Bioactive substances produced by *Burkholderia* sp. with antifungal action in *Candida* spp

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ABSTRACT

Yeasts of the genus *Candida* are commensals, colonizing the gastrointestinal and genital tract. Accounting for 15% of hospital acquired infections, they are considered a pathogen of clinical importance. The emergence of fungal infections and the occurrence of intrinsic and acquired resistance have reflected in the increased search for new antimicrobials. The objective of this study was to evaluate the antifungal activity of extracellular substances produced by *Burkholderia* sp. strain RV7S3, for yeast control of the genus *Candida*. The substance responsible for the antifungal activity was identified and characterized biochemically, its activity was evaluated by agar diffusion tests, minimum inhibitory concentration (MIC), action effect on biofilm formation, and hemolytic activity. The data suggested that the antifungal substance is a hydrolase that exhibits lipolytic activity. The lowest concentration of this enzyme, capable of inhibiting 90% of fungal growth, was 0.38 µg.mL^{-1}. The agar diffusion test showed inhibition halo formation of fungal growth with a diameter of 10 mm or greater, presenting 17.5 ± 0.5 mm. The substance showed low hemolytic activity and reduced biofilm cell viability, demonstrating its potential as an antifungal agent.

Key words: Yeasts, antimicrobial production, lipase, antifungals.

INTRODUCTION

Yeasts of the genus *Candida* can cause various infectious processes in humans, which vary according to host-microbiota relationships. These microorganisms are commensal, although in certain individuals and specific situations, they can become pathogenic, being responsible for superficial and systemic fungal infections (Silva et al., 2012). The risk factors that contribute to these infections are the use of broad-spectrum antibiotics, corticosteroids, chemotherapy, dialysis and the use of long-term catheters (Pfaller et al., 2014).

Candida albicans is the most frequently described species in cases of infections, accounting for 70% of clinical cases, followed by *C. tropicalis*, *C. glabrata*, *C. krusei* and *C. dublinienses*. In Brazil, the prevalence of urine isolates ranges from 35 to 70% for *C. albicans*, 5 to 52% for *C. tropicalis* and 7 to 9% for *C. glabrata* (Rodrigues et al., 2011).

The frequency of bloodstream infection by *Candida* sp. has increased worldwide, although this episode is most notable in North America, where these fungi are the fourth most common cause of this disease in tertiary hospitals. *C. albicans* is the most common agent, followed by *C. glabrata* (Pfaller et al., 2014).

Treatment of candidiasis is difficult due to the limited number of antifungal agents available, and the remarkable capacity of these microorganisms to acquire resistance to such drugs. Polyene and azole antifungals are the main therapeutic resources in the treatment of candidiasis, but non-*albicans* species such as *C. glabrata* and *C. krusei* naturally present reduced sensitivity to fluconazole (França et al., 2008).

To reduce the resistance problem, some measures should be taken, such as the control of the drug use, research on the functioning of the genetic resistance mechanisms and the potential targets for the development of new antifungal agents, thus offering an appropriate and efficient treatment with higher specificity (Nascimento et al., 2000).

It is known that soils harbor a great diversity of microbial species fundamental to the functioning of this ecosystem. In this context, bacteria are important sources of new drugs and other metabolites of industrial interest (Zhang and Xu 2008).

Antagonist bacteria are possible sources of research for new drugs with antimicrobial activity, since the metabolites they produce can inhibit the growth of other bacteria, filamentous fungi, yeasts and protozoa (Cain et al., 2000). It is essential to expand the natural product research area and increase economic incentives for academic research, attracting investment from pharmaceutical industries (Mossialos et al., 2010; Braine 2011). In this sense, the Laboratory of Microbial Ecology of the State University of Londrina / PR Brazil has sought to find compounds produced by microorganisms with antibiotic activity against phytopathogens (Oliveira et al., 2011) and pathogens of human importance. Following this line of research, this work had the objective of evaluating the antifungal activity of extracellular substances produced by the gram-negative bacteria, *Burkholderia* sp., strain RV7S3, in the control of yeasts of the genus *Candida*.

MATERIALS AND METHODS

Yeasts

Eight clinical isolates of yeasts of the species *Candida albicans*, *Candida tropicalis*, *Candida glabrata* and *Candida krusei* from patients with diabetes, human immunodeficiency virus (HIV) and vaginal candidiasis from the collection of the Laboratory of Molecular Biology of Microorganisms of the State University of Londrina were cultivated in sabouraud broth and agar at 37 ºC for 24 hours. The isolates were stored in sabouraud broth containing 40% glycerol at - 20 ºC and in liquid nitrogen.

Antifungal producing bacteria

The antagonistic bacteria of the genus *Burkholderia* (strain RV7S3), which produce antifungal substances, was isolated from soil samples of grass rhizosphere. Identification of the genus was performed from the sequencing of the 16S rRNA gene. The bacteria were stored in nutrient broth containing 40% glycerol at -20 °C and in liquid nitrogen. The antifungal activity of *Burkholderia* RV7S3 strain was initially evaluated against the eight clinical isolates of *Candida* spp. In the antagonism test, the RV7S3 strain was inoculated to the center of petri dishes containing nutrient agar added with $CuCl_2.2H_2O$ (100 $mg.L^{-1}$), and incubated at 28 °C for 48 hours. After the incubation period, a suspension on sabouraud agar of each yeast was poured separately by the pour plate method on *Burkholderia* culture, and incubated at 37 °C for 24 hours. The size of the halo formed around the *Burkholderia* colony was evaluated.

Obtaining bioactive substances from cultures of *Burkholderia* sp.

RV7S3 strain of *Burkholderia* sp. was inoculated on nutrient agar supplemented with 100 $mg.L^{-1}$ $CuCl_2.2H_2O$ and incubated at 28 °C for 24 hours. Cells were collected from the second subculture, resuspended in sterile saline solution (0.85%) at OD_{590nm}= 0.390, corresponding to a cell density of $1x10^8$ $CFU.mL^{-1}$, and 100 $\mu L.L^{-1}$ of this suspension was transferred into nutrient broth added with 100 $mg.L^{-1}$ of $CuCl_2.2H_2O$. The culture was incubated at 28 °C for 72 hours under an aeration system with a flow rate of 170 $L.min^{-1}$ of sterile atmospheric air.

After the incubation period, the cells were centrifuged (9.000 rpm/20 min, 4 °C), the supernatant was concentrated in rotary evaporator (Büchi® R 215) at 10% volume, and then liquid-liquid partition was performed with the supernatant concentrated in the ratio 1: 2 (v/v) in 100 ml aliquots in a separation funnel with ethyl acetate (crude extract). The partition aqueous phase was fractionated and precipitated with ammonium sulfate 20% (Sigma) (partially purified extract). The pellet was resuspended in minimal volume of phosphate-NaCl buffer 0.85% (PBS) pH 7.4, placed on a dialysis membrane and dialyzed in water deionized at 4 °C for 24 hours.

A volume 10 mL of the precipitated extract was centrifuged at 10.000 rpm/10 min, filtered in 0.45 µm membrane (Milipore®) and applied to SUPERDEX 75 10/300 GL GE column, flowing from 0.5 $mL.min^{-1}$ and pressure of 1.5 MPa. The fractions collected were lyophilized, resuspended in 1 mL of PBS pH 7.4 and tested for biological activity against *Candida* spp. The crude and partially purified extracts were sterilized by filtration using 0.22 µm cellulose acetate membranes (Millipore®).

For the protein concentration determination of the enzymatic extracts obtained (crude and partially purified), the technique described by Bradford (1976) was used, based on bovine serum albumin.

Analysis of polypeptide in polyacrylamide gel

Samples of crude and partially purified extract were analyzed by polyacrylamide gel electrophoresis under denaturing (SDS-PAGE 12%) and non-denaturing conditions (PAGE 10%), following the methodology described by Laemmli (1970). After electrophoresis, the gels were stained in silver solution (Blum et al., 1987).

Evaluation of antifungal activity by diffusion in agar

The antifungal activity evaluation of the RV7S3 strain of *Burkholderia* sp. was performed by the agar diffusion method. The yeasts were resuspended in 0.85% (w/v) NaCl at the concentration of 10^6 $cells.mL^1$, estimated by direct counting of the cells in Neubauer's Chamber. An aliquot 0.1 ml of this suspension was inoculated onto sabouraud agar (Fluka ®) by the pour plate technique, and the culture was punched into equidistant wells of 9 mm diameter, in which 150 µL of the crude extract was added and partially purified separately. As control, nutrient broth (CN) with $CuCl_2.2H_2O$ was added. Plates were incubated at 37 °C for 24 hours, and the diameter of growth inhibition halos (mm) were measured. All tests were performed in duplicate.

As a control of antifungal quality produced from *Burkholderia* sp strain RV7S3, db 59 strain of yeast *C. glabrata* was used, since it showed high sensitivity to polypeptide with antifungal activity in the direct antagonism test.

Determination of minimum inhibitory concentration (MIC)

The MIC was determined by the broth microdilution method in 96-well microplates, as proposed by CLSI (2008), with modifications. An inoculum of 100 ul of *C. glabrata* (db 59) and *C. tropicalis* were added to each well together with the antifungal substance at concentrations of 0.0112 to 3.04 µg/mL. Controls were performed using the Fluconazole antifungal (Pharmascience®) of 0.125 to 64 µg/mL, culture medium sterility, inoculum growth and sterility of the antifungal substance. The microplates were incubated at 37 °C for 24 hours, when cell growth was observed. This was revealed by the addition of 10 µL of a solution of 1% 2, 3, 5-triphenyl tetrazolium chloride (TTC) and incubated for 20 min at 37 °C. All tests were performed in duplicate.

Biochemical characterization of bioactive substances with antifungal action

Determination of lipolytic activity of the bioactive polypeptide

A reaction mixture was prepared with 1 mL of ρ-nitrophenyl palmitate solution (ρ-NPP) in 3 mg/mL isopropanol and dissolved in 10 mL of a solution 450 mL of 50 mM Tris-HCl pH 8.5 buffer containing 2 g of Triton X-100. The mixture was homogenized at room temperature until complete dissolution of the substrate. An aliquot 0.3 mL of 1:10 diluted enzyme extract was incubated at 37 °C for 10 min and added to 0.7 mL of the reactive mixture, remaining under the assay conditions for 3 min. To the blank, 1 mL of 50 mM Tris-HCl buffer pH 8.5 was added; in the test control, 0.7 mL of buffer and 0.3 mL of enzyme extract were added; in the control of substrate, 0.3 mL of buffer and 0.7 mL of reactive mixture were added. Hydrolysis of substrate ρ-nitrophenyl palmitate (ρ-NPP) by lipase in aqueous media generated the release of the chromogenic substance ρ-nitrophenol, which was defined as the release of 1 µmol ρ-nitrophenol per minute of reaction per mL of the enzyme extract under the reaction conditions.

Evaluation of lipolytic activity through the zymography technique

The zymogram was performed according to the methodology described by Castro-Ochoa et al. (2005) with modifications. For lipolytic activity (bacterial agar 15 g, olive oil 2% (m/v),30 mL of culture medium was prepared, Tween 80 0.001 g, rhodamine B 0.001%, 1000 mL H_2O) spilled onto the petri dish, and the native electrophoresis gel containing the precipitated material was deposited on the medium. The plates were incubated at 55 °C for 18 hours. Lipolytic activity was determined by halo formation revealed by exposure to 365 nm UV-A light.

Effect of pH on the stability of bioactive substances

An aliquot of 1 mL of the precipitated extract was treated with 2 mL of the following sodium acetate buffer pH= 3.6, 4.0, 4.5, 5.0 and 5.6, sodium phosphate buffer: pH= 6.0, 6.5, 7.0, 7.5 and 8.0 and Tris buffer-HCl: pH= 8.5 and 9.0, and incubated at 25 °C for 24 hours at 120 rpm. After this period, the enzymatic activity was determined according to the methodology described in the item determination of the lipolytic activity of the bioactive polypeptide. Concomitantly, biological activity test was performed using the agar diffusion method, in the same buffer solutions.

Thermostability

Aliquots of the precipitated extract were maintained under different temperature conditions, heated in a water bath at 37, 70, 80, 110 and 120 °C for 30 min, and cooled to -20 °C and 4 °C for 24 hours. The antifungal activity was analyzed by the agar diffusion method as described above.

Evaluation of the antimicrobial activity of bioactive substances

During the purification process, the obtained fractions were evaluated through tests performed in 96- well microdilution plates in broth medium. The inoculum was prepared from colonies of *C. glabrata* (db) 59.

Detection of hemolytic activity of precipitated extract

The hemolytic activity was verified in 96-well U-bottom plates, following a technique described by Bohach et al. (1988) using sheep red blood cells, which were washed three times with 0.05 M PBS pH 7.2 at a concentration of 1% (v/v). About 50 µL of the red blood cell suspension was added to each well plate. The precipitated extract was added at concentrations ranging from 0.0112 to 3.04 µg/mL. Distilled water was used as a positive control of hemolysis, and PBS as a negative control. The plates were incubated in a BOD oven at 37 °C for 1 hour, and after that period, they were taken to the refrigerator (4 °C) for 12 hours. All tests were performed in triplicate.

Antifungal activity of the precipitated extract on the biofilm formation of *Candida glabrata*

The antifungal activity of the precipitated extract on the biofilm cell viability was analyzed by the XTT method (2, 3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide) and fluorescence microscopy through LIVE/DEAD® Yeast Viability Kit.

The XTT method was performed on 96-well microplates. *C. glabrata* was cultured on sabouraud agar at 37 °C for 18 hours. After the incubation period, the cell density was adjusted to a $D.O_{520nm}$= 0.380. An aliquot 100 µL of cell suspension was added to each well of the plate, and incubated at 37 °C for 1.5 hour/75 rpm. After that time, well contents were aspirated and the wells washed once with PBS.

150 µL of RPMI 1640 was added to each well, and then incubated at 37 °C for 24 hours/75 rpm. After incubation, the wells were washed once with sterile PBS, and the precipitate extract at concentrations of 0.059 to 30.4 µg/mL in duplicate was added and incubated at 37 °C for 48 hours. A cell suspension without antifungal substance was considered a positive control. The readings were performed in a spectrophotometer (Asys HiTech UVM 340), λ = 490 nm.

Fluorescence microscopy for biofilm viability analysis was performed on 24-well plates containing circular coverslips. The inoculum and the biofilm preparation were the same used by the XTT method. Aliquot of precipitated extract at the concentration of 7.6 µg/mL was added in duplicate. The procedures used for the coloration of the coverslips by fluorescence at formed and treated biofilms followed the manufacturer's recommendations.

RESULTS AND DISCUSSION

Lipases are found in several organisms, including plants, animals, fungi and bacteria, although the main source for industrial application is microorganisms, among which the most important are the bacteria of genus *Burkholderia* (Padilha et al., 2010).

Lipases (triacylglycerol acylhydrolases) are enzymes belonging to the hydrolases family, catalyze the hydrolysis of long chains of acylglycerols, with acyl chain consisting of more than 10 carbon atoms forming fatty acids and glycerol, acting at the oil / water interface (Chia-Feng et al., 2012; Messias et al., 2011). They have been used extensively in various processes such as detergent composition, food additives to modify and enhance organoleptic properties, in the treatment of effluents, in textile, cosmetics and other applications. In the pharmaceutical industry, they have been used in the synthesis of intermediates of anti-inflamatory drugs. Lipase applications vary according to the type of reaction catalyzed, e.g., in transesterification reactions, they are used to convert oil into biodiesel (Chia-Feng et al., 2012).

Lipases are used by microorganisms as a defense mechanism, due to their phospholipidic action. When secreted, they provide competition with the microbiota, facilitate the digestion of lipids and released free fatty acids, aiding in cell-cell and host-cell adhesion (Messias et al., 2011).

From the precipitation, 20% ammonium sulfate, 0.45 g/L of partially purified bioactive substance were produced. The antifungal protein extract presented lipolytic activity of 820 IU/mL. In the qualitative evaluation of the lipase activity by ultraviolet light irradiation of the culture media containing the rhodamine B dye, the presence of the compound fluorescence formed between the free fatty acids in the medium and the dye was observed.

Lipase production by *Burkholderia* sp. was also described by other authors. Chia-Feng et al. (2012) observed that strains of *Burkholderia* sp. produced lipases that presented lower activity when compared to the results obtained in this work (45.8 UII/mL and 122.3 UII/mL, although in different culture media and culture time). On the other hand, Shu et al. (2009) reported lipase production by *B. cepacia*. However, they identified the activity through olive oil hydrolysis in the plaque test containing the Rhodamine B.

The enzymatic extract was more active between pH 4.0-4.5 (Figure 1), although bacterial lipases generally presented a neutral or basic character. Nevertheless, some *Pseudomonas* species had an optimum pH of 4.8 (Gupta et al., 2004), similar to the polypeptide optimal pH analyzed in this study.

The pH changes the stability of the enzyme due to modifications in the pH alter the ionic character of the amine and carboxylic groups of the protein, affecting the catalytic site and the enzyme conformation. These conformational changes influence the enzyme-substrate interaction affecting enzyme stability (Gupta et al., 2004).

The antifungal substance showed to be thermostable presenting higher biological activity at 37 °C and 70 °C, although still remained active at the other tested temperatures, but with less activity. (Figure 2). Thermal stability is an important requirement for lipase commercialization, and it generally presents an optimum temperature between 30-60 °C (Gupta et al., 2004).

Depending on the source, lipase activity is maintained in ambient temperature and 70 °C, but with optimum activity ranging between 30 and 40 °C, varying its thermostability depending on the origin. Microbial lipases are the ones with the best thermal stability (Castro et al., 2004).

Three active fractions were detected in the molecular exclusion chromatography (Figure 3), the fractions that showed biological activity were identified and fractionated (Figure 4). An efficient and low cost methodology was employed.

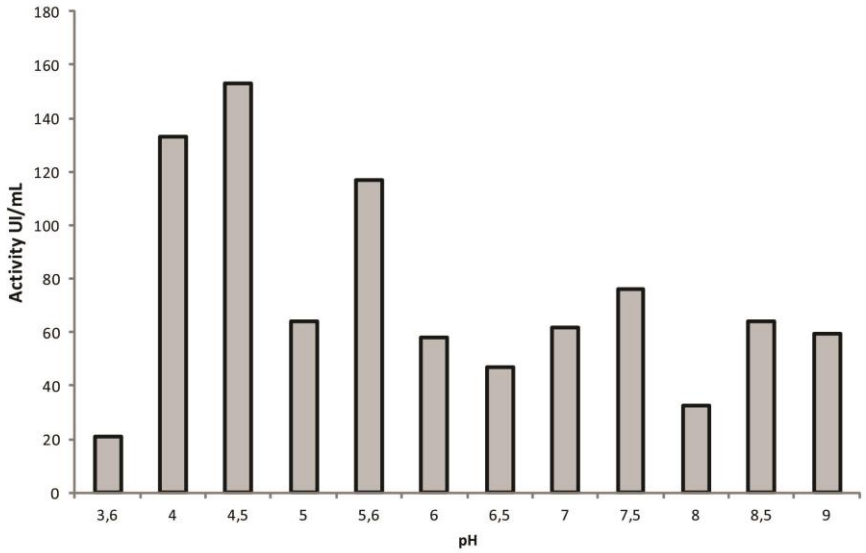

Figure 1. Ph effect on the enzymatic activity of the precipitated extract produced by Burkholderia sp.

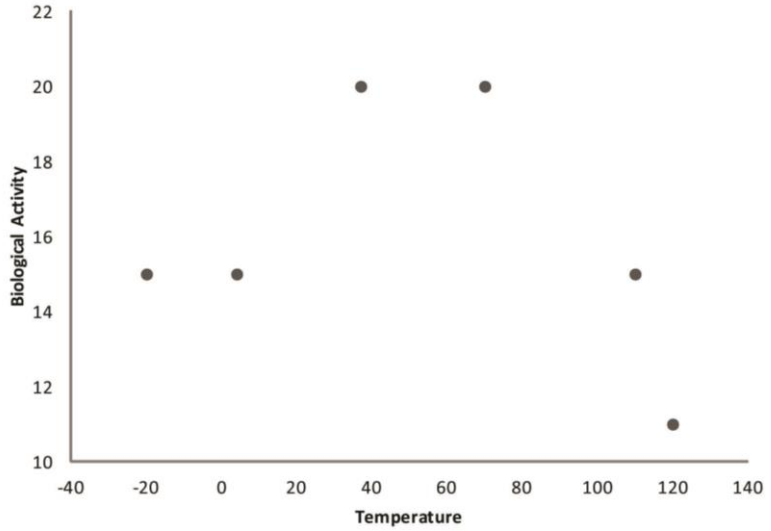

Figure 2. Thermostability test of the crude protein extract produced by Burkholderia sp.

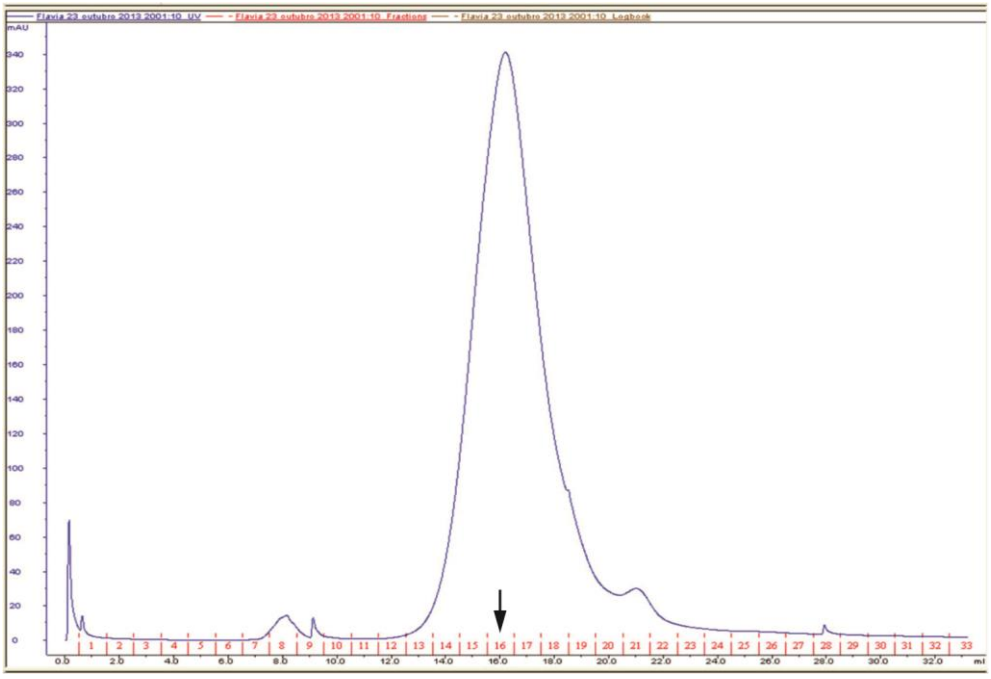

Figure 3. Chromatographic profile of molecular exclusion using Superdex 75 gel filtration column in FPLC and PBS pH 7.4. Fractions with antifungal activity found in tubes, 14, 15 and 16 (1 mL per tube) (indicated by arrow).

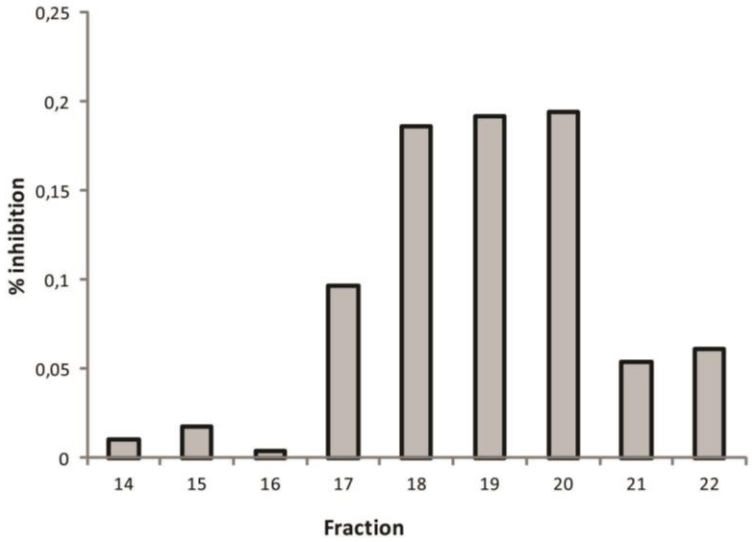

Figure 4. Biological activity of the fractions tested with spectrophotometer reading λ = 530 nm. Fractions with antifungal activity found in tubes, 14, 15 and 16.

The molecular weight of the semi-purified substance was estimated to be 26-37 kDa. Padilha et al. (2010) detected lipases with molecular mass ranging from 20 to 75 kDa, in *Burkholderia cepacia*.

For the crude extract and precipitated extract respectively for *C. glabrata*, inhibition halos of 17.5 ± 0.5 mm and 19 ± 1 mm were observed.

The antifungal substance MIC with the *C. glabrata* and *C. tropicalis* strains was 0.38 μg/mL for both strains and ≤0.25 μg/mL for the fluconazole used as control.

Species of *Candida* non-*albicans* present higher MIC values for azoles than *C. albicans* in hospitals in Latin America (Godoy et al., 2003). Isolates of *C. glabrata* versus fluconazole presented MIC50 and MIC90 values of 2 and 4 μg/mL, respectively. Substance antifungal activity obtained from bacteria was also described by Spadari (2013). In his work, the author described the activity of bioactive substance produced by actinomycetes in *Candida* and dermatophytes, presenting inhibition halo of 8.75 mm.

As for the hemolysis test of the supernatant and enzymatic extract precipitated with ammonium sulphate (20%), only concentrations 1.52 and 3.04 μg/mL showed hemolytic activity (Table 1).

Table 1. Hemolytic Titration assay.

Concentration (μg mL^{-1})	Supernatant RV_7S_3	Precipitated extract 20%
3,04	+	+
1,52	+	+
0,76	-	-
0,38	-	-
0,19	-	-
0,095	-	-
0,0475	-	-

(+) presence of hemolysis (-) absence of hemolysis.

The antifungal substance at the concentration of 7.6 μg/mL (12.5%) was active against *C. glabrata* biofilm cells, demonstrating cell viability reduction when compared to the positive control of the reaction without precipitated extract (Figure 5), comparing the images of the positive control with the treated biofilm (Figure 6).

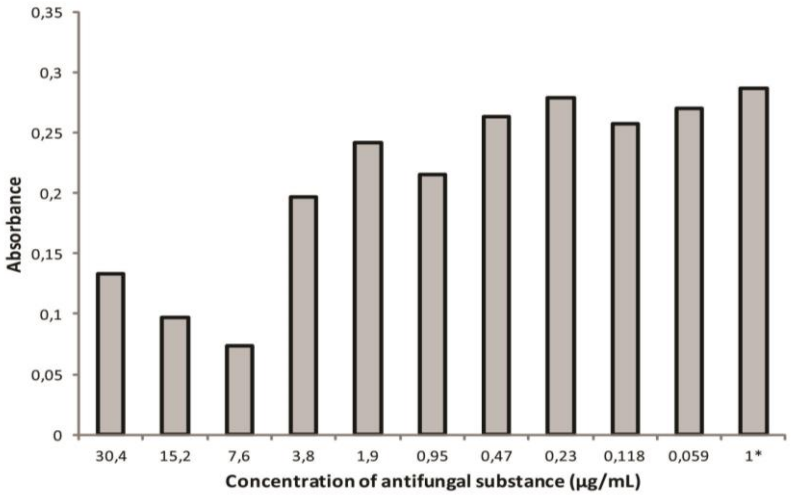

Figure 5. Antifungal activity on biofilm formation by the XTT method. 1*Growth control

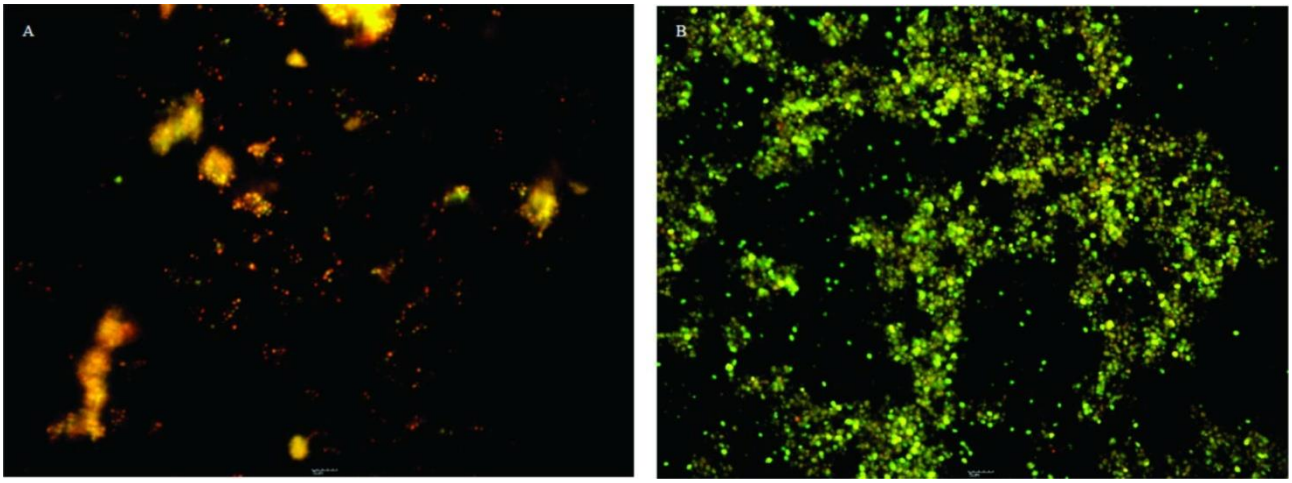

Figure 6. Antifungal activity of the precipitated extract on biofilm of *C. glabrata* by fluorescence microscopy. A. Biofilm treated with 7.6 µg mL^{-1} protein extract (24h). B. Positive control without treatment with the protein extract.

CONCLUSIONS

The results of the biochemical characterization of the antifungal substance produced by Burkholderia sp strain RV7S3 suggest a lipase activity which is thermostable and has an optimum pH between 4.0 and 4.5. The substance presented antifungal activity both in planktonic cells and in *C. glabrata* biofilm at concentrations that do not cause hemolysis in sheep blood. Taken together, these results suggest the likely hydrolase potential as an antifungal agent.

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