The efficacy of photodynamic inactivation with laser diode on *Staphylococcus aureus* biofilm with various ages of biofilm

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Abstract

Biofilms are able to cause microorganisms to be 80% more resistant to antibiotics. The extracelullar polymeric substance (EPS) in biofilm functions to protect bacteria, making it difficult for antibiotics to penetrate the biofilm layer. This study aims to determine the effectiveness of photodynamic inactivation with blue diode laser to reduce *Staphylococcus aureus* biofilm at various ages of biofilms. The light source is a 403 nm blue diode laser with an energy power of about 27.65 ± 0.01 mW. The study was designed with two groups: Group C was the untreated control group with variations in age of biofilms (0; 6; 11; 17; 24; 32; 40 and 48) hours; Group T was a laser treatment group with variations in age of biofilm and energy density (4.23; 8.46; 12.70; 16.93 and 21.16) J/cm^2. Biofilm reduction measurement method using ELISA test was performed to calculate OD_{595} value. The statistical analysis results of variance showed that there was an influence of biofilm age and irradiation energy density of laser on biofilm reduction. Optical density analysis showed the most optimum biofilm reduction happened when biofilm age is perfectly constructed (about 17 hours) and with 91% reduction. The longer biofilm age lived among those biofilms, the greater the reduction. The results of the Scanning Microscope Electron and fluorescent microscope measurement showed destruction site of the EPS biofilm and bacterial cell death. So, the activated photodynamic with 403 nm laser diode is effective to reduce the *Staphylococcus aureus* biofilm in the maturation phase.

Introduction

Staphylococcus aureus (*S. aureus*) is a Gram-positive form of cocci.[1] This bacterium can cause bacteremia, endocarditis, skin and soft tissue infections, osteomyelitis, septic arthritis, prosthetic device infections, and lung infections.[1,2] Generally, treatment of infections caused by *S. aureus* are systemically done with antibiotics. The use of specific antibiotics to bacteria causes unfavorable environmental conditions that stimulates bacteria to form biofilms.[3] Biofilm is a collection of microorganisms that are bound irreversibly and enclosed in a matrix of Extracellular Polymeric Substances (EPS) that is produced to increase the bacterial survival.[4,5] The National Institutes of Health states that more than 65% of the microbial infections were related to biofilms.[6]

EPS covers about 50% to 90% of the total biofilm forming components, and 15% of the volume is a collection of microorganisms.[7] EPS is a polysaccharide produced by bacteria themselves, that is able to protect the bacteria and has an adhesive function between bacteria. Each bacterium produces different EPS so that each biofilm has different chemical and physical properties.[6] The formation of biofilms is divided into several stages, including the attachment to a surface, the production phase of Extracellular Polymeric Substances (EPS), the initial growth stage of the biofilm layer, the maturation stage of the biofilm layer, and the detachment or release of bacterial cells from the biofilm.[4] The formation of biofilms starts from free-living planktonic cells that attach to a surface. Planktonic cells will multiply and form a thin layer of biofilm. At the time of its growth, bacteria will produce EPS and form a micro-colony to form a biofilm layer. The longer life of biofilm, the more bacterial cells and the amount of EPS that causes the biofilm layer to thicken.[7] Furthermore, biofilm will go through a process of maturation.

Biofilms in planktonic form are able to cause microorganisms to be 80% more resistant compared to antibiotics due to genetic exchange and the formation of resistance genes.[7,8] The Euro-World Health Organization reports that biofilm resistance is difficult to manage and requires new antibiotics, but the development of resistance is rapid because the new antibiotics have been discovered.[9] An increase in the infection also occurs if the growth of biofilms in the body is increasing.[10] Extracellular polysaccharide production will increase along with the longer life of biofilms which causes biofilms become thicker.[11] The EPS biofilm functions to protect bacteria, making it difficult for antibiotics to penetrate the biofilm layer.[12] The problem of biofilm resistance requires other more effective methods to reduce biofilm, one of which is Photodynamic Inactivation (PDI).

PDI is a method of inactivation of microbes that utilizes light and light-absorbing substances (photosensitisers).[13] The photoinactivation mechanism involves the photosensitization process, which is the process of absorption of light by photosensitizing molecules (porphyrins) in bacteria, which in turn activates the chemical reaction to produce various reactive oxygen species. Photosensitization depends on the type and quantity of photosensitizer and the suitability of the light spectrum with the

photosensitizer absorption spectrum. At the molecular level, irradiation begins with photophysical in the form of light absorption, which then activates photosensitizer molecules, followed by photochemical reactions that produce various reactive oxygen species. Reactive oxygen causes lipid and protein peroxidation, which subsequently induce cell lysis or inactivation of the membrane transport system and the enzyme system in the bacterial cell.

The interaction between light and biological material causes absorption events. The occurrence of light absorption is more relevant in the loss of light intensity during the penetration process. Absorption ratio depends on intensity. The ability of matter to absorb light has several influence factors, including the wavelength of light, the thickness of the material, and the concentration of absorbent agents. The process of absorption of light in a matter is formulated systematically in the Beer-Lambert law shown in Eqs. 1-4.

$$I_x = I_0 e^{-(\varepsilon c l)} \qquad (1)$$

$$\frac{I_0}{q I_x} = exp^{-(\varepsilon c l)} \qquad (2)$$

$$\ln \frac{I_0}{I_x} = \varepsilon c l \qquad (3)$$

$$A = \varepsilon c l \qquad (4)$$

I_x is intensity at depth x, I_0 is the initial intensity, ε is the molar absorptivity coefficient, c is concentration, l is the thickness of the absorbent medium, and A is absorbance.

Bacteria are able to produce endogenous porphyrin compounds, which become photosensitizer agents that are sensitive to light. Light Amplification by Stimulated Emission of Radiation (Laser) is a monochromatic, coherent and focused light-producing device that is caused by stimulated emission of radiation. Lasers have been widely applied to both biomodulation therapy[14] and antimicrobials. The combination of light at certain wavelengths with photosensitizers in antimicrobial photodynamics will produce radical reactive oxygen species (ROS) products that can cause bacterial cell lysis.[15] Zanin *et al.* conducted a study to demonstrate the effectiveness of PDI in reducing the biofilm of *Streptococcus mutans* bacteria using LEDs and HeNe lasers.[16] The biofilm used has a variation in age of 3 days, 7 days and 10 days. Zanin *et al.* found that a longer life span of biofilms

brought a decrease the percentage of bacterial deaths.[16] Also in the study of Quishida *et al.* also showed that exposure to LED light with a radiation density of 37.50 J/cm^2 was able to inactivate the biofilms of *Candida albicans*, *Candida glabrata*, and *Streptococcus mutans* at the age of 24 and 48 hours. The three bacteria showed a decrease in viability per biofilm age. At the age of 24 hours there was a decrease in biofilm viability more significantly than at 48 hours.[17] Astuti's research on the *Candida albicans* biofilm showed the effectiveness of using a 450 nm diode laser 6.13 J/cm^2 with exogenous photosensitisers to increase bacterial reduction at 24 hours of biofilm.

The aim of the present study is to determine the effectiveness of the energy density of blue diode laser exposure to reduce the *S. aureus* biofilm. This study considered variations in the age of biofilms and variations in the density of laser radiation energy.

Materials and Method

According to the microtiter method Stepanovic *et al.*[18], the manufacture of strains of the bacteria *S. aureus* was made on Tryptic Soy Broth (TSB) media and added subsequently 2 ml of 2% sucrose.[19] Cultured bacteria were grown on 96-well microplates of 100 µL per well then shake the culture until biomass formed which has the same cell density in the well and then incubated at 37ºC for 48 hours.[20]

Treatment of bacteria biofilm

The light source used is a blue diode laser with a wavelength (403,000±0.007) nm and power (27.65±0.01) mW, suitable with the endogenous photosensitizer spectrum of bacteria.[15] The characterization results showed the stability of the laser intensity at (300,000±0.005) seconds after being turned on. Laser irradiation on biofilms was carried out for 30 s, 60 s, 90 s, 120 s and 150 s. The value of the density of the laser energy is calculated using Eq. 5.

$$\text{Energy density}\left(\frac{J}{cm^2}\right) = \frac{Power}{Beam\ area} \qquad (5)$$
$$x\ time\ irradiation$$

Staphylococcus aureus biofilm

Bacterial isolate *S. aureus* (ATCC 25923) was obtained from the Surabaya Medical Center Laboratory. The isolate was grown on Tryptic Soy Agar (TSA) (OXOID CM0131B) media for 24 hours in an incubator at 37ºC. Culture was made in Tryptic Soy Broth (TSB) media

(microbiology VM631759410) with 0.5 optical density.

The microtiter method according to Stepanovic *et al.* begins with the manufacture of *S. aureus* bacteria on TSB media.[18] Furthermore 2 mL of 2% sucrose was added into the bacterial culture.[19] One hundred µL bacterial culture was added into each well of 96-well microplate. The microplate was shaken until biomass formed then incubated at 37ºC for 48 hours.[20]

Treatment of bacteria biofilm

Biofilm samples were divided into 5 laser treatment groups. Group B1: treatment with laser diode exposure 30 seconds (4.23 J/cm^2); Grup B2: treatment with laser diode exposure 60 seconds (8.46 J/cm^2); Group B3: treatment with laser diode exposure 90 seconds (12.70 J/cm^2); Group B4: treatment with laser diode exposure 120 seconds (16.93 J/cm^2); and Group B5: treatment with laser diode exposure 150 seconds (21.16 J/cm^2). After laser exposure, the biofim sample was re-incubated at 37ºC for 24 hours. The samples were washed 3 times with sterile Phosphate Buffered Saline (PBS) using a micropipette then added with 2% cristal violet.[18] Samples were washed again with distilled water until clean and fixed. After that, the sample was added with 100 µL 33% Glacial Acetic Acid for the dye resolubilization. The sample at this point was ready to be analyzed for OD$_{595}$ using the ELISA Bio Rad EIA reader 2550.

SEM analysis

S. aureus biofilm was grown on the glass cover. Culture was made from 9 ml TSB media, 2 ml of 2% sucrose, and 2 ml of collagen. 1000 µL culture was added to the glass cover in a petri dish. The sample was incubated for 0 hours, 24 hours and 48 hours and subsequently exposed to the laser for 150 seconds in the treatment group. Samples were washed with 10% PBS 3 times and fixed. Then the sample was cleaned with 10% ethanol and re-fixated.

Fluorescence analysis

S. aureus biofilm was grown on the glass cover. Culture was made from 9 ml TSB media, 2 ml of 2% sucrose, and 2 mL of collagen. 1000 µL culture was added to the glass cover in a petri dish. The sample was incubated for 0 hours, 24 hours and 48 hours and subsequently exposed to the laser for 150 seconds in the treatment group. Samples were washed with 10% PBS 3 times and fixed. The samples were stained with a Propidium Iodine (PI) dye solution made from 45 ml of sterile aquades, 5 ml of 10% ethanol and 1 mL of propidium iodine

then the samples were re-fixed.

Statistical analysis

Calculation of many reduction bacterial colonies is carried out with Eq. 6.[21]

$$\% \text{ colony} = \left| \frac{\Sigma \text{ Control colony} - \Sigma \text{ Treatment colony}}{\Sigma \text{ Control colony}} \right| \times 100\% \quad (6)$$

Statistical analysis was performed with the help of IBM SPSS 21 (Statistical Package and Service Solutions) software using a two way ANOVA test with equal variances assumed tuckey P<0.05.

Results and Discussion

This research uses a blue laser diode as the light source. Laser characterization results show that the laser used has a wavelength of $(403,000 \pm 0.007)$ nm. At 1 cm distance, the laser emits a beam with an area of (0.20 ± 01) cm^2 so that it has approached the area of the microplate of the sample place. Power measurements were also carried out at a distance of 1cm which amounted to (27.65 ± 0.01) mW. The temperature of the laser output ranges at room temperature that is (32.190 ± 0.001) °C so it is assumed that during the process, the temperature does not effected on bacterial death due to photochemical processes. Power stability occurs at a time $(300,000 \pm 0.005)$ seconds so that before the sample is exposed to the laser, the laser is switched on first $(300,000 \pm 0.005)$ seconds. The energy density is known by using equation (1) which is in the 30 seconds at 4.23 J/cm^2, 60 seconds at 8.46 J/cm^2, 90 seconds at 12.70 J/cm^2, 120 seconds at 16.93 J/cm^2 and 150 seconds is 21.16 J/cm^2.

The photosensitizer agent used is porphyrin which has naturally been produced by bacteria. Porphyrin is a macromolecule involved in the biosynthesis of several biological molecules consisting of four pyrrole rings connected by a methine bridge (=CH−).[22] Porphyrin has absorbance at a wavelength of 390-750 nm with a sorle band at the wavelength region of 390-450 nm and Q-band at 500-750 nm.[22] Staphylococcal strains produce coproporphyrins around 68.3-74.6% so that they are sufficient for the photoinactivation process.[23] S. aureus bacterial absorbance test was performed to determine the absorption spectrum of bacteria. The test was carried out using a UV-Vis spectrophotometer which produced a maximum wavelength value of 406 nm

shown in Figure 1.

This study uses 2 types of sample groups namely the treatment group that will experience laser exposure and the control group. Each sample group had biofilm age variations, namely 8 hours, 16 hours, 24 hours, 32 hours, 40 hours and 48 hours. Specifically for the treatment group, each age of the biofilm will be given variations of laser exposure for 30, 60, 90, 120 and 150 seconds. OD_{595} results from the control and treatment groups will be calculated using Equation 2 so that bacterial death is known. The results of the calculation of bacterial death from the ELISA treatment test were analyzed with the help of the IBM SPSS 21 software which resulted in a value in the Kolmogorov-Smirnov normality test of 0.579 (P>0.05) meaning that the data was non-distribution. Then homogeneity test is performed to determine the level of homogeneity in each data using the test of

equality of error variances. Homogeneity test produces model significance α=0.054 (α>0.05) which indicates that the data is homogeneous. The mortality rate graph is shown in Figure 2 and Figure 3 below.

The biofilm culture used had a log CFU/ml value of 13.65 which would be used for all sample groups. The incubation process caused an increase in OD595 values in all samples at each age indicated by a black line. The black line also shows the results of the control group without laser scanning. Laser exposure caused a reduction in the log CFU/mL value in the treatment group as evidenced by the difference in the log CFU/mL value of the control group.

Figure 2 showed the rate of bacterial death based on the age of the biofilm and length of irradiation. In Figure 4.3 diagram it is known that the rate of bacterial death on biofilms is inversely proportional to the age
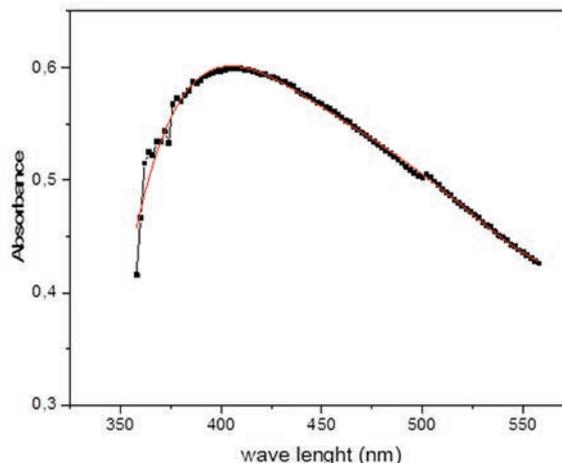
Figure 1. The absorbance results of the *S. aureus* bacteria.
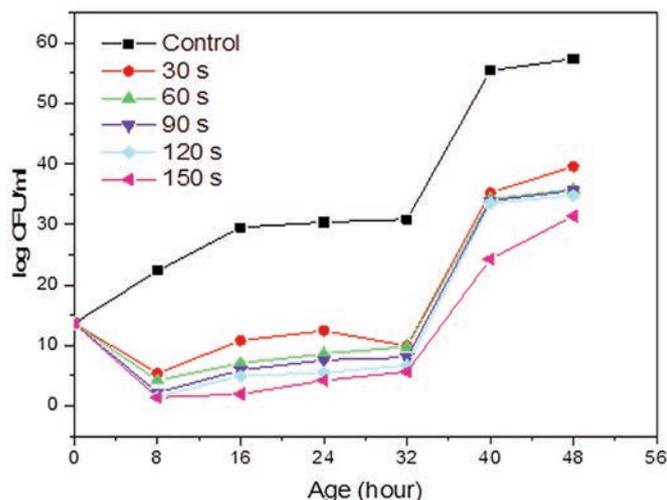
Figure 2. ELISA's analysis of graphs in the control and treatment groups at each biofim age.

of biofilms. The longer the life of biofilms is, the lower death rate of bacteria, and the longer irradiation, the higher the death rate. ELISA's test results show that laser exposure can reduce *S. aureus* biofilms. At 150 seconds exposure with a laser radiation energy density of 21.16 J/cm^2, causing the highest biofilm mortality at each age, such as 8 hours biofilm at 93.62%, biofilm 16 hours 93.31%, biofilms 24 hours 86.09%, 32-hour biofilm 81.56%, 40-hour 56.16% biofilm and 48-hour 45.30% biofilm. At the age of 8 hours biofilm with radiation energy density 21.16 J/cm^2 has the highest mortality rate of 93.62% while at 48 hours of radiation exposure energy capacity of 4.23 J/cm^2 has the lowest mortality rate of 31.07%.

The photodynamic mechanism starts from photophysics, namely the absorption of light by photosensitizer photoactive molecules, causing the electrons to be excited. Unstable electrons tend to return to the ground state through several mechanisms such as Intersystem Crossing (ICS) which occurs when the energy level of the singlet excited state overlaps with the triplet state level accompanied by a reversal of electron spin in the molecule.[14] Unstable molecules can interact with surrounding molecules through photochemical processes.[24]

Photochemistry plays a role in changes in energy and electronic structure due to excitation events.[12] Type I photochemical events produce superoxide molecules whereas in type II they produce singlet oxygen which both can interact with
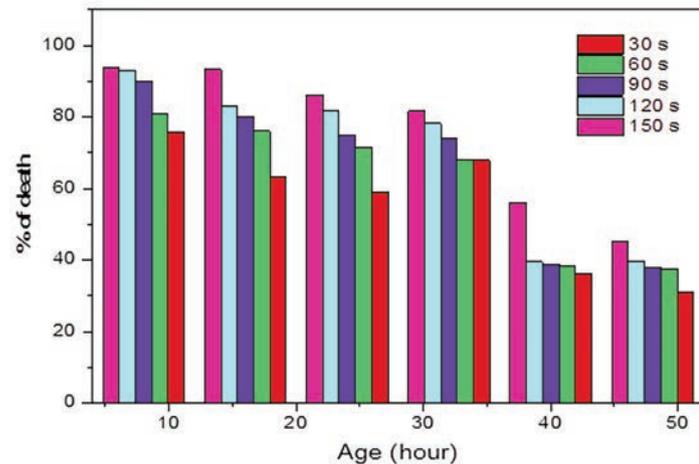
Figure 3. Bacterial death rate at each biofilm age.
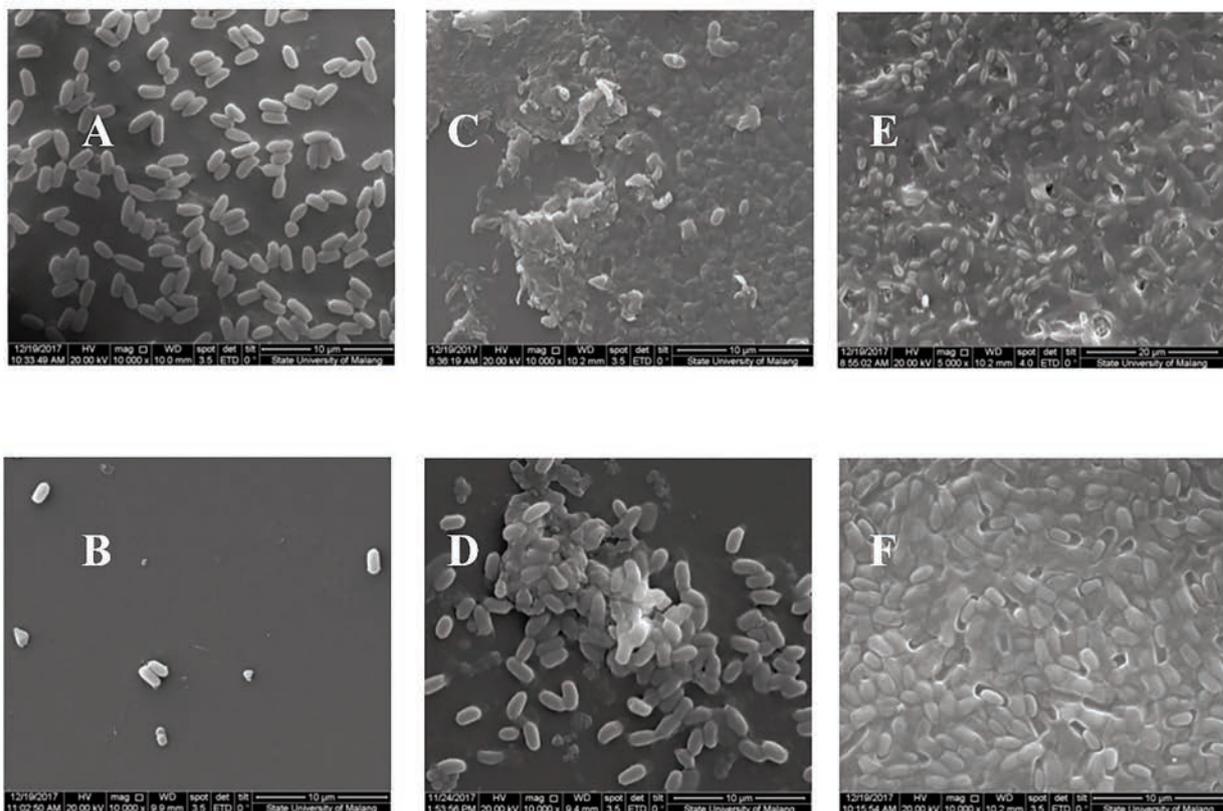
Figure 4. The results of SEM analysis at (a) 0 hour age biofilm control group (b) 0 hour age biofilm treatment group (c) 24 hour age biofilm control group (d) 24 hour age biofilm treatment group (e) 48 hour age biofilm control group (f)) 48-hour-old biofilm treatment group

biomolecules so that they are damaged.[25] Type I and type II photochemistry occur in parallel depending on the photosensitizer used and oxygen concentration but type 2 II photochemistry occurs more dominant.[26] The interaction of reactive oxygen with biomolecules causes photobiology. Reactive oxygen is radical so that it can damage cells. It is also able to initiate changes in unsaturated fatty acids to form lipid peroxide (monodiadehyde) which will change the structure of amino acids and proteins chemically.[27] On biofilms, reactive oxygen causes damage to intracellular biofilms, lipids, and biofilm matrices.[28] In this study, bacterial death in biofilms was demonstrated by a decrease in the ELISA OD595 test value in the treatment group. Decreasing in density indicates cell loss from biofilms.[29] Reactive oxygen can damage DNA, lipids, proteins and membranes in bacteria.[28] Excessive reactive oxygen in cells causes DNA damage significantly and permanently.[28] If reactive oxygen interacts with proteins, it causes damage to aromatic amino acids.[28]

Bacteria are protected by an outer membrane called a cell wall. Damage to the bacterial outer membrane due to reactive oxygen causes disruption of membrane permeability. Increased outer membrane permeability causes metabolite leakage resulting in cell surface disturbance.[28] Reactive oxy-

gen also causes changes in the biofilm matrix that is composed of polysaccharides resulting in changes in biofilm aggregate size.[28-30] The change in biofilm aggregate size depends on the amount of radiation energy density and biofilm characteristics.[2] Laser exposure can cause damage to biofilms that are characterized by morphological changes in biofilms and dead bacterial cells.[30] The SEM test was carried out to determine the morphological changes in the biofilm due to laser exposure shown in Figure 4.

In Figure 4, it can be seen that the bacterial cell is not ocular but round. Monteiro et al, 2015,[31] revealed that there were changes in the morphology of *S. aureus* cells from round to oval due to cell cycles so that *S. aureus* bacteria were not spherical at all stages of the cycle. Cells will elongate at certain time intervals through peptidoglycan synthesis and remodeling.[31] Peptidoglycan is a polysaccharide that is rigid as a constituent component of bacterial cell walls to maintain cell integrity.[32] Peptidoglycan synthesis, old peptidoglycan autolysis or a combination of both can cause cell enlargement.[32] When the division of stem cells, cells do not enlarge significantly, but there is a variation in the volume of cells that is the re-formation of the septum for division in each daughter cell.[31] Formation of the septum requires a

short time of <2 minutes so that there is a sudden change that results in duplicate incompatible cell volumes.[31]

The SEM analysis showed that in the 0 hour sample the cells were in the form of planktonics. Laser exposure on planktonic cells causes a bacterial death which is more significant than in the form of biofilms, which are samples aged 24 hours and 48 hours. The formation of biofilms at 24 hours and 48 hours is marked by the presence of EPS matrix covering the cell surface. Laser exposure causes damage to the polysaccharide making up the biofilm matrix.[28] Biofilm matrix has pores (water channel) as a place of diffusion of nutrients, oxygen or antimicrobial agents.[4] The SEM test results of this study indicate that the formation of pores on 48-hour biofilms is greater than on 24-hour biofilms, and laser exposure also results in an increase in the number of pores. SEM test results are able to identify cell death, characterized by cell lysis. In the 24-hour and 48-hour biofilm samples there was no cell lysis but this condition could not be justified that the bacterial cells in the biofilm did not die, so in this study a fluorescent test was performed to determine bacterial death visually.

In the fluorescent analysis, bacterial cells that have died can absorb propidium iodine dye so that if tested with a
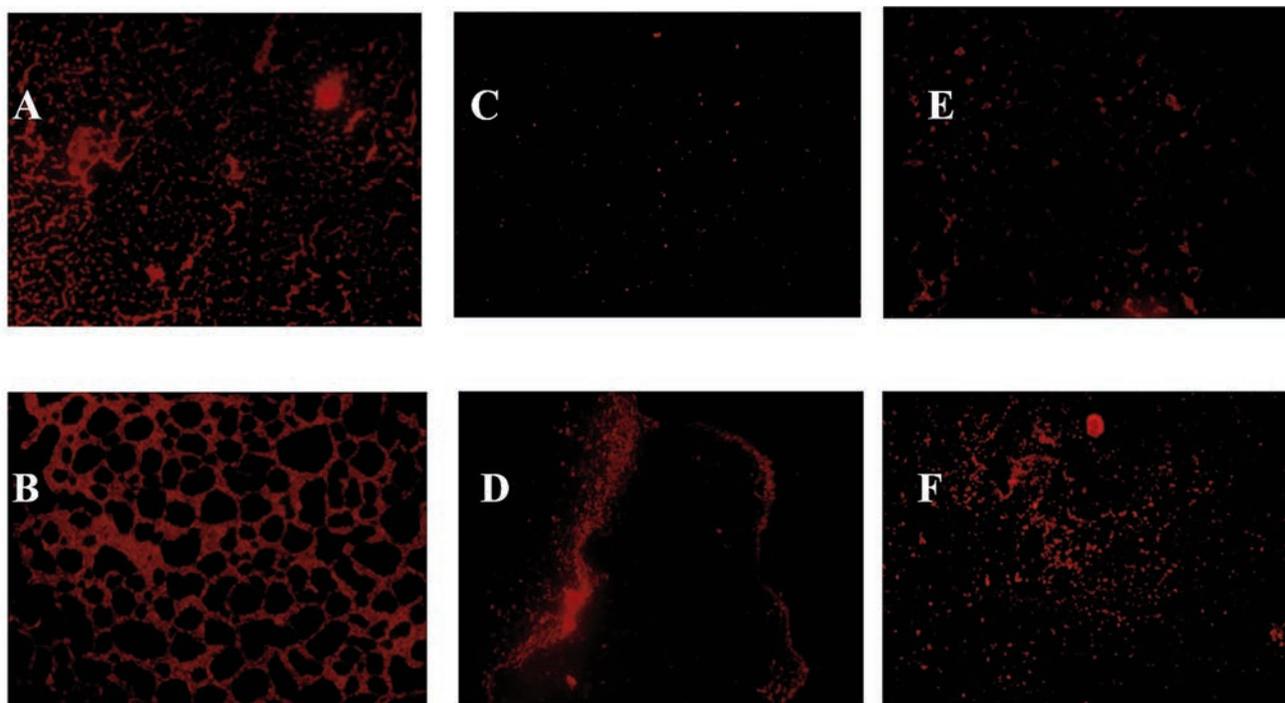
Figure 5. The results of fluorescence analysis at (a) 0 hour age biofilm control group (b) 0 hour age biofilm treatment group (c) 24 hour age biofilm control group (d) 24 hour age biofilm treatment group (e) 48 hour age biofilm control group (f)) 48-hour-old biofilm treatment group

fluorescent microscope will show the presence of bacterial cells that are marked by the presence of red dots. Propidium iodine is a fluorogenic compound that can bind to nucleic acids so that a fluorescent test will produce fluorescence emissions that are comparable to cell DNA.[33] Propidium iodine compounds are only able to interact with damaged cell DNA.[33] Fluorescent test results are shown in Figure 5. Fluorescent images are analyzed with the ImageJ application. Dead cells are counted using the Threshold menu on ImageJ. The results of ImageJ showed that in the 0 hour biofilm there was a reduction in bacterial cells by 1448 cells, a 24 hour biofilm 663 cells and a 48 hour biofilm 221 cells. Reduction of dead bacterial cells each biofilm age decreases with increasing biofilm age. The photodynamic mechanism is related to the absorbance of light, which is affected by the depth of the material. In biofilms, the longer the life of the polysaccharide extracellular substance production increases so that the biofilm layer is thicker.[2] So it can be said that the age of biofilms can affect the ability of light penetration. Research by Moormeier *et al.*[34] showed that *Staphylococcus aureus* has several stages for the formation of biofilms, divided into 4 times, namely 0 hours, 6 hours, 11 hours and 17 hours. At 0 hours it shows the attachment of planktonic cells, in the next 6 hours the cells undergo multiplication, 11 hours, the cells will start to form biofilms called exodus and 17 hours have formed mature biofilms.[34] So in this study a SEM test was performed on each of the biofilm developments shown in Figure 6.

In the SEM test results the 0 hour sample shows the presence of planktonic cells that have not yet formed a colony, whereas in the 6 hour sample a cell colony is formed which is characterized by a collection of cells that are attached to each other. In the 11 hour sample, an EPS matrix was formed which was marked by the presence of a thin layer on the bacterial cell while at 17 hours, the EPS layer appeared to be thicker and a water channel was formed (pore). The SEM test results in Figure 6 show that at the age of biofilm 0 hours, 6 hours and 11 hours biofilm has not formed completely so that in the ELISA test sample biofilm 8 hours and 16 hours also cannot be said to be perfect (mature) biofilms. ELISA's test results showed that at a meeting energy level of 21.16 J/cm², the most efficient rate of bacterial mortality was at 24 hours and 32 hours of biofilm. Whereas at the age of 40 hours and 48 hours has a low bacterial death rate due to laser exposure of 21.16 J/cm² so it is not efficient to reduce biofilms at that age. So the longer the life of the biofilm will reduce the rate of bacterial death. Wang *et al.* and Astuty *et al.* reported that the age of biofilms could affect the decrease in bacterial mortality due to exposure to light due to limited light penetration on biofilms.[35,36]

Conclusions

Blue laser diode (403,000 ± 0.007) nm with power (27.65 ± 0.01) mW can reduce the biofilm of *S. aureus* bacteria at each various age. Radiation energy density of 21.16 J / cm2 already given the most efficient rate of bacterial death at 24 hours and 32 hours of biofilms aged. In 8 hours and 16 hours of biofilms aged, mature biofilms have not formed even though the rate of bacterial death is high, while at 40 hoursand 48 hours, mature biofilms have been formed, but the resulted death rate is low. Reduction
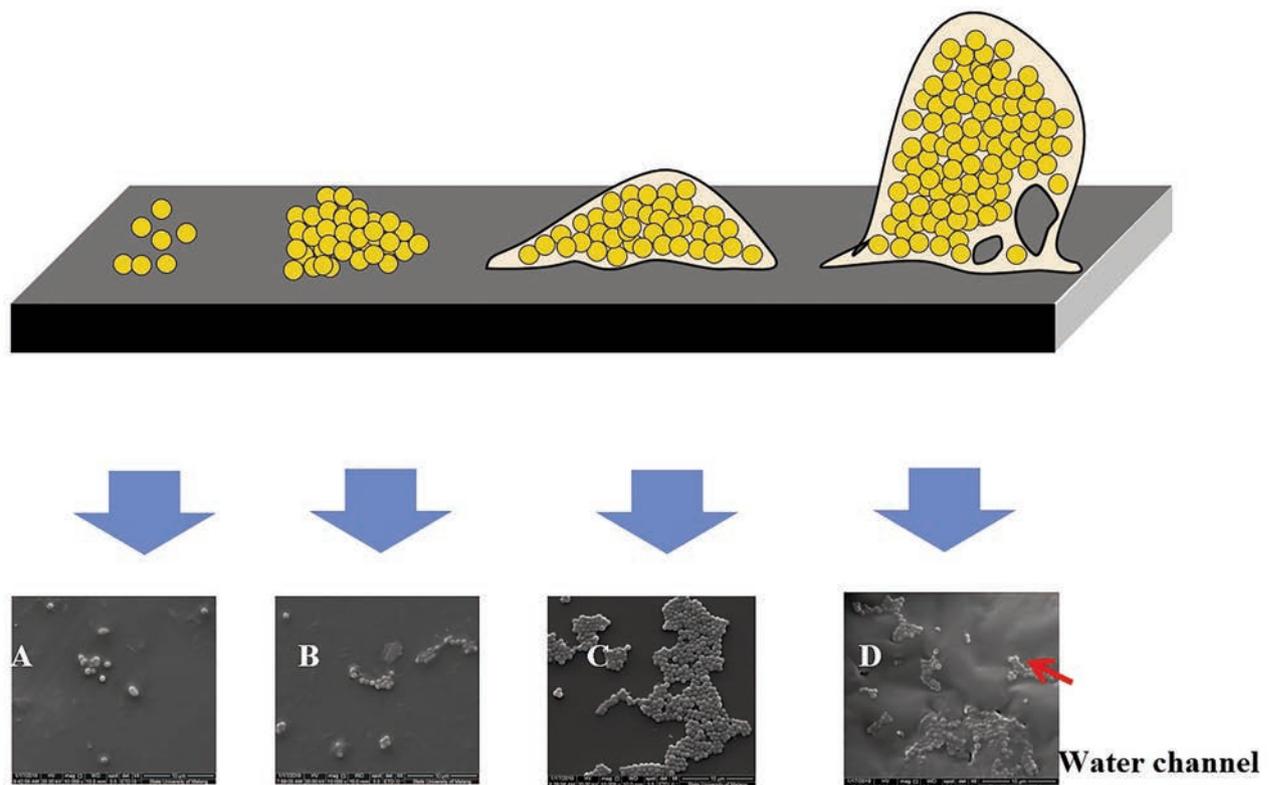
Figure 6. The result of SEM analysis for the development of *S. aureus* at (a) 0 hour biofilm, (b) 6 hours, (c) 11 hours, and (d) 17 hours.

of dead bacterial cells each biofilm age decreases with the increase in biofilm age. The photodynamic mechanism is related to the absorbance of light, which is affected by the depth of the material. In biofilms, the longer life of the polysaccharide extracellular substance production increases so that the biofilm layer is thicker. So it can be said that the age of biofilms can affect the ability of light penetration and biofilm reduction.

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