the laboratory.

#### Isolate revivification

*Streptomyces sp 3400* conserved in a cryotube at  $-20^{\circ}\text{C}$  was taken out of the freezer, put in an ice container and picked quickly in SCA medium previously poured onto Petri plates. The culture was then incubated at  $30^{\circ}\text{C}$  during 7 days, colonies growth and cultural aspects were observed.

This isolate is selected among the 8 active isolates from ginger associated actinomycetes against *Candida albicans* for its high ability to inhibit the fungal development (with an inhibition zone diameter of 59mm) [9, 10]. This investigation was, thus, conducted to extend knowledge on secondary metabolites produced by *Streptomyces sp 3400*.

### 2.2. *Candida Albicans*

The test-pathogen yeast was obtained from fungal strain collection of LBM (Laboratoire de formation et de recherche en Biologie Médicale), Antananarivo-Madagascar. It was isolated from a vaginal swab of a woman infected by recidivate candidiasis. Cultural characters of the pathogen yeast on Sabouraud agar showed round, convex and white colonies (figure 1), while its microscopic aspect showed ovoid cells without filamentous form (figure 2).



Figure 1. Cultural characters of multidrug resistant *Candida albicans*.



Figure 2. Microscopic aspect of multidrug resistant *Candida albicans* (LEICA X40).

### 2.3. Antifungal Test

Antifungal activity of *Streptomyces sp* 3400 was assessed using agar cylinder technique as described by Andriambelison *et al.* [10] and agar diffusion method according to Veron *et al.* [11]. Actinomycete cylinders of 6mm in diameter cultured on sporulation agar for 7 days were introduced into wells created in Mueller-Hinton agar, previously inoculated with  $10^5$  ufc/ml of the test-yeast. Plate was kept at room temperature for 30 min to allow antifungal pre-diffusion and then, incubated at 37°C for 24h. Test was done in triplicate; Nystatin (NY 100 000) and Ketoconazol (KET 50) were used as standards. Antifungal activity was estimated by the measure of inhibition zone around the actinomycete cylinders and the disks.

### 2.4. Optimization of Antifungal Metabolites Production

#### 2.4.1. Effect of Culture Media

The influence of culture media on antifungal metabolites production by *Streptomyces sp* 3400 was carried out using five solid media: Starch Casein agar (soluble starch 10g, casein hydrolysate 1g,  $K_2HPO_4$  0.5g, agar 15g, distilled water 1000ml), Bennett agar (anhydrous D-glucose 10g, casaminoacids 2g, yeast extract 1g, meat extract 1g, agar 15g, distilled water 1000ml), ISP2 agar (yeast extract 4g, D-glucose 4g, malt extract 10g, agar 20g, distilled water 1000ml), Sporulation agar (yeast extract 1g, meat extract 1g, tryptose 2g, Fe  $SO_4$  traces, glucose 10g, agar 15g, distilled water 1000ml) and Nutrient agar (meat peptone 4.3g, casein peptone 4.3g, Na Cl 6.4g, agar 15g, distilled water 1000ml) and five liquid media: starch casein broth, Bennett broth, ISP2 broth, sporulation broth and nutrient broth. Actinomycete cultures were incubated for 7 days at 30°C for both media and under shaking at 150 rpm for liquid cultures. Actinomycete growth was noted and the production of antifungal metabolites was assessed by antifungal test according to the methods described previously for solid media.

For liquid media, antifungal metabolites production was determined using agar diffusion method. The cultures were, firstly, centrifuged at 10 000xg for 20 min; the supernatants and pellets were then subjected to antifungal test. Disks of 6mm in diameter was soaked with 20µl of supernatants for each media, dried and put into Mueller-Hinton medium previously inoculated with *Candida albicans* suspension. On the other side, 5ml of ethanol 90° were added to each pellet and 20µl of the extraction solvent were tipped onto sterile disks (6mm). After incubation at 37°C for 24h, antifungal activity was indicated by the measure of inhibition halo around the disks.

#### 2.4.2. Effect of Extraction Solvents

The effect of different solvents on antifungal metabolites extraction was investigated using solvents with different polarities: hexane, ethyl acetate, butanol and ethanol, antifungal activity was estimated by antifungal test using disks method. For that, antifungal metabolites were extracted

using each of the tested solvents from a 7 days old actinomycete grown on the best medium tested above for antifungal activity. Extracts were, thereafter, filtered and disks soaked with 20µl of filtrates from each solvent were put onto Mueller-Hinton agar previously inoculated with the test-pathogen yeast. Antifungal activity was appreciated according to the diameter values of the inhibition zone around the disks.

#### 2.4.3. Effect of Culture Conditions

This assay was carried out by growing up *Streptomyces sp* 3400 on adequate medium deduced from previous experimentations for 7 days under different culture conditions as the temperature and the pH. A temperature range from 4°C to 46°C and a pH range from 5 to 11 (adjusted with H Cl 1N and Na OH 1N) were tested. Isolate development was noted and antifungal activity was evaluated using agar cylinder diffusion methods as described previously.

### 2.5. Chemical Characterization of Antifungal Metabolites from *Streptomyces sp* 3400

#### 2.5.1. Liquid-liquid Fractionation

*Streptomyces sp* 3400 crude extract from optimized parameters for antifungal metabolites production was subjected to liquid-liquid fractionation in the purpose to separate different compounds in the extract according to their affinity to solvents with increasing polarities.

Crude extract was dissolved in distilled water in a separating funnel and the solution obtained has undergone an exhaustion volume by volume liquid-liquid fractionation with increasing polarity organic solvents (hexane, dichloromethane, butanol). After decantation, all organic and aqueous fractions were recovered and evaporated. Fractionation yield was calculated as follows:

$$\text{Yield (\%)} = \frac{\text{Weight of obtained fractions (mg)}}{\text{Weight of crude extract (mg)}} \times 100$$

All fractions obtained were tested for their antifungal activity according to the disk method described above.

#### 2.5.2. Thin Layer Chromatography of Active Fraction

Bioactive compounds from the active fraction were separated by TLC method using a mixture of dichloromethane, methanol and water (v/v/v, 8/2/0,2) as solvent system. The chromatogram was then revealed according two methods:

1. Observation under UV light in which active molecules appears in bands or spots under UV light at specific wavelengths of 254nm and 365nm.
2. Spraying with sulfuric vanillin in which active compounds appears in light spots.

Frontal references (Rf) of different detected spots were calculated using the following formula:

$$R_f = \frac{\text{Distance of solute}}{\text{Distance of solvent}}$$

### 2.5.3. Bioautography of Active Extract

Bioautography method was adopted in order to assess microbiological activity of the chromatogram. After separation of active molecules by TLC, a portion of the chromatogram (6mm in width and 9mm in length) was put onto plate containing Mueller-Hinton agar previously inoculated with the pathogen yeast. Plate was incubated at 37°C for 24h and antifungal activity was expressed by the measure of inhibition zones appeared on either side of the chromatogram portion.

### 2.5.4. Chemical Screening of *Streptomyces sp 3400* Active Extract by TLC Colorimetric Method

The compounds in the active fraction were separated using the same technique of TLC as cited above. Besides, chemical family of the detected compounds were revealed by specific reagents such as sulfuric anisaldehyde 5% for terpenes and saponines detection, Dragendorff for alkaloids confirmation, K OH ethanolic solution 5% for anthracene derivatives and coumarines, Lieberman burchard reagent for triterpenes detection and NP/PEG (natural products-polyethylenglycol) reagent for flavonoids presence.

### 2.5.5. Purification of Antifungal Metabolites

A preparative TLC was performed according to the same TLC method described above to purify active molecules from active fraction. Molecule bands were then scratched and put into pure methanol. Thereafter, methanolic suspensions were diluted, filtered with millipore filter (0,45µm) and subjected to antifungal test using disk method in order to confirm antifungal activity of obtained molecules.

### 2.5.6. Determination of Minimal Inhibitory Concentration (MIC) and Minimal Fungicidal Concentration (MFC) of Active Molecules

MIC and MFC of active molecules were assessed by microdilution technique from initial concentrations of 272µg/µl for methanolic fraction 1 (MF 1) and 200µg/µl for methanolic fraction 2 (MF 2). MIC was defined as the lowest concentration of antifungal molecules able to inhibit any visible growth to the naked eye while MFC was deduced as the lowest concentration of antifungal metabolites for which 0,01% to 0,1% of yeast cells survived after culture on Mueller-Hinton agar plates of all limpid liquid cultures from MIC evaluation.

### 2.5.7. Stability of Active Molecules

In the purpose to investigate the stability of the active molecules, methanolic fractions were treated to different variations of parameters such as the temperature (-20°C, 4°C, 15°C, 30°C, 37°C, 46°C) and the light (75W light bulb, ambient light, darkness) for 24h. Antifungal activity of methanolic fractions for each treatment was assessed according to the disk method.

### 2.5.8. UV- visible Spectra of Bioactive Molecules

The polyenic or non-polyenic nature of the two active compounds obtained was performed by spectrophotometry detecting UV-light absorption at wavelengths from 200nm to

500nm. Polyenes were characterized by three peaks serial and their type was assessed according to Lindenfelser *et al.*'s criteria [12].

Table 1. Maxima absorption spectra of different polyenes types.

Type of polyenes	Maxima absorption spectra (nm)
Tetraene	290 - 291, 303 - 306, 318 - 320
Pentaene	318 - 324, 333 - 338, 346 - 358
Hexaene	339 - 341, 356 - 358, 377 - 380
Heptaene	358 - 365, 376 - 380, 399 - 405

## 3. Results

### 3.1. Morphology of *Streptomyces sp 3400* JX826625 After Revivification

Macroscopic aspect of the isolate after 7 days of culture at 30°C showed circular and convex colonies with irregular contour and an average size of 3mm. The aerial mycelium was whitish, thick forming powdery surface while the vegetative mycelium was brownish and adhered to the gelose (figure 3). Microscopic observation of the strain showed vegetative mycelium with fragmented hyphae and aerial mycelium formed by ten rectiflexible spores chains (figure 4). The strain didn't produce any diffusible pigment and isolated spores are non-motile.

Compared to its aspect after its isolation in 2012, some morphological modifications were observed. *Streptomyces sp 3400* JX826625 in SCA medium showed large size colonies of 3mm to 5mm in diameter, opaque, bumpy, compact; dry with radial furrows and irregular shape (figure 5). The aerial mycelium was white and less developed while the back of the colony was brown. Microscopic aspects of the strain show long, thin and branched filament vegetative mycelium, but not sporulated, nor fragmented. The aerial hyphae were, conversely, characterized by chains of 2 to 4 spores (figure 6).



Figure 3. Culture of *Streptomyces sp* on SCA medium in 2019.



Figure 4. Microscopic aspect of *Streptomyces sp* in 2019.



Figure 5. Culture of *Streptomyces sp* on SCA medium in 2012 [10].



Figure 6. Microscopic aspect of *Streptomyces sp* in 2012 [10].

### 3.2. Antifungal Activity

The results of *Streptomyces sp* 3400 antifungal test revealed that the actinomycete strain was active against *Candida albicans* with an inhibition zone diameter of 25mm. The two antifungal standards (NY 100 000 and KET 50) were both, conversely, inactive against the pathogen yeast (figure 7).

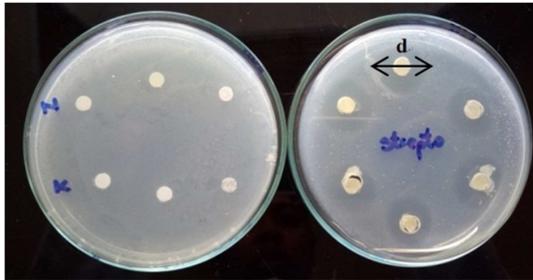


Figure 7. Antifungal activity of *Streptomyces sp* 3400, the nystatin and the ketoconazole.

N: nystatin (100 000UI), K: Ketoconazole (50), d: diameter of inhibition zone.

### 3.3. Optimization of Antifungal Metabolites Production

#### 3.3.1. Influence of the Culture Media

The results obtained showed that the media used for *Streptomyces sp* culture during 7 days at 30°C were favorable for its growth except nutrient agar and nutrient broth media where any growth wasn't observed (data non shown).

##### i. Solid media

Antifungal test of *Streptomyces sp* grown on different culture media against test-yeast mentioned that three media among the five tested allowed the production of antifungal metabolites. The best antifungal activity was obtained from *Streptomyces sp* grown on sporulation agar medium with an inhibition zone diameter of 25mm around the actinomycete

agar cylinder (figure 8).

##### ii. Liquid media

The pellets were active than the supernatants, more antifungal activity was recorded for Bennett broth pellet (10mm) than supernatant (6mm) and sporulation broth pellet (20mm) than supernatant (10mm). The best activity was obtained with sporulation broth pellet showing an inhibition zone diameter of 20mm (figure 8).

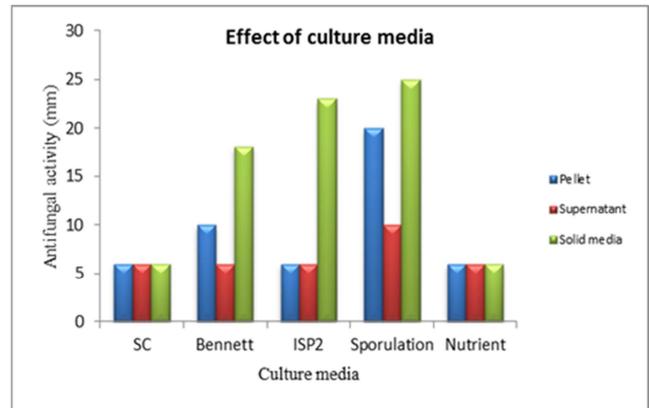


Figure 8. Effect of culture media on *Streptomyces sp* antifungal activity.

#### 3.3.2. Influence of the Solvents on Antifungal Metabolites Extraction

Among the four solvents used to extract antifungal metabolites from *Streptomyces sp*, ethyl acetate and butanol extracts displayed activity on the test pathogen. Though, high activity was recorded with the butanol extract which the diameter value of the inhibition zone was 17mm (figure 9).

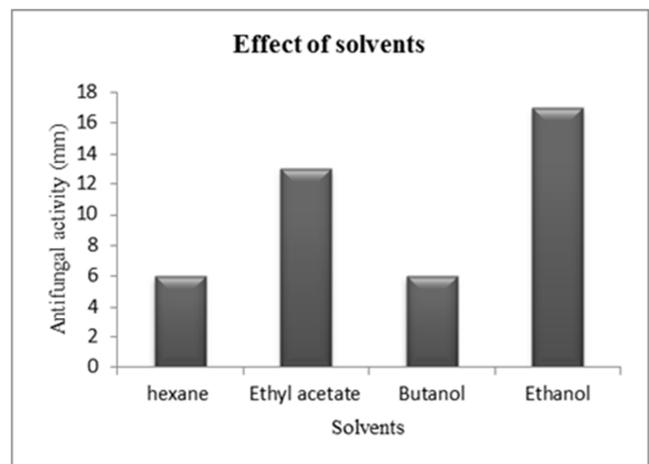


Figure 9. Effect of solvents on antifungal metabolites extraction.

#### 3.3.3. Influence of the Temperature

It was observed that the temperatures 25°C and 30°C were favorable for actinomycete strain development. Nevertheless, the best antifungal activity (21mm) was observed with the temperature 30°C (figure 10).

#### 3.3.4. Influence of the pH

The results showed that pH variation didn't affect actinomycete growth; an abundant growth was emphasized

for all tested pH. Furthermore, a brown diffusible pigment which intensified was observed when pH became basic and it didn't influence the growth or the antifungal activity of the strain. The isolate grown on sporulation agar medium at pH=5,13 showed high antifungal activity on the pathogen yeast with an inhibition zone diameter of 23mm (figure 11).

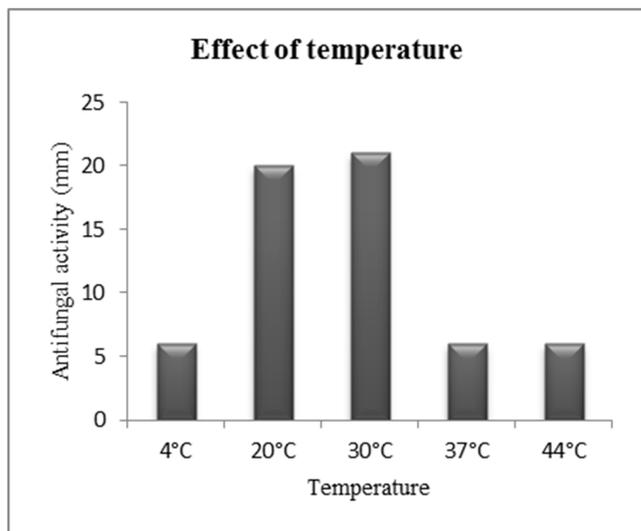


Figure 10. Effect of temperature on *Streptomyces sp* antifungal activity.

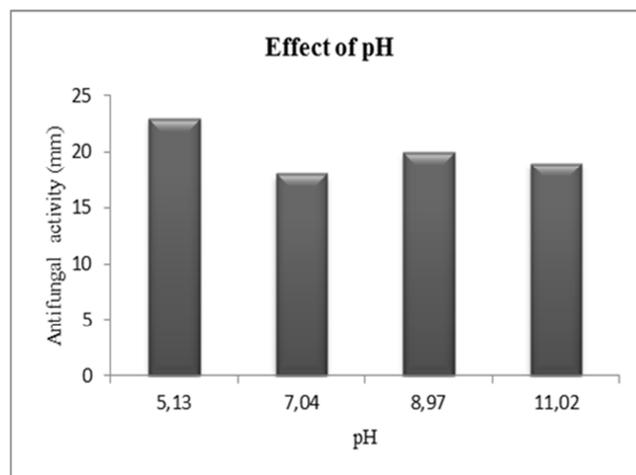


Figure 11. Effect of pH on *Streptomyces sp* antifungal activity.

### 3.4. Production of Antifungal Metabolites from *Streptomyces sp* 3400

From these results, the following parameters were maintained to produce antifungal metabolites from *Streptomyces sp* 3400: the isolate was grown on sporulation agar medium at pH 5,13 for 7 days at 30°C and the butanol was used as extraction solvent of antifungal metabolites.

Table 2. Characteristics and yield of fractions from *Streptomyces sp* butanol crude extract.

Fractions	Color	Weight of fractionated extract (mg)	Yield (%)	Antifungal activity (mm)
Hexane fraction	Yellowish	60,60	25,23	6
Dichloromethane fraction	Black brownish	116,4	48,46	12
Butanol fraction	Yellow	25,8	10,74	19
Aqueous fraction	Uncolored	30	12,49	6

### 3.5. Chemical Investigation of Antifungal Metabolites Produced by *Streptomyces sp* 3400

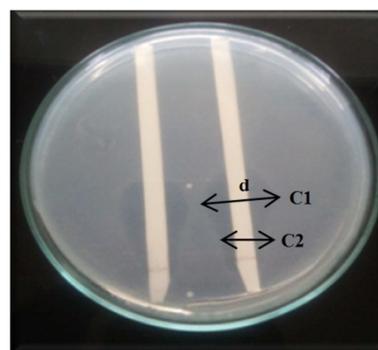
#### 3.5.1. Liquid-liquid Fractionation

This process allowed to obtain four fractions (hexane fraction, dichloromethane fraction, butanol fraction and aqueous fraction) from *Streptomyces sp* butanol crude extract. The characteristics, the yield and the antifungal activity of each fraction obtained were summarized in the table 2. From 0,300g of crude extract, high yield was obtained with dichloromethane fraction (48,46%); DCM and butanol extracts exhibited activity against the pathogen yeast but butanol extract was more active with a diameter of inhibition zone of 19mm than DCM extract (12mm).

#### 3.5.2. Chemical Screening of the Most Active Fraction

The separation of butanol fraction compounds by TLC method showed that active fraction contained 10 compounds which characteristics were shown in the table 3. The bioautography of the active fraction demonstrated that two compounds with R<sub>f</sub> of 0,023 and 0,178 respectively on the plate displayed antifungal activity. The values of inhibition zone diameters exhibited by these two substances were, respectively, 19mm and 10mm (figure 12). Chemical screening of the butanolic fraction using TLC colorimetry

method revealed that it contained terpenes, alkaloids, anthracene derivatives and coumarins (table 4).



C1: compound 1, C2: compound 2, d: diameter of inhibition zone.

Figure 12. Bioautography of the chromatogram from compounds separation of the butanolic fraction.

#### 3.5.3. Purification of the Active Compounds

The preparative TLC of the butanol fraction allowed to obtain 10 bands which were recovered into methanol. The results of antifungal activity of the 10 methanol fractions obtained showed that 2 fractions were active against *Candida albicans* with inhibition zone diameters of 17mm and 19mm respectively.

### 3.5.4. MIC and MFC Values of the Active Compounds

The results obtained from the dilution technique and the culture of the pathogen yeast on Mueller-Hinton agar indicated that MIC of the methanolic fraction 1 (MF1) was estimated at 1,562µg/ml and that of the methanolic extract 2 (MF2) was 17µg/ml. Moreover, MFC were 3,125µg/ml and 34µg/ml for MF1 and MF2, respectively.

### 3.5.5. Stability of the Active Compounds

The results of temperature and light influences investigation on the stability of the two active compounds isolated, summarized in the table 5 emphasized that any loss of antifungal activity was not recorded for all tested parameters. However, a decrease of antifungal activity was noted when the temperature increased and when the compounds were exposed to different sources of light for 24h.

For temperature assay, antifungal activity was stable from ambient temperature to 46°C (16mm) for MF1; at 37°C and 46°C (12mm) for MF2.

For light assay, antifungal activity was weak when fractions were exposed to 75W light bulb (11mm for MF1 and 14mm for MF2).

Hence, it would be deduced from these results that the best storage condition of MF1 and MF2 was the freezing temperature (-20°C).

### 3.5.6. UV Visible Spectra of the Active Molecules

According to Lindenfelser *et al.*'s criteria [12], UV visible spectra of the two methanolic fractions MF1 and MF2 showed the presence of polyene characteristic peaks recorded at 360nm, 380nm and 405nm, belonging to heptaene group. Other non-polyene molecules were also recorded for both

compounds; they were recorded at 299nm for MF1 (figure 13) and at 321nm for MF2 (figure 14).

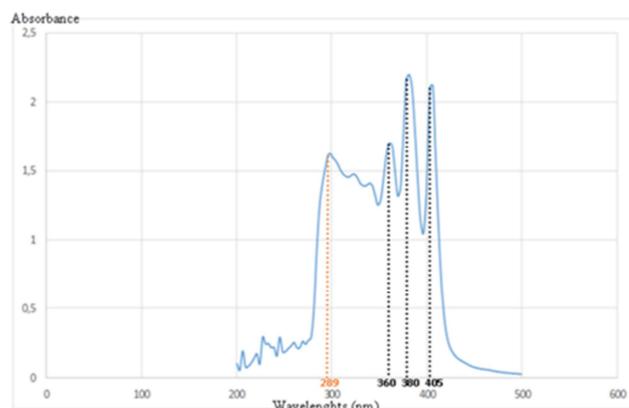


Figure 13. UV- visible spectra of methanolic fraction 1 (MF1).

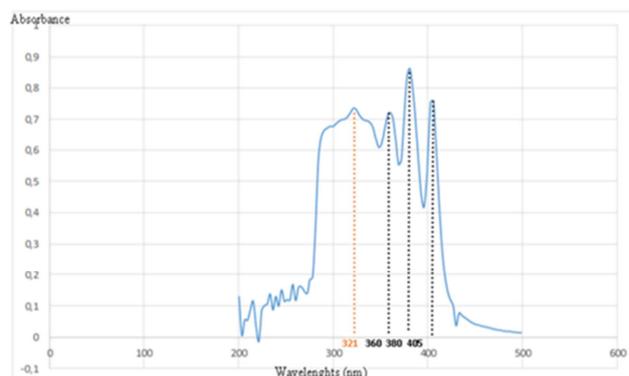


Figure 14. UV- visible spectra of methanolic fraction 2 (MF2).

Table 3. Characteristics of butanol extract compounds by TLC method.

Bands	UV 254nm		UV 365nm		Sulfure vanilline	
	Rf	Color	Rf	Color	Rf	Color
1	0.940	Grey	0.952	Blue fluorescence	0.952	Blue
2	—	—	0.928	Orange	0.928	Violet
3	0.857	White	0.857	White	—	—
4	—	—	0.75	Yellow	0.75	Red
5	0.535	Black	0.535	Red brown	0.535	Red
6	0.416	Grey	0.416	Yellow	—	—
7	—	—	0.214	Yellow fluorescence	0.214	Yellow
8	0.202	Dark blue	0.178	Orange brown	—	—
9	—	—	0.154	Violet fluorescence	—	—
10	0.023	Orange	0.023	Yellow	—	—

UV: ultra-violet light, Rf: frontal reference.

Table 4. Chemical screening of butanolic fraction.

Chemical family	Reagents	Observation		Rf
		Color	Results	
Terpenes	Sulfure anisaldehyde (5%)	Red brown	+	0.75
Triterpenes	Lieberman- Burchard	-	-	-
Alkaloids	Dragendorff	Orange	+	0.535
		Orange	+	0.154
Flavonoids	NP/PEG	-	-	-

Chemical family	Reagents	Observation		Rf
		Color	Results	
Anthracene derivatives and coumarins	Ethanol solution of KOH (5%)	Blue fluorescence	+ (coumarins)	0.952
		Yellow fluorescence	+ (anthracene derivatives)	0.214
		Violet		0.178*
		Yellow fluorescence	+ (anthracene derivatives)	0.154
		Yellow fluorescence	+ (anthracene derivatives)	0.023*

\*Rf of compounds with antifungal activity, +: positive, -: negative.

Table 5. Influence of the temperature and the light source on active molecules.

Parameters		Antifungal activity (mm)	
		MF1	MF2
Temperature	-20°C	18	17
	4°C	17	13
	19°C	16	15
	30°C	16	14
	37°C	16	12
	46°C	16	12
Light	A	14	11
	B	15	13
	C	16	12

MF: methanolic fraction, A: 75W light bulb, B: ambient light, C: darkness.

## 4. Discussion

The potential of *Streptomyces sp* 3400 JX826625 to inhibit multidrug resistant *Candida albicans* growth and chemical characteristics of the antifungal metabolites were demonstrated in this work. The revivification of the actinomycete strain after a long cryoconservation period for 7 years allowed noting some morphological modifications. Any morphologic change or polymorphism of actinomycetes strains after or during their conservation hasn't been yet emphasized in the literature; however, this modification in our opinion promoted a quick development of the strain and confirmed the good preservation by cryoconservation of *Streptomyces sp* 3400 JX826625.

Multiresistance of *Candida albicans* to usual antifungal drugs (azoles, echinocandines) was demonstrated in several works [13-15]. In this study, the pathogen isolated from a vaginal swab was resistant to the nystatin (100 000) and the ketoconazole (50) confirming, thus, disease recidivism (candidiasis) for the patient. On the other hand, actinomycetes strain tested (*Streptomyces sp* 3400) was demonstrated to be active against the multiresistant pathogen isolated. In fact, the reputation of actinomycetes as promising candidate to produce antifungal drugs is yet confirmed. As example, a recent work conducted by Ahmed *et al.* [16] led to isolate the Novonestmycins A and B, two non-polyenic antifungal derivatives from *Streptomyces sp* Z26.

Regarding the optimization of antifungal metabolites production parameters, the best antifungal production was obtained with the culture of *Streptomyces sp* on sporulation agar medium at pH 5,13 for 7 days at 30°C and the butanol was the best solvent for antifungal metabolites extraction. Generally, the production of secondary metabolites from

actinomycetes strains is performed with liquid media [17, 18]; this work used conversely fermentation on solid media due to the high antifungal activity obtained. Moreover, the simplicity and the efficiency of this method were emphasized in some investigations as the absence of mycelia fragmentation promoting antibiotics production [19]. The components of culture media were also demonstrated to impact secondary metabolites production by actinomycetes [20- 22]. In fact, they vary with the actinomycetes needs for their development; in this study, the richness of sporulation agar medium in carbohydrates (glucose), proteins (meat extract, yeast extract, tryptose) and mineral elements (Fe SO<sub>4</sub>) could promote antifungal metabolites production. Likewise, *Streptomyces griseus*, for the production of streptomycin and its growth; used as carbon sources in preference order the glucose, the mannose, the starch, the dextrin and the mannitol [23]. These results don't corroborate with some findings which indicated that a catabolic repression inhibited the biosynthesis of secondary metabolites when a carbon source promptly assimilable such as the glucose was used [24]. For the secondary metabolites extraction, it depends of metabolites affinity to extraction solvents. In this work, the butanol (polar solvent) was the best solvent for antifungal metabolites extraction; suggesting that the antifungal metabolites extracted were polar. This is in accordance with that found by Forar *et al.* [25] who reported that the butanol was a good solvent for the extraction of active compounds produced by certain actinomycetes, among a range of solvents with different polarities (petroleum ether, n-hexane, chloroform, diethyl ether, ethyl acetate, butyl acetate, benzene, n-butanol, ethanol) tested for their extraction ability.

Besides, culture conditions of the actinomycetes strains

constitute determinant factors in secondary metabolites production. The best activity, in our case, was obtained by incubating the culture on sporulation agar medium slightly acid (pH=5,13) at 30°C for 7 days. This is different to the most of the cases occurred in which antimicrobial activity of secondary metabolites was optimal at neutral pH [26, 27]. The presence of a diffusible pigment was also noted during this study when the pH increased. Some works demonstrated that certain actinomycetes produced pigmented active secondary metabolites showing often antibiotic properties such as phenazines (red-brown), prodigiosins (red) or carotenoids (yellow or orange) [28-30]. In our case, the nature of the pigments is yet unidentified but it is known that its diffusion is stimulated by the pH.

Chemical investigation of *Streptomyces sp* butanol crude extract showed that the different steps of crude extract compounds separation (liquid-liquid fractionation, TLC, bioautography and preparative TLC) allowed isolating two active products showing both antifungal activity against multiresistant *Candida albicans*. TLC colorimetric method revealed that the butanol fraction contained alkaloids, terpenes, coumarins and anthracene derivatives. Indeed, antifungal properties of these compounds in microorganisms were demonstrated in several works: tropane alkaloids of *Streptomyces spp* isolated from *Datura stramonium* [31], anthracene derivatives produced by an endophytic fungus, *Stemphylium globuliferum* from *Mentha pulegium* [32] and coumarins' antifungal activity of *Streptomyces aureofaciens* reported by Taechowisan *et al.* [33]. Furthermore, the low MIC value of the compound MF1 would allow to suggest its use as a promising antifungal. The nature assessment of the two active compounds obtained revealed that they contained both polyenic molecules belonging to heptaene group and non-polyenic molecules. Polyenes constitute one of the important classes of antifungals used in mycosis treatment and they are evaluated in many investigations. Numerous polyenic antifungal metabolites belonging to different groups were identified in actinomycetes: linear polyenes such as mediomycins A and B, clethramycin produced by *Streptomyces mediocidicus* [34], heptaene polyenes from *Streptomyces sampsonii* GS 1322 [35]. Otherwise, the search of non-polyenic antifungal molecules to fight against mycosis affections recognized particular interest these last years. Ahmed *et al.* [16] isolated two non-polyenic antifungals derivatives, the novonestmycines A and B from *Streptomyces sp. Z26*. The same authors [8] in 2018 isolated another non-polyenic antifungal, the antimycin A19 produced by *Streptomyces albidoflavus* AS25. Polyenes or non-polyenes metabolites can be both exerted antifungal activity and thus, benefic for mycosis treatment. Their use depends then of their action mechanism towards the target pathogen and especially of their toxicity towards the treated organism.

The stability investigation of the two compounds obtained showed that any antifungal activity loss wasn't observed for all tested parameters, they were stable at high temperatures in the

range from 20°C to 46°C for 24h. Augustine *et al.* [26] obtained similar results by testing the effect of temperature variation (30°C to 80°C) on antifungal activity of a non-polyene antibiotic produced by *Streptomyces albidoflavus* PU 23.

## 5. Conclusion

From this work, it would be concluded that *Streptomyces sp* 3400 JX826625 butanol crude extract possessed interesting antifungal activity against multidrug resistant *Candida albicans* isolated from a woman infected by recidivate candidiasis. Furthermore, the nature of the active compounds was reported. The next step of this study will consider completing chemical investigation of the two isolated compounds by structural elucidation and identification of the antifungal molecules. The toxicity of the isolated molecules will be also assessed in the purpose to propose an alternative for producing efficient antifungal drugs against multidrug resistant *Candida albicans*.

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