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Study on the Effect of the Antifungal Extract from *Bacillus* sp. on the Physicochemical Properties of *Candida albicans*

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ABSTRACT

Candida albicans is the most common fungal pathogen of humans; this infectious agent can adhere and colonize several surfaces to establish many dangerous infections. The effect of methanol extract from *Bacillus* sp. isolated from *Calotropis procera* Ait. rhizosphere on the physicochemical characteristics of *Candida albicans* cell surface was investigated. The Lifshitz-van der Waals (γ^{LW}), acid-base (surface tension components ΔGiwi, electron donor (γ^-) and electron acceptor (γ^+) parameters of the yeast cell surface were assessed using contact angle measurement. Results showed higher antifungal activity of methanol extract against tested yeast. Regarding contact angle measurements, cell surface of control or untreated *C. albicans* showed a hydrophilic character (ΔGiwi = 19.96 mJ m^{-2}), a strong electron donor character (γ^- = 48.4 mJ m^{-2}) and a weak electron acceptor character (γ^+ = 5.8 mJ m^{-2}). After limited exposure to the antifungal extract, the treated cell surface has become more hydrophilic quantitatively. Moreover, the results showed an increase of the electron donor character and a decrease of the electron acceptor character. However, non-significant modifications on the physicochemical characteristics of cell surface between exposures for 1 and 2 h to the extract were found. The present investigation may provide information that could be used to alter or modify the adherence of *C. albicans* to biotic and abiotic surfaces.

Key words: *Bacillus* sp., antifungal activity, physicochemical characteristics, *Candida albicans*, contact angle

INTRODUCTION

Candida albicans which is the most common microorganism implicated on fungal infections in human, represent nowadays a serious problem on public health (Papon *et al*., 2013). A varied range of biomaterials are used in clinical practice and they often represent a risk factor for the development of nosocomial *Candida* infections (Press *et al*., 2014). Biofilm formation occurs as a consequence of the device being colonized by the microorganisms. Adhesion is an essential step in colonization and development of infection, its role in the pathogenesis of several diseases by fungus is widely acknowledged. This capacity to adhere to biotic or abiotic surfaces depend significantly

on the physicochemical properties of substratum and the microbial surface such as hydrophobicity/ hydrophilicity (Klotz *et al*., 1985), surface charge and electron donor-electron acceptor (acid-base) properties (Hamadi and Latrache, 2008).

Several studies that has been focused on microbial natural products have demonstrate the ability of *Bacillus* sp., to produce a widely range of antifungal metabolites (Beric *et al*., 2012; Ren *et al*., 2013; Tan *et al*., 2013). Some have been reported as biosurfactants such as surfactin and iturin which may be adsorbed on microbial surfaces and modify their hydrophobicity (Ahimou *et al*., 2000). The potential of plant extracts and phytochemical compounds to modify the physicochemical characteristics of *C. albicans* and other microorganisms was extensively studied (Annuk *et al*., 1999; Polaquini *et al*., 2006; Razak *et al*., 2006). While, few studies have been reported with microbial extracts. For this reason, the present study aims to evaluate the effect of bioactive extract obtained from solid-state fermentation of *Bacillus* spp. (Cp-LMA-9) strain isolated by our laboratory from *Calotropis procera* rhizosphere on the physicochemical characteristics of the yeast cell surface.

MATERIALS AND METHODS

Microbial fermentation and extraction: *Bacillus* sp., Cp-LMA-9 strain was isolated by our laboratory from *Calotropis procera* Ait. rhizosphere and selected for its excellent antifungal activity against C YMA (Yeast extract 1 g L^{-1}, malt extract 20 g L^{-1} and agar 20 g L^{-1}) at 30°C for 48 h. After incubation, biomass was discarded and solid medium was extracted by maceration using *C. albicans*. Based on the 16 S rRNA gene sequence analysis, it was identified as *Bacillus* sp. A solid-state fermentation of this isolate was performed on yeast extract-malt extract-agar methanol. The mixture was filtered and evaporated under vacuum. Finally, the extract was resuspended in distilled water.

Antifungal bioassay: The methanol extract was tested for its antifungal activity against *C. albicans* ATCC 10231_using disk diffusion method according to NCCLS (2004) with slight modifications. Briefly, *C. albicans* was cultivated on Potato-Dextrose-Broth at 30°C at 125 rpm for 48 h. Microbial inoculum was prepared in sterile saline, adjusted to 0.5 McFarland scale and used to inoculate PDA Plates. Filter paper (5 mm in diameter) were impregnated with 10 μL of methanol extract dissolved in distilled water (0.6 mg $disk^{-1}$). The Petri dishes were incubated at 30°C and the inhibition zones were measured after 24-48 h of incubation.

Preparation of strain: The method used for measuring contact angles on microbial layers has been described by Hamadi and Latrache (2008). Briefly, 500 mL of YPG broth medium (Yeast extract 10 g L^{-1}, Peptone 10 g L^{-1} and glucose 20 g L^{-1}, pH = 7) were inoculated with a 48 h old culture of *C. albicans* ATCC 10231 and incubated under shaking conditions at 30°C for 48 h. Fungal cell were harvested in three tubes by centrifugation and washed tree times with sterile solution of KNO_3(0.1 M). Then, the pellets were resuspended in 10 mL of sterile KNO_3 (0.1 M) and the extract was added on two tubes to a final concentration of 30 mg mL^{-1}. The tubes were mixed by vortexing and incubated at 30°C for 1 and 2 h. The control was performed with KNO_3 alone.

Finally the fungal cell suspension of each tube was deposited onto a 0.45 μm cellulose acetate filter (Sartorius) by first washing the filter with 10 mL of distilled water for wetting and then 10 mL of the cell suspension was added obtaining a thick lawn of cell after filtration. The rainy

Table 1: Surface energy of contact angle liquids[a]

Liquids	γ^{LW} (mJ m^{-2})	γ^{+} (mJ m^{-2})	γ^{-} (mJ m^{-2})
Water	21.8	25.5	25.5
Formamide	39.0	2.3	39.6
Diiodomethane	50.5	0.0	0.0

[a]Results found by Van Oss *et al*. (1988)

filters were placed prudently on a glass support with double-sided sticky tape and allowed to air dry. Then, the contact angle measurements were made on each filter.

Contact angle measurements: Contact angle measurements for *C. albicans* ATCC 10231 surface were performed by using a goniometer (GBX Instruments, France) by the sessile drop method (Sadiki *et al*., 2014). Three measurements of contact angles were made on each surface of cell using two polar liquid (water and formamide) and one non-polar liquid (diiodomethane) with known energy characteristics (Table 1).

Surface tension components and hydrophobicity: The Fungal cell surface hydrophobicity was assessed through contact angle measurements in accordance with the approach of Van Oss *et al*. (1988). In this approach, the degree of hydrophobicity of a given material is expressed as the free energy of interaction between two entities (i) of that surface when contacted with water (w): The ΔGiwi. If the interaction between the two entities is stronger than the interaction of each entity with water, the surface is considered hydrophobic (ΔGiwi>0); conversely, for a hydrophilic surface (ΔGiwi>0). The ΔGiwi is calculated through the surface tension components of the interacting entities, according to the following equation:

$$\Delta Giwi = -2\gamma_{iw}$$
$$= -2\left[\left((\gamma_i^{LW})^{1/2} - (\gamma_w^{LW})^{1/2}\right)^2 + 2\left((\gamma_i^+\gamma_i^-)^{1/2} + (\gamma_w^+\gamma_w^-)^{1/2} - (\gamma_i^+\gamma_w^-)^{1/2} - (\gamma_w^+\gamma_i^-)^{1/2}\right)\right]$$

where, γ^{LW} accounts for the Lifshitz-van der Waals component of the surface free energy and γ^{+} and γ^{-} are the electron acceptor and electron donor parameters, respectively, of the Lewis acid-base component (γ^{AB}) with:

$$\gamma_S^{AB} = 2(\gamma_S^-\gamma_S^+)^{1/2}$$

The surface energy components of a fungal cell surface are obtained by measuring the contact angles of three pure liquids (one apolar and two polar) with well-known surface energy components (Van Oss, 1994), followed by the simultaneous resolution of three equations of the following form:

$$\gamma_S^{AB} = 2(\gamma_S^-\gamma_S^+)^{1/2}$$

Statistical analysis: All data were subjected to one-way ANOVA test, performed with the software package statgraphics Centurion XIV. Differences were considered significant at the $p<0.05$ level of probability.

RESULTS

***In vitro* antifungal bioactivity:** The antifungal activity of methanol extract from *Bacillus* spp. isolate was assessed using disk diffusion method. As shown in Fig. 1, the extract exhibited a higher antifungal effect against *C. albicans* ATCC 10231 with an inhibition zone of 25.0±1 mm in diameter.

Cell surface hydrophobicity of *C. albicans* before and after treatment: The sessile drop technique was used to characterize the surface properties of *C. albicans* ATCC 10231. The hydrophobicity and the surface energy of the yeast surface were assessed through contact angle measurements and calculation was done in accordance with Van Oss approach (Van Oss *et al*., 1988). According to Van Oss and Giese (1995) the surface hydrophobicity can be showed qualitatively by the water contact angle with higher values indicating a more hydrophobic surface ($\theta w(°) > 50$). Vogler (1998) sets the limit at 65°. As shown in Table 2, the water contact angle obtained for untreated *C. albicans* surface are lower than 50 ($\theta w(°) = 20.3$) indicating qualitatively the hydrophilic character of the fungal cell surface. Moreover, the positive value of free energy ($\Delta Giwi = 19.96$ mJ m^{-2}) (Table 3) showed also quantitatively that the yeast surface was hydrophilic.

After exposure to the antifungal extract obtained from solid-state fermentation of *Bacillus* spp. for 1 and 2 h, the water contact angle was decreased to 19.1° and 16.6°, respectively compared with control or untreated yeast. In addition, the free energy confirmed that the yeast surface has become more and more hydrophilic quantitatively after exposure to antifungal extract for 1 and 2 h with values of 27.4 and 27.09 mJ m^{-2}, respectively. Whereas, no significant modification was noted between the free energy of the yeast surface treated for 1 h and that treated for 2 h.

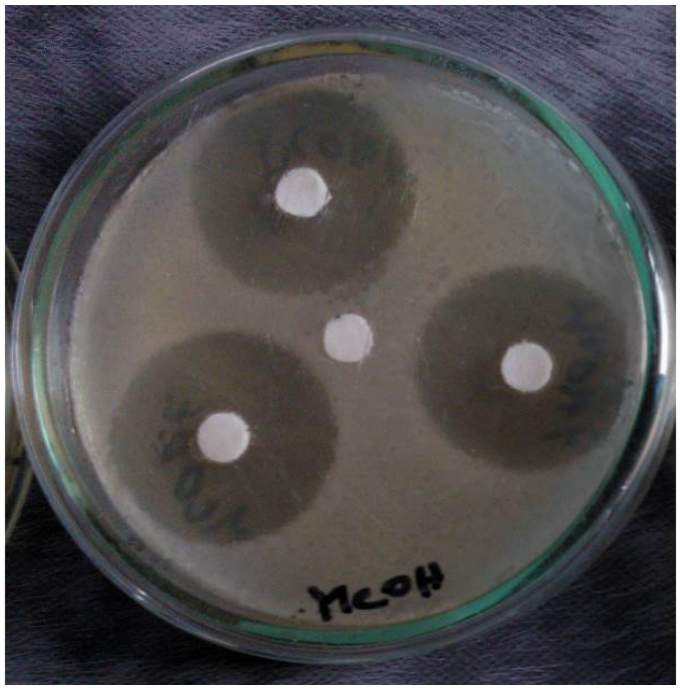

Fig. 1: Antifungal activity of methanol extract from *Bacillus* spp. (Cp-LMA-9) against *Candida albicans*

Table 2: Contact angles of water (θ_W), formamide (θ_F) and diiodomethane (θ_D) of treated and untreated *Candadida albicans* ATCC 10231

	Contact angles (°)		
Treatments	θ_w (°)	θ_F (°)	θ_D (°)
Control	20.3±0.11	16.9±0.10	66.8±0.10
Treated (1 h)	19.1±0.30	22.3±0.28	59.7±0.92
Treated (2 h)	16.6±0.36	18.8±0.46	58.6±0.64

Table 3: Lifshitz-van derWaals (γLW) component and electron donor (γ-) and electron acceptor (γ+) parameters and free energy of interaction (ΔGiwi) of treated and untreated *Candadida albicans* ATCC 10231

Treatments	γ^{LW} (mJ m^{-2})	γ^{+} (mJ m^{-2})	γ^{-} (mJ m^{-2})	ΔGiwi (mJ m^{-2})
Control	24.39±0.34	5.97±0.02	48.72±0.13	19.93±0.13
Treated (1 h)	28.70±0.53	3.39±0.23	52.86±0.45	27.54±0.82
Treated (2 h)	29.28±0.38	3.55±0.09	52.93±0.31	27.05±0.36

Electron donor and electron acceptor of *C. albicans* before and after treatment: Regarding the electron donor and electron acceptor characters, it can be seen that untreated *C. albicans* cells surface showed a high electron donor character ($\gamma^- = 48.4$ mJ m^{-2}) (Table 3) which was much higher than electron acceptor (γ+). Therefore, the untreated yeast cells are predominantly electron donors. Regarding the Lifshit Van Der Waals component, untreated yeast cells showed a value of 24.7 mJ m^{-2}.

After exposure for 1 and 2 h to the antifungal extract, the electron donor parameter (γ^-) was increased significantly compared with untreated yeast, indicating that, the cells surface of *C. albicans* became more electron donor. While, the electron acceptor character was decreased significantly. Moreover, it can be noted from Table 3, the yeast treated with antifungal extract has given values of the Lifshit Van Der Waals component significantly more than that of untreated cells. However, no significant modifications were noted for the Lifshit Van Der Waals, the electron acceptor (γ+) and the electron donor components between treatment for 1 and 2 h.

DISCUSSION

Strains belonging from *Bacillus* genus are known for their ability to produce a multiple antimicrobial molecules such us surfactin, iturin, fengymycin bacilysocin, fengycin and others (Ahimou *et al*., 2000; Loeffler *et al*., 1986; Tamehiro *et al*., 2002). Some of this bioactive compounds especially lipopeptides exhibit a surface-active properties such as surfactin, iturin and fengycin groups (Razafindralambo *et al*., 1998; Thimon *et al*., 1992).

This study demonstrate that antifungal extract from *Bacillus* sp. isolate affect the physicochemical properties of *C. albicans* cells surface. Firstly, we found similarly to Henriques *et al*. (2002) that untreated *C. albicans* cells surface are hydrophilic. Moreover, Blanco *et al*. (2008) found that *C. albicans* is always hydrophilic compared to *Candida dubliniensis* using the Microbial Adhesion to Hydrocarbons (MATH) method.

Several techniques have been used to determine the degree of hydrophobicity of microbial cells or particulate materials. Microbial adherence to hydrocarbons (MATH), hydrophobic chromatography, salting-out aggregation, partitioning of cells in two-phase systems and contact angle measurements have been used for this purpose (Geertsema-Doornbusch *et al*., 1993; Oliveira *et al*., 2001; Van Loosdrecht *et al*., 1989). They concluded that water contact angle measurements technique was the best method for the quantification of cell hydrophobicity.

Hazen *et al*. (2000) tested influence of fluconazole on cell surface hydrophobicity of *C. albicans* and showed that hydrophilic strains remained hydrophilic after treatment. Previously data showed that hydrophobic cells of C. *albicans* were more resistant than hydrophilic cells to phagocytic killing

(Antley and Hazen, 1988). In addition, it has been reported that hydrophobic yeast cells produce germ tubes sooner than hydrophilic yeast cells. These germ tubes and extracellular materials make them less susceptible to killing during subsequent encounters with phagocytes and more capable of successful colonization and dissemination (Antley and Hazen, 1988; Sobel *et al*., 1984).

Previously published data showed that the hydrophobicity of solid surfaces influences adhesion of bacteria, eukaryotic cells and proteins. Therefore, bacteria and other microorganisms have developed many multiple performances to use the hydrophobic effect in order to adhere to substrata. In fact, there are convincing reasons to believe that the hydrophobic effect may be the primary driving force for the adhesion of most pathogens (Oliveira *et al*., 2001). Ellepola and Samaranayake (1998) demonstrate the existence of a highly significant positive correlation between the relative surface hydrophobicity of *C. albicans* and adhesion to buccal epithelial cells and showed a significant reduction in hydrophobicity of the yeast cells after limited exposure to the antifungal agents (nystatin and ketoconazole). However, the cells surface hydrophobicity of the yeast cells was not found affected by fluconazole and 5-fluorocytosine (Ellepola and Samaranayake, 1998).

Regarding the electron donor and electron acceptor characters, Similar results were found by Henriques *et al*. (2002) for untreated *C. albicans* cells surface using contact angle measurements technique with water, formamide and l-bromonaphthalene. Moreover, recent study reported that several actinomycetes strains were predominantly electron donor and weak electron acceptor (Maataoui *et al*., 2014). It was reported previously by several authors that microbial cells surfaces are predominantly electron donating but sometimes also sizeable electron-accepting cells surfaces can be found (Van der Mei *et al*., 1998).

Previously published data reported that the high γ^- and low $\gamma+$ values are typical of the electron-donating characteristic of hydrophilic bacteria, while the low γ^- and low $\gamma+$ values are characteristic of hydrophobic bacteria (Feng *et al*., 2013; Van der Mei *et al*., 1998). It has been showed that the net surface charge of *C. albicans* clearly influenced adherence to plastic surfaces and that more positively charged *C. albicans* are considerably more adherent than more negatively charged yeast (Klotz *et al*., 1985).

CONCLUSION

In conclusion, this study demonstrated that the *Bacillus* spp. antifungal extract is able to affect the physicochemical properties of *C. albicans*. Indeed, it decrease significantly the hydrophobicity of *C. albicans* cells surface and increase the electron donor character of this strain. As proved previously, the decrease of the cells surface hydrophobicity and the enhancing of negative charge surface of *C. albicans* contribute to reduce its ability to adhere and colonize the biotic and abiotic surfaces. For this reason, these findings provide important information about the antifungal compounds presents in the studied extract which could be a promising and alternative agents for use to modulate or affect the adherence of *C. albicans* to different surfaces of many research scientific area.

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