ANTIFUNGAL ACTIVITY OF *BACILLUS* SPP. ISOLATED FROM *CALOTROPIS PROCERA* AIT. RHIZOSPHERE AGAINST *CANDIDA ALBICANS*

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ABSTRACT

Objective: This study aimed to investigate the antifungal activity of a microbial strain isolated from a non-exploited habitat (southeast region of Morocco) against *Candida albicans.*

Methods: The antifungal producing strains were isolated from the rhizosphere of *Calotropis procera* Ait. The isolate (Cp-LMA-9) was selected based on the primary antimicrobial screening. The optimization of the culture media for an antifungal production, and the extraction of bioactive metabolites were performed. The bioautography and a partial characterization of these metabolites were also done.

Results: Based on the molecular identification, it was identified as *Bacillus* spp. with a similarity percentage of 98% with *Bacillus tequilensis* and *Bacillus subtilis*. The maximum antifungal production against *C. albicans* was observed with malt extract-yeast extract-agar by solid-state fermentation and malt extract-yeast extract-broth under stationary conditions. Antifungal fraction was extracted successfully from the solid-state fermentation by acetone and methanol. It maintained the anti-*Candida* activity after heat treatment (autoclaving at 121°C for 15 minutes and boiling for 30 minutes), within a pH range of 2-10, and after treatment with proteolytic enzymes.

Conclusion: The bacterium isolated and selected exhibit a remarkable antifungal effect against *C. albicans*. The resistance of the antifungal metabolites extracted to heat, alkaline, and acidic conditions and to proteolitic enzymes making theme a promising antifungal compounds for the control of *Candida* infections.

Keywords: Antifungal activity, *Bacillus* spp, *C. albicans*, Rhizosphere, Molecular identification.

INTRODUCTION

Candidiasis is a common infection of the skin, oral cavity and esophagus, gastrointestinal tract, vagina and vascular system of humans. *Candida albicans* is the most implicated agent on human's candidiasis. Habitually, this yeast act as common saprophytic microorganisms of the normal human microflora [1]. However, it can submit a transition from commensal to a pathogenic phase to become an opportunistic pathogen [2]. Although, most infections occur in immunocompromised patients, and they range from minor to severe and fatal infections [3]. Nowadays, incidence of *C. albicans* acquiring resistance to antifungal like azole has increased dramatically [4,5]. This resistance to currently available antifungal drugs is a highly relevant factor because it is often associated with high morbidity and mortality [4]. For this reason, intensified research efforts are needed to develop new drugs with minimal side-effects for use in human and veterinary medicine [6].

The Gram-positive bacterium *Bacillus* spp. produces a large number of bioactive metabolites, which exhibit a potent antibacterial and/or antifungal activity such as surfactin, iturinic, fengymycin, bacilysocin, and fengycin [7-9]. For 50 years, the potential of *Bacillus subtilis* to produce antibiotics has been recognized with peptide antibiotics representing the predominant class [10]. The possibility of screening for a new *Bacillus* spp. antibiotic producers is considered to be one of the major interests in antibiotics research [10].

Several researchers obtained bioactive substances from different strains of the *Bacillus* genus using different culture media and extracted by different methods depending on the nature of bioactive molecules. Peptide antibiotics were extracted mostly by ammonium sulfate precipitation [11,12], pH precipitation [13], or organic solvents [14]. Other authors extracted non-peptide bioactive molecules, such as cispentactin, using the ion-exchange chromatography [15].

In general, different culture media were used for the production of bioactive molecules by *Bacillus* strains. The most used ones are; brain heart infusion (BHI), nutrient broth (NB), trypticase soya broth (TSB) and Luria Bertani (LB). The bioactive metabolites may be produced in solid-state and liquid fermentation [13,16-19].

This present work was designed in an attempt to develop a safe and more powerful antifungal agent from the microorganisms living in plant rhizosphere mostly from non-exploited habitats. For this reason, this is 1st time that the rhizosphere of *Calotropis procera* Ait. is used for the isolation of antimicrobials producing strains. A bacterial strain identified as *Bacillus* spp was selected for this study basing on its remarkable antifungal activity against *C. albicans*.

METHODS

Isolation and purification of strain

The sample was collected from the rhizospheric soil of *C. procera*. Ait growing in the southeast region of Morocco using the method of Pochon and Tardieux [20]. Two grams of the soil sample were suspended in 20 ml of sterile distilled water, homogenized by vortexing and serially diluted up to 10^{-5}. Then, 100 μl from each dilution were spread over the surface of potato dextrose agar (PDA) and yeast extract-malt extract-agar (YMA) culture medium (yeast extract 1 g/L, malt extract 20 g/L and agar 20 g/L; pH=7±0.2) [21]. Plates were incubated at 30°C for 7 days. Microbial strains were purified by streaking technique and conserved as slants on their isolation culture media at 4°C and on

glycerol 20% (v/v) at −20°C. The isolate Cp-LMA-9 was selected for this study for its remarkable anti-*Candida* activity.

In vitro evaluation of the antifungal activity
Agar-plug diffusion method
The primary antimicrobial screening test and the effect of solid media on the antifungal production were performed using the agar-plug diffusion method [22,23]. Briefly, the isolates were grown on their isolation media. After 14 days of incubation, three plugs (10 mm in diameter) were cut and placed on the surface of PDA plates that were seeded with *C. albicans* ATCC 10231 (previously prepared and adjusted to 0.5 McFarland). Plates were first kept at 4°C for at least 2 hrs to allow the diffusion of the produced bioactive molecules, and then incubated at 30°C. Inhibition zones were determined after 24-48 hrs of incubation.

Disk-diffusion method
Unlike solid state fermentation, the evaluation of the antifungal production in liquid fermentation was done by the disk diffusion method according to Gajbhiye *et al.* [24]. Briefly, PDA plates were inoculated with *C. albicans* ATCC 10231 (0.5 McFarland size inoculum). The paper disks (6 mm in diameter) were deposited on the surface of the inoculated plates and impregnated with 10 μl of the supernatant. Plates were first kept at 4°C for at least 2 hrs, and then incubated at 30°C. Inhibition zones were determined after 24-48 hrs of incubation.

Molecular identification of the producer
Molecular characterization was done using 16S rRNA gene sequence. To this aim, total genomic DNA was isolated from the isolate (Cp-LMA-9) using thermal shock according to Mostakim *et al.* [25]. Then, a fragment of 16S rRNA gene was polymerase chain reaction (PCR) - amplified using the primers fD1 (5'AGAGTTTGATCCTGGCTCAG3') and Rs16 (5'TACGGCTACCTTGTTACGAC TT3'). The PCR mixture contained 1.5 mm $MgCl_2$, 200 μm of each dNTPs (Promega, Madison, USA), 1 μM of each primer (Metabion, Bengaluru, India), 4 μl of taq buffer (×5) and 1 unit of taq polymerase (GoTaq Gold; Promega). To this mixture, 2 μl of the DNA template were added. In the control tube, 2 μl of ultrapure water were added instead of DNA. The total reaction volume was 20 μl. The reaction was amplified in a thermal cycler (TECHNE, UK). The PCR conditions were as follow: Denaturation at 94°C for 4 minutes, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 1 minute. A final extension step at 72°C for 10 minutes was also included. PCR product was separated in a 1% agarose gel and was subsequently visualized by ultraviolet (UV).

DNA sequencing was performed using applied biosystems 3130 according to the manufacturer instructions. Sequence similarity searches were compared to the database presented at the GenBank using the online sequence analysis resources basic local alignment search tool (BLAST).

Biochemical tests
The gram stain was first performed. Then, the isolate was tested for biochemical characteristics using the analytical profile index (API) system (API 20 E, bioMérieux) [26]. The catalase test was also tested. The growth in NB at different pH (4, 5, 6, 7, 8 and 9); the growth at 50°C and the growth in NB containing 0, 5, 7 and 10% (w/v) of NaCl were also tested according to Schleifer [27].

Effect of culture media on the antifungal production
Submerged fermentation
The liquid fermentation was performed using four culture media, LB broth, NB, TSB and malt extract-yeast extract-broth (YMB: 0.1% yeast extract and 2% malt extract). Flasks containing 25 ml of each culture medium adjusted at pH 7±0.2 were inoculated with 48 hrs old culture of the isolate and incubated at 30°C without and with agitation (125 rpm) for 48 hrs. After incubation, aliquots of 10 ml were taken, centrifuged at 4500 rpm for 15 minutes. The supernatants were tested using disk diffusion method as described previously.

Solid-state fermentation
The solid-state fermentation was performed on plates containing the four culture media described previously supplemented with bacteriological agar at 2% (w/v). pH of each medium was adjusted at 7±0.2 before adding the agar. Plates were streaked with 48 hrs old culture of the isolate. Incubation was made at 30°C for 48 hrs. Antifungal production on the solid-state fermentation was tested by agar-plug diffusion method as described previously.

Extraction of the antifungal fraction
Fermentation was carried out on Petri dishes (9 cm in diameter) containing 25 ml of solid culture medium (YMA). After 48 hrs of incubation at 30°C, the extraction was carried out by maceration using different solvents. The solvents used, were hexane, chloroform, ethyl acetate, dichloromethane, acetone, n-butanol, and methanol to determine the best solvent for the extraction of the antifungal compound(s). The solvents were evaporated under vacuum, and the residues were resuspended in 1 ml of dimethyl sulfoxide and tested against *C. albicans* using disk-diffusion method.

Sensitivity of the antifungal molecule(s) to proteolytic enzymes, heat, and pH
The extract recovered in water was subjected to heat treatment to test the thermal stability of the active substance(s). 10 μl of the extract was incubated for 30 minutes at 100°C and autoclaved (121°C for 15 minutes). For pH stability, NaOH (1N) and HCl (1N) solutions were used to achieve different pH values. After 2 hrs of incubation at 37°C, pH was adjusted at 7±0.2. In addition, the sensitivity of antifungal molecule(s) to proteinase K and trypsin was also tested as follow: 100 μl of the extract recovered in water were mixed with each enzyme at the final concentration of 1 mg/ml. Then, the mixture was incubated for 2 hrs at 37°C [11]. After treatments, antifungal activity was assessed by disk-diffusion method. The positive control and all treated extract were assessed at a concentration of 0.6 mg/disk of the extract.

Thin layer chromatography (TLC) and bioautography
The acetone extract from solid-state fermentation was subjected to TLC with dichloromethane:methanol:water (65:32:3 v/v) as a development solvent. The bioautography was performed with *C. albicans* ATCC 10231. TLC plates were placed in bioassay plates and overlaid with PDA (0.7% [w/v] of agar), which had been seeded with *C. albicans*. The inhibitory zone was visible after 24 hrs of incubation at 30°C.

RESULTS

Isolation and identification of the antifungal producer
All isolates were grown on their isolation culture media, and a primary antimicrobial screening was conducted using agar-plug diffusion method (data not shown). The most potent bacterial isolate against *C. albicans* ATCC 10231 has been selected for this study. Fig. 1 illustrates the anti-*Candida* activity of the isolate Cp-LMA-9. The results of biochemical and cultural characterization of this isolate are presented in Table 1.

The isolate Cp-LMA-9 was rod-shaped Gram-positive bacterium showing positive reaction in catalase test. The isolate was capable of hydrolyzing gelatin, positive for citrate utilization and negative for nitrate reduction and H_2S production. It was able to grow at 50°C, at pH range of 6-9 and at NaCl concentration from 0% to 5%.

The molecular identification of the strain selected was carried out by PCR amplifying and sequencing a fragment of 16S rRNA gene. The obtained sequence was compared with the data presented in the Genbank using BLAST research program. It was found that the fragment amplified showed high levels of sequence similarity (98%) to species of *Bacillus tequilensis* (accession no JN660079.1) and *B. subtilis* (accession no JF834076.2).

Effect of different culture media on antifungal production
Different culture media were examined to obtain the highest antifungal effect against *C. albicans* from the isolate studied. The antifungal

activity was tested using agar-plug diffusion method for solid-state fermentation and disk-diffusion method for submerged fermentation. As shown in Table 2, the highest antifungal effect was found for (YMA) compared to (NA) and (LB Agar). While, no antifungal production has been detected for (TSA). Regarding the submerged fermentation under stationary conditions, (YMB) gave the maximum antifungal production, which was 2 times higher than that produced in (LB broth) and (NB). However, under shaking conditions, no antifungal production
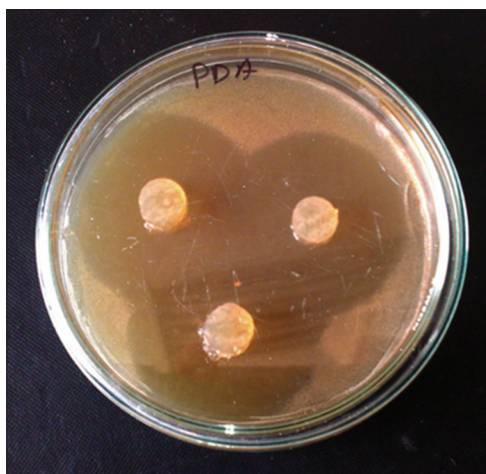
was detected for (NB), (LB broth) and (TSB). In the case of (YMB), the antifungal production was slightly lower than that found under stationary conditions. Overall, the maximum microbial growth was observed under shaking conditions for all media used in submerged fermentation.

Extraction and partial characterization of the anti-*Candida* fraction
The extraction of bioactive fraction was carried out using different organic solvents from the solid culture medium (YMA). The results were represented in Table 3. Among these solvents only acetone and methanol crude extracts showed antifungal activity against *C. albicans*. This finding indicates that the antifungal metabolite(s) produced by the strain studied are highly polar.

Effect of heat, pH and proteolytic enzymes on the antifungal fraction
Peptide antibiotics represent the predominant class of the antibiotics produced by *Bacillus* strains [10]. For this reason, the effect of heat, pH and proteolytic enzymes (proteinase K and trypsin) on the activity of the antifungal metabolite(s) produced by *Bacillus* spp. isolate was studied. The results showed that the anti-*Candida* activity of the bioactive metabolites was not affected after exposure to trypsin and proteinase K. The active fraction was also resistant to boiling and autoclaving as well as to acidic and alkaline treatment (Table 4), raising the possibility that we are in the case of a non-peptide bioactive molecule(s).

Fig. 1: Primary antifungal screening of the isolate Cp-LMA-9 against *Candida albicans* using agar-plug diffusion method

Table 1: Biochemical and cultural characterization of the isolate Cp-LMA-9

Test	Result
Biochemical tests	
β-galactosidase	+
Arginine dihydrolase	−
Lysine decarboxylase	−
Ornithine decarboxylase	−
Citrate utilization	+
H$_2$S production	−
Urease	−
Tryptophane deaminase	−
Indole production	−
Voges–Proskauer	+
Hydrolysis of gelatin	+
D-glucose	−
D-Mannitol	−
Innositol	−
D-sorbitol	−
L-rhamnose	−
D-Sucrose	−
D-Melibiose	−
Amygdalin	−
L-arabinose	−
Nitrate reduction	−
Catalase	+
Growth at 50°C	+
Growth in NaCl%	
0	+
5	+
7	−
10	−
Growth at pH	
4	−
5	+
6	+
7	+
8	+
9	−

Table 2: Effect of nature and different culture media on the antifungal production against *C. albicans*

Culture media	Antifungal activity (inhibition zones (mm))		
	Broth		Solid
	With agitation	Without agitation	
YM	15.66±1.52	22±3	36.33±1.15
TS	-	-	-
LB	-	10.66±0.57	11.33±0.57
N	-	10±1	11.66±1.15

YM: Yeast and malt extract, TS: Trypticase soya, LB: Luria bertani, N: Nutrient, *C. albicans: Candida albicans*

Table 3: Antifungal activity of extracts against *C. albicans*

Solvent	Inhibition zone (mm)
Hexane	-
Chloroform	-
Dichloromethane	-
Ethyl acetate	-
Acetone	33.66±0.57
n-butanol	-
Methanol	30.00±1.73

C. albicans: Candida albicans

Table 4: Sensitivity of extract of isolate Cp-LMA-9 to heat, pH and proteolytic enzymes

Treatment	Inhibition zone (mm)
Positive control	23.33±0.57
Heat	
100°C for 30 minutes	21.33±0.57
Autoclaving (121°C for 15 min)	20.66±0.57
pH	
2±0.2	23.33±0.57
7±0.2	23.33±0.57
10±0.2	23.33±0.57
Enzymes	
Trypsin	20.66±0.57
Proteinase K	21.33±0.57

TLC/bioautography bioassay

The results presented in Fig. 2 showed the TLC of acetone extract after UV detection. This TLC plate was used for the TLC bioautographic overlay assay which will continue to prove useful in the assessment of antimicrobial activity of natural products extracts [28]. One active spot was detected using dichloromethane:methanol:water (65:32:3 v/v) as solvent system with a retardation factor of 0.7.

DISCUSSION

Bacterial isolate selected basing on its high anti-*Candida* activity was identified as member of *Bacillus* genus with a high similarity to *B. subtilis* and B. tequilensis. This later is a spore forming Bacillus isolated from a sample taken from an approximately 2000-year-old shaft-tomb located in the Mexican state of Jalisco [29]. This strain is closely related to *B. subtilis* and it is very difficult to differentiate between these two strains. Advanced investigation including DNA-DNA hybridization, whole-cell fatty acids profile, pulsed-field gel electrophoresis of NotI-digested DNA should be used for this purpose [29].

Bacillus spp. strains produce a wide range of antifungal metabolites on (BHI), (NB), (TSB), (LB) and other synthetic culture media. However, This is the 1st time that the antifungal production by *Bacillus* spp. was reported in malt extract-yeast extract culture medium. Moreover, unlike the results of Kumar *et al.* [17]. Who found the maximum antifungal production by *B. subtilis* in TSB medium, no antifungal production was observed by our isolate in this culture medium. The production of bioactive metabolites was previously performed in both broth and solid culture media [13,16-19]. In fact, it has been found that microbial growth and antifungal production is not supported in aeration and submerged cultural conditions in the case of actinomycetes [30]. Similarly, Kumar *et al.* observed the maximum antifungal production by *B. subtilis* under stationary conditions, which are in accordance with our findings [17].

The extraction of bioactive fraction was carried out by methanol and acetone indicating its high polarity. Ethyl acetate and chloroform have been previously used to extract the antifungal fraction produced by *Bacillus* strains [16,17]. Hence, the variability of the efficient solvents and the method of extraction reported by several studies for the extraction of the antifungal compounds produced by *Bacillus* spp. strains resulted from their chemical multiplicity (peptide, polyketide lipopeptide, phospholipid and others) [8-11].

CONCLUSION

Promising antifungal activity against *C. albicans* of *Bacillus* spp. isolated from *C. procera* Ait. rhizosphere has been investigated in this work. It is

necessary to emphasize that maximum antifungal production was found on (YMA) culture medium, which is usually used for cultivating the fungi. This finding suggests that this bioactive compound(s) might be a novel antifungal drug. Bioactive fraction was extracted successfully from solid-state fermentation by acetone and methanol. A major advantage of this fraction is its stability to heat, pH and proteolytic enzymes, which widen its potential industrial importance. Further investigations are needed to complete the identification of the producer as well as for the purification and the chemical characterization of this antifungal compound.

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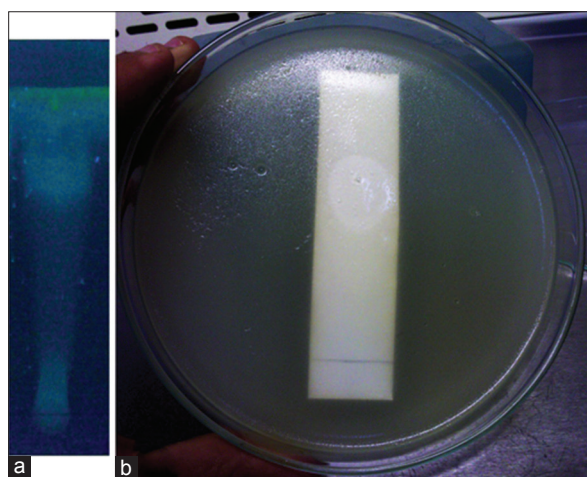
Fig. 2: (a) Thin layer chromatography of acetone extract of isolate Cp-LMA-9 visualized by ultraviolet, (b) bioautography using *Candida albicans* as microorganism test

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