Hydrolytic activity and biofilm formation in clinical isolates of *Candida albicans*: the effect of changing pH and temperature

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Summary

Candida albicans is the most frequently isolated opportunistic yeast in hospitals; consistently responsible for invasive fungal infections. The formation of biofilms and the activity of hydrolytic enzymes are two major virulence factors contributing to the pathogenicity of this species. This study aimed to highlight the activity of hydrolytic enzymes in isolated strains of *C. albicans* which form biofilms, as well as the effect of the change in pH and temperature on their synthesis.The capacity to form biofilms was determined using the crystal violet technique. The synthesis of phospholipase was determined by the plate method using egg yolk culture medium. For the proteinase activity, agar plates containing bovine albumin serum was used. However, esterase, coagulase and hemolysin were evaluated using the opacity test, the con-

ventional tube test and the sheep blood plaque test, respectively. In addition, phospholipase, proteinase and esterase activities were assessed under different conditions of temperature and pH. The isolated strains of *C. albicans* were able to form biofilms and synthesize phospholipase, proteinase, esterase, coagulase and hemolysin; the activities of these enzymes vary differently from one strain to another. *C. albicans* further exhibited hydrolytic activities. The interaction significance between the strains, pH and temperature depends on the type of enzymes. This draws attention to the importance of these enzymes to better understand the relationship between the pathogenesis and the virulent process of this species.

Key words
Candida albicans, Biofilms, hydrolytic enzymes, temperature, pH

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1. Introduction:

The frequency of fungal infections has increased dramatically over the past years; these infections are a major cause of morbidity and mortality.[1,2] *Candida albicans* is frequently isolated in the hospital and is the most incriminated in invasive mycosis.[3,4] Several virulence factors are attributed to the pathogenicity of *C. albicans*, such as adhesion to host cells and/or medical devices, biofilm formation, growth capacity under varying conditions and secretion of hydrolytic enzymes.[5-7] Indeed, hydrolytic enzymes play an important role because they facilitate the adhesion and penetration of tissues and therefore the invasion of the host.[8,9] Several studies have demonstrated the proportional relationship between enzyme synthesis and their hydrolytic activity with the increase in the pathogenic potential of *C. albicans*.[10-12] The level of expression of these enzymes may correlate with severity in case of *Candida* infection.[13] Phospholipase participates in *C. albicans* invasion of the host tissue by disrupting the membrane of the epithelial cells and enabling hyphae penetration into the cytoplasm.[14] The expression of aspartyl proteinases is considered one of the most important virulence factors in *Candida sp*;[15,16] this enzyme destroys some important immune proteins in host cells, such as immunoglobulin, mucin and pepstatin A.[17] On the other hand, hydrolytic enzymes are extremely sensitive to environmental conditions, especially temperature and pH.[18] Fungal lipase activity is optimal at a neutral to slightly acidic pH,[19] while Aspartic proteinases are generally active only in acid pH ranges, which are an advantage to the growth of *C. albicans* in some host tissues, such as vaginal mucosa.[10] Furthermore, the pathogenicity of *Candida spp.* can also be attributed to hemolysin activity, in particular in disseminated candidiasis.[16,20] This study aimed, in addition to assessing the potential of the isolated strains to form biofilms, to test in vitro isolates of *C. albicans* for the synthesis of phospholipases (Plp), proteinases (Prt), esterases (Est), coagulases (Cgl) and hemolysin (Hls); and then to determine the effect of temperature and pH change on the hydrolytic activity of Plp, Prt and Est.

2. Material and methods

A collection of 14 strains *C. albicans* was used in this study; they were isolated by Touil et al.[21] from two dif-

ferent medical devices (central venous catheters and urinary catheters) which were removed from hospitalized patients in the departments of medical neurology, ortho-traumatology and anesthesia in the University Hospital of Tlemcen-Algeria. It should be noted that the reference strain, *C. albicans* ATCC10231, was used for the positive control of the following tests. All tests were done in triplicate.

2.1. Biofilms formation

Candida albicans isolates used have already been subjected to the biofilm formation test by Touil et al.[21] For confirmation, this potential has been replicated again using the crystal violet method.[22] Briefly, after incubation at 30°C for 24 h, *C. albicans* cells were washed three times with phosphate buffered saline (PBS) and then adjusted to10^6 cells/mL in RPMI 1640. From this suspension, 200 µL were distributed into microtiter plate (96 wells) and then incubated at 37°C for 24 h. The wells were then washed three times with PBS, and 100 µL of methanol (99%) were added to each well. After further incubation for 15 min, the wells were washed and then 100 µL of crystal violet solution were added to them, and the plates were incubated for 20 minutes at room temperature. To dissolve the crystal violet linked to the biofilms, 150 µL of acetic acid (33%) were added. Finally, the biofilm formation potential was assessed by measuring the optical density with a microplate reader (RT-2100C) at 570 nm. According to the absorbance, the strains were grouped as high production of biofilms (OD> 1.39), moderate production of biofilms (OD = 0.40 to 0.99), low production of biofilms (OD = 0.20 to 0.39) and no production of biofilms (OD <0.20).

2.2. Enzymatic activity

The formation of a halo (hydrolysis zone) around the colonies of isolates indicates enzymatic activity. The results were expressed qualitatively as levels of enzyme activities (high, medium, weak or no activity). These were evaluated by the Pz ratio which was calculated as the diameter of zone of hydrolysis divided by the diameter of the microbial colony.[23] Based on the Pz values, five classes have been described for enzyme activity, as follows: *Pz* = 1 means that the activity is negative. Whereas a value of *Pz* included between 0.99 and 0.90 indicates a low activity. If 0.89 <*Pz*> 0.80, the enzymatic activity is weak. For 0.79 <*Pz*> 0.70, the enzymatic activity is called moderate and for *Pz*< 0.70, the activity is described as large.[23]

Phospholipase

Plp activity was determined using the method of Tsang et al.[16] with some modifications. For this, the following medium was prepared as follows; Sabouraud dextrose agar (13.0 g), NaCl (11.7 g), $CaCl_2$ (0.11 g) and distilled water (184 mL) were mixed and sterilized using autoclave. The egg yolk was centrifuged at 3000g for 10 minutes at 4°C and then, under aseptic conditions, 20 mL of supernatant were added to the previously prepared medium. Seeding was carried out by depositing 10 µL of *C. albicans* suspension (10^8cells/mL) on the egg-yolk-agar poured into Petri dishes, and then incubated at 37°C for 72 h.

Proteinase

The production of Prt was determined according to the method of Aoki et al.[24] In brief, 60 mL of a sterile solution (pH 3.5) containing $MgSO_4$ (0.04 g), K_2HPO_4 (0.5 g), NaCl (1 g), yeast extract (0.2 g), glucose (4 g) and 0.05 g of Bovine Serum Albumin (BSA) were mixed with 140 mL of molten agar-agar and then poured into Petri dishes. 10 µL of the inoculum (10^6cells/ml) were deposited on the agar plate, and the incubation was then made at 37°C for 7 days.

Esterase

Est activity was evaluated according to Slifkin.[25] Briefly, the following medium was prepared. 10 g of peptone, 5 g of NaCl, 0.1 g of $CaCl_2$, 15 g of agar-agar and distilled water (qs 1 L). Once autoclave sterilization was done, the medium was cooled to 50°C and then, 5mL of tween 80 were added to it. A spot of 10 µL of the inoculum were deposited on the medium in Petri dishes which were incubated at 30°C for 10 days. The presence of a transparent halo around the colony indicates positive activity.

Coagulase

From a pre-culture of *C. albicans* (10^8 cells/mL), 0.1 mL was added to the tubes containing 500 µL of human serum. The tubes were then incubated at 37°C. The formation of clots was verified after 2, 4, 6 and 24 hours. Clots indicate the enzymatic activity of Cgl.[26]

Hemolysin

To determine the Hls activity, 7 mL of fresh sheep blood were added to 100 mL of Sabouraud dextrose agar medium which was supplemented with glucose (3%). Then, 10 µL of the inoculum (10^8cells/mL) were deposited on the prepared agar plates in Petri dishes. The incubation was done at 37°C for 48 h.[27] The presence of a distinctive translucent halo around the colony indicates positive hemolytic activity. A totally translucent ring indicates β-Hls, while an incomplete ring (greenish-black) reflects α-Hls. Conversely, the absence of a ring around the colony reflects the γ-Hls activity and therefore, no synthesis of the enzyme.

2.3. Effect of temperature and pH on Plp, Prt and Est activities

According to Sardi et al.;[1] Dabiri et al;[28] Pandey et al.,[29] Plp, Prt and Est are the most interesting microbial enzymes in medical pathology; these enzymes are in fact involved in the invasion of the microorganism into host tissues, consequently its pathogenesis. Because of this, the previous tests were carried out under different pH and temperature conditions; Plp and Est activities were tested at 30°C then at 37°C with respect pH of 5 and 7.[16,25] However, Prt activity was tested at the same previous temperatures but at pH 7 and 3.5, as recommended byAoki et al.[24]

2.4. Statistical analysis

Statistical software Gen Stat Discovery Edition 3 was used for data analysis. The results were presented as means ± standard error of means. The comparison between means was statistically studied using analysis of variance (ANOVA) and Duncan's Multiple Range Test. The significance level was set at *P* value <0.01.

3. Results and discussion

3.1. Biofilms formation

This study aimed to determine the pathogenesis factors attributed to the formation of biofilms and to enzymatic activities of *C. albicans* isolates. According to the results of the crystal violet test, isolates were able to form these structures with varying levels; 53.33% of them were highly biofilm-forming, while 46.66% were moderate biofilmogenic. These results confirm those of Touil et al.[21] who have already demonstrated this power in these same strains. Other studies have shown that clinical strains of *C. albicans* have the potential to form biofilms.[30-32]

3.2. Enzymatic activity

As far as the synthesis of hydrolytic enzymes is concerned, 73.33%, 93.33% and 86.66% of *C. albicans* isolates were respectively positive for the synthesis of Plp, Prt and Est. Similarly, Cgl and Hls activities were observed in 80% and 86.66% of isolates, respectively. With reference to the Pz values obtained, the activities of these enzymes differ from one strain to another. Indeed, high activity was observed for Plp, Prt and Est, respectively in 20%, 40% and 80% of the isolated strains. In contrast, 33.33% and 20% of the strains exhibited moderate activity for Plp and Prt, respectively. According to figure 1, these two enzymes were recorded with weak activities respectively in 13.33% and 33.33% of the isolates;while no more than 26.66%, 6.66% and 13.33% of isolates showed no activities for Plp, Prt and Est, in that order.

Statistical analysis showed that only A11 and A13 presented significant reduced enzymatic activities than the reference strain (figure 1). On the other hand,

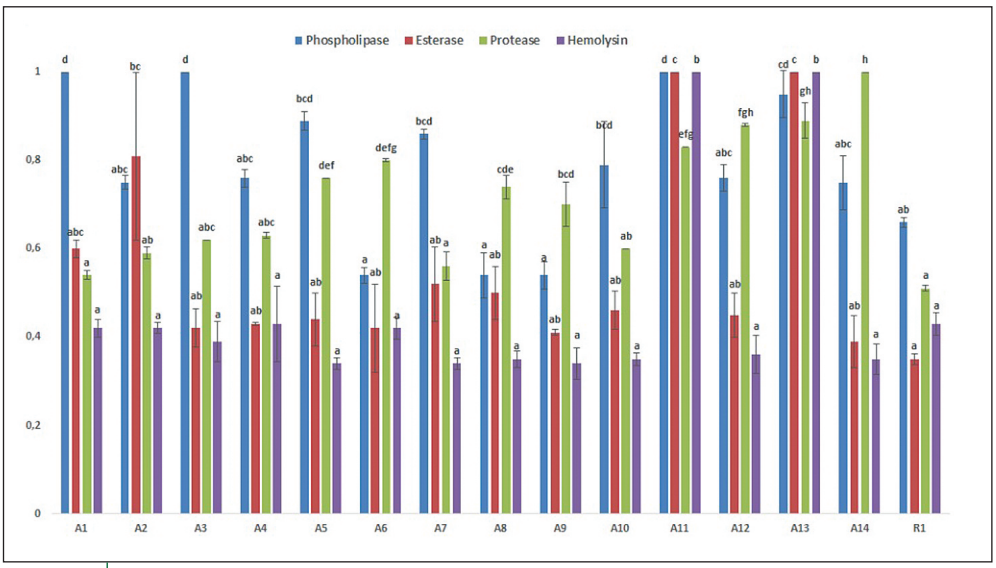

Figure 1 Mean production of hydrolytic enzymes in *Candida albicans* strains according to Pz values. Non-identical letters for the same enzymatic activity indicate significant differences between the means according to Duncan's mean separation test at the critical threshold of 0.01. Vertical bars show standard errors. A1-A14: isolated strains, R1: *C. albicans* ATCC10231.

ten isolated strains (A2, A4 - A10, A12 and A14) had no significant difference with regard to the Plp activity compared to *C. albicans* ATCC 10231. For the Prt activity, A1-A4, A7 and A10 had no significant difference with that of the reference strain. However, only three isolated strains (A2, A11 and A13) showed a significant difference with regard to the Est activity of R1. Except A11 and A13 which remained negative for certain enzymatic activities (Pz=1), all isolates had hemolysin activity which did not differ significantly from that of R1. All of these results suggest that the majority of isolates had active hydrolytic potential; in other words, the expression of these activities seems to be considered as a virulence factor attributed to these strains. (Figure 1)

An emphasis on hydrolytic enzymes produced by *Candida* spp. can, in fact, help in understanding the disease process better as these enzymes have activity on a wide array of host substrates,[8] but the quantity and the potential of the enzymatic activity are different.[33,34] It seem that the type of incorporated medium, the time of incubation, anatomically distinct sites of *Candida* isolates, the type of biological samples tested and the patient groups affect the levels of extracellular enzyme activities.[35]

Similarly to the results of this study, Chin et al.[36] reported that the activity of Plp was detected in 75% of their *C. albicans* isolates. For Pinto et al.[37] and Ramos et al.,[38] respectively 99.4% and 100% of their isolates had positive Plp activity. Furthermore, high levels of Prt activity had also been reported by many studies.[7,38-41] The Est activity, another virulence factor analyzed in this study, was detected in 86.66% of isolates. These results were in agreement with those obtained by others.[25,34,42] At the opposite, Deepa et al.[39] and Noori et al.[43] respectively showed that no more than 59% and 68.2% of *C. albicans* produced Est. Moreover, in this study, the synthesis of hemolysin was observed in 86.66% of isolated clinical strains, this result is lower than that reported by Sachin et al.[44] Additionally, high hemolytic activity has been observed in clinical strains of C. albicans.[14,27,34,39,40] Conversely to the results obtained by the coagulase test, Padmajakshi et al.[45] reported that the activity of this enzyme was observed only in 3% of *C. albicans* strains. In addition, Yigit et al.[46] did not record any coagulase activity using human serum; but 45.3% of the same strains were positive for the synthesis of this enzyme using rabbit serum. It should be remembered that the results obtained in this study were tested with human serum.

3.3. Effect of pH and temperature on the synthesis of Plp, Prt and Est

According to the results obtained, A6, A8 and A9 showed high Plp activity during their incubation whatever the pH (5 or 7) and the temperature (30°C or 37°C). A2 and A7 had maintained their hydrolytic activities unchanged, respectively moderate and weak (Pz = 0,73 ± 0,01 and 0,83 ± 0,01), despite the change in incubation conditions. However, no Plp activity was observed for A11 and A13. Conversely, the rest of the strains had variable enzymatic activities depending on the pH and the incubation temperature. Regarding proteinases, no strain showed activity at pH=7 whatever the incubation temperature; these results showed that this pH value was not favorable to that hydrolytic activity. At pH 3.5, however, A4, A10, A11 and A13 maintained their activities unchanged despite the change in temperatures; but for the other isolates, variation in the enzymatic activity was observed when the pH and/or the incubation temperature changed. Referring to figure 2, 85.71% of the strains had a high esterase activity whatever the experimental conditions of pH and incubation temperature. The exception was observed with A11 and A13 for which no esterase activity was recorded. (Figure 2)

According to figures 2 and 3, *C. albicans ATCC10231* (R1) showed high activity for the enzymes studied, although Plp activity was moderate at pH=7 and 30°C

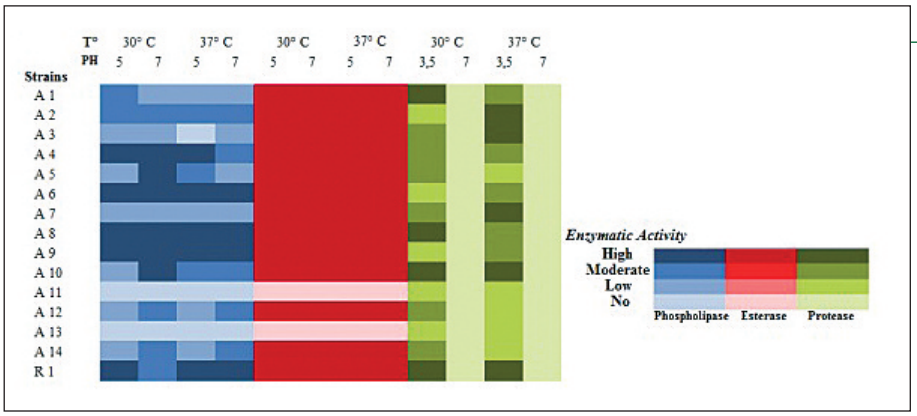

Figure 2

Levels of hydrolytic activities of Plp, Prt and Est of *C. albicans* under different pH and temperature conditions.

(Pz value=0.75 ± 0.01). Additionally, after changes in pH and incubation temperature, the results showed a significant difference in the Plp and Prt activities of all the isolates compared to R1 (P< 0.01). However, this observation was not recorded in the same way for Est activity in A1, A3, A6, A7, A8, A10, A12 and A14 which was not significant compared to the reference strain.

Overall, despite the fact that the enzymatic activities were kept unchanged for certain strains, the results obtained suggest that *C. albicans* has variable Plp, Prt activities depending on pH and incubation temperature; indeed, the strains-pH-temperature interaction was not significant (P<0.01). For Est, neither pH nor incubation temperature had any influence on its activity level; the significance of strains-pH-temperature interaction was observed (P=0.187). (Figure 3)

According to several studies, environmental factors such as pH and temperature influence the virulence of *Candida*.[47-50] Indeed, Mukherjee et al.[51] detected that phospholipase B was expressed differently under varying environmental and physiological conditions and that the optimal pH was 5.9 with temperature of 30 and 37°C; on the other hand Samaranayake et al.[52] have shown that *C. albicans* secretes Plp only within a pH range of 3.6 to 4.7. Conversely, for Bornscheuer et al.,[53] microbial lipases are active over a wide range of pH (7 to 9) and temperature (30 to 40°C). According to White and Agabian,[54] pH affects the expression levels of the aspartic proteinase (Sap) isoenzymes of this species. Indeed, Sap1, Sap2 and Sap3 have the highest activity at low pH, while Sap4, Sap5 and Sap6 are more active at high pH.[55,56] This difference in pH gives *C. albicans* a range of proteolytic activity from 2 to 7, which may be essential for this yeast during its existence in the vaginal mucosa (acid pH) or the oral cavity (neutral pH).[57,58] On the other hand, it has been demonstrated *in vitro* that SAP2 is the main gene expressed by *C. albicans* at 30 and 37°C.[59] In contrary, Monod et al.[60] revealed that the optimal expression temperature of SAP8 was 25°C, which suggests that its expression is temperature dependent. However, the expression of SAP9 and SAP10 seems to be independent of environmental conditions.[61,62] Other studies reported that 100% of *C. albicans* isolates produced Est at pH 6.8 and that these results were similar at 30°C and 35°C.[25,63] It should be noted that controversial results were reported by various works, Chi and his colleagues[64] indicated that *Candida* sp. produced these enzymes at temperatures ranging from 30°C to 40°C and a pH at 6 to 8. For Alami et al.,[65] these environmental conditions vary from 27°C to 45°C and from 4.5 to 7 for the pH. In contrast, Korbekandi et al.[66] revealed that the optimal temperature and pH for Est activity were 30°C and 7, respectively.

4. Conclusion

The aggressiveness of *C. albicans* is multifactorial; virulence and pathogenesis of this species are attributed to the effect of biofilm formation and hydrolytic activity. The present study showed that isolates of *C. albicans* were capable of forming biofilms in vitro and also exhibited hydrolytic activities of Plp, Prt, Est, Cgl and Hls. These potentials vary from one strain to another and have been recorded in all isolated strains at variable rates. Moreover, the hydrolytic activity was influenced by the pH and/or the incubation temperature; changing these settings affects activities levels of Plp, Prt; but Est activity had not been influenced.

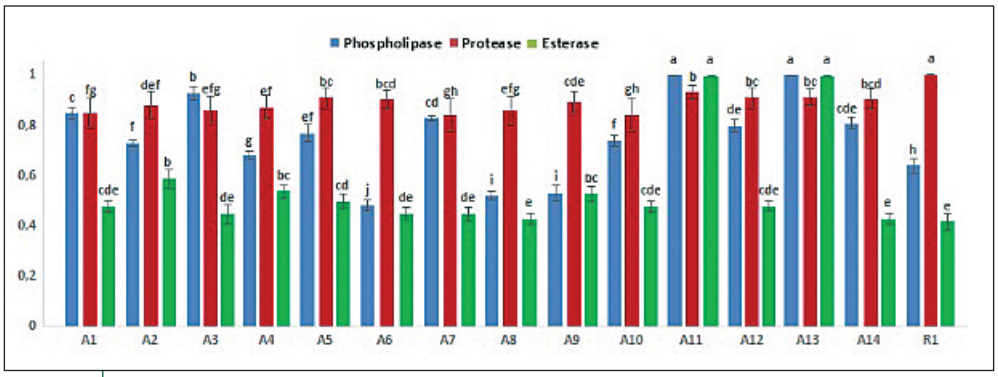

Figure 3 Averages of Pz values of Plp, Prt and Est activities in *C. albicans* at different pH and incubation temperatures. Non-identical letters for the same enzymatic activity indicate significant differences between the means according to Duncan's mean separation test at the critical threshold of 0.01.Vertical bars show standard errors. A1-A14: isolated strains, R1: *C. albicans* ATCC1023.

This may explain the fact that, the significance of strains-pH-temperature interaction depends on the type of enzyme. The importance of these enzymes as biochemical virulence factors of *C. albicans* partly explains its invasive opportunistic nature, because they further accentuate the virulence of this species.

Περίληψη

Υδρολυτική δράση και δημιουργία βιομεμβράνης κλινικών στελεχών *Candida albicans*: ποια η επίδραση της αλλαγής pH και θερμοκρασίας;

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Η *Candida albicans* είναι το συχνότερο είδος ευκαιριακού μύκητα απομωνούμενου στο νοσοκομειακό περιβάλλον και υπεύθυνη για διεισδυτικές μυκητικές λοιμώξεις. Η δημιουργία βιομεμβρανών (biofilms) και η παραγωγή υδρολυτικών ενζύμων είναι δύο κύριοι παράγοντες λοιμογονικότητας του συγκεκριμένου είδους. Η παρούσα μελέτη σκοπό είχε τη διερεύνηση της δράσης των υδρολυτικών ενζύμων κλινικών στελεχών *C. albicans* που παρήγαγαν βιομεβράνη, καθώς και η επίδραση των αλλαγών pH και θερμοκρασίας στη σύνθεσή τους. Η ικανότητα δημιουργίας βιομεμβράνης μελετήθηκε με την τεχνική χρωματισμού με κρυσταλλικό ιώδες. Για τη μελέτη των ενζύμων ακολουθήθηκαν οι παρακάτω τεχνικές: για τη σύνθεση της φωσφολιπάσης η καλλιέργεια σε καλλιεργητικό υλικό που περιείχε κρόκο αυγού, για τη δράση της πρωτεϊνάσης η καλλιέργεια σε άγαρ που περιείχε βόειο λευκωματίνη ορού, για την παραγωγή εστεράσης η χρήση της δοκιμής θολερότητας, για την παραγωγή κοαγκουλάσης η δοκιμή σε σωληνάριο και για την παραγωγή αιμολυσίνης η καλλιέργεια σε άγαρ με ερυθρά προβάτου. Επιπλέον, η δραστικότητα φωσφολιπάσης, πρωτεϊνάσης και εστεράσης εκτιμήθηκε υπό διαφορετικές συνθήκες θερμοκρασίας και pH. Τα απομονωμένα στελέχη *C. albicans* κατάφεραν να σχηματίσουν βιομεμβράνη και να συνθέσουν φωσφολιπάση, πρωτεϊνάση, εστεράση, κοαγκουλάση και αιμολυσίνη. Παρατηρήθηκαν διαφορές ως προς τη δραστικότητα αυτών των ενζύμων ανάλογα με το στέλεχος. Παράλληλα τα υπό μελέτη στελέχη παρουσίασαν υδρολυτική δράση. Η αλληλεπίδραση μεταξύ των στελεχών, του pH και της θερμοκρασίας εξαρτάται από τον τύπο των ενζύμων, γεγονός που εφιστά την προσοχή μας σχετικά με την καλύτερη κατανόηση της σχέσης μεταξύ αυτών των ενζύμων και της παθογένειας / μολυσματικής δράσης της *C. albicans*.

Λέξεις κλειδιά

Candida albicans, βιομεβράνη, υδρολυτικά ένζυμα, θερμοκρασία, pH

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