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Cell progression of biofilm formation in *Candida albicans* and estimation of aspartic proteinase activity

Rowida Ghareb Elshazly*[1], Shaimaa Selmi Sobieh[1], Sanaa Sobhy Zaki[1], Sahar Abdel Fattah Abdel Tawab[1]

[1]Botany Department, Faculty of Women for Arts, Science and Education, Ain Shams University, Cairo, Egypt.

Abstract:

Yeast cells are commonly used as powerful model system to study morphological phenotype and their processes. The study of morphogenesis of different modes of growth in yeast during biofilm development is an important issue. Cell progression is important tool to understand morphological transition and virulence in *C. albicans*. Biofilm formation and aspartic proteinase activity are two major important virulence factors associated with the pathogenicity of *C. albicans*. This study aimed to highlight the formation of biofilm, monitor the development of biofilm progression and estimate the activity of aspartic proteinase. Biofilm formation was assessed by Eliza plate and monitored at different pH values, different incubation time and different media. Biofilm development was visualized microscopically. Cell division and mitotic phases in yeast cells were studied and photographed. The production of aspartic proteinase was assessed by bovine serum albumin agar plates and evaluated at different pH values and different incubation time intervals. Results revealed that *C. albicans* was positive for biofilm formation and aspartic proteinase production. pH and different media had marked significant effect on biofilm formation. Additionally, the transition from simple yeast budding form to a complex filament structure was time dependent. Moreover, pH showed significant effect on aspartic proteinase activity.

Keywords: *Candida albicans*, Morphogenesis, Biofilm formation, Aspartic proteinase activity, Mitosis.

1. Introduction

Candida albicans is an opportunistic fungus that associated with several causes of mortality worldwide in individuals with immunocompromised system. It is the most frequently isolated species in both superficial and systemic infections and is classified as the third most pathogen isolated from bloodstream in hospitalized patients **[1,2,3]**. Morphological transformation, biofilm formation and the secretion of hydrolytic enzymes were considered the virulence factors associated with *C. albicans* pathogenicity mechanism. These factors increase the ability of *C. albicans* to infect different host sites **[4,5]**. *Candida albicans* is a polymorphic

***Corresponding author:** Rowida Ghareb Elshazly, Botany Department, Faculty of Women for Arts, Science and Education, Ain-Shams University, Cairo, Egypt.

E-mail: rowida.Elshazly@women.asu.edu.eg

fungus that grows either as ovoid budding yeast form, as elongated cells with constrictions at the septa (pseudohyphae) or as parallel walled true hyphae **[4,6]**. Polymorphic nature of *C. albicans* has a major effect on virulence and is essential during the switching from a commensal to a pathogenic state growth **[4,7]**. Morphogenesis regulation and cell progression linked with cell cycle control, which depended on specific cyclins **[8,9,10,11]**.

The ability to form biofilm on biotic or abiotic surface is one of the major important virulence factors because of its complex three-dimension structures consisted of a community of microbes embedded within an extracellular matrix (ECM), which provides protection to the microbes **[12,13,14,15]**. The transformation from planktonic cells to biofilm is contributed to a complex remodeling of morphological behavior supported by myriad change in genes expression **[16,17]**. pH and nutrient availability are the most important variables on the transition from planktonic cells to the biofilm cells **[18,19]**. *Candida albicans* undergoes transcriptional reprogramming and cellular progression in the host to make adaptation to different conditions that differ in pH, nutrient availability, CO_2 and O_2 levels **[7,20]**. Aspartic proteinase is another important virulence factor, promoting the invasion of host tissue through the degradation of cell surface protein **[21,22]**. Secreted aspartic proteinase encoded by SAP family which included ten genes; *SAP*1–10 **[23,24]** and is sensitive to environmental factors such as pH and temperature **[25]**.

The objective of this study was to detect biofilm formation and aspartic proteinase production by *C. albicans*. As well as, the evaluation of biofilm formation at different environmental conditions as pH, incubation time and culture media. Visualization of morphological progression of biofilm formation stages to follow up morphology regeneration. Determination of percentage of cell division and different phases of *C. albicans* cells to track the proliferation process. Moreover, evaluation of secreted aspartic proteinase activity at different pH values and different incubation time intervals.

2. Materials and methods

2.1. Yeast culture

Clinical identified *Candida albicans* strain obtained from Nephrology Department at Theodor Bilharz Research Institule Hospital, Giza, Egypt was used in this study **[26]** and it was identified using; chromogenic medium (CHROM agar *Candida*), Cornmeal-Tween 80 agar and germ tube formation tests in addition to biochemical identification by assimilation and fermentation tests **[27]**. Working culture was kept at $4^{O}C$ on universal agar slants **[28]**. For

long-term preservation, the culture was stored in vial tubes containing 1ml aliquot plus 20% glycerol at -20^{o}C.

2.2. Estimation of biofilm formation by the crystal violet method

Biofilm formation was assessed by using flat pre-sterilized polystyrene 96-well microplates **[29,30]** with slight modifications. *Candida albicans* strain was grown at 37°C for 24 h on Sabouraud Dextrose Agar (SDA). Then it was inoculated into a flask containing Sabouraud Dextrose Broth (SDB) with 8% glucose. The turbidity of the suspension was adjusted to $3x10^{7}$colony forming unit\ml (cfu/ml) as determined by comparative plate count and spectrophotometric reading using spectronic 21 **(Bauch and Lomb, New York, USA)** spectrophotometer. The flask was diluted at a ratio of 1:20 by using SDB with 8% glucose. A volume of 200 µl of this dilution was added to single well of the sterile microplate. After incubation at 37°C for 48 h, the broth was discarded and wells were washed with 200 µl phosphate-buffered saline (PBS) **(Caisson laboratories, lnc, USA)**. Adhered cells were stained with 200 µl of 0.1% crystal violet **(BioShop Canada lnc.)** for 20 min then washed with PBS. The crystal violet is solubilized by adding 200 µl of acetone: ethanol mixture (20:80 v/v) for 10 min and the absorbance was read at 450 nm by an Elisa reader **(Biotek ELx808, USA)**. Negative control is the medium without inoculum.

The biofilm formation was recorded as described by Rodrigues *et al*. [31]:

Negative; no biofilm formation ($OD_s = OD_{nc}$)

Weak biofilm formation ($OD_{nc} < OD_s \leq 2OD_{nc}$)

Moderate biofilm formation ($2OD_{nc} < OD_s \leq 4OD_{nc}$)

Strong biofilm formation ($4OD_{nc} < OD_s$)

Where OD_{nc} is the absorbance of negative control (medium without inoculum) and OD_s is the absorbance of sample.

2.3. Evaluation and optimization of factors affecting biofilm formation

All studied factors were carried out by **[29,30]** with slight modifications.

2.3.1. Effect of different pH values

Estimation of biofilm formation was carried out using SDB with 8% glucose at different pH values ranging from 4 to 8 (0.5 interval).

2.3.2. Effect of incubation time

The optimum pH of *C. albicans* was obtained from the previous experiment and was used to establish the effect of different incubation time intervals (90 min, 8 h, 24 h and 48 h) on biofilm formation.

2.3.3. Effect of culture media

The optimum pH and incubation time of *C. albicans* that obtained from the previous experiments were used to estimate the effect of different culture media, Sabouraud Dextrose Broth (SDB) with 8% glucose and Roswell Park Memorial Institute (RPMI 1640) **(Caisson laboratories, lnc, USA)** on biofilm formation.

2.4. Visualization of biofilms development by light microscope

For visualization of biofilm phases, first biofilm formation was assessed according to **Shin *et al*. [32]** with modification. Overnight grown yeast cells on Sabouraud Dextrose Agar (SDA) were washed with 5 ml of sterile saline, centrifuged at 5000 g for 10 min and the pellet of yeast cells was resuspended in Sabouraud Dextrose Broth (SDB) with 8% glucose at pH 4 in a turbidity equivalent to $3x10^7$ cfu/ml. A volume of 0.5 ml of the suspension was inoculated into a polystyrene tubes containing 4.5 ml of SDB. After incubation at 37°C for 90 min, 8 h, 24 h and 48 h, the broth cultures were discarded, then the internal walls of the tubes were washed with distilled water and then, the adhered layers of biofilm forming *C. albicans* on the internal walls and the bottom of the tubes were collected according to **Samaranayake *et al*. [33]** by adding 1ml PBS in the tubes and vortexed at 180 rpm for 2 min to disperse the biofilm then, 200 µl of this suspension were placed on glass slides and covered with glass covers. The morphology of biofilm formation stages of *C. albicans* was visualized by the light microscope **(Olympus, BX50, Japan)**, with magnification of 1000 x. The actual size of an object is calculated by ocular and stage micrometer.

The studied images were calibrated by magnification formula in biology:

Actual size of an object = length of the object ÷ magnification

https://www.biomadam.com/how-to-calculate-magnification-in-biology

2.5. Determination of percentage of cell division

Grown *Candida albicans* in SDB with 8% glucose at pH 4 after 48 h of incubation was used to determine percentage of cell division for yeast cells, by staining yeast chromosomes suspension by aceto-carmine stain according to **Nagel [34]**. Then, a drop of *Candida albicans*

suspension was placed on a glass slide to which a drop of aceto-carmine stain was added and covered with a glass cover. Then examined under the light microscope (**Olympus, BX50, Japan**) with magnification of 1000x and photographed.

Percentage of cell division was calculated as follow:

$$\% \text{ of cell division} = \frac{\text{No. of dividing cells}}{\text{No. of total cells}} \times 100$$

Percentage of each dividing phase was calculated as follow:

$$\% \text{ of dividing phase} = \frac{\text{No. of phase cells}}{\text{No. of dividing cells}} \times 100$$

2.6. Determination of secreted aspartic proteinase activity

Candida albicans was tested for production of SAP using bovine serum albumin (BSA) agar medium. The production of SAP was determined according to **[35,36]**. *Candida albicans* strain was precultured on Yeast Extract Peptone Dextrose **(**YEPD) agar at 37°C for 24 h. Sterile filter paper disks with a diameter of 6 mm were dipped into a suspension of yeast at density of 3x 10^7 yeast cells/ml in YEPD broth and applied to plates of bovine serum albumin medium. Plates were incubated at 28°C for 7 days. After that, plates were fixed for 10 min with 20% trichloroacetic acid and stained for 15 min with 1.25% amido black. Then, decolourization was performed using15% acetic acid for 72 h with several changes.

Enzyme activity was recorded according to the following criteria:

(-): Negative; no clear zone around the growth

(+): Mild activity; the clear zone around the growth was 1-2mm

(++): Strong activity; the clear zone around the growth was 3-5 mm

2.7. Evaluation and optimization of factors affecting secreted aspartic proteinase production

All studied factors were carried out according to **[35,36]**.

2.7.1. Effect of different pH values on secreted aspartic proteinase activity

Secreted aspartic proteinase activity was carried out using bovine serum albumin medium with different pH values ranging from 4 to 8 (0.5 interval).

2.7.2. Effect of different incubation time on secreted aspartic proteinase activity

The optimum pH of *C. albicans* that was obtained from the previous experiment was used to establish the effect of different incubation time intervals (2, 3, 4, and 5 days) on secreted aspartic proteinase activity.

2.8. Statistical analysis

All experiments were performed in triplicate, and the recorded data were expressed as mean ± standard deviation. One-Way ANOVA according to SPSS software Version 16.0 **[37]** followed by A post –hoc test (Duncan test) was applied to estimate particular differences in means. Moreover, the significant difference between the means of two interventions were assessed using independent sampled t- test. Statistically, significant difference was accepted for $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$.

3. Results and discussion

3.1. Biofilm formation

The present result reveals that *C. albicans* was able to form strong positive biofilm at OD 0.738 compared to negative control at OD 0.047. According to several studies, most infections caused by *C. albicans* were contributed to biofilm formation **[15,38]**. This result agrees with **Tiwari *et al*. [39]**, who found that all studied isolates of *C. albicans* were positive for biofilm formation. Moreover, **[40,41,42]** found that *C. albicans* was able to form biofilm.

3.2. Effect of different pH values on biofilm formation

The present results show fluctuating pattern of biofilm formation at different pH values, *C. albicans* showed statistically marked significant difference in biofilm formation in a wide range of pH values (4:8) with 0.5 interval ($p^{***} < 0.001$). *Candida albicans* showed optimal and highest biofilm formation at pH 4 **(Fig. 1)**. According to several studies, the formation of biofilm is very complex and affected by pH, nutrient availability and other factors. **[18,19,43]**. pH change induced signal pathways which allow the microorganism to sense and adapt to different pH values **[44]**. *Candida* had the ability to proliferate in media with a wide pH ranging from 2 to 10 **[44,45]**, and this dynamic character plays a key role in changing gene expression and morphology transition **[43,46]**. These results are in agreement with **[47,48]** who observed that an acidic pH had a significant impact on biofilm formation by *Candida* species. Moreover,

[49] reported that *C. albicans* in artificial urine at acidic pH form slightly higher biofilm than those formed in neutral pH.

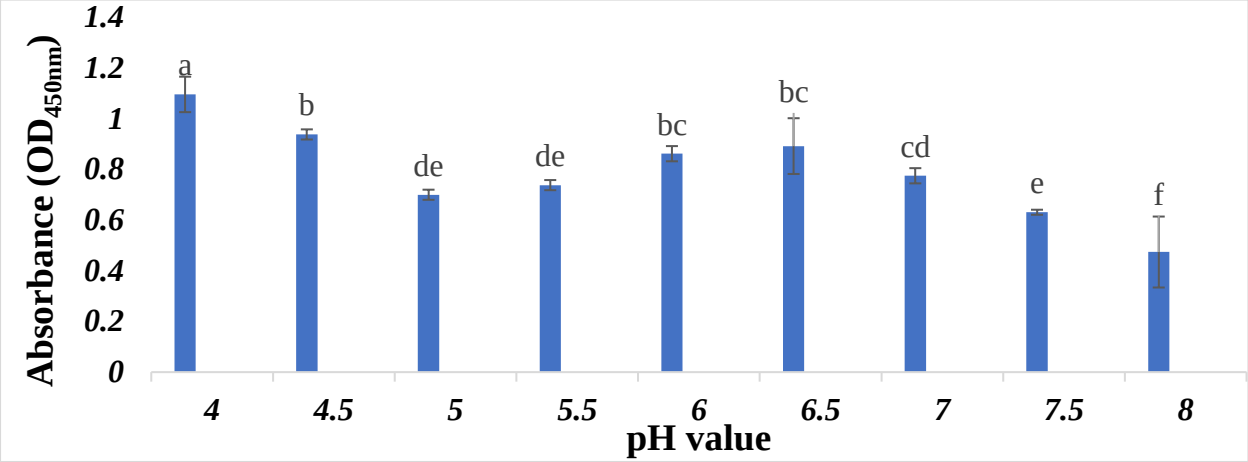


Fig. 1. Effect of different pH values on biofilm formation by *C. albicans*.

Non-identical letters indicate significant differences between the means according to Duncan's test.

3.3. Effect of incubation time on biofilm formation

The current results reveal that the biofilm formation was directly proportion to incubation time. *Candida albicans* formed weak biofilm at 90 min, moderate biofilm at 8 h and strong biofilm at 24 and 48 h. Incubation time had a marked significant effect on the biofilm forming *C. albicans* ($p^{***} < 0.001$) **(Fig. 2)**. According to previous studies, the development of biofilm depended on incubation time and occurred in a sequential process over a period of 24–48 h, the first step was adhesion of yeast cells to a surface, followed by the initiation step, cells were proliferated and formed hyphae then maturation step that was followed by the production and accumulation of extracellular matrix and finally dispersal phase, when non adhered yeast cells were released to form new biofilms **[50,51]**. These results are in accordance with **Pumeesat *et al.* [52]** who found that the biofilm formation gradually increased by increasing time (2, 4, 6, 8, 24 and 48 h) at 37°C. Moreover, **[53]** found that the biofilms formed by *C. albicans* at different time (0, 12, 24 and 48 h) increased by increasing incubation time. As well, **[54]** reported that incubation time intervals had a significant effect on the biofilm formation.

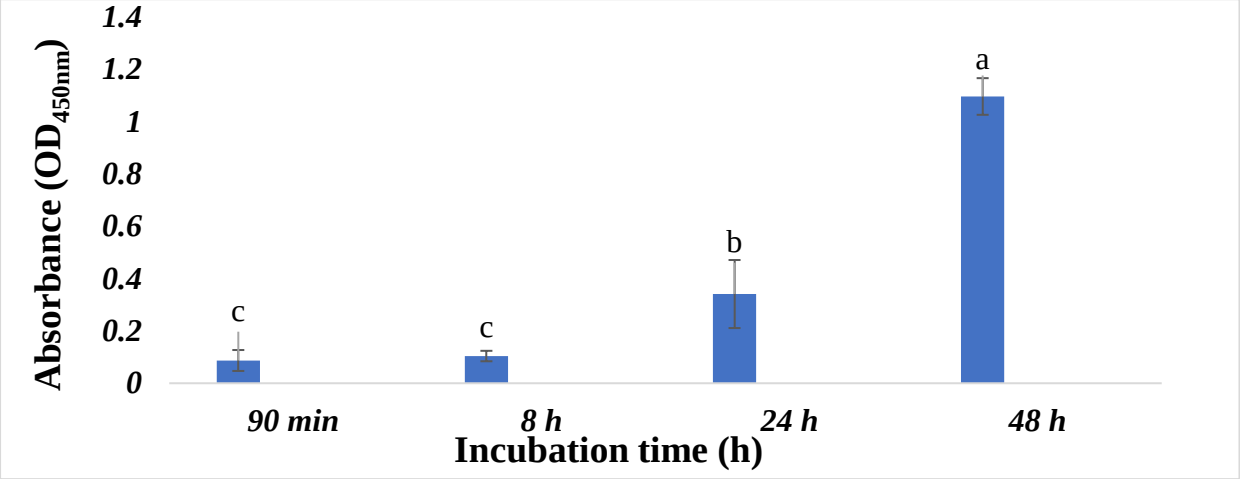


Fig. 2. Effect of incubation time on biofilm formation by *C. albicans*.

Non-identical letters indicate significant differences between the means according to Duncan's test.

2.4. Effect of culture media on biofilm formation

The present results indicate that Sabouraud Dextrose Broth (SDB) medium with 8% glucose showed maximum value of biofilm formation. Culture media showed marked significant effect on biofilm forming *C. albicans* (p*** < 0.001) **(Fig. 3)**. According to previous studies, biofilm formation is affected by nutrient availability **[19,55]**. Sabouraud Dextrose Broth medium (SDB) with 80 g/l glucose contains a high content of glucose which considered the energy source for the growth of microorganisms and peptone that provides nitrogen, vitamins, minerals, amino acids and growth factors as reported by **[56]**, while RPMI 1640 medium contains low content of glucose (2 g/L glucose) and a high content of amino acids: (L-Glutamine, L-Arginine, L- Asparagine) as well as vitamins and inorganic salts. These results are in accordance with **Hosida *et al.* [57]**, who found that biofilm formed on SDB medium by crystal violet and colony forming unit (CFU) assay was higher than RPMI1640 medium. Conflicting evidence on this result proved by **[55,56]**, who found that RPMI 1640 medium formed a significantly higher biofilm than SDB in *Candida* species. This contrary result in the present study may be due to differences in the strain and culture conditions.

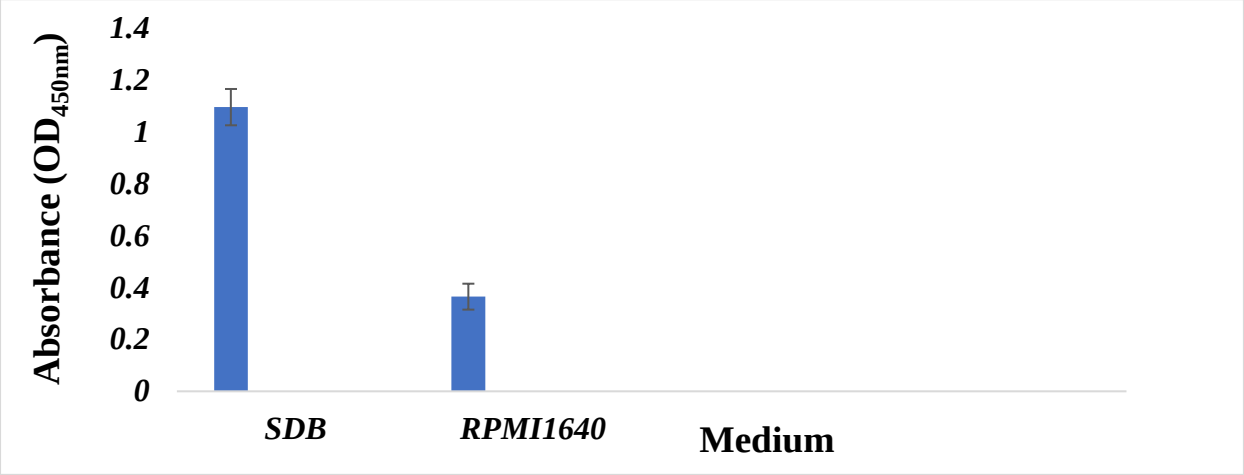
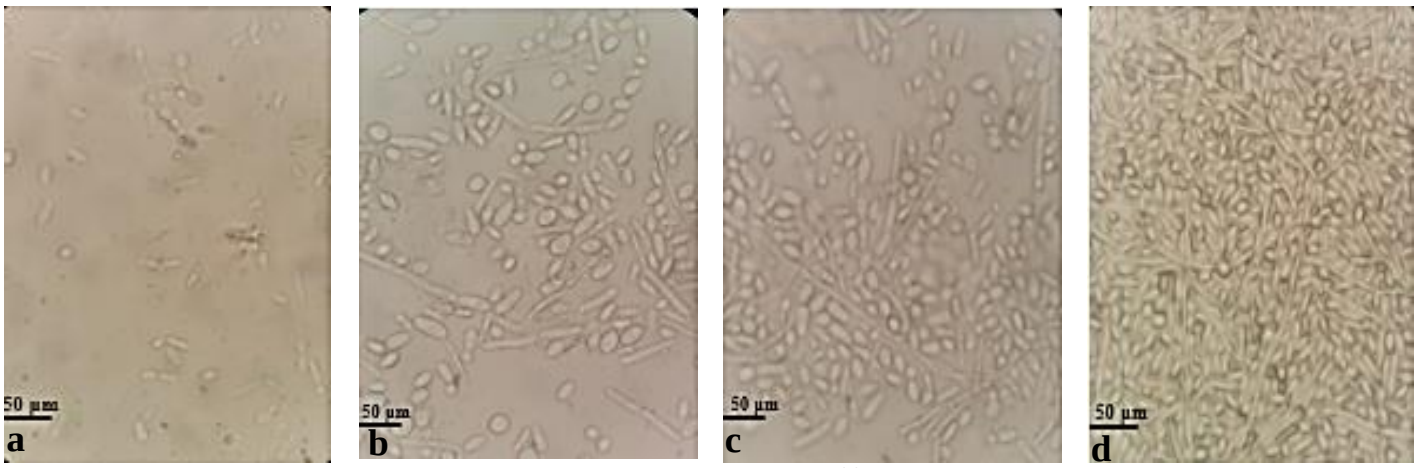


Fig. 3. Effect of culture media on biofilm formation by *C. albicans*.

SDB: Sabouraud Dextrose Broth with 8%glucose
RPMI1640: Roswell Park Memorial Institute
$p < 0.001$

2.5. Morphology progression of biofilm by the light microscope

The present results are confirmed by observing the morphological progression of the formed biofilm of *C. albicans* that was grown on SDB with 8% glucose at pH 4 for 90 min, 8 h, 24 h and 48 h by the light microscope. *Candida albicans* appeared as yeast with germ tubes after initial adhesion time 90 min **(Fig. 4a)**, ovoid yeast, and elongated yeast (pseudohyphae) after 8 h **(Fig. 4b)**, the biofilm characterized by yeast cells, pseudohyphae and true hyphae after 24 h **(Fig. 4c)** and finally, a dense network of biofilm with yeast, pseudohyphae and true hyphae with extracellular matrix (ECM) was observed after 48 h **(Fig. 4d)**. These results are in agreement with **Bonfim-Mendonça *et al.* [24]**, who found that recurrent vulvovaginal candidiasis appears as yeast form after 2 h and increased in filamentation after 6 h. in addition, **[58]** noted that the number of germ tube formation after 2 h decreased since germ tubes formed was transformed to pseudo hyphae and hyphae. As well, **[33]** found that after 90 min, *C. albicans* form microcolonies with germ tubes, after 24 h, the biofilm was consisted of yeast and hyphal cells and after 48 h thin biofilm was formed with extracellular material using scanning electron microscope. Also, **[59]** found that at time of 0-2 h *C. albicans* was found as yeast form and that the amount of cell growth and extracellular matrix increased by increasing incubation time. Moreover, **[60]** stated that after 2, 6 and 8 h of incubation biofilm was consisted of yeast and hyphal cells.



Actual size of oval yeast = 3 µm

Actual size of pseudohyphae = 20 µm

Actual size of filament = 30 µm

Actual size of oval yeast= 2 µm

Fig. 4. Morphology of *C. albicans* visualized by light microscope at a magnification of 1000x.

(a): yeast cells with germ tubes after 90 minutes of incubation, **(b**): yeast cells and pseudohyphae after 8 hours of incubation **(c):** yeast cells with pseudo and true hyphae after 24 hours of incubation and **(d):** network of yeast, pseudo and true hyphae with extracellular matrix after 48 hours of incubation. Scale 50 µm.

2.6. Determination of the percentage of cell division and different phases of *C. albicans*

Staining of yeast chromosomes showed the dividing cells with different phases **(Fig.5)**. The percentage of cell division of *C. albicans* was 12.6. Additionally, studying the percentage of several mitotic phases revealed that yeast cells were accumulated in metaphase on the expense of the other phases. Percentage of metaphase was 84.13, while percentage of prophase, anaphase and telophase were 7.94, 4.76 and 3.17 respectively **(Fig.6)**. This result reveals that yeast cell division occurs in normal sequence and most of dividing cells were at metaphase stage. According to previous studies, mitosis is a crucial cellular process that ensures faithful transmission of genetic material to the following generation **[61]**. Asexual reproduction of budding yeast would lead one to anticipate that its nuclear and mitotic division will display specific unique phenomena. Despite the extensive research that has been done on the yeast cytology, there is still no consensus on this issue since the yeast cell is so small, making it difficult to accurately observe even the smallest details. **[34]**. However, **Milne *et al*. [62]** indicated that the regulation of mitosis in *C. albicans* is poorly understood, and the circuitry governing the transition from metaphase to anaphase, mitotic exit and during transformation

from planktonic cells to biofilm mass and vice versa has not been clearly described. Yeast mitotic division will need extensive studies.

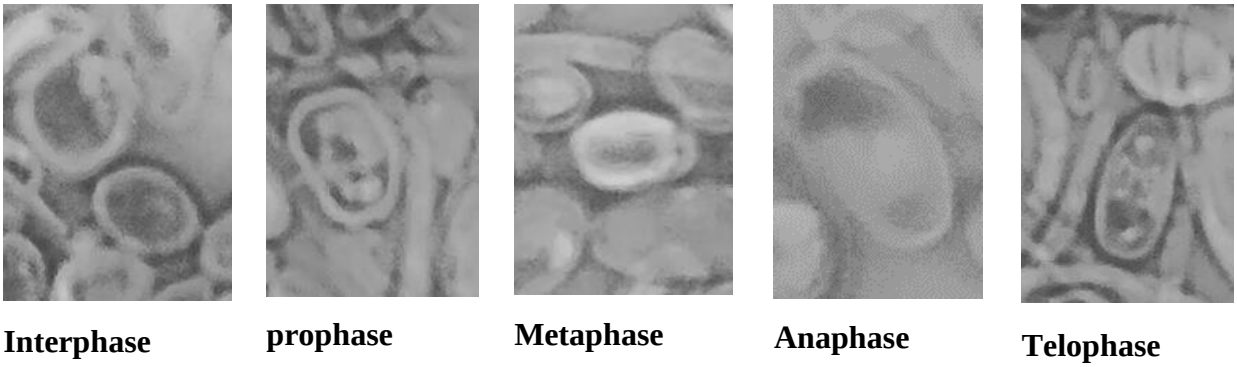


Fig. 5. Different phases of *C. albicans* cell division.

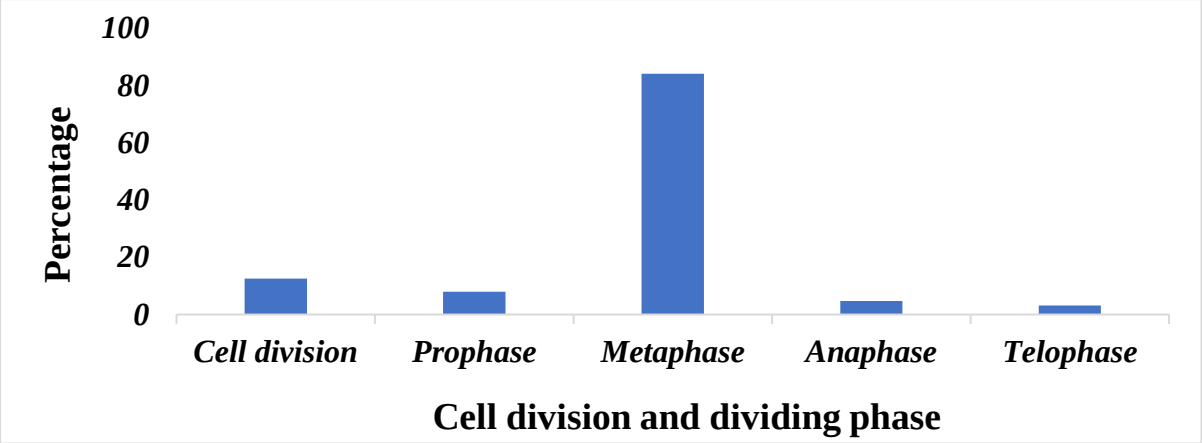


Fig.6. Percentage of cell division and different phases of *C. albicans*.

3.7. Secreted aspartic proteinase activity

Secreted aspartic proteinase activity assessed by bovine serum albumin agar plate method. The present result reveal that *C. albicans* was positive for SAP production with clear zone around the growth of 0.17 cm. According to previous studies, the production of aspartic proteinase enzyme by fungi increased penetration of the host tissue and colonization **[44,63]**. This finding is consistent with **Tefiani *et al.* [64]**, who indicated that all the *C. albicans* strains were positive protease activity. As well, **[65]** found that *C. albicans* produced SAP.

3.8. Effect of different pH values on secreted aspartic proteinase activity

The present results show marked significant difference in SAP activity at different pH values ($p^{***} < 0.001$) and SAP activity decreases with increasing pH value.

pH 4 was the optimum pH for SAP production where *C. albicans* showed strong (++) SAP activity (**Fig. 7)**. According to previous studies, SAP activity was affected by pH, where Sap1, Sap2 and Sap3 had optimum activity at lower pH and Sap4, Sap5 and Sap6 had optimum activity at higher pH **[25,66]**. This variation in pH gives *C. albicans* ability to proliferate in different host niches. These results are in agreement with **Tefiani *et al*. [25]**, who found that proteinase had activity at pH 3.5 and no *C. albicans* strains showed activity at pH 7 and there was variation in the activity of the enzyme when the pH changed. As well, **[47]**, found that the highest production of proteinase was at pH 4.0 and the lowest was at pH 5.5 followed by pH 7.0.

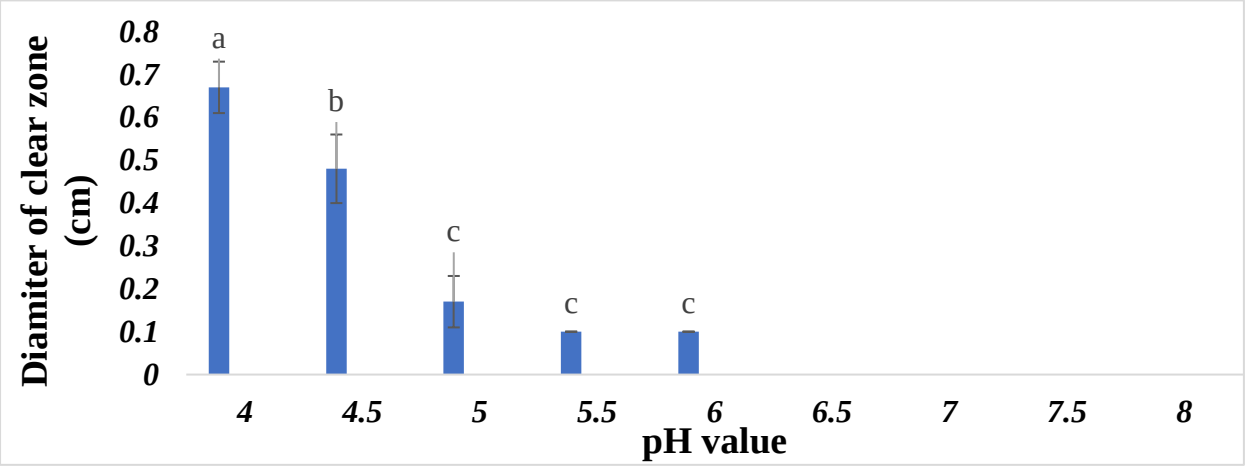


Fig. 7. Effect of pH values on secreted aspartic proteinase activity by *C. albicans*.

Non-identical letters in the same strain indicates significant differences between the means according to Duncan's test.

3.9. Effect of different incubation time on secreted aspartic proteinase activity

The current results reveal that no clear measurement of secreted aspartic proteinase activity was observed after 2, 3, 4 and 5 days of incubation. This result confirmed that secreted aspartic proteinase takes 7 days as optimum incubation period for activity as reported by **[35,36]**.

4. Conclusion

Cell progression of *Candida albicans* during biofilm formation is affected by different factors like pH, incubation time and nutrient medium. Sabouraud Dextrose Broth (SDB) medium with high glucose content at pH 4 increased *C. albicans* opportunity to form significant biofilm. Additionally, microscopic investigation indicated the relationship between morphological changes during biofilm development and experimental time. Cytological

results showed different phases of cell division with accumulation of most yeast cells at metaphase. As well, *Candida albicans* showed highest aspartic proteinase activity at pH 4 and that the secreted aspartic proteinase production was pH and time dependent.

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الملخص العربي

تطور الخلية لتكوين الغشاء الحيوى فى جنس الكانديدا البيكانز وتقدير نشاط إنزيم الاسبارتيك بروتينيز

رويدا غريب الشاذلى[1], شيماء سلمى صبيح[1] , سناء صبحي زكى[1] , سحر عبد الفتاح عبد التواب[1]

[1]قسم النبات، كلية البنات للأداب و العلوم و التربية، جامعة عين شمس، القاهرة، جمهورية مصر العربية

الملخص العربي

تستخدم خلايا الخميرة على نطاق واسع كنظام نموذجي لدراسة تطور الخلايا في الشكل الظاهرى والعمليات الحيوية المصاحبة. وتعتبر دراسة الشكل الظاهرى للعديد من أطوار النمو لخلايا الخميرة أثناء مختلف مراحل تكوين الغشاء الحيوى أمرا حيويا حيث يعد تتبع التطور الظاهرى لخلايا الكانديدا البيكانز أداة هامة لفهم تحولها ظاهريا من شكل لأخر وكذلك لمعرفة مدى ضراوتها. كما يعد تكوين الغشاء الحيوى و نشاط إنزيم الاسبارتيك بروتينيز من أهم عوامل الضراوة المرتبطة بإمراضية الكانديدا البيكانز. هدفت هذه الدراسة إلى تسليط الضوء على تكوين الغشاء الحيوى وتتبع مراحل تطوره. كما هدفت هذه الدراسة أيضا إلى تقدير نشاط إنزيم الأسبارتيك بروتينيز. تم تقييم تكوين الغشاء الحيوى بجهاز الاليزا باستخدام أطباق الإليزا عند العديد من قيم الأس الهيدروجيني ووقت التحضين ونوعين مختلفين من البيئات الغذائية وتم تصوير مراحل تطور الغشاء الحيوى مجهريا. كما تم دراسة وتصوير الإنقسام الخلوى و مراحله المختلفة في خلايا الخميرة. كذلك تم تقييم إنتاج انزيم الأسبارتيك بروتينيز باستخدام بيئة الألبومين البقرى وتقييم انتاجه عند العديد من قيم الأس الهيدروجيني ووقت التحضين. أظهرت النتائج قدرة الكانديدا البيكانز على تكوين الغشاء الحيوى وانتاج إنزيم الأسبارتيك بروتينيز. كما أظهرت النتائج ان للأس الهيدروجيني والبيئات الغذائية المختلفة تأثيرا ملحوظا على تكوين الغشاء الحيوى. بالإضافة إلى ذلك، أظهرت النتائج ان التحول في الشكل الظاهري للخميرة من خلايا بسيطة إلى خيوط معقدة التركيب يعتمد على زمن تحضينها. علاوة على ذلك، أوضحت النتائج أن للأس الهيدروجيني تأثيرًا معنويًا على نشاط إنزيم الأسبارتيك بروتينيز.