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Efficacy of CNC-Diode Laser Combine with Chlorophylls to Eliminate *Staphylococcus aureus* Biofilm

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***Abstract*—** The clinical manifestations of infection by bacteria forming biofilms is their resistance to antibiotic treatment. So, we need an alternative method that is both effective and selective for killing of *Staphylococcus aureus* (*S. aureus*) biofilm bacteria. One of which is a method of photodynamic (PDT) by utilizing light and photosensitizer. The purpose of this study is to build and determine the efficacy of CNC-laser diode, combine with/without chlorophyll to eliminate the microbial *S. aureus* biofilm. CNC (Computer Numerical Control) laser instrument consisted of light source (diode laser), sample holder and controller to control of the intensity values, the number of microplate lines, time duration of irradiation, the stepper motor rotation according to predetermined coordinates, and control the fan. Fan control is aimedto maintain inside temperature of the sample box at room temperature. The characterization of CNC-laser showed the stability of laser power and temperature during irradiation. The 405 nm laser treatment group combine with and without chlorophylls resulted in statistically non-significant efficacy of log CFU ($p<0.05$) compared to each other. Best efficacy was shown under laser irradiation time of 75 seconds with endogenous PS with the efficacy of 18% and chlorophylls of 22.28%. The result of biofilm viewing of confocal microscopy shows that CNC-laser treatment with exogenous PS (chlorophylls) could eliminate the *S. aureus* biofilm. So CNC-diode laser combine with chlorophylls can eliminate Staphylococcus biofilm.

Keywords— CNC-laser diode, efficacy, Biofilm, chlorophyll, Staphylococcus aureus

I. INTRODUCTION

Bacteria that can produce biofilms are associated with chronic infectious diseases. *Staphylococcus aureus* (*S. aureus*) is a normal flora that lives in commensal on the skin, nasal passages or humans throat [1]. In abnormal conditions, these bacteria can cause some diseases from mild skin diseases such as dermatitis, acne vulgaris, cellulitis folliculitis to severe illnesses such as pneumonia, meningitis, osteomyelitis endocarditis, toxic shock syndrome and septicemia [2],[3].

Biofilm is a group of microbial cells irreversibly attached to biological or inanimate surfaces and encased in a matrix of Extracellular Polymeric Substances (EPS) generates its own [4],[5]. This formation allows bacteria to withstand the extreme environment harmfully, survive against antibiotics, disinfectants, even able resistant to immune-system host [5]. The clinical manifestations of infection by bacteria forming biofilms is their resistance to antibiotic treatment. Antibiotic therapy only kill bacterial cells planktonic while the bacteria in the biofilm will survive and develop, and will release from planktonic cells out of the biofilm formation [6]. So, we need an alternative treatment having effective and selective ability for eliminating *S. aureus* biofilm; it is a photodynamic therapy (PDT) [7].

PDT utilizes radical oxygen species that generated from the stimulation of the photosensitizer (PS) by a suitable light source. In the past, PS activation was achieved *via* a variety of light sources, LED and any various of laser [8]. High power lasers have a complicated system and expensive. However, diode laser has a more simple system, small dimensions, and inexpensive [9]. The choice of PS used in dentistry is strongly dependent on the light source used. Laser diode produces light with a spectral range, based on the composition of the semiconductor material. The use of PDT laser in vitro has been widely investigated. Research results demonstrated that PDT can eliminate the bacterial biofilms [10],[11].

Number of PS on PDT can be increased by administering

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exogenous PS. Exogenous PS is PS that is added to enhance the concentration and absorption of light. Efficacy of PDT depends on the type and concentration of PS, quantum yield, output power and irradiation time of diode laser [12].

Organic PS is extracted from natural materials such as chlorophyll from green plants and photosynthetic bacteria. Chlorophylls have physical properties similar to porphyrins, but they have wider absorption area and higher intensity than porphyrins. Chlorophylls are more applicable to be developed for PDT in tumors and cancer [13],[14]. High energy absorption during the process of photosynthesis is caused by the presence of relatively long excitation phase of chlorophyll ($\leq 10^{-8}$seconds). The longer phase of singlet excitation of chlorophyll, the higher the electronic energy conversion from the basic level to an excited triplet. Excess energy at the level of the excited triplet chlorophyll gives an opportunity to transfer its energy to nearby oxygen molecules. This reaction produces ROS [15].

The purpose of this study was to build and determine the efficacy of CNC-laser diode combine without/with chlorophyll to eliminate the microbial *S. aureus* biofilm.

II. METHODS

A. Bacterial Strain

Bacterial isolate of *Staphylococcus aureus* (ATCC 28923), obtained from the Faculty of Veterinary Medicine - Airlangga University. The isolates were grown in TSA - *Tryptic soy agar* (Oxoid, UK), taken and placed in TSB *Triptic soy broth* solution (Merk, Germany) and for 24 hours incubated until optical density value of OD_{600}nm = 0.5 is reached, which is equal to ~ 10^6 CFU/mL.

Biofilm cultures were taken of 100 mL in each well in 96 well microplate. Each well was added 20µL of 2% sucrose and placed on a shaker for 4 hours. Sample culture cleaned with Phosphate Buffered Saline pH 7.4 three times and placed in the incubator for 48 hours.

B. Materials

Chlorophyll was extracted from Alfalfa leaves or *Medicago sativa L* (K-Link liquid, Indonesia) with a concentration of 1.6 mg/ml diluted with sterile normal saline. The absorption spectrum of chlorophyll measured using Shimadzu UV-VIS 1800 spectrometer.

C. CNC Laser for Treatment

The schematically set up of treatment shown in Figure 1. The block diagram of CNC (Computer Numerical Control) laser instrument for irradiation treatment is shown in Figure 2. It comprises of light source and controller. The light source used to excite photosensitizer in photodynamic mode is a diode laser output wavelengths of 405 nm. The power output is 82.22±0.11 mW focal spot ®xed: 0.3 cm^2.

The CNC-laser instrument consists of the sample holder and the microcontroller. Sample holder consists of: 1) a light source (laser diode) (2) laser driver to limit the current and voltage to the laser. (3) a temperature sensor serves to detect the temperature during the process of irradiation. (4) fans serve to control the temperature to remain constant. If the room temperature irradiation> 27ºC, the fan will turn on. It remains silent if the room temperature irradiation ≤ 27ºC. (5) The stepper motor serves to move the microplate in the X and Y coordinates and set the height of the laser on the coordinate Z.

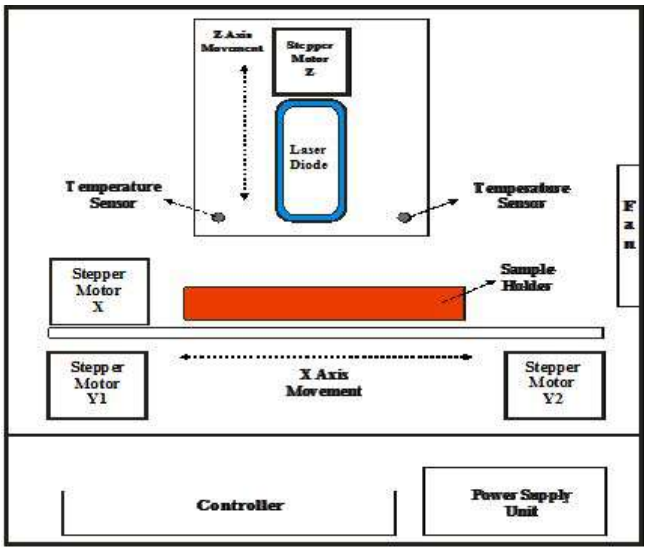

Fig. 1. The schematic CNC laser for irradiation treatment

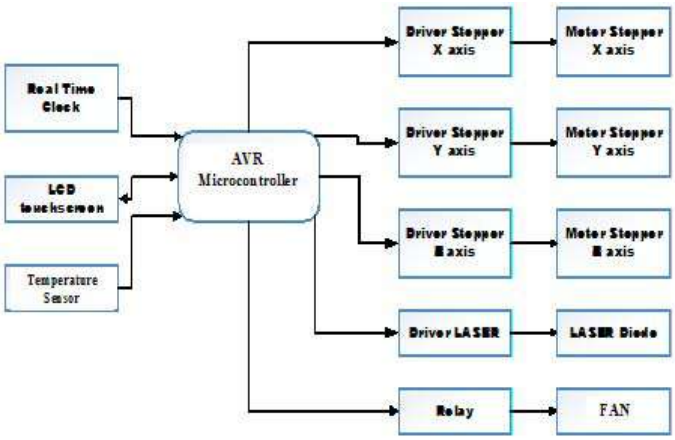

Fig. 2. The block diagram performance of CNC laser for irradiation treatment

The function of the microcontroller is (1) to control the intensity values, the number of microplate lines and time duration of irradiation. (2) to counts time according to a predetermined of time duration. (3) to control the stepper motor rotation according to predetermined coordinates. (4) to activate and deactivate the fan to keep sample box temperature ata room temperature.

D. Sample Treatments

To determine the antimicrobial effect of treatments on *S. aureus* biofilm, the samples were distributed into 4 groups as follow: (1) Groups A (treated with laser), (2) Groups B

photodynamic (treated with laser and chlorophylls), (3) Group C^- (negative control, no exposure to either chlorophylls), (4) Group C^+ (treated only with chlorophylls). For each group, the experiment was repeated at least 3 times. Treatments of samples done in a completely dark room.

E. Crystal Violet Assay

After treatments, samples were cleaned with PBS for 3 times and then incubated for 24 hours. A 150µL of 0.2% crystal violet dyes were then applied and incubated for 15 minutes. Samples were then cleaned with distilled water for three times, and then 50µL 33% glacial acetic acid (GAA) were given. Measurements were then carried out using microplate reader S/N 17539 (Bio-rad, US) at 595nm. Irradiation times are 0; 15s; 30s; 45s; 60s; 75s. For each group, the experiment was repeated at least 4 times. Samples treatments were done in completely dark room

F. Biofilm View in Confocal Laser Scanning Microscopy

Morphology of biofilm is visualized by confocal laser scanning microscopy (CLSM). A sample of CLSM was used a cover glass 1.5cm×1.5 cm collagen-containing and taken place in a petri dish with 6cm diameter that filled 3 mL TSA. Biofilm was taken 2 mL culture and stored in an incubator for 48 hours. After treatment, sample was rinsed by 10% PBS at pH 7.4 three times. The sample was dyed using 2% Acridine Orange (Sigma, Aldrich) for 30 minutes and rinsed using PBS pH 7.4 once. The sample was ready to be measured using Confocal Microscopy PV1000 (Olympus) with 200x magnification. The 3D images morphology was visualized by Bioview3D. Image of CLSM is computed by COMSTAT ver2.1 via ImageJ software [16].

G. Statistical analysis

The results were log-transformed and analyzed by analysis of variance (ANOVA). A p-value ≤ 0.05 was considered to indicate a statistically significant difference. The % efficacy of log CFU/mL from samples treatments defined as:

$$\% \text{ Efficacy} = | (\Sigma \text{ necrosis colony} / \Sigma \text{ control colony}) | \times 100\%.$$

III. RESULTS AND DISCUSSION

The characteristic of output power of the CNC laser instrument for irradiation treatment is depicted in Figure 3. Figure 4 shows the temperature measurements during irradiation. It can be seen that the temperature is below 45ºC. This temperature is still in the range of optimum growth of *S. aureus* so that the bacterial death not caused by an increase in temperature due to irradiation.

PS used in this study is chlorophyll from an extract of Alfalfa leaves or *Medicago sativa L.* with 1.6 mg/ml concentration. The absorption spectrum of chlorophyll was measured using a UV-Visible spectrophotometer. The absorption spectrum of chlorophylls is shown in Figure 5.

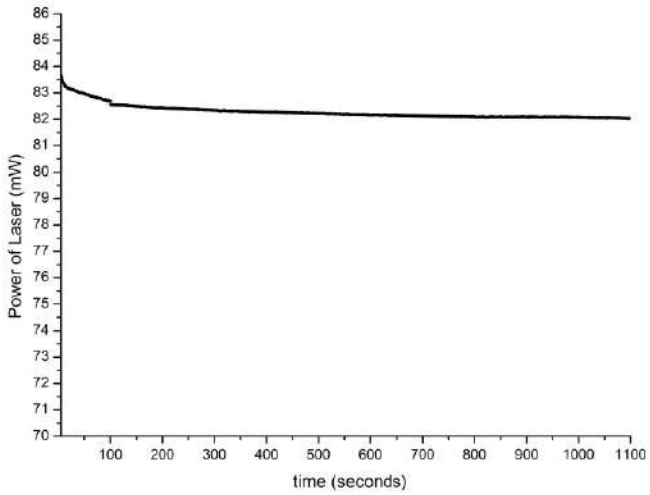

Fig. 3. The steady state of Power of Laser.

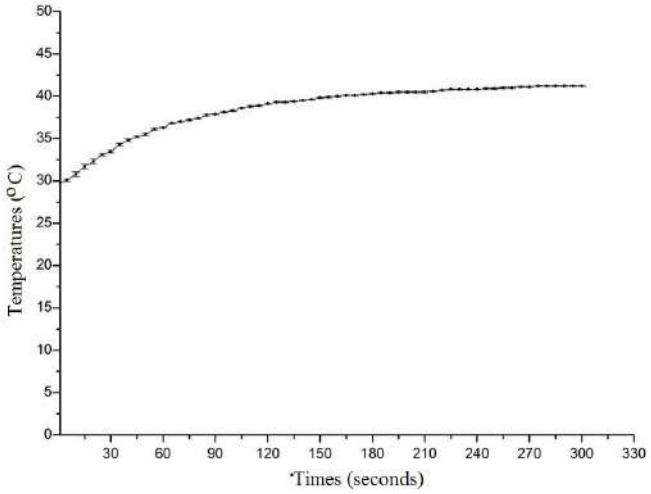

Fig. 4. Temperatures measurement of Laser

The CNC-Diode laser had benefit to control microplate position and real time irradiation. Microplate position and laser position had controlled by a motor servo, and they gave benefit to give distance irradiation accurately. Laser position could accurately move in every well of the microplate. The user could easily use it with touchscreen and understanding of language performance.

The absorption percentage of PS will affect the production of ROS. The concentration of oxygen and PS, the effectiveness of the light source holds an important role in the success of the PDT [17]. The absorption spectrum of exogenous PS from Alfalfa leaves or *Medicago sativa L.* indicates good absorption at a wavelength of blue and red. Based on these results, the quantum yield of the laser diode at 405 nm [18] is 92.97%.

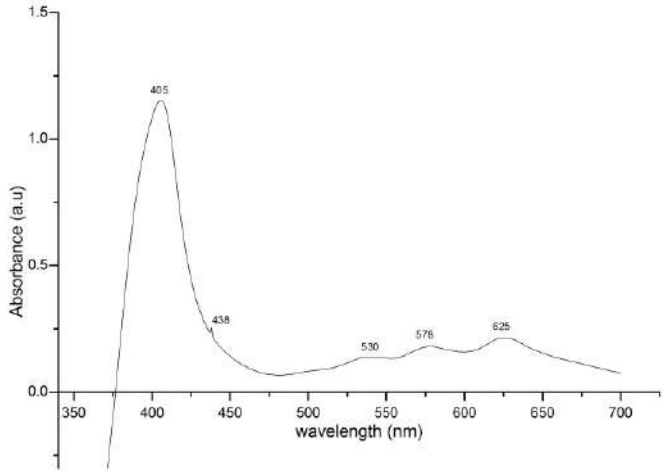

Fig. 5. The absorption spectrum of chlorophyll.

Results of PDT treatment with endogen PS is shown in Figure. 6. The laser at 405 nm treatment group resulted in a not significant efficacy of log CFU/mL ($p<0.05$) compared to each other. Best efficacy was shown under laser irradiation time 75 seconds with efficacy 18.95%.

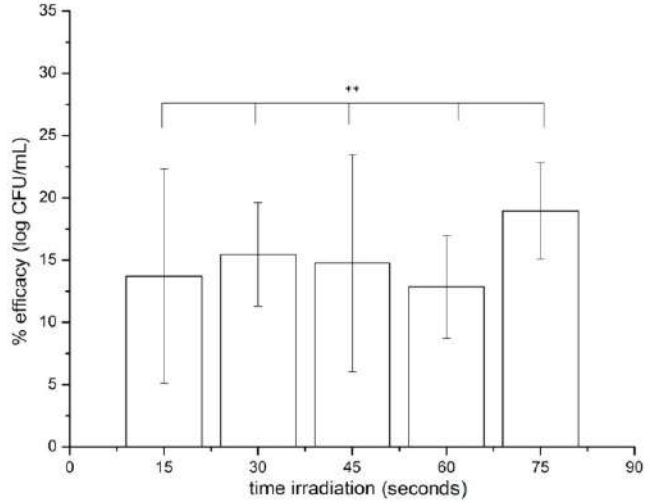

Fig. 6. Results of PDT treatment with endogenous PS. Marker ** shows not significant level in ANOVA one-way test $p>0.05$.

The endogenous porphyrin of *S. aureus* consists of Uroporphyrin (89%)[19] and Coproporphyrin III (68.3 - 74.6%)[20]. The absorbance of this porphyrin is in the blue region (405 nm), so suitable for laser spectrum.

Results of PDT treatment with exogenous PS is shown in Fig 7. The laser 405 nm treatment group resulted in a non-significant efficacy of log CFU ($p<0.05$) compared to each other. Best efficacy was shown under laser irradiation time 75s with efficacy 22.28%.

Results of biofilm viewing of confocal microscopy for the control group (without and with exogenous PS) and CNC-laser treatment group (without and with exogenous PS) are shown in Fig.8. They had difference morphology and adding of chlorophyll gave less thickness and surface on biofilm. Table 1. showed the parameter measurement of biofilm after treatment.

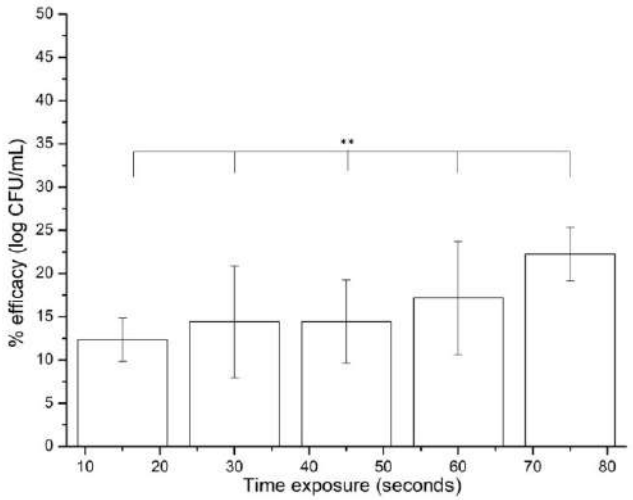

Fig. 7. Results of PDT treatment with exogenous PS. Marker ** shows not significant level in ANOVA one-way test $p>0.05$.

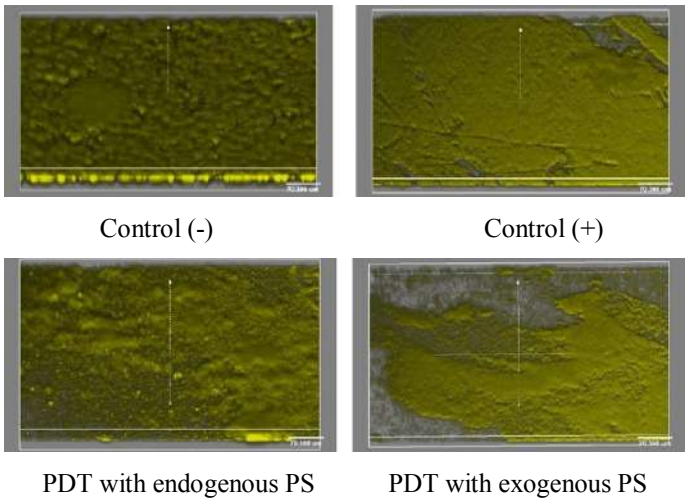

Fig. 8. Biofilm viewing of confocal microscopy using Bioview3D

Results of confocal laser scanning microscopy is an image that will show some parameters and properties of biofilms [21]. Biomass of biofilm showed turbidity or the ratio between volume and surface area. Biomass measurement in each treatment showed that the irradiation of laser and the addition of exogenous photosensitizer provide a decrease of biomass. The maximum thickness of biofilm showed a maximum altitude of community bacteria that are able to produce. The average height biofilm is a normalized height of biofilm. From the results of measurements of heights, laser irradiation and the addition of the sensitizer able to reduce the height of the biofilm. Roughness state has a relationship with the height of the biofilm. This parameter is the distance of biomass each other or can be inferred roughness biofilm. Biofilm surface area is the region that occupied by a community of bacteria.

TABLE I. PARAMETER MEASUREMENT OF BIOFILM

Parameter	Control (-)	Control (+)	PDT (-)*	PDT(+)**
Biomass ($\mu m^3/\mu m^2$)	15.25	8.09	9.19	5.37
Max Thick (μm)	57	41	33	26
Roughness State	1.05	1.08	1.10	1.07
Surface area (μm^2)	16.02×10^6	9.02×10^6	9.41×10^6	6.12×10^6
Average Thickness (entire surface (μm))	23.68	15.89	13.11	9.87

PDT (-)* = PDT with endogenous PS
PDT (+)** = PDT with exogenous PS

The result of biofilm viewing of confocal microscopy showed that CNC-laser treatment with exogenous PS (chlorophylls) could eliminate the *S. aureus* biofilm.

IV. CONCLUSION

The characterization of CNC-laser showed the stability of laser power and temperature during irradiation. The 405 nm laser treatment combine with and without chlorophylls resulted in the non-significant efficacy of log CFU ($p<0.05$) compared to each other. Best efficacy was shown under laser irradiation time of 75 seconds with endogenous PS efficacy of 18 % and chlorophylls 22.28%. The confocal microscopy image of the biofilm shows that CNC-laser treatment with exogenous PS (chlorophylls) could eliminate the *S. aureus* biofilm. So CNC-diode laser combine with chlorophylls can eliminate *S.aureus* biofilm.

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