IOP Conf. Series: Journal of Physics: Conf. Series **853** (2017) 012026 doi:10.1088/1742-6596/853/1/012026

The effectiveness of laser diode induction to *Carica Papaya L.* chlorophyll extract to be ROS generating in the photodynamic inactivation mechanisms for *C.albicans* biofilms

S Dewi Astuty[1,3,*] and A Baktir[2]

[1] Student of Doctoral Program, Faculty of Science and Technology, Universitas Airlangga, Surabaya, East Java, Indonesia
[2]Department of Chemistry, Universitas Airlangga, Surabaya, East Java, Indonesia
[3]Department of Physics,Faculty of Science and Technology, Universitas Hasanuddin, Makassar, South Sulawesi, Indonesia

*Corresponding email address: tuty_ilyas@yahoo.com

Abstract. Research on the effectiveness of photo inactivation of *C.albicans* biofilms led by a-PDT system mediated by chlorophyll-diode-laser-induced was done. This research was done using *in vitro* technique in order to effectively determine chlorophyll extract of ROS-generated *Carica Papaya L.* using *in situ* technique. This technique induced laser diode on different dose and *C. albicans* with reduced degree. This research is a preliminary study in efforts to find anew sensitizer agent candidate made of chlorophyll extract and antifungal of *Carica Papaya L.* The effectiveness of eradication has been tested with MDA's content and OD of biomass biofilms as well as analyzed using ANOVA and Tukey Test (α=0.05).The characteristic of chlorophyll extract of *Carica Papaya L.* has maximum absorptions on blue areas (λ_{max}= 420 nm) and red areas (λ_{max}= 670 nm). The MIC value of *Carica Papaya L.*'s*chlorophyll* extract against *C. albicans* planktonic and biofilms cell is 63.8 μM and 31.9 μM respectively. The result shows that treatment using laser which was combined with chlorophyll extract is more effective than that with laser only or chlorophyll extract only. The treatment using laser combined with chlorophyll extract obtained more than 65% (α=0.05) (more than that of negative control) for P_2L_1 group with OD_{595} 0.915. The MDA's content showed that group of laser which was mediated with chlorophyll extract had larger values than group of laser or chlorophyll extract only.

1. Introduction

Photo inactivation is part of photodynamic therapy (PDT), known as a-PDT (antimicrobial photodynamic therapy) [1,2]. Photo inactivation aims to inhibit the growth of pathogenic microbes which cause infections in human body. Mechanisms of photo inactivation based on interaction between photons of light with molecules of the sensitizer produce some kinds of ROS (reactive oxygen singlet). ROS is reactive compounds produced from the reaction of the radical compound substrate triplet sensitizer to molecular oxygen, whereas singlet of oxygen molecules formed from the reaction of triplet sensitizer to molecular oxygen. Both types of reactive compounds contribute to oxidation biomolecule by hydrolysis cell mechanisms (Figure 1.).

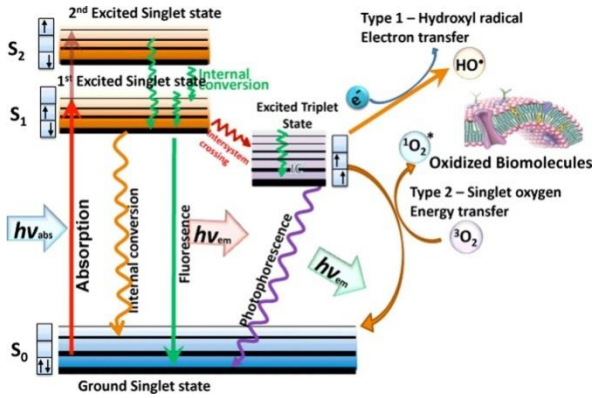

Figure 1. Photoinactivation mechanisms [3]

While the amount of ROS accumulated in a tissue such as the cell membrane or cell wall of microbes, hydrolysis, and ion exchange out and into the cells causes an imbalance of cellular metabolism and DNA chain termination, it also causes cell death by necrosis or apoptosis.

Photons of light absorbed by a molecule or tissue cause multiple interactions depending on the power density of photons of light: photochemical interaction (0.01 to 50 W/cm^2), photothermal (10^0-10^6 W/cm^2), photoablation (10^7-10^8 W/cm^2), plasma-induced ablation (10^{11}-10^{18} W/cm^2), photodisruption (10^{16} W/cm^2) [4]. The interaction of photo inactivation for *C.albicans* is expected to occur through the transfer of photochemical interaction outer electrons in the molecule guanine group so that the electrons become unstable and tend to form free radicals which in turn will induce cell death [4]. In order to avoid excessive thermal effects, the power density of 10-100 mW/cm^{2}is used [5].

C.albicans is microbial pathogens with a high level of virulence, known as triggers infection called candidiasis [6]. Candidiasis can occur in the mouth, gastrointestinal tract, and respiratory and genital mucosa [7] called systemic candidiasis. Systemic candidiasis can be associated with opportunistic nature of *C.albicans* as likely to be severe if patients experience pathologicalimmune-compromise such as HIV-AIDS patients. Pathogenicity degree of *C.albicans* is caused by several factors: the process of adhesion and invasion, toxin secretion of enzymes and metabolites, dimorphism properties, and the phenotype of switching from white to opaque and biofilm formation [8]. Biofilms are considered as a major mediator of infection, with an estimated 80% incidence of infections associated with biofilm formation [9,10].

Morphology of *C.albicans* cell includes the type of eukaryotic organisms with complex structures and has a cell membrane with a more rigid and thicker cell structure. The existence of the extracellular matrix in the form of a layer biofilm β-1.3 glucan improves the rigidity and thickness of *C.albicans* [11,12]. Eradication of pathogenic microbe photo inactivation *C.albicans* through a mechanical consistent with the wavelength of red light was conducted in order to get deeper tissue penetration.

Chlorophyll and its derivatives are potential new photosensitizers in cancer therapy [13,14] because they have similar structure of Heme blood that they can easily accumulate in the body, select only tissue cancer for its hydrophilic properties, and is rapidly cleared from normal tissue because of hydrolytic properties. The main properties of chlorophyll in photosynthesis is a high energy absorption therefore it is also potential as a photosensitizer in photodynamic therapy. The important characteristic of chlorophyll for photodynamic mechanisms is the lifetime of triplet state of a-PDT which is one of photo dynamics mechanisms for inhibition growth of microbial pathogen.

One of the plants which contain high levels of chlorophyll content is medicinal plant [15] with antifungal substances such of flavonoids, saponins, tannins, and papain. Chlorophyll extract of *Carica Papaya L.* has a molecular structure similar to the porphyrin, and it has two characteristic wavelength of maximum absorption in the region: blue and red areas.

This study aims to analyze characterization and effectiveness chlorophyll extract of *Carica Papaya L.,* related to reduction of OD values and MDA's values on photo inactivation mechanisms against *C. albicans* biofilms growths.

2. Method

2.1. Isolation of chlorophyll from Carica Papaya L..

The mixtures for isolation of chlorophyll by maceration methods were made of methanol and petroleum ether in a 2:1 ratio, and they were also re-extracted with petroleum ether and diethyl ether in 1:1 ratio. The chlorophyll fraction used column chromatography step gradient polarity methods with silica gel with the adsorbent and *n*-hexane/acetone mixture as the eluent with ratio from 1:0 to final 1:1 [16].

2.2. Characterization of extract chlorophyll of Carica Papaya L..

This step included several tests such as stability test absorbs spectrum in different temperature of storage (4^0C, 27^0C, 60^0C) and pH of solutions(pH 4.5 – 7.5) [17], phytochemical test on the content of flavonoids, saponins, and tannins, the MIC and MFC test to determine the optimal concentration in photo inactivation treatment. The concentration series was made of 4.3, 8.5, 21.3, 31.9, 42.5, 63.8, and 170 μM (mixture of chlorophyll extract 2% and 20% distilled water).Purity test was done using TLC (*thin layer chromatogram*) compared to *standard chlorophyll TLC 6%chl-a* [16].

2.3. Culture and biofilms preparation.

C. albicans was reactivated by cultivation in PDA media incubation for one night with 37^0C temperature before experiment. One dose of a culture was inoculated into 10 mL of BHI-B and was grown aerobically at 37^0C for one night and shaken at 160 rpm. The aliquot was harvested after centrifugation at10000 rpm and 4^0C for 15 min, washed twice with PBS (sterile pH 7.4), and re suspended in PBS for concentration of 0.5 McFarland (10^7 CFU/mL) with Densichek Calibration Standard. 100 μL of aliquot was next transferred into 96-well microtiter plate (the well number filled aliquot was three times of the number of all treatment groups) and incubated for 1.5 hour for adhesion phase at 37^0C and shaken at 160 rpm. After adhesion phase, the supernatant was removed from the well plate and gently washed twice with 150 μL of PBS to remove non adherent cells. Next, 200 μL of BHI-B, containing 8% glucose, was transferred to each well and incubated and shaken at 160 rpm and 37^0C for 48 hours. Then, biofilms were grown on well plate and washed twice with PBS to remove nonadherent cells [18,19]. For treatment group of chlorophyll extract alone, 100 μL of PBS for both concentrates prepared was transferred into well filled with biofilms and was incubated at room temperature for 2 hours before irradiation with time of illuminating difference. It is the same procedure for positive control group using fluconazole. After all treatment groups had been finished (including illumination), all of biofilms on each well were washed twice with PBS then staining was conducted with 200 μL of crystal violet0.1%. It was next incubated at room temperature, washed twice again with PBS, and added 200 μL of ethanol 98%. 100 μL of aliquot which had been through staining phase was then transferred into new 96-well microtiter plate and measured for its turbidity using Elisa readerλ_{595}.

All of treatment groups should be processed separately to avoid difference of start treatment, especially on time of biofilm incubation. It can also be overcome by creating a biofilm in 96-well separately for each treatment group such as the photosensitizer group alone and the combination of laser-sensitizer group, which was added to chlorophyll extract and incubated simultaneously before the irradiation can be done in parallel. Then other treatment groups (laser alone, positive and negative controls) were stored at refrigerator to slow down the growth of biofilms before all of treatment groups proceeded through phases of staining and reading of their OD.

2.4. Parameters of light and photo inactivation methods

The distance of illuminating of 450 nm of Blue Laser diode with 53.880 mW of output power was 3 cm, whereas its irradiation durations were 60, 30, and 15 seconds (marked as treatment L_1, L_2, and L_3 respectively). Concentration of chlorophyll extract based on MIC biofilms values (P_2 = 31.8 μM)was then compared to another concentration (P_1 = 42.5 μM). The treatment group as much as 13 each in triple namely: (P_1-P_2) photosensitizer only (2 groups), (L_1-L_3) laser only (3 groups), (P_1L_1-P_2L_3) laser combine photosensitizer (6 groups), (F+) positive control groups with antifungal only, and (B-) negative control groups with no biofilms.

2.5. Measurement levels of ROS formation,

In order to measure the levels of ROS formation, this study used MDA-TBA (Malondialdehyda-2Thiobarbituric acid) method. MDA is the number of radicals formed in the sample due to a particular treatment. MDA test aims to detect how much free radical compound accumulated in animal tissues after extreme treatments whereas in the system photo inactivation radical compounds are formed through a photochemical process after the photosensitizer absorbs the light in photophysics process. Radicals formed from a chain of chemical reactions can be assumed as a result of free radicals oxidative stress that it can be measured by MDA method. Chlorophyll extract was made in several rows of isolated and irradiated concentration with a laser diode which has been characterized previously by the length of irradiation time(irradiation dose determination). 0.5 μL of sterile PBS was mixed with 0.05 gr of test samples vortex and centrifuged drying (20 minutes, 10000 rpm, 4^0C) obtained filtrate (f_1). 250 μL of f_1 was mixed with 4.75 mL of sterile PBS vortex and centrifuged (15 minutes, 3000 rpm)obtained f_2. 4 mL of f_2 was mixed with 1 mL TCA vortex and centrifuged (15 minutes, 3000 rpm) obtained f_3. Finally, 4 mL of f_3 was mixed with 1 mL TBA vortex, incubated twice (80^0C for 15 minutes than room temperature for 60 minutes), and centrifuged (15 minutes, 3000 rpm) obtained f_4. The last filtrate determined the absorbance with UV-Vis spectroscopy whose absorbance value was detected as MDA's content on test sample.

2.6. The analysis

The data treatment was analyzed with ANOVA test to significantly determine the difference among treatments and with Tukey test whose degree of confidence was above 90%.

3. Results

The isolation of *Carica Papaya L.*'schlorophyll extract showed that chl-*a*, chl-*b*, and chl-total with Argon formula(1949) were 4.431 mg/L, 4.631 mg/L, and 14.058 mg/L. The characteristic of chlorophyll is similar to porphyrin as a dye with an absorbance spectrum with two major absorption bands in the visible range, namely Q band (red area) and Soret band (blue area) [20]. The maximum of absorption obtained two peaks at blue and red areas which could be viewed from the wavelength. The absorbance value at blue areas on 414 nm of wavelength was 0.233 au and in red areas on 670 nm of wavelength was 0.123 au. These were higher than the chlorophyll standard which are 420 nm, A= 0.123 au (blue areas) and 670 nm, A=0.093 au (red areas) (Figure 2.). The literature shows that maximum absorption wavelength for acetone solution were Chl-a with 662, 430, Chl-b with 645.5, and 456.9 nm [16,21].

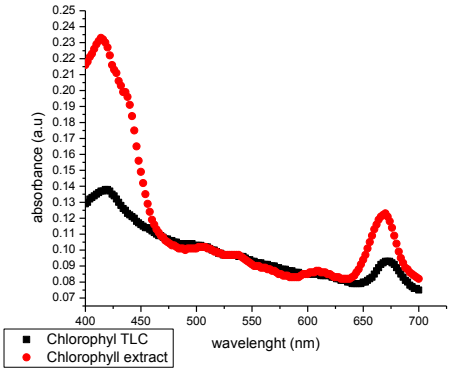

Figure 2. Chlorophyll Absorption Spectrum

HPLC analysis, when starting the retention time of *Carica Papaya L.*'schlorophyll extract is 2.8 minutes (Area=186.9 mAU.s) compared to the standard which is 3.0 minutes (Area=388.5 mAU.s) (flow rate 1 mL/minutes, pressure 35 bar, λ_{ref} 220 nm). In a study was mentioned for chl-*a* and chl-*b* had 13.6 minutes and 9 minutes of retention time [16].

The stability properties of chlorophyll extract of *Carica Papaya L.* have been tested with different storage temperature and pH conditions against maximum absorption wavelength. Acid of conditions had nosignificant effect to changes of maximum absorption spectrum. However, absorbing values relatively decreased because the acid condition made more dilute solution. Despite storage temperature stability, the solutions of chlorophyll extract was not stable for thermal storage above 60^0C because the solution changing color became clear, and it meant there was degradation. Even so, maximum absorption wavelength did not change. The absorption values of *Carica Papaya L.*'s chlorophyll extract storage against temperature of 4^0C, 27^0C, and 60^0C are 0.717, 0.697, and 0.731 (for λ_m=668 nm), and 2.824, 2.523, and 2.340 (for λ_m=415 nm) respectively. A study by Araujo(1995) mentioned the value of molar absorption coefficient against room temperature and refrigerator storage with the dark condition after 84 days with the value are 82.29 L.mmol^{-1}.cm^{-1} (refrigerator) and 80.73 L.mmol^{-1}.cm^{-1} (room temperature) at λ_{665} did not change significantly [22].

The MIC values were needed in determining optimum concentration of *Carica Papaya L.*'s chlorophyll extract (sensitizer) for a-PDT. They were needed to streamline the mechanism of photo inactivation in a-PDT that when applied as a sensitizer, they only act as alight absorber which then produced ROS compound. The result of MIC test showed that the MIC values against *C. albicans* planktonic and biofilms cell are 63,8µM and 31.9 µM respectively. There was reduction about 50% of biofilms concentration compared to that of planktonic cell which was applied to a-PDT mechanisms in which MIC value of biofilms was (31.9 µM) combining with another concentration.

Photo inactivation mechanisms which were applied against biofilms' growth showed effective combination between blue light of laser diode and *Carica Papaya L.*'s chlorophyll extract.

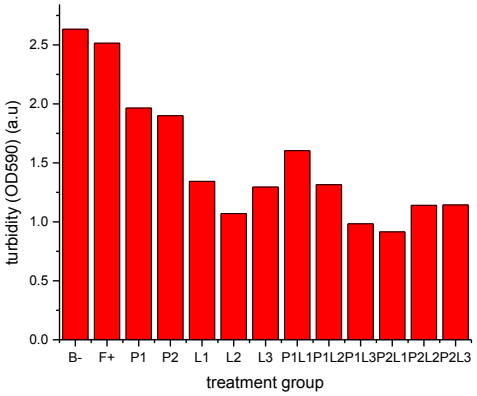

Figure 3. a-PDT mechanisms compared to control

The low turbidity means treatment was done more effectively than in high turbidity. The value of higher OD_{595} showed much biomass which was detected using crystal-violet staining. In this study, the turbidity was impacted by surviving cell in biofilms. The turbidity of negative control (B- treatment group) was 2.632 au, for positive control with antifungal conventional by fluconazole (F+ treatment group)which was 2.515 au (4.46% reduction), for treatment chlorophyll extract only treatment group P_2 (31.8 μM) was 1.899 au (48.97% reduction), for treatment lower than P_1 (42.5 μM) was 1.965 au (27.86% reduction), for treatment laser only treatment group L_2 (30 s) was 1.070 au (59.35% reduction), for treatment lower than L_1 (60 s)was 1.343 au (48.97% reduction), and for treatment group L_3 (15 s)was 1.294 au (50.84 % reduction). For combination treatment of laser and chlorophyll extract (a-PDT mechanisms), the turbidity for P_2L_1 treatment group with 0.915 (65.22 % reduction) was lower than other treatment groups on combination laser with exogenous photosensitizer.
The MDA's value had not shown variability which was significant among groups. The MDA's value of photosensitizer only and laser only groups showed similar value of 6.4906 nmol/mL. However, for laser-mediated chlorophyll group showed higher value than that of photosensitizer only or laser only groups which was about 7.2134 nmol/mL. Based on MDA's value, in this study was noted that radical compound can be detected using MDA's content test, and it can also be proven that there was an interaction between light and a sensitizer in order to produce a radical compound.

4. Discussion

The clinical application of photodynamic therapy with *in vitro* technique on wound infection was carried out. In some microorganisms, it has been proven effective in reducing gram-positive and gram-negative bacteria using a blue light with or without the addition of exogenous photosensitizer [20,23]. In contrast to bacteria in general, microbial *C.albicans* with more complex and thick structures is more effectively inactivated by red light [5] because it has a strong penetrating power. MB dye induced by 660 nm of diode laser for 98 seconds could more effectively reduce *C.albicans* biofilm formed in association with other microbes compared to TBO dye [24].Something similar was also reported that 630 nm of LED with a dose of 30 J/cm^{2}was no less effective photosensitizers to kill planktonic cells of *C.albicans* [25]. MB dye with 660 nm of Laser diode capable of producing the type of ROS sodium dodecyl sulphate, caffeine, and hydrogen peroxide [26] and 660 nm of the low-power laser 100 mW are more reactive against gram-positive bacteria rather than against yeast and gram-negative bacteria [27].

The next stage of further research, that the relation with one of the characteristics of the biofilm is limited zone of oxygen in the layer [28], which is the resistance of photodynamic therapy such as photo inactivation [29]. Limitations of oxygen in suspension and biofilm have been investigated inrelevance to the viability reduction of cells in applications of photo inactivation [20,30] which was

allegedly affected by the lack of effective ROS, where the availability of sufficient oxygen into research opportunities especially for applications against *C.albicans* biofilm.

5. Conclusion

Carica Papaya L.'s chlorophyll extract has been isolated with antifungal active substance content, and absorbent molecules with specific characteristics of the two intensities are Soret band and Q band which area potential photosensitizers in photo inactivation specifically for eradication against *C.albicans* biofilm. Furthermore, combining 450 nm of laser diode L_1 27.84 J/cm^2 (60 s) with *Carica Papaya L.*'s chlorophyll extract P_2 (31.875 μM) is more effective in inhibiting 65% growth of *C. albicans* biofilms.

Acknowledgments

We would like to give thanks to Suhariningsih as my promotor and to my co-promotor, Suryani Dyah and Afaf Baktir for all motivation, support, and advice in finishing this paper. This research is part of my great doctoral research and its preliminary study. We would also like to give thanks to DIKTI who has given financial support for Disertation Doctoral Research (PDD) in 2017. Finally, we would like to give thanks to all colleagues at RSKI and Biochemical Laboratory, of whom I cannot mention one by one, for support for instruments used in this research.

References

[1] Ruiz-González R, Agut M, Reddi E, Nonell S 2015 Article A Comparative Study on Two Cationic Porphycenes Photophysical and Antimicrobial Photoinactivation Evaluation *International Journal of Molecular Science* **16** pp 27072–27086
[2] Dai T, Huang Y, Hamblin 2009 Review : Photodynamic Therapy for Localized Infections - State of The Art Photodiagnosis and Photodynamic Therapy **6** pp 170-188
[3] Silva Z S, Sandra K, Kristianne P, Ying-Ying H, Michael R 2015 Animal models for photodynamic therapy (PDT) *Bioscience Reports* **13** 35 (6)
[4] Niemz M H 2007 *Laser-Tissue Interaction, Fundamentals and Applications* Third enlarged edition Springer-Verlag Berlin.
[5] Calzavara-Pinton P G, Rossi M T, Ortel B 2011 Photodynamic Inactivation of Microbial Pathogen : Medical and Environmental Applications, Chapter 14 : Photodynamic Antifungal Chemotherapy *European Society for Photobiology* pp 361-375
[6] McGinnis M R and Tyring S K 1998 *General Concepts of Mycology* Medical Microbiology 4th ed.
[7] Samaranayake Y H *et al.* 2005 In Vitro Method To Study Antifungal Perfusion In Candida Biofilms *J. Clin. Microbiol.* **43** (2) pp 818-825.
[8] Mayer F, Wilson D, and Hube B 2013 Candida Albicans Pathogenicity Mechanisms *Virulence Landes Bioscience* **4**:2 pp 119–128
[9] Archer N K *et al.* 2011 Staphylococcus aureus biofilms Properties, regulation and roles in human disease Virulence *Landes Bioscience Special Focus* **2**:5 pp 445-459
[10] Melo *et al.* 2013 Photodynamic inactivation of biofilm: taking a lightly colored approach to stubborn infection *NIH Public Access Expert Rev Anti Infect Ther.* **11**(7) pp 669–693
[11] Stubss H J *et al.* 1999 Hydrolase and transferase activities of the ß-1, 3-exoglucanase of Candida albicans *Eur. J. Biochem.* **263** pp 889-895
[12] Baktir A *et al.* 2012 Novel Materials for Eradicationof Biofilm Extracell Matrix of pathogenic candida *J. of Materials science and Engineering B2* **12** pp 650-658
[13] Indrawati R *et al.* 2010 Perkembangan Sensitizer pada Terapi Fotodinamik Tumor dan kanker Hingga Penuntunan Nanopartikel (Nanoparticulate Targeting) Dengan Antibodi Monoklonal *Indonesian J. of Cancer* **4**(3) pp 101-110
[14] Brandis A S *et al.* 2006 *Chlorophylls and Bacteriochlorophylls : Biochemistry, Biophysics, Functions and Applications,* pp. 461-483, Springer, Printed in The Netherlands.

[15] Setiari N and Nurchayati Y 2009 Eksplorasi Kandungan Klorofil pada beberapa Sayuran Hijau sebagai Alternatif Bahan Dasar Food Supplement J. BIOMA **11** (1) pp 6-10
[16] Milenkovic S M *et al.* 2012 The Identification of Chlorophyll and Its Derivatives in he Pigment Mixtures : HPLC-Chromtography, Visible and Mass Spectroscopy Studies *Advanced Technology* **1**(1) pp 16-24
[17] Budiyanto AW, Notosudarmo, Limantara L 2008 Pengaruh Pengasaman terhadap Fotodegradasi Klorofil a *J. Matematika dan Sains* **13** (3) pp 66-75
[18] Dovigo L N, J.C. Carmello, C.A. de Souza Costa, C.E. Vergani, I.L. Burnetti, V.S. Bagnato, A.C. Pavarina 2013 Curcumin-Mediated Photodynamic inactivation of Candida albicans in a murine model of oral candidiasis *Medical Mycology J.* **51** pp 243-251
[19] Andrade A.P.D. Ribeiro, L.N. Dovigo, I.L. Brunetti, E.T. Giampaolo, V.S. Bagnato, A.C. Pavarina, 2013, Effect of different pre-irradiation times on curcumin mediated photodynamic therapy against planktonic cultures and biofilms of Candida spp, archives of oral biology, **58** pp. 200-210.
[20] Astuti S D 2011 *Potensi* Light Emitting Diode (LED) Biru Untuk Fotoinaktivasi Bakteri Staphylococcus Aureus dengan Porfirin Endogen *Disertasi, Program Doktor MIPA, Program Pascasarjana Univeritas Airlangga*
[21] Jeffrey S W *et al.* 1996 *Phytoplankton Pigments in Ocenography : Guidelines to Modern Methods,* UNESCO Publishing, Paris, p.1.
[22] Araujo P W and Brereton R G 1995 Stability Studies of Chlorophyll a Using Different Storage systems *Analytical Proc. Including Analytical Communications* **32** pp 415-417
[23] Dai T *et al.* 2012 *Concepts and Principles of Photodynamic Therapy As An Alternative Antifungal Discovery Platform* Front. Microbiol.
[24] Pereira C A *et al.* 2011 *Original* Article : Susceptibility of Candida albicans, Staphylococcus aureus, and Streptococcus mutans Biofilms to Photodynamic Inactivation : an in vitro Study *Laser Medical Science* **26** pp 341-348.
[25] Matthes D F P *et al.* 2012 Photosensitization of In Vitro Biofilms Formed on Denture Base Resin *The J. of Prosthetic Dentistry* **112** (3) pp 632-637
[26] Kato L T *et al.* 2013 Antimicrobial Photodynamic Inactivation Inhibits C.albicans Virulence factors and Reduces In Vitro Pathogenicity *Antimicrobial Agents and chemotherapy* **57** (1) pp 445-451
[27] DeOliveira B P *et al.* 2014 Original Article : In Vitro Antimicrobial Photoinactivation With Methylene Blue in Different Microorganisms *Brazilian J. Oral Sciences* **13** (1) pp 53-57
[28] Walters M C *et al.* 2003 Contributions of antibiotic penetration, oxygen limitation, and low metabolic activity to tolerance of *Pseudomonas aeruginosa* biofilms to ciprofloxacin and tobramycin. *Antimicrob. Agents Chemother*. **47** pp 317–323
[29] Taraszkiewicz A *et al.* 2013 Review Article Innovative strategies to overcome biofilm resistance *BioMed Research International*
[30] Maclean M *et al.* 2008 The Role of Oxygen in The Visible-Light Inactivation of Staphylococcus Aureus *J. of Photochemistry and Photobiology B : Biology* **92** pp 180-184
[31] Grossweiner L I 2005. *The Science of Phototherapy: An Introduction* Springer USA.
[32] Prasad P N 2003 *Introduction to Biophotonic* Wiley Interscience John Wiley and Sons, Inc, Pub.