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FULL PAPER

Candida albicans biofilm development under increased temperature

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SUMMARY

C. albicans is one of the most important species of fungi known to produce biofilms on installed medical devices. The environment surrounding the fungi influences the development of the biofilm. Temperature is known to affect the yeast-to-hypha transition of *C. albicans,* but the impact of this factor on biofilm formation is still not understood. This study aimed to investigate the effects of temperature (42°C versus 37°C) on the formation of *C. albicans* biofilms. Three reference *C. albicans* strains were used: SC 5314, ATCC 90028, and ATCC 96901. Biofilm development was monitored in a series of time intervals, 2, 4, 6, 8, 24, and 48 h, at both 37°C and 42°C. Biofilm formation under each condition was evaluated by scanning electron microscopy, crystal violet staining, and 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-(phenylamino)-carbonyl-2H-tetrazoliumhydroxide reduction assay. Our results demonstrated that at 42°C, tested strains of *C. albicans* could produce a biofilm, but the mass, thickness, and metabolic activity were lower than those of the biofilm formed at 37°C.

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INTRODUCTION

Biofilm formation is one of the most important virulent factors of *Candida albicans* because its resistance to several antifungal drugs makes it difficult to eradicate (Seneviratne *et al.,* 2008), it affects the host immune responses (Nett, 2016), and it leads to medical device failures, requiring invasive removal procedures (Kojic and Darouiche, 2004). *C. albicans* biofilms are known to form in several medical devices, such as central venous catheters, prosthetic heart valves, artificial voice prostheses, and intrauterine catheters (Kumamoto, 2002; Kojic and Darouiche, 2004). In recent years, bloodstream infection caused by *Candida* spp. has been widely diagnosed, especially in immunocompromised patients (Sims *et al.,* 2005; Low and Rotstein, 2011; Anwar *et al.*, 2012). Biofilm-forming *Candida* infections (candidemia) affect the clinical outcome of patients, and are often associated with higher mortality rates (Tumbarello *et al.*, 2007; Tumbarello *et al.,* 2012).

Biofilms are defined as a microbial community attached to the surface of several materials. These microbial cells secrete and are surrounded by a matrix of extracellular polymeric substances (EPS), and typically display different phenotypic characteristics from their planktonic counterparts (Seneviratne *et al.,* 2008; Mathe and Van Dijck, 2013). Mature *C. albicans* biofilms are generally composed of yeast, pseudohyphae, hyphae, and EPS (Ramage *et al.,* 2001). A previous study has shown that the morphogenesis of *C. albicans* plays a role in the formation and persistence of adherent cells, resulting in the different architecture within each layer of the *C. albicans* biofilm (Baillie and Douglas, 1999).

Several factors influence *C. albicans* biofilm formation. For example, surface materials, medium composition, and nutrient and culture conditions (such as CO_2 levels) all lead to different type of biofilms (Hawser and Douglas, 1994; Daniels *et al.,* 2013). The effects of temperature on biofilm formation have been studied in bacteria such as *Pseudomonas aeruginosa, Klebsiella pneumoniae* and *Vibrio cholerae* (Hoštacká *et al.,* 2010). *C. albicans* can grow at 42-45°C (Pinjon *et al.,* 1998). Although *C. albicans* biofilm development models have been studied by several groups of researchers (Chandra *et al.,* 2001; Jin *et al.*, 2003; Melo *et al.*, 2011; Montelongo-Jauregui *et al.,* 2016), the effects of temperature, especially at 42 °C, have not been observed. The aim of this study was to investigate *C. albicans* biofilm formation at two different culture temperatures. The data from this investigation may provide a better understanding of *C. albicans* biofilm formation, thereby leading to the development of better methods for controlling this organism in critical medical environments.

MATERIALS AND METHODS

Candida albicans strains

Three strains of *C. albicans* were purchased from American Type Culture Collection (ATCC) for this study: *C. albicans* SC 5314, a well-characterized biofilm-producing strain; *C. albicans* ATCC 90028, an antifungal susceptibility testing strain; and *C. albicans* ATCC 96901, a fluconazole-resistant strain.

Key words:
Biofilm, Candida albicans, Crystal violet staining, XTT.

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Media and growth conditions
All three *C. albicans* reference strains were propagated on Sabouraud dextrose agar (SDA) at 37°C overnight. Subsequently, cells were cultured in Yeast Peptone Dextrose (YPD) broth (2% peptone, 1% yeast extract, and 2% glucose) overnight at 30°C to produce a culture of yeast cells.

Biofilm formation
C. albicans biofilm development for the above three reference strains was performed as previously described by Ramage *et al.* (2001). One-hundred microliters of a suspension containing 10^6 cells/ml in RPMI 1640 (with L-glutamine) (Gibco, USA), buffered to pH 7 with 3-(*N*-morpholino) propanesulfonic acid (MOPS, 0.165 M) (Fisher Scientific, China) was seeded into polystyrene 96-well flat-bottomed microtiter plates and incubated over a series of time intervals (2, 4, 6, 8, 24, and 48 h) at 37°C and 42°C. A well containing only medium acted as a negative control.

Scanning electron microscopy
Biofilm formation of each *C. albicans* strain was achieved on a 13 mm Thermanox plastic coverslip (Nunc) in 24-well cell culture plates (Ramage *et al.,* 2002). Standardized cell suspensions (2 ml of a suspension containing 1×10^6 cells/ml in RPMI 1640) were seeded on discs and incubated in a series of time intervals (2, 4, 6, 8, 24, and 48 h) at 37°C and 42°C. The coverslip discs were removed at each indicated time and washed with sterile PBS. The discs were placed in a fixative reagent (2.5% glutaraldehyde in sucrose phosphate buffer, pH 7.4) for 1 h. The samples were washed three times with sucrose phosphate buffer for 10 min each. The biofilm was immediately dehydrated in a series of ethanol washes (once each with 30%, 50%, 70%, and 90% ethanol for 10 min, and then twice with 100% ethanol for 10 min). Finally, the samples were dried in a desiccator.
The biofilm samples were coated with gold and observed under scanning electron microscopy (SEM) (JEOL JSM-6610LV, Japan). During development, the fungal biofilm spread to cover the disc, forming a multilayer microbial community. Therefore, a semi-quantitative assessment of the *C. albicans* biofilm development was measured by the H-score assessment, which uses percent coverage area of the biofilm (determined by the ImageJ program) multiplied by the layer score of biofilm thickness (1 = one layer, 2 = two layers, and 3 = three or more layers of biofilm thickness). ImageJ program was downloaded from https://imagej.nih.gov/ij/download.html. Ten fields of biofilms were randomly observed and captured at 1,000x magnification. The results are displayed as mean H-score and standard deviation.

Measurement of C. albicans biofilm development using crystal violet staining
Biofilm development was quantified by a slight modification to the crystal violet (CV) staining procedure, as previously described by others (Jin *et al.,* 2003; Melo *et al.*, 2011). The wells were washed twice with 200 µl sterile PBS and then air-dried for 45 min. The prewashed wells were stained with 110 µl 0.4% aqueous CV solution for 45 min. Stained wells were washed five times with 350 µl sterile distilled water, and then destained with 200 µl 95% ethanol for 45 min. After that, 100 µl of destaining solution was transferred to a new 96-well flat-bottom microtiter plate and absorbance values were measured with a microtiter plate reader at 595 nm (Tecan, Sunrise, Austria). The absorbance values of control wells were subtracted from those of test wells to eliminate background interference. Experiments were performed on two separate occasions with at least eight replicates for each occasion.

Measurement of C. albicans biofilm development by XTT reduction assay
Biofilm formation for each strain and condition was monitored by tetrazolium salt 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-(phenylamino)-carbonyl-2H-tetrazoliumhydroxide, or XTT (Sigma, USA) reduction (Melo *et al.*, 2011). After biofilm formation at the indicated time, the medium was aspirated and non-adherent cells were removed by thoroughly washing the biofilms twice with 200 µl PBS. Then, 200 µl of PBS and 12 µl of the XTT-menadione solution were added to each well**.** The XTT solution (1 mg/ml in PBS) was prepared and sterilized by filtering through a 0.22 µm pore-size filter, and then maintained at -70°C. The XTT-menadione solution was freshly prepared on the day of testing by mixing XTT (1 mg/ml in PBS) and the menadione solution (0.4 mM in acetone) at a ratio of 5:1 by volume (Jin *et al.,* 2003).
The plates were incubated in the dark for 2 h at 37°C. Next, 100 µl of solution were transferred to a new microtiter plate and the color change was measured with a microtiter plate reader at 490 nm. The experiments were performed twice on separate occasions with at least eight replicates each time. The absorbance values of negative control wells were subtracted from those of the test wells to eliminate background, and the arithmetic mean of the absorbance values was calculated.

Statistical analysis
The independent t-test and Mann–Whitney test were used to analyze the data. A p-value of <0.05 was considered statistically significant.

RESULTS

Scanning electron microscopy
Figure 1 displays the structure of the *C. albicans* biofilm at intervals of 2, 4, 6, 8, 24, and 48 h after incubation at 37°C and 42°C, as observed by SEM. At 2 h, *C. albicans* formed long hyphae and attached to the surface of the plastic coverslip forming a single layer of growth (thickness score =1). At 4 h, two layers of biofilms were formed (thickness score =2). Subsequently (6-48 h), multiple layers of biofilm growth, which consists of hyphae, pseudohyphae, and yeast cells, were observed at both 37°C and 42°C (thickness score =3) for all three tested strains.
In addition, we tried to quantify biofilm development with SEM observations using the H-score determination. *Figure 2* illustrates the mean H-score and standard deviation of all three *C. albicans* biofilms. H-scores were calculated by multiplying percent coverage area of the biofilm by the layer score of the *C. albicans* biofilm. The percent coverage area of the biofilm came from the ImageJ program. The results showed that the H-score of all studied strains gradually increased by time interval at both 37°C and 42°C. The mean H-score of biofilm development at 37°C was significantly higher than that at 42°C ($p<0.05$) in *C. albicans* SC 5314 at 4 h (55.28±2.72 *vs* 43.01±4.13), 6 h (225.61±13.24 *vs* 191.24±17.73), and 48 h (288.01±6.97 *vs* 254.34±8.78). In ATCC 90028, biofilm development at 37°C

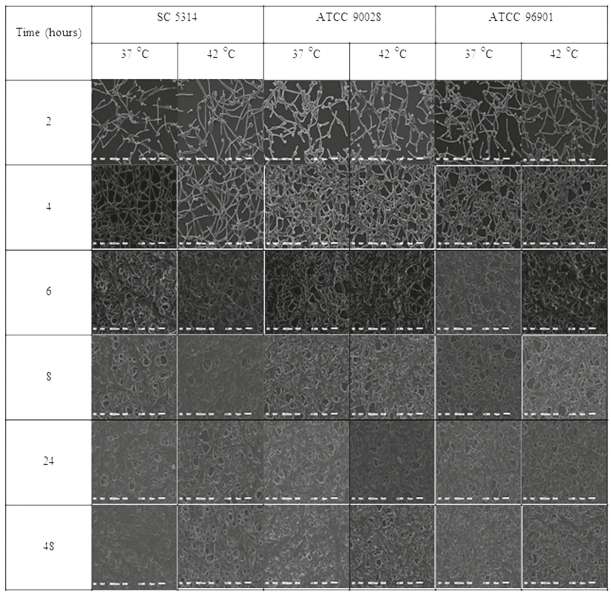

Figure 1 - *Biofilm development of 3* C. albicans *reference strains, SC 5314, ATCC 90028, and ATCC 96901, incubated at 37°C and 42°C for 2, 4, 6, 8, 24, and 48 h, as observed under a scanning electron microscope.*

versus 42°C was significantly higher at 8 h (233.90±8.95 *vs* 215.33±13.55) and 48 h (264.72±19.88 vs 241.71±11.62). For strain ATCC 96091, biofilm development at 37°C versus 42°C was significantly higher at 6 h (201.05±8.45 *vs* 193.07±9.37), 8 h (239.46±9.28 *vs* 220.7±11.05), 24 h (244.51±11.40 vs 226.0±11.42), and 48 h (270.08±15.75 *vs* 255.21±9.98). However, at the beginning of the incubation (2 h), the H-score at 42°C was significantly higher than that at 37°C ($p<0.05$) in ATCC 90028 (25.45±2.17 *vs* 32.52±4.02) and ATCC 96901 (26.11±3.54 vs 30.23±2.26).

Quantification of biofilm by Crystal violet

Biofilm development for three strains of *C. albicans* was investigated under the incubation conditions of 37°C and 42°C, for 2, 4, 6, 8, 24, and 48 h. Biofilm production of all three *C. albicans* reference strains gradually increased in a time-dependent manner. *Table 1* shows the mean A_{595} for biofilm mass observations using crystal violet staining. For *C. albicans* SC 5314, biofilm production at 37°C was significantly higher than at 42°C for each time interval (2, 4, 6, 8, 24, and 48 h) ($p<0.05$). These same significant patterns of biofilm development were observed for *C. albicans* ATCC 90028 ($p<0.05$) at each indicated time (*Table 1*).

Biofilm development for strain ATCC 96901 did not show significant differences in growth between temperatures at the 2 h or 6 h time interval; however, the mean A_{595} at 37°C versus 42°C was significantly higher ($p<0.05$) at 4

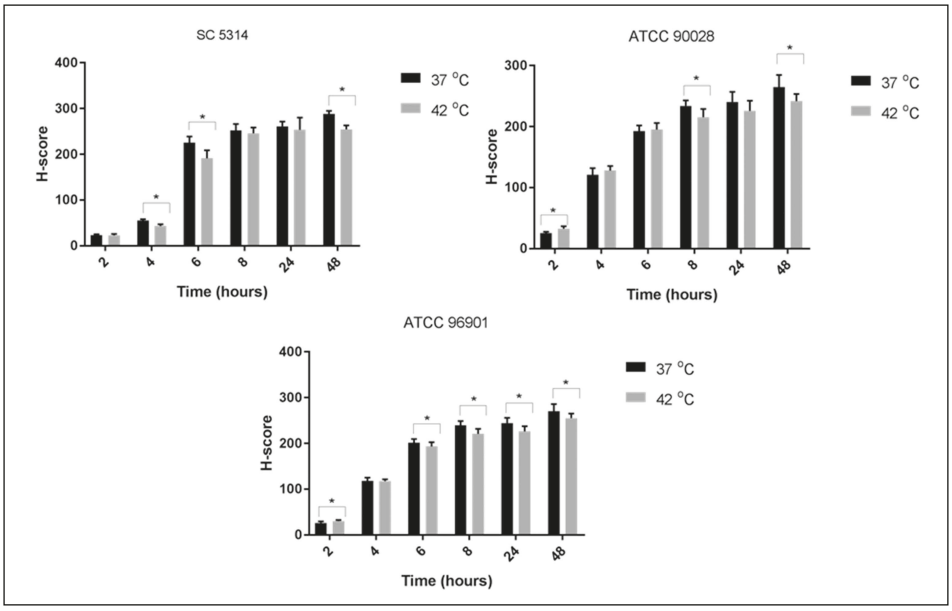

Figure 2 - *Biofilm development of the 3* C. albicans *reference strains incubated at 37 °C and 42°C for 2, 4, 6, 8, 24 and 48 h as analyzed by H-score. Results are presented as means ± standard deviations (error bar). *indicates a significant difference (P value<0.05).*

Table 1 - *Biofilm formation of the three* C. albicans *reference strains incubated at 37 °C and 42 °C for 2, 4, 6, 8, 24, and 48 h as examined by crystal violet staining. Results are presented as mean A_{595} ± standard deviations.*

Time (hours)	*SC 5314*		*ATCC 90028*		*ATCC 96901*	
	37 °C	*42 °C*	*37 °C*	*42°C*	*37 °C*	*42 °C*
2	0.19±0.11	0.08±0.03*	0.26±0.11	0.15±0.03*	0.23±0.09	0.2±0.06
4	0.43±0.12	0.23±0.13*	0.54±0.1	0.38±0.12*	0.43±0.03	0.38±0.09*
6	0.65±0.07	0.42±0.23*	0.64±0.09	0.50±0.19*	0.56±0.07	0.58±0.11
8	0.91±0.19	0.51±0.27*	0.87±0.13	0.60±0.23*	0.79±0.11	0.68±0.12*
24	1.04±0.17	0.86±0.18*	0.91±0.12	0.61±0.12*	0.92±0.14	0.78±0.07*
48	1.49±0.24	1.17±0.16*	1.33±0.17	0.88±0.13*	1.17±0.18	1.03±0.13*

*indicates a significant difference (*P* value <0.05)

Table 2 - *Biofilm metabolic activity of* C. albicans *reference strains, SC 5314, ATCC 90028, and ATCC 96901, incubated at 37°C and 42°C for 2, 4, 6, 8, 24, and 48 h metabolic activity, as determined by XTT assay. Results are presented as mean* A_{490} *± standard deviations.*

Time (hours)	SC 5314		ATCC 90028		ATCC 96901	
	37 °C	42 °C	37 °C	42°C	37 °C	42 °C
2	0.03±0.01	0.03±0.02	0.02±0.01	0.03±0.01	0.04±0.02	0.05±0.02
4	0.04±0.01	0.03±0.01	0.04±0.01	0.03±0.01	0.04±0.01	0.05±0.02
6	0.10±0.03	0.05±0.02*	0.11±0.02	0.04±0.02*	0.13±0.02	0.07±0.03*
8	0.14±0.05	0.06±0.02*	0.13±0.05	0.06±0.02*	0.13±0.03	0.1±0.04*
24	0.17±0.07	0.09±0.03*	0.15±0.05	0.07±0.03*	0.16±0.06	0.11±0.04*
48	0.22±0.04	0.15±0.03*	0.21±0.04	0.12±0.02*	0.25±0.04	0.19±0.05*

**indicates a significant difference (P value <0.05)*

(0.43±0.03 *vs* 0.38±0.09), 8 (0.79±0.11 *vs* 0.68±0.12), 24 (0.92±0.14 *vs* 0.78±0.07), and 48 h (1.17±0.18 *vs* 1.03±0.13) (*Table 1*).

XTT reduction assay for C. albicans *biofilm development*

Biofilm metabolic activity for each *C. albicans* strain, incubated at 37°C or 42°C, was measured by an XTT reduction assay at six incubation times: 2, 4, 6, 8, 24, and 48 h (*Table 2*). The results showed that at 37°C versus 42°C, biofilm metabolic activity was significantly higher for all three strains, especially during the latter growth stages of 6, 8, 24, and 48 h ($p<0.05$).

DISCUSSION

Previous studies have shown that *C. albicans* biofilm development starts from yeast cells attaching to a material surface where microcolonies begin to form (early phase). Next, long hyphae and EPS develop (intermediate phase), and finally, a mature biofilm composed of yeast, hyphae, pseudohyphae and EPS forms (Chandra *et al.,* 2001). Our study showed that the structure of *C. albicans* biofilms observed by SEM did not differ at the two different temperatures. Hyphae were observed in the early phases of incubation (2 h). After that time, the plastic coverslips were covered with multiple layers of fungal cells and mature biofilms were produced. We tried to quantify these biofilms by H-score. The results showed that all studied strains displayed higher H-scores at 37°C versus 42°C. This indicates that *C. albicans* can produce biofilms at 42°C, but the area and the thickness of biofilm development are much lower than those at 37°C.

In addition, we measured biofilm production by CV staining. This method determines biofilm bulk because it stains all cells in the biofilm community (Melo *et al.,* 2011). From our CV results, biofilm bulk at 42°C was lower than at 37°C (2-48 h), except *C. albicans* ATCC 96901 in during early growth (2 h incubation). In addition, the XTT technique is widely used for evaluating biofilm activity (Ramage *et al.,* 2002; Jin *et al.,* 2003; Melo *et al.,* 2011). XTT indicates biofilm cell viability because mitochondrial dehydrogenase in live cells can reduce XTT to formazan (Jin *et al.,* 2003). Our study showed that biofilms (6-48 h) growing at 37°C showed higher levels of XTT metabolic activity than at 42°C.

The effects of temperature on biofilm formation have been widely studied, particularly in bacteria (Else *et al.,* 2003; Giaouris *et al.,* 2005; Jerônimo *et al.,* 2012). However, the ability of *C. albicans* to form biofilms at temperatures greater than 37°C has not been reported. Previous work has shown that *C. albicans* can grow at 42-45°C (Pinjon *et al.,* 1998), but this was not translated to biofilm growth and development. Temperature influences yeast-to-hypha transition of *C. albicans*. Nadeem *et al.* (2013) studied the effects of temperature on germ tube formation in *C. albicans* showing that at 40°C, germ tubes could be produced in the early 1.5 h of growth, but then decreased at 34°C. From our SEM observations, after 2 h at both 37°C and 42°C, long filaments were observed on the plastic coverslip. This implies that *C. albicans* can retain morphological plasticity under non-optimal conditions of 42°C.

Our study did not determine the adherence capabilities of *C. albicans* under the tested temperatures, but previous investigations illustrated greater adherent abilities of *C. albicans* on vaginal epithelial cells at 40°C compared to 25°C, but decreased at 40°C compared to 37°C (Karam El-Din *et al.,* 2012). From our studies, we could not say that the adherent ability at 42°C was higher or lower than at 37°C, but results from the H-score, CV, and XTT assays showed that at 42°C, *C. albicans* could adhere well enough to the plastic coverslip to produce biofilms subsequently. Temperatures are known to affect bacterial biofilm formation and have been shown to decrease biofilm production at 37°C compared to 30°C in some strains of *P. aeruginosa*, *K. pneumoniae,* and *V. cholerae*, whereas other tested strains were not affected by this temperature change (Hoštacká *et al.,* 2010).

In conclusion, high growth temperatures (42°C) do have an effect on the biofilm formation of *C. albicans*. Although bulk, thickness, and metabolic activity of the biofilm at 42°C is lower than at 37°C, biofilms can still be formed at this temperature.

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