Absorbance thickness in the formation of biofilm produced by *Candida albicans* due to glucose, lactose, protein (soy) and iron induction

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Abstract

Candida albicans (*C. albicans*) is a major cause of *Candidiasis* in the oral cavity. Carbohydrates and protein are required by the fungI to grow when it is infecting the soft tissues of the oral cavity. Whereas in *Candidiasis* therapy, reducing the carbohydrate diet and increasing intake of iron and vitamins must be done to inhibit the growth of *C. albicans* in the soft tissue of the oral cavity. The aim of this study to determine the thickness of absorbance in the formation of biofilm from *C. albicans* induced by various materials such as Glucose, Lactose, Protein (Soy) and Iron. The experimental study grow *C. albicans* on Saboraud Dextrose Agar with 4 types of treatment as follow group A, the *C. albicans* is induced by 5% Glucose, group B is induced by 5% lactose, group C is induced by soy protein and group D is induced by Iron (5% $FeCl_2$). Whereas group E is the growth control of *C. albicans* without any induction. Each treatment was replicated with 6 times, then stained with Crystal Violet. A microplate assay was used to carry out tests. The average of Optical Density (OD) is read with a wavelength of 492 each group as follow A = 0.197; B = 0.279; C = 0.297; D = 0.177 and E = 0.053. *C. albicans* biofilm induced by protein and lactose materials had much thicker OD than induced by glucose and iron. The conclusion of this study that the thickness of absorbance in the formation of *C. albicans* biofilms is influenced by the demand of the growth factors of *C. albicans*.

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Introduction

The formation of biofilm is a matrix that is thought to be the cause of the development of an infectious disease.[1] It is because biofilms are materials that can protect the microbes living in them against both the physical and chemical disturbances from the environment.[2,3,4] These microbes can develop and spread after undergoing a maturation stage. The formation of biofilms, especially in the organs of the human body, is prevented from experiencing maturation.[5]

The beginning of the occurance of biofilm is the formation of a glucan layer which envelops from one planktonic microbial (bacteria) cell used to attach to the organ to be infected and then to coagregate with other microbes.[6,7] The glucan layer of the individual cells formed can be influenced by the presence of nutrients available in the environment.[8,9] Biofilm formed by these microbes might be used to detect what nutrients are available around it. A proof is highly necessary to carry out by giving various inducers to a particular type of microbe to be analyzed for the biofilm formation that occurs in the microbes with different inducers.[10]

A type of microbes called *Candida albicans* (*C. albicans*), as a cause of infection of *Oral Candidiasis*, was used in this study. *C. albicans* was induced with various inducers commonly found in the daily food intakes. Microassay plate examination was used to see the formation of biofilm (glucan layer) from several inducers to produce biofilm formation from *C. albicans* and the thickness of biofilm proteins formed by *C. albicans* with these various inducers.[11,12] In this study, the inducer to stimulate the biofilm formation from *C.*

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albicans were lactose, glucose, iron and soy protein.

Exopolysaccharide (EPS/glucan) is a major component of glycocalyx biofilms which form the mucus layers. After EPS is fully dehydrated, the dominant content of glycocalyx is water. In most microbial species, glycocalyx is dominated by anionic and is used as a collection point for important minerals and nutrients from the surrounding environment. Beside that glycocalyx also serves to protect microbes contained in the glycocalix of biofilms against some environmental threats, including: biocides, antibiotics, antibodies, surfactants, bacteriophages. In general, glycocalyx can create a three-dimensional force field that surrounds the microbial cells, serves as an anchor, and protects the microbes that are bound to the surface.[8]

The thickness of the biofilm formation of *C. albicans* clinically signifies some virulence and infection resistance. In the oral cavity the formation of *C. albicans* biofilm is triggered by the presence of serum and saliva. The functions of biofilm formation for microbes include protection against antibiotics, disinfectant and dynamic environment, intercellular communication in stimulation of ups and downs of regulation. An expression of genes to form biofilms quickly can occur due to the temporary adaptations like the phenotypic variations; and the ability to survive in nutrient deficiencies. Approximately 99% of the world's bacterial/ microbial population is present in the form of biofilms with very large variations in growth stages and biofilm formation.[2]

C. albicans cells consist of 6 layers (from outside to inside), and they are fibrillar layer, mannoprotein, β glucan, β glucan-chitin, mannoprotein, and plasma membrane. *C. albicans* biofilm formation consists of 4 stages as follow spherical yeast cell, proliferation to form a basal layer of anchor cells, growth of pseudo hyphae and hyphae (producing extracellular matrix) and the slow spread of yeast-shaped cells from biofilms to new places.[13]

Material and Methods

C. albicans (local isolat) is cultured on *Sabouraud Dextrose Broth* (=SDB-Oxoid) which is 48 hours of age and the size of *Mc. Farland* 3. A sample of 24 wells was placed on Microassay plate and divided into 4 groups. Each group consisted of 6 samples of *C. albicans* which were induced with a variation of 4 ingredients. Group (1) was the growth group of *C. albicans* biofilm induced by 5% glucose (SIGMA). Group (2) was the growth group of *C. albicans* biofilm induced by 5% lactose (SIGMA). Group (3) was the growth group of *C. albicans* biofilm induced by soy protein (Trypticase Soy Broth, TSB-Oxoid). While group (5) was the growth group of *C. albicans* biofilm induced by 5% $FeCl_2$ (Choice Chem LTD). Solution of 30% glacial Acetic Acid (Merck) was used to control OD reading. All sample groups were incubated for 24 hours at room temperature. After 24 hours, the centrifuge (Fisher Scientific) of those samples was carried out with a speed of 3,000 rpm for 10 minutes. Then, pellets were taken as much as 5 µl in each sample and added by 125 µl of 0.1% cristal violet solution in each Microassay plate. Next, they were incubated again for 10-15 minutes at room temperature. After that, the microassay plate was rinsed 3-4 times with Phosphate Buffer Saline (=PBS-SIGMA) solution. Then the Microassay plate was turned upside down and dried for a few nights. After that 125 µl of 30% Glacial Acetic Acid was added into each microassay plate well to dissolve the Crystal Violet (SIGMA). Microassay plates were incubated for 10-15 minutes at room temperature, and then transferred to a new Microassay plate (Polypropilene-Costar) with a flat bottom. An absorbance measurement at the plate reader (Biorad) with a wavelength of 492 nm was carried out by using Glacial Acetic Acid as a control.[14]

Results

There are 4 groups of *C. albicans* with 4 different inducers: 5% Glucose, 5% Lactose, Soy Protein and Iron (5% $FeCl_2$), each group is replicated by 6 times. Based on the reading by using a plate reader (ELISA reader) with a wavelength of 492 nm, the results obtained ranging from the largest to the smallest are as follows: inductions of Soy Protein (0.297); Lactose (0.279); glucose (0.197) and iron (0.177). Solution of 30% Glacial Acetic Acid is used to control.

Replication	Glu	Lact	Prot	Fe	Control of 30% As. Glacial Acetate
1	0.172	0.247	0.226	0.175	
2	0.162	0.231	0.237	0.158	
3	0.236	0.358	0.403	0.179	0.053
4	0.200	0.312	0.342	0.164	
5	0.210	0.252	0.248	0.184	
6	0.203	0.276	0.327	0.201	
$\bar{x}$	0.197	0.279	0.297	0.177	0.053

Table 1. Results of Reading of OD Candida albicans biofilm with various inducers (492 nm)

Discussion

Biofilm formation in Soy Protein and Lactose-induced *C. albicans* has a greater thickness than of glucose and iron-induced. This can be described by the following explanation as below.

Glucose induction

C. albicans grown on Microassay plate, induced with 5% glucose was incubated for 24 hours, and read with a plate reader with a wavelength of 492 nm produce the biofilm formation with OD = 0.197. The density of *C. albicans* with biofilm is ±0.144 thicker compared with 30% Glacial acetic acid control with OD = 0.053.

The previous research of biofilm matrix revealed that the results of glucose synthesis in the form of β → 1,3 glucan together with the mannan-glucan complex for the development and maintenance of biofilm structures. This synthesis of polysaccharide matrix is regulated by an independent pathway that mediates cell wall synthesis. Glucose β → 1,3 in the *C. albicans* biofilm matrix has important roles in eliminating anti-fungi, inhibiting neutrophil ROS production and hiding fungal cells from neutrophil recognition.[15] It means that certain levels of glucose are needed by *C. albicans* to produce biofilm formation.

Lactose induction

The growth of *C. albicans* in Microassay plate induced with 5% Lactose and incubated for 24 hours was carried out, and then read with a plate reader with a wavelength of 492 nm resulted in the biofilm formation with OD = 0.279. This density of *C. albicans* biofilm induced by 5% Lactose is ±0.226 thicker than that of 30% Glacial acetic acid control with OD = 0.053. This density is also higher than that of *C. albicans* induced by glucose (= 0.197).

Lactose is a disaccharide carbohydrate which is a combination of glucose and galactose. Galactose is the best inducer to form *C. albicans* biofilm growth, while glucose itself is also a form of monosaccharide carbohydrate which can induce the best formation of *C. albicans* biofilm after Galactose. Lactose is a combination of 2 monosaccharides, namely galactose and glucose so that it can be well understood that the density (OD) of lactose is higher than glucose.

At the beginning, the growth of the formation of *C. albicans* biofilm induced by glucose was faster than that of *C. albicans* biofilm induced by galactose. However, after further incubation (1-3 days), the growth rate of *C. albicans* induced by Galactose is faster than that of Glucose induction. According to the previous research, Galactose could alter the components of the outer surface of the wall of *C. albicans* which could cause an increase in fibrillar mannoprotein synthesis. This additional adhesin (fibrillar mannoprotein) is thought to facilitate *C. albicans* attachment and biofilm formation in subsequent processes. When it is compared with the growth of *C. albicans* cells induced with other carbohydrates, the cell growth of *C. albicans* induced by galactose shows the highest hydrophobicity making it more likely for an aggregate growth in the liquid media.[16]

Protein induction

The growth of *C. albicans* in Microassay plate induced by Soy Protein (using TSB = Trypticase Soy Broth media) and incubated for 24 hours was carried out, and then read by using a plate reader with a wavelength of 492 nm, and produced the formation of the biofilm with OD = 0.297. This density is the highest when it is compared to the other inducers (5% Glucose; 5% Lactose, and 5% $FeCl_2$). If it is compared with the density control of 30% Glacial acetic acid with OD = 0.053, the density of the biofilm of *C. albicans* induced by Soy Protein is ± 0.244 thicker.

According to the previous studies, the amount of protein in *C. albicans* biofilm is, ± 55% of the extracellular dry weight, greater

than the mass content of carbohydrates. From the protein extraction and the matrix of glycoprotein biofilm identified by two-dimensional electrophoresis gel technique and mass spectrometry there are similarities between the protein matrix and protein components in the liquid supernatant of planktonic culture. The protein is involved in the degradation of the matrix to increase the biofilm formation[17]. These results seem to indicate that ingredients secreted during the growth on the artificial media in the planktonic conditions will form parts of the matrix during the growth of a biofilm. It is the growth model most likely to occur during the infection process. The proteins found in the biofilm matrix include several predictions to form part of the glycoprotein.

As stated by Pierce (2017), that it was understandable that if OD of *C. albicans* biofilm induced with protein had the highest density because 55% of the composition of *C. albicans* biofilm is protein so that this protein was used to form biofilms.

Iron induction

C. albicans in Microassay plate induced with 5% $FeCl_2$ (iron) and incubated for 24 hours was carried out, and then read by using a plate reader with a wavelength of 492 nm, and produced the formation of biofilm with OD = 0.177. This density was the lowest when it was compared to the density of the other inducers (5% Glucose; 5% Lactose and Soy Protein). If it was compared with the density control of 30% Glacial acetic acid with OD = 0.053, the density of *C. albicans* biofilm induced with iron was ±0.124 thicker.

Iron is an important micronutrient needed by almost all organisms, especially as a cofactor in the metabolic function. Iron, however, can contribute and receive electrons, and participate in the formation of free radicals. Therefore, the presence of iron in the host cell is limited in number. Iron has an important role in forming natural resistance to infections in humans.[18] Pathogenic microorganisms also use iron from the host cells so that there is an iron competition between pathogens and host cells. It is an important aspect that must be highly considered in infectious diseases. In mammals the iron requirements needed /absorbed is very limited so that the availability of free iron is also limited for the pathogens. In this case, iron serves as a defense against infection.

Adaptation to survive in the host cells is done by *Candida* cells by obtaining the thinning iron from the environment of the host. The availability of iron can function as an adaptive signal for pathogens to induce the expression of virulence. The study showed that iron had an important role in the virulence of *C. Albicans.*[19] Iron is required by living things, both host and pathogen (*C. albicans*), but in small amounts. If the amount exceeds the required standards, it will result in toxicity.

Conclusions

Absorbance thickness (density) in the formation of *C.albicans* biofilm with various inducers (Glucose, Lactose, Soy Protein and Iron) can affect the density of the biofilm formation. The inducers in these concentrations are also needed for the growth/formation of *C. albicans* biofilms.

Conflict of Interest

The authors state that there were no conflicts of interest related to this study.

References

1. Miranti A, Ariadna AD, Sri AS. Candida albicans biofilm profiles on various denture base materials. J Int Dent and Med Res 2018;11(1):191-6.
2. Garrett TR, Bhakoo M, Zhang Z. Bacterial adhesion and biofilms on surfaces. Nat Sci 2008;18:1049-56.
3. Daniel L, Hera V, and Roberto K. Biofilms. Cold Spring Harb Perspect Biol 2010; 2(7):a000398.
4. Flemming HC and Wingender J. The biofilm matrix. Nat Rev Microbiol 2010;8(9):623–33.
5. Lars DR and Douglas B. Weibel. Physicochemical regulation of biofilm formation. MRS Bull 2011;36(5): 347–55.
6. Kaitlin FM, Robert Z, and David RA. Fungal Super Glue: The biofilm matrix and its composition, assembly, and functions. PLoS Pathog 2016;12(9): e1005828.
7. Karin S, Alex HR, and David GD. Biofilms and biocomplexity. Microbe 2007;2(7):347-53.
8. Dunne WM. Bacterial adhesion: seen any good biofilms lately?. Clin Microbio Rev 2002;2:155–66.
9. Lovegrove A, Edwards CH, Noni ID, et al. Role of polysaccharides in food, digestion, and health. Crit Rev Food Sci Nutr 2017;57(2):237–53.
10. Karimi A, Karig D, Kumar A, and Ardekani AM. Interplay of physical mechanisms and biofilm processes: review of microfluidic methods. Lab Chip. 2015;15(1):23–42.
11. Ken W, Yanling C and Maria S. A method for quantitative determination of biofilm viability. J Funct Biomater. 2012, 3, 418-31.

12. Afreenish H, Javaid U, Fatima K, Maria O, Ali K, Muhammad I. Evaluation of different detection methods of biofilm formation in the clinical isolates. Braz J Infect Dis 2011;15(4):305-11.
13. Clarissa JN and Alexander DJ. Candida albicans biofilms and human disease. Annu Rev Microbiol 2015;69:71–92.
14. O'Toole GA. Microtiter dish biofilm formation assay. J Vis Exp 2011; 47:2437.
15. Sheppard DC and Howell PL. Biofilm exopolysaccharides of pathogenic fungi: lessons from bacteria. The J Bio Chem 2016;291(24):12529-37.
16. Michelle DL, TaeHyung K, Sonja ED. Candida albicans is resistant to polyglutamine aggregation and toxicity. Genes Genomes Genetics 2017;7(1): 95-108.
17. Pierce CG, Vila T, Romo JA, et al. The candida albicans biofilm matrix: composition, structure and function. J of Fungi 2017;3:14.
18. James EC and Eric PS. Iron in Infection and Immunity. Cell Host Microbe 2013;13(5): 509–19.
19. Hameed S, Prasad T, Banerjee D, et al. Iron deprivation induces EFG1 mediated hyphal development in Candida albicans without affecting biofilm formation. FEMS Yeast Res 2008;8(5):744-55.