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Research Article

Influence of the Environment on Biofilm Formation *Candida albicans* of Vulvovaginal Candidiasis Isolate Patient

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ABSTRACT

Context: *Candida albicans* is a type of fungus that can produce biofilms and may cause Vulvovaginal Candidiasis (VVC) disease. We investigated the effect of environment on biofilm formation of *C. albicans* patient isolates and ATCC 14053. Biofilm formation is influenced by several factors such as environments and nutrients. Objectives: To investigated the effect of environment on biofilm formation of *C. albicans* patient isolates and ATCC 14053. **Methods**: The samples using *C. albicans* ATCC 14053, *C. albicans,* which may form biofilms, was isolated from patient Dermatology and Venereology and Obstetrics and Gynecology from a hospital in Malang. TCP (A tissue Culture Plate) is the biofilm formation method used. **Results**: Biofilm formation took 48-72 hours at 25 °C and 96-120 hours at 37 °C. Based on the result biofilm formation of *C. albicans* is influenced by environmental factors and characterized by a high OD value. **Conclusions**: Biofilm formation is accelerated in temperature incubation needed at 25 °C for 48-72 hours, using biomass 10^7 CFU/mL, nutrition using Potato Dextrose Broth media and 1% glucose, and the solvent of 30% acetic acid to obtain acid condition.

Key words: *Biofilm formation; Candida albicans; sabourau dextrose broth; potato dextrose broth; vulvovaginal candidiasis*

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INTRODUCTION

VVC (Vulvovaginal Candidiasis) is one of the most frequent infections in women. This infection is most common in women of childbearing age, and about 40-50% of them will have a recurrence or second infection.[1] VVC is the second most prevalent vaginal infection.[1,2] Dr. Soetomo general hospital Surabaya collected 242 new VVC patients between 2007 and 2009 based on morbidity from the Division of Sexually Transmitted Infections (STI), Skin and Sexual Health outpatient unit.[3] *Candida albicans* species are the most common cause of VVC, accounting for 80-90% of cases, followed by *Candida nonalbicans* species, including *Candida parapsilosis*, *Candida tropicalis*, *Candida krusei*, and *Candida glabrata* which also cause VVC but are more resistant to conventional treatment.[1,2]

Candida albicans is a fungus's ability to form biofilms. Biofilm is a collection of microorganism cells that attach to the surface of both biotic and abiotic surfaces and produces their matrix, Extracellular Polysaccharide Substances (EPS). EPS contains polysaccharides, proteins, and DNA.[4] Biofilm formation is a matrix that is thought to be the source of disease development. It is because the biofilm is a substance that can protect microbes or act as a self-defense mechanism for cells living within it against physical and chemical disruptions from the environment.[5] Attachment, proliferation, biofilm formation, biofilm maturation, and release are the stages of biofilm formation in *C. albicans*.[6]

One or two planktonic cells connect to a surface to start the creation of biofilms, followed by their multiplication and appearance of a biofilm monolayer. According to Hidayati and Liuwan (2019), biofilm formation begins with a reversible attachment and then progresses to an irreversible attachment.[7] Following the completion of the first layer of biofilm, cells of the same or other species will be withdrawn from the biofilm. Biofilm begins as a tiny layer, then becomes thicker and thicker until it resembles a mushroom or a tower. Biofilm cells produce EPS, which attach bacteria to form microcolonies on a surface. The bacterial biofilm thickens after the microcolony formation stage and becomes a three-dimensional structure with enveloped cells connected. Following that, biofilm-related genes are expressed.[8]

Some bacterial cells switch to planktonic growth when the biofilm matures. These scattered cells explore new surfaces and attach themselves to them. As a result, dispersal marks both the end of the biofilm life cycle and the start of other life cycles. The final stage of biofilm formation is the dissemination of single-cell bacteria from the biofilm to a new environment.[9] Attachment efficiency, cycle stages, physicochemical environment, mechanical factors and shear forces, substratum, genotype, and nutrient sources are all elements that influence biofilms formation.[10] One of the most important is a source of nutrients, as microorganisms require nutrients to survive. Thus, this research aims to see how nutritional differences affect the biofilm formation of *C. albicans*. So that it can be used as a reference for future researchers related to the problem of Vulvovaginal candidiasis or those associated with *C. albicans* infection with more specific parameters such as protein and EPS production level.

MATERIAL AND METHODS

Chemicals and reagents

C. albicans ATCC 14053, *C. albicans*, which may form biofilms, was isolated from patient Dermatology and Venereology and Obstetrics and Gynecology from a

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hospital in Malang, 1% glucose, PBS (Phosphate Buffer Saline), 96% ethanol (Merck), 30% acetic acid (Merck), 0.1% crystal violet, SBD (Sabouraud Dextrose Broth) media (Himedia), PDB (Potato Dextrose Broth) media (Himedia), and a sterile 96-wells Microplate (Biologix).

Preparation of *Candida albicans* suspension

The rejuvenated *C. albicans* culture was suspended in 10 mL of media media (SDB, SDB and 1% glucose, PDB, PDB and 1% glucose) and homogenized with a vortex. Furthermore, using a 625nm wavelength UV-VIS Spectrophotometer, read the absorbance value in the range of 0.08-0.13 (1x10^8 CFU/mL) (equivalent to 0.5 McFarland). A 1:10 dilution ratio was used to obtain 1x10^7 CFU/mL from the suspension.[11,12]

Candida albicans biofilm formation test

The biofilm formation test was carried out based on the coloring crystal violet method conducted by O'Toole (2011).[12] Added a total of 100 µL *C. albicans* suspension and 100 µL of a media (SDB, SDB and 1% glucose, PDB, PDB and 1% glucose) to the test well and added 200 µL of a media (SDB, SDB and 1% glucose, PDB, PDB and 1% glucose) to the well media control on a sterile 96-wells Microplate. The microplate was closed and incubated at 25 °C and 37 °C for 24, 48, 72, 96, and 120 hours without being stirred. After incubation, biofilm formation was measured. The fluid in the microplate was discarded and washed using PBS three times to remove planktonic cells that were not attached to the microplate and then dried. After that, a dye solution of 200 µL 0.1% crystal violet was placed in each well of the microplate to stain biofilm biomass, which was then incubated for 15 minutes at room temperature. The microplate was washed three times with PBS to remove the cell colors but was not attached to the microplate and dried at room temperature. Next, the biofilm was dissolved in 200 µL of 96% ethanol or 30% acetic acid. After that, incubated for 15 minutes a room temperature, using a microplate reader, the solution's absorbance was measured at 595nm, which is the wavelength of crystal violet.[12,13]

Biofilm formation can be seen by comparing the OD (Optical Density) value with OD-cutoff. The OD-cutoff value is calculated using the following formula: OD-cutoff = OD average control media plus (3x standard deviation OD-control media). Ability formed biofilms on *C. albicans* ATCC 14053 and 4 other groups where OD ≤ ODc means no biofilm is formed, ODc ≤ OD ≥ 2 x ODc means weak biofilm formed, 2 x ODc ≤ OD ≥ 4 x Odc means biofilm is formed with strong medium and 4 x ODc ≤ OD means strong biofilm is formed.[14]

Statistical analysis

This research is descriptive research that will be presented in table format. Data analysis of this research was conducted qualitatively. Data analysis is qualitative, which means that the outcome is descriptive.

RESULTS

Using the *Tissue Culture Plate* method, this research will examine the effect of variations in nutrition (SDB, SDB and 1% glucose, PDB, PDB and 1% glucose) and environment (temperature and incubation time, biomass, and solvent of crystal violet) on the biofilm formation of *C. albicans*. By comparing the OD values of five treatment samples, namely, control media that were purposefully not given *C. albicans* isolates for comparison control, ATCC 14053 was a pure culture of *C. albicans*, and four isolates of *C. albicans* were taken from a candidiasis patient from a hospital, the results can be seen as follows. Tables 1-4 shows the mean value of OD on influence environment. Biofilm formation can be seen by comparing the OD value with OD-cutoff. Tables 5-8 shows the five samples that were developed using four different treatments or different growth media (SDB media, SDB and 1% glucose, PDB, and PDB and 1% glucose).

Biofilm formation at 25 °C for 48 and 72 hours with 96% ethanol and biomass 10^7 CFU/mL and 10^8 CFU/mL has moderate-strong biofilm formation, while biofilm formation with time incubation of 24, 96, and 120 hours has weak-moderate biofilm formation (Table 1). Biofilm formation was likewise not seen on sample CP-4 with biomass 10^8 CFU/mL after 96 and 120 hours of incubation.

Strong biofilm formation was seen at a temperature of 25 °C using 30% acetic acid and biomass 10^7 CFU/mL and 10^8 CFU/mL on incubation for 48 hours (Table 2). Moderate-strong category biofilm formation was seen at time incubation of 72-120 hours, while weak biofilm formation was seen at 24 hours, and two treatments had no biofilm formation. Two samples have biofilm formation category weak on time incubation of 72 and 96 hours in the biofilm formation with biomass 10^7 CFU/mL.

Biofilm formation incubation time of 24 hours with 96% ethanol and biomass 10^7 CFU/mL and 10^8 CFU/mL almost all treatments do not form a biofilm, in comparison, incubation time of 48 and 72 hours has category weak–moderate, and testing at 96 and 120 hours has formation biofilm moderate–strong (Table 3).

Biofilm formation using 30% acetic acid and biomass 10^7 CFU/mL and 10^8 CFU/ mL at 37 °C incubation of 96 and 120 hours has a moderate–strong category (Table 4). Testing at 48 and 72 hours with biofilm formation is weak–moderate, and at the same time, incubation at 24 hours almost all do not form a biofilm. Only three treatments with category weak on biomass 10^7 CFU/mL.

Biofilm formation of *C. albicans* on SDB media after 24 hours of incubation, with the CP-1 sample showing strong biofilm formation categories and four samples (ATCC 14053, CP-2, CP-3, and CP-4) showing weak biofilm formation categories (Table 5). The incubation time was 48 hours, with the CP-1 sample falling into a strong biofilm category, two samples showing weak biofilm formation, and two samples (CP-3 and CP-4) showing no biofilm formation. While the incubation time was 72 hours, three samples (ATCC 14053, CP-1, and CP-3) had strong biofilm formation, and two samples (CP-2 and CP-4) had moderate biofilm formation. Compared to other samples and control media that did not exhibit biofilm formation, the CP-1 sample with an incubation time of 24-48 hours showed a strong biofilm formation category.

The incubation time of 24 hours of *C. albicans* biofilm with SDB and 1% glucose media. The CP-1 sample had a strong biofilm formation category, the ATCC 14053 sample had a moderate biofilm category, and three other samples (CP-2, CP-3, and CP-4) had a weak biofilm formation category (Table 6). After 48 hours of incubation, the CP-1 sample had a strong biofilm formation category, the CP-3 sample had a moderate category, and three samples (ATCC 14053, CP-2, and CP-4) had a weak biofilm formation category. Furthermore, after 72 hours of incubation, the CP-1 sample dominated with a strong biofilm formation category, followed by two samples (CP-2 and CP-3) in the moderate category, and two samples (ATCC 14053 and CP-4) had a weak biofilm formation category. When compared to other samples and control media that did not have biofilm formation, the CP-1 sample showed a strong biofilm formation category after an incubation time of 24-48 hours.

The incubation time of 24 hours, a PC-3 sample with strong biofilm formation results, three samples (ATCC 14053, CP-1, and CP-4) with moderate formation category, and a CP-2 sample with weak formation category were the results of biofilm formation utilizing PDB media (Table 7). The incubation time of 48 and 72 hours from the five showed similar biofilm formation results, with three samples (ATCC 14053, CP-1, and CP-4) with strong biofilm formation categories and two samples (CP-2 and CP-4) had a moderate biofilm formation category. Compared to other samples in PDB media testing, the CP-3 sample

Table 1. Biofilm formation at 25 C using 96% Ethanol with biomass of 10^7 CFU/mL and 10^8 CFU/mL

Sample	The average OD value of the biomass was 10^7 CFU/mL ± SD.					
	0 Hours	24 Hours	48 Hours	72 Hours	96 Hours	120 Hours
MC[a]	0.135 ± 0.002	0.209 ± 0.010	0.280 ± 0.008	0.321 ± 0.007	0.313 ± 0.012	0.209 ± 0.009
ATCC 14053	0.125 ± 0.003	0.766 ± 0.005	2.397 ± 0.027	2.273 ± 0.022	0.399 ± 0.006	0.405 ± 0.013
CP[b]-1	0.117 ± 0.002	0.753 ± 0.012	2.236 ± 0.023	3.323 ± 0.035	0.834 ± 0.018	0.405 ± 0.009
CP[b] -2	0.139 ± 0.004	0.772 ± 0.008	1.466 ± 0.030	1.629 ± 0.016	0.445 ± 0.012	0.431 ± 0.014
CP[b] -3	0.131 ± 0.008	0.475 ± 0.006	1.630 ± 0.015	2.036 ± 0.048	0.489 ± 0.008	0.468 ± 0.012
CP[b] -4	0.136 ± 0.008	0.467 ± 0.008	0.800 ± 0.013	0.720 ± 0.016	0.410 ± 0.006	0.410 ± 0.005
Sample	**The average OD value of the biomass was 10^8 CFU/mL ± SD.**					
	0 Hours	**24 Hours**	**48 Hours**	**72 Hours**	**96 Hours**	**120 Hours**
MC[a]	0.148 ± 0.009	0.267 ± 0.004	0.316 ± 0.024	0.378 ± 0.007	0.257 ± 0.010	0.287 ± 0.009
ATCC 14053	0.161 ± 0.022	0.700 ± 0.006	1.593 ± 0.032	2.729 ± 0.059	0.827 ± 0.004	0.408 ± 0.004
CP[b] -1	0.168 ± 0.009	0.665 ± 0.004	1.614 ± 0.002	2.589 ± 0.069	0.809 ± 0.009	0.817 ± 0.006
CP[b] -2	0.160 ± 0.008	0.414 ± 0.004	1.178 ± 0.009	1.510 ± 0.016	0.442 ± 0.020	0.413 ± 0.008
CP[b] -3	0.151 ± 0.011	0.423 ± 0.010	1.331± 0.009	1.058 ± 0.018	0.434 ± 0.010	0.827 ± 0.011
CP[b] -4	0.133 ± 0.003	0.437 ± 0.010	1.726 ± 0.063	1.593 ± 0.025	0.242 ± 0.008	0.303 ± 0.019

[a,b] Means MC: Media Control, CP: Candidiasis Patient

Table 2. Biofilm formation at 25 °C using 30% acetic acid with biomass 10^7 CFU/mL and 10^8 CFU/mL.

Sample	The average OD value of the biomass was 10^7 CFU/mL ± SD.					
	0 Hours	24 Hours	48 Hours	72 Hours	96 Hours	120 Hours
MC[a]	0.092 ± 0.010	0.312 ± 0.010	0.156 ± 0.008	0.205 ± 0.015	0.282 ± 0.009	0.225 ± 0.021
ATCC 14053	0.097 ± 0.003	0.545 ± 0.009	2.189 ± 0.035	1.408 ± 0.032	0.874 ± 0.013	1.652 ± 0.073
CP[b] -1	0.101 ± 0.006	0.489 ± 0.006	0.893 ± 0.023	3.021 ± 0.044	0.877 ± 0.022	2.846 ± 0.091
CP[b] -2	0.097 ± 0.009	0.537 ± 0.015	1.671 ± 0.059	1.337 ± 0.034	0.880 ± 0.025	0.947 ± 0.078
CP[b] -3	0.097 ± 0.011	0.249 ± 0.008	1.647 ± 0.051	2.245 ± 0.027	0.480 ± 0.025	1.729 ± 0.097
CP[b] -4	0.092 ± 0.005	0.370 ± 0.006	0.662 ± 0.029	0.407 ± 0.033	0.811 ± 0.018	0.946 ± 0.056
Sample	**The average OD value of the biomass was 10^8 CFU/mL ± SD.**					
	0 Hours	**24 Hours**	**48 Hours**	**72 Hours**	**96 Hours**	**120 Hours**
MC[a]	0.074 ± 0.002	0.264 ± 0.008	0.152 ± 0.001	0.199 ± 0.015	0.215 ± 0.013	0.282 ± 0.019
ATCC 14053	0.085 ± 0.015	0.449 ± 0.010	2.151 ± 0.037	0.976 ± 0.038	0.671 ± 0.018	1.374 ± 0.052
CP[b] -1	0.081 ± 0.012	0.429 ± 0.002	3.134 ± 0.039	1.544 ± 0.028	0.626 ± 0.005	0.893 ± 0.049
CP[b] -2	0.092 ± 0.007	0.660 ± 0.019	1.284 ± 0.059	0.973 ± 0.059	1.157 ± 0.015	0.846 ± 0.064
CP[b] -3	0.089 ± 0.008	0.392 ± 0.015	1.061 ± 0.033	0.753 ± 0.039	0.664 ± 0.020	0.951 ± 0.051
CP[b] -4	0.085 ± 0.007	0.237 ± 0.005	2.183 ± 0.060	1.492 ± 0.023	1.246 ± 0.046	1.024 ± 0.062

[a,b] Means MC: Media Control, CP: Candidiasis Patient

Table 3. Biofilm formation at 37 °C using 96% ethanol with biomass 10^7 CFU/mL and 10^8 CFU/mL.

Sample	The average OD value of the biomass was 10^7 CFU/mL ± SD.					
	0 Hours	24 Hours	48 Hours	72 Hours	96 Hours	120 Hours
MC[a]	0.135 ± 0.002	0.249 ± 0.015	0.299 ± 0.017	0.314 ± 0.010	0.248 ± 0.006	0.234 ± 0.013
ATCC 14053	0.125 ± 0.003	0.283 ± 0.017	0.633 ± 0.018	0.470 ± 0.023	0.633 ± 0.049	1.133 ± 0.014
CP[b] -1	0.117 ± 0.002	0.275 ± 0.009	0.517 ± 0.003	0.712 ± 0.059	1.269 ± 0.022	0.871 ± 0.024
CP[b] -2	0.139 ± 0.004	0.275 ± 0.010	0.581 ± 0.011	0.761 ± 0.022	1.578 ± 0.011	0.762 ± 0.021
CP[b] -3	0.131 ± 0.008	0.281 ± 0.009	0.453 ± 0.014	0.512 ± 0.024	0.585 ± 0.011	1.099 ± 0.014
CP[b] -4	0.136 ± 0.008	0.284 ± 0.004	0.478 ± 0.026	0.758 ± 0.014	1.094 ± 0.007	0.809 ± 0.007
Sample	**The average OD value of the biomass was 10^8 CFU/mL ± SD.**					
	0 Hours	**24 Hours**	**48 Hours**	**72 Hours**	**96 Hours**	**120 Hours**
MC[a]	0.148 ± 0.009	0.242 ± 0.004	0.375 ± 0.004	0.259 ± 0.007	0.274 ± 0.011	0.180 ± 0.003
ATCC 14053	0.161 ± 0.022	0.252 ± 0.004	0.614 ± 0.048	0.446 ± 0.021	0.714 ± 0.009	0.815 ± 0.008
CP[b] -1	0.168 ± 0.009	0.252 ± 0.011	0.698 ± 0.040	0.315 ± 0.011	0.715 ± 0.011	0.778 ± 0.041
CP[b] -2	0.160 ± 0.008	0.380 ± 0.006	0.927 ± 0.053	0.582 ± 0.012	1.376 ± 0.008	0.582 ± 0.012
CP[b] -3	0.151 ± 0.011	0.226 ± 0.009	0.506 ± 0.047	0.298 ± 0.004	0.623 ± 0.011	0.806 ± 0.018
CP[b] -4	0.133 ± 0.003	0.242 ± 0.003	0.522 ± 0.019	0.385 ± 0.009	0.688 ± 0.005	0.908 ± 0.034

[a,b] Means MC: Media Control, CP: Candidiasis Patient

Table 4. Biofilm formation at 37 °C using 30% acetic acid and biomass 10^7 CFU/mL and 10^8 CFU/mL.

Sample	The average OD value of the biomass was 10^7 CFU/ml ± SD.					
	0 Hours	24 Hours	48 Hours	72 Hours	96 Hours	120 Hours
MC[a]	0.092 ± 0.010	0.265 ± 0.005	0.129 ± 0.002	0.158 ± 0.011	0.243 ± 0.004	0.219 ± 0.009
ATCC 14053	0.097 ± 0.003	0.427 ± 0.005	0.330 ± 0.010	0.606 ± 0.042	1.158 ± 0.004	0.543 ± 0.015
CP[b] -1	0.101 ± 0.006	0.408 ± 0.024	0.363 ± 0.021	0.554 ± 0.006	1.076 ± 0.006	0.537 ± 0.033
CP[b] -2	0.097 ± 0.009	0.440 ± 0.009	0.350 ± 0.009	0.434 ± 0.005	1.182 ± 0.018	0.749 ± 0.044
CP[b] -3	0.097 ± 0.011	0.211 ± 0.006	0.252 ± 0.009	0.334 ± 0.018	0.641 ± 0.012	1.132 ± 0.005
CP[b] -4	0.092 ± 0.005	0.277 ± 0.003	0.296 ± 0.020	0.424 ± 0.028	1.037 ± 0.014	0.815 ± 0.018
Sample	**The average OD value of the biomass was 10^8 CFU/ml ± SD.**					
	0 Hours	**24 Hours**	**48 Hours**	**72 Hours**	**96 Hours**	**120 Hours**
MC[a]	0.074 ± 0.002	0.269 ± 0.006	0.165 ± 0.010	0.142 ± 0.007	0.244 ± 0.004	0.190 ± 0.012
ATCC 14053	0.085 ± 0.015	0.214 ± 0.009	0.205 ± 0.013	0.356 ± 0.012	1.113 ± 0.062	0.548 ± 0.019
CP[b] -1	0.081 ± 0.012	0.281 ± 0.006	0.294 ± 0.008	0.386 ± 0.023	1.287 ± 0.075	0.524 ± 0.017
CP[b] -2	0.092 ± 0.007	0.275 ± 0.002	0.310 ± 0.005	0.467 ± 0.032	1.264 ± 0.026	0.570 ± 0.025
CP[b] -3	0.089 ± 0.008	0.275 ± 0.006	0.267 ± 0.014	0.372 ± 0.018	1.128 ± 0.033	0.538 ± 0.005
CP[b] -4	0.085 ± 0.007	0.273 ± 0.007	0.277 ± 0.011	0.392 ± 0.015	1.182 ± 0.059	0.548 ± 0.010

[a,b] Means MC: Media Control, CP: Candidiasis Patient

Table 5. Sabouraud Dextrose Broth (SDB) media for biofilm formation.

Sample	The average OD value of the biomass was 10^7 CFU/mL ± SD.			
	0 Hours	24 Hours	48 Hours	72 Hours
MC[a]	0.084 ± 0.003	0.108 ± 0.009	0.166 ± 0.032	0.097 ± 0.014
ATCC 14053	0.081 ± 0.002	0.161 ± 0.060	0.325 ± 0.070	0.705 ± 0.115
CP[b] -1	0.082 ± 0.004	0.722 ± 0.099	1.521 ± 0.236	2.608 ± 0.228
CP[b] -2	0.089 ± 0.012	0.164 ± 0.027	0.324 ± 0.060	0.439 ± 0.045
CP[b] -3	0.093 ± 0.005	0.159 ± 0.016	0.193 ± 0.013	0.602 ± 0.071
CP[b] -4	0.094 ± 0.006	0.268 ± 0.059	0.245 ± 0.045	0.390 ± 0.043

[a,b] Means MC: Media Control, CP: Candidiasis Patient

Table 6. Sabouraud Dextrose Broth (SDB) media and 1% glucose for biofilm formation.

Sample	The average OD value of the biomass was 10^7 CFU/mL ± SD.			
	0 Hours	24 Hours	48 Hours	72 Hours
MC[a]	0.087 ± 0.003	0.125 ± 0.020	0.118 ± 0.010	0.124 ± 0.018
ATCC 14053	0.095 ± 0.006	0.373 ± 0.047	0.247 ± 0.040	0.239 ± 0.019
CP[b] -1	0.093 ± 0.007	0.765 ± 0.144	2.465 ± 0.391	3.349 ± 0.179
CP[b] -2	0.094 ± 0.007	0.259 ± 0.025	0.214 ± 0.043	0.411 ± 0.065
CP[b] -3	0.097 ± 0.011	0.269 ± 0.038	0.475 ± 0.030	0.472 ± 0.083
CP[b] -4	0.092 ± 0.005	0.282 ± 0.058	0.205 ± 0.038	0.217 ± 0.027

[a,b] Means MC: Media Control, CP: Candidiasis Patient

Table 7. Potato Dextrose Broth (PDB) media for biofilm formation.

Sample	The average OD value of the biomass was 10^7 CFU/mL ± SD.			
	0 Hours	24 Hours	48 Hours	72 Hours
MC[a]	0.085 ± 0.002	0.111 ± 0.017	0.116 ± 0.013	0.108 ± 0.020
ATCC 14053	0.094 ± 0.005	0.362 ± 0.018	0.967 ± 0.084	0.872 ± 0.160
CP[b] -1	0.092 ± 0.009	0.434 ± 0.063	0.641 ± 0.107	0.709 ± 0.089
CP[b] -2	0.101 ± 0.008	0.264 ± 0.043	0.440 ± 0.079	0.504 ± 0.047
CP[b] -3	0.101 ± 0.006	0.750 ± 0.101	0.998 ± 0.158	1.228 ± 0.159
CP[b] -4	0.096 ± 0.004	0.484 ± 0.081	0.563 ± 0.087	0.452 ± 0.074

[a,b] Means MC: Media Control, CP: Candidiasis Patient

Table 8. Potato Dextrose Broth (PDB) media and 1% glucose for biofilm formation.

Sample	The average OD value of the biomass was 10^7 CFU/mL ± SD.			
	0 Hours	24 Hours	48 Hours	72 Hours
MC[a]	0.067 ± 0.005	0.113 ± 0.013	0.116 ± 0.010	0.137 ± 0.014
ATCC 14053	0.072 ± 0.005	0.431 ± 0.091	1.067 ± 0.219	1.767 ± 0.275
CP[b] -1	0.077 ± 0.007	0.522 ± 0.061	1.138 ± 0.226	1.540 ± 0.274
CP[b] -2	0.075 ± 0.010	0.833 ± 0.116	1.208 ±0.195	1.553 ± 0.319
CP[b] -3	0.080 ± 0.008	0.427 ± 0.087	0.852 ± 0.152	1.140 ±0.068
CP[b] -4	0.073 ± 0.004	0.349 ± 0.059	0.817 ± 0.023	0.741 ± 0.092

[a,b] Means MC: Media Control, CP: Candidiasis Patient

exhibited a strong category of biofilm formation with an incubation time of 24-72 hours. Biofilm formation was not detected in the control media group.

The results of biofilm formation with PDB media and 1% glucose showed After 24 hours of incubation, the CP-2 sample had a strong biofilm formation category, whereas the other four samples (ATCC 14053, CP-1, CP-3, CP-4) had a moderate biofilm formation category (Table 8). Furthermore, each sample showed the same results after 48 and 72 hours of incubation, with four samples (ATCC 14053, CP-1, CP-2, CP-3, and CP-4) having a strong biofilm formation category. The CP-2 sample had strong biofilm formation in PDB and 1% glucose media with an incubation time of 24-72 hours, but the other four samples had similar results when compared to the control media that did not have biofilm formation an incubation time of 48-72.

DISCUSSION

This research aimed to see how the influence of the environment (temperature and time incubation, biomass, nutrition, as well as solvent of crystal violet) affects biofilm formation in *Candida albicans*. Candidiasis vulvovaginal is a fungus infection of the vaginal mucosal organs that affects 80-90% of women. VVC disease is the second most common type of vaginal infection after total vaginal infection.[15,16] Infections caused by fungi will be more common in people who are immunocompromised or have a weak immune response, making them pathological. *C. albicans* will develop a community that is structured, coordinated, and functioning in an extracellular secretory matrix in pathogenicity species. Biofilm formation is associated with high antifungal resistance.[16,17]

Based on testing of eight treatments on environmental influences, the formation of biofilms in the time range isolation of 24-120 hours has a cycle growth from no biofilm formation or else from phase moderate to no biofilm formation. According to this research, high biofilm formation occurs after 48-72 hours of incubation at 25 °C and 96-120 hours at 37 °C, with a high OD value compared to the OD-cutoff score. Meanwhile, the results of testing on four media treatments had differences in the development of *C. albicans* biofilms. It corresponds to the *C. albicans* biofilm formation cycle, including the attachment cell host, microcolony formation, maturation, and experience spread phases (Figure 1).

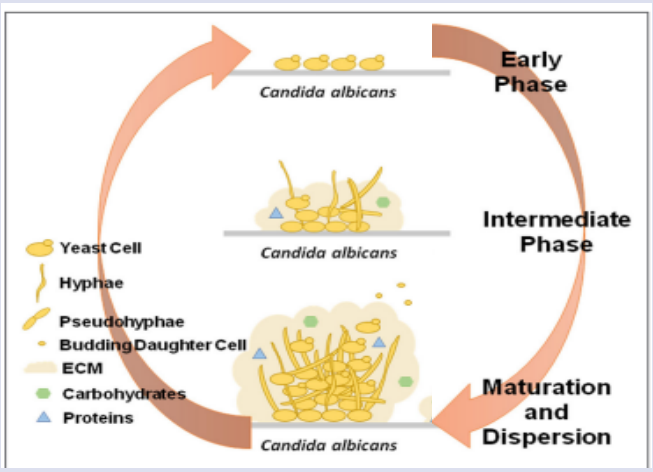

Figure 1. *Candida albicans* biofilm formation cycle. Source: Data taken from Cavalheiro and Teixeira, 2018.

The attachment cell host or substrate phase of *C. albicans* biofilm formation was followed by the initiation or intermediate phase, in which cells proliferate and produce the hyphae that form a monolayer. The detachment phase, which occurs before this process, is essential for developing a strong attachment phase. The GPI cell surface is encoded by the active genes ALS1, ALS3, and HWP1, which are necessary for normal biofilm formation. The release of biomaterials that are gene transcription active is the beginning step in this process. This process can be triggered by a variety of factors such as temperature, serum, amino acid availability, pH level, and CO2 presence.[6,18] The initial phase is then followed by the maturation phase, during which time cells get attached to the extracellular polymer, and finally, the deployment phase, during which time fungi are released into a fresh biofilm. The attachment phase can take up to 12 hours, followed by the initiation phase, during which time hyphae grow, the maturation phase, during which time cells embed hyphae, and the distribution phase, which can last up to 72 hours.[19,20] According to Hamzah *et al*, (2021), 48 hours phase of biofilm formation of *C. albicans* takes longer than the 24 hours phase, contains more and organized some other forms of a 3D group that will communicate with one another when there are object foreigners who will enter the community and mature *C. albicans* biofilm containing cell in form yeast nor hyphae which insert and tightly bound to the material the usual extracellular fibrous.[21] Because it had reached the maturity phase, the incubation time of 72 to 120 hours in the reseach resulted in a high biofilm formation rate. According to Oliveira's research, high biofilm formation values were seen at incubation times of 72, 96, 120, and 144 hours, where it entered the maturation phase.[22]

According to the results of this research, *C. albicans* biofilm growth from maturation to the deployment hyphae phase results in no forming biofilms following a high biofilm growth phase.[20] In addition to the influence of the environment on the formation of biofilms on *C. albicans*. Different nutrient content in the growth medium greatly influences the growth of microorganisms. Nutrients include carbohydrates, protein, carbon, nitrogen, and glucose. Then, growth media is needed to study the properties of a microorganism.[15,23]

In addition to incubation time considerations, environmental parameters affecting biofilm formation include incubation temperature, isolate amount, and crystal violet solvent. Compared to biomass 10^8 CFU/mL, biofilm formation was higher on biomass 10^7 CFU/mL, resulting in a high OD value. The presence of turbidity or many microorganisms in it can indicate a high OD value or a lot of growing microorganisms when coloring crystal violet is done.[24] Because of competing nutrients on the SDB media used, the OD value for both isolate amounts can change. Biofilm growth and formation are heavily influenced by medium nutrition. Nutrition also has an impact on biofilm structure.[25]

Temperature influences the transition hyphae of *C. albicans* in the biofilm formation process.[26,27] This research does not mention that a temperature of 25 °C is better than 37 °C. However, the method of Tissue Culture Plate, which occurred at 25 °C, has a high OD value. Temperature incubation of 25 °C is better in biofilm formation. However, this has not yet been reported. Glyoxalase/glx3, an abundant protein present in the extracellular matrix of biofilms, was used by Cabello *et al* (2019) to quantify the impact of temperature on *C. albicans* biofilm formation.[28] The result showed that a temperature of 28 °C influenced biofilm formation more than a temperature of 37 °C, with significant development of the glx2 mutant. Pumessat's research found that *C. albicans* may produce biofilm at temperatures of 37 °C and 42°C on biofilm formation.[26] The result showed that 42 °C has higher biofilm formation. But the mass, thickness, and activity metabolism of the biofilm formed at 37 °C is lower. *C. albicans* is a species pathogen that will proliferate at temperatures between 25–37 °C.[29] The *C. albicans*

detachment process, which is essential for biofilm formation, is also thought to occur before the biofilm formation process.[6,18]

Solvent plays an essential function in this research as a biocatalyst, such as substrate biotransformation or carrier substrate phase, making it easier to observe biofilm formation outcomes. The viability of the cell is also affected by the solvent via the permeability membrane.[30] This research showed that 30% acetic acid could catalyze *C. albicans* biofilm, resulting in a high OD value. *C. albicans* can grow faster in an acidic environment and with standard or alkaline pH. *C. albicans* can form exposure to chitin and β-glucan on the cell wall in an acidic environment to increase the cause of inflammation and protect the yeast from the environment.[31,32] Then, this research found different results between samples of *C. albicans*, which were caused by the development of biofilms in conjunction with an increase in infection clinical in cells host, allowing it to be concluded that infection clinical differences between VVC patients influence biofilm formation.[21]

Meanwhile, the result of different media on *C. albicans* biofilm formation, SDB media contains carbohydrates and proteins, essential nutrients for fungal growth. Because of its simple formulation, this medium is commonly used as a culture medium. Protein nutrition in the media is an energy source, whereas carbohydrate nutrition supports fungal growth.[33,34] Five samples treated with control media were purposefully not given *C. albicans* isolate for comparison control when observing biofilm formation of *C. albicans* with SDB medium. Five samples with the strong biofilm formation category had the biggest OD value of 2.608 ± 0.228. Two samples with the moderate biofilm formation category, six samples in the weak biofilm formation category, and two other samples that did not have biofilm formation from the incubation time of 24 to 72 hours. The results of SDB media with 1% glucose added showed that three samples had a strong biofilm formation category with the biggest OD value of 3.349 ± 0.179. Four samples had a moderate biofilm formation category, and eight samples had weak biofilm formation from the incubation time of 24 to 72 hours.

In these two observations, it can be concluded that SDB media with 1% glucose addition has a faster biofilm formation than SDB alone, presumably due to the influence of 1% glucose added nutrition. One form of monosaccharide that provides energy for the metabolic process of a fungus and serves as a medium for fungal growth and development is glucose[35,36] According to research by Getas *et al.* (2014), the addition of 3 g of glucose can make the incubation time of the fungus *C. albicans* faster and colony growth more fertile.[35] The addition of 5% glucose in SDA media for observing the biofilm formation of *C. albicans* had better increase than the addition of 10%.[37]

PDA (Potato Dextrose Agar) media is included in semi-synthetic media because it's composed of natural ingredients (potatoes) and synthetic materials (dextrose and agar). In addition to having elements that compact the PDA medium, potatoes are a source of dextrose, sugar, vitamins, and energy. This medium is needed in mushroom breeding in the laboratory.[38,39] In addition, PDA media has a simple nutritional formulation and components in the medium.[40]

The results showed that biofilm formation of *C. albicans* with PDA media from 5 samples treated with control media was intentionally not given *C. albicans* isolate for comparison control. It shows that with an incubation time of 24-72 hours, seven samples had a strong biofilm formation category with the biggest OD value of 1.228 ± 0.159, seven samples had a moderate biofilm formation category, and one sample had a weak biofilm formation category. While the results of the research using PDA media with 1% glucose added, the results of *C. albicans* biofilm formation from all samples were in a strong category, namely 11 samples, and the biggest OD value was 1.767 ± 0.275, and 4 samples were in the moderate biofilm growth category. From the two treatments, it can be concluded that the addition of PDA media with 1% glucose resulted in better and faster biofilm formation of *C. albicans*. Until now, no research has been reported on PDB media with 1% glucose addition, but this PDB medium is often used in various studies on growing microorganisms, one of which is the observation of biofilm formation from *C. albicans*.[41]

Comparing biofilm formation of *C. albicans* in both PDA and SDB media with or without the addition of 1% glucose, it can conclude that PDA media with the addition of 1% glucose has better and faster biofilm formation results and more fertile colony growth. It is characterized by constant average OD values with a strong biofilm formation category. This can occur in addition to of 1% glucose due to differences in the content of nutrients in the media for biofilm formation of *C. albicans*. PDA contains natural ingredients of potato as a source of carbon, vitamins, and energy, while the composition of SDA is a source of synthetic carbohydrates. This research added only 1% glucose media because the higher the glucose concentration in the fungal media, it would disturb the balance between cells and fungi and the external environment.[35,36] Then all treatments produced different results with an incubation time of 24-72 hours because of the isotonic state of the medium. There was a fluid balance so that the growth of *C. albicans* became stable and tended to increase. On the other hand, the hypertonic state of the medium can cause a decrease in yield.[37]

CONCLUSION

The environment includes temperature and time incubation, biomass, nutrition, and solvent of crystal violet influences *C. albicans* biofilm formation. The temperature incubation for *C. albicans* biofilm needed 25 °C at 48-72 hours with biomass was 10^7 CFU/mL. The media is composed of Potato Dextrose Broth media and 1% glucose. We used 30% acetic acid solvent to obtain the acid condition.

CONFLICTS OF INTEREST

The authors have not disclosed any financial conflict of interest.

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