ICOWOBAS

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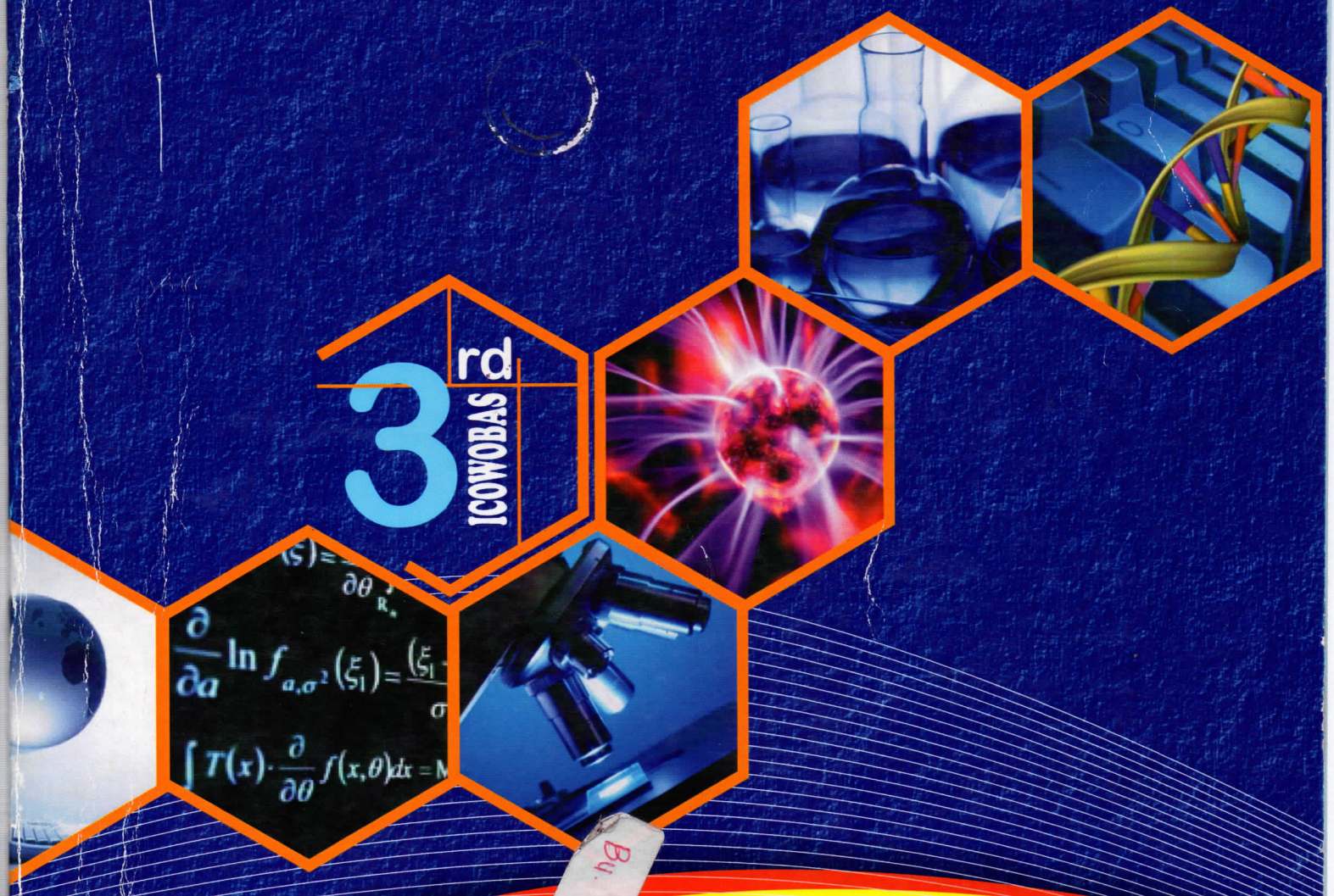
Enabling Research Innovation on Sciences and Technology to Meet Global Challenges

Surabaya, Indonesia, September 21st - 23rd, 2011



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PROCEEDING

THIRD INTERNATIONAL CONFERENCE AND WORKSHOP ON BASIC AND APPLIED SCIENCES

ENABLING RESEARCH INNOVATION ON SCIENCES AND TECHNOLOGY TO MEET GLOBAL CHALLENGES

SEPTEMBER 21st - 23st

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FOREWORD BY THE DEAN
Faculty of Science and Technology, Airlangga University, Indonesia

Assalamu' alaikum warahmatullahi wabarakatuh

On behalf of all member of The Faculty of Science and Technology, Airlangga University, It gives me a great pleasure to extend my sincere and warm welcome to the participants of the International Conference and Workshop on Basic and Applied Sciences /ICOWOBAS 2011, in Elmi Hotel, Surabaya. It is an honor and joy for us to be host of this congres, where all participants have a chance to present and discuss our knowledge about development of basic and applied science.

The International Conference and Workshop on Basic and Applied Sciences 2011 is organized as implementation of existing collaboration between Airlangga University with Universiti Technology Malaysia (UTM), in order to promote the development of science and their prospects in industry application.

Basic science is become basic for development of multi applied science and has grown very rapidly into more specified fields, including biotechnology, material science, techno biomedical, informatics, computer and Biophysics.

Hopefully, this conference provides a great opportunity for lecturers, researchers and industries to build better communications. A better communication between university and industry is one of the best ways to share the development of research in the basic and applied science which will be used in industries.

Finally let me congratulate to all of you once again for organizing and participating in this conference. Also to the committee members, speakers, moderators, editor boards, sponsors and participants for your kind contributions, I would like to express my gratitude for all the hard work on the succeeding this congres. Hopefully the new friendship and new collaboration will start from this moment, and for UTM's delegates please do not forget to pleasure by visiting a nice places for examples new Suramadu Bridge and other places that Surabaya can be offered.

Thank to Allah SWT for His blessing so far.

Win Darmanto

FOREWORD

International Conference and Workshops on the Basic and Applied Sciences (ICOWOBAS) is organized as implementation of existing collaboration between Airlangga University with Universiti Teknologi of Malaysia (UTM) in order to promote the development of sciences and their prospect of application in industry. The programs of this activity are the scientific program involves the presentation of paper and poster in the area of chemistry, biology, physics, and mathematics and their applications. It also conducts the workshop program that will be presented the current issues in biological product. Thus it invited many participants as academic researcher, scientist, industrial practitioners, industrial professionals, government officers, students and other participants. The meeting intends to bring together researchers, scientists, and scholars to exchange and share their experiences, new ideas, and research results in related fields and discuss the practical challenges encountered and the solutions adopted.

These proceedings hold the full papers presented at the 3rd ICOWOBAS. The conference took place in Surabaya (Indonesia) at the Mercure Grand Mirama Hotel Convention Centre, September 21-22, 2011, and the workshops were conducted at the Faculty of Sciences and Technology, Airlangga University, September 22-23, 2011.

Highlights of the conference include: Prof. Dr. Zuhaimy Ismail (Faculty of Science/ Mathematics, University Teknologi Malaysia), Dr. Yoshiaki Takaya (Faculty of Pharmacy, Meijo University, Japan), Prof. Dr. Noriah Bidin (Faculty of Science/Physics, University Teknologi Malaysia) and Dr. Xavier Daniel, Ph.D (Lab. Cell and Molecular Biology, JUNINE Neuchatel, Switzerland) as plenary speakers.

In total, we received 144 submissions for oral publications and 39 for posters, and 137 submissions for publication-based full papers. Of these, the numbers of acceptances were 139 conference, and 39 poster publication. As the acceptance rates illustrate, competition was stiff, and the accepted submissions reflected high rates of reviewer enthusiasm. By design, these are lightly reviewed and almost always accepted.

ICOWOBAS is a lot of work. We could not have done it without help from many people. We would especially like to thank:

- The Scientific Board of ICOWOBAS, for inviting us to chair the meeting
- The Rector of Airlangga University, for permiting and supporting us to conduct the conference
- Our colleagues in the Faculty of Sceinces and Technology Airlangga University, for their help in the conference organization
- The Program Committee, for managing the review process
- The Scientific Committee for assisting in selecting debates and symposia
- The Dean of Faculty of Sceinces and Technology Airlangga University, for his support in the conference planning
- The Local Committee, for organizing and handling the conference
- The 11 reviewers, for providing professional reviews.
- Our sponsors: Diastika Biotekindo, Pupuk Kaltim and dr. Agus Subagjo SpJP (K) FIHA.

And above all, the authors, symposium participants, and attendees.

Surabaya, September 21, 2011

Hery Purnobasuki, PhD.
ICOWOBAS 2011 Chairperson

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Department of Mathematics, Faculty of Science, Universiti Teknologi Malaysia
"Optimal Design Of Parameter Estimation In Double Exponential Smoothing Using Genetic Algorithms And Other Evolution Programs"

Dr. Yoshiaki Takaya
Natural Product Chemistry, Faculty of Pharmacy-Meijo Univ-Japan
"A Stilbenetetramer from Corks of *Vitis vinifera* 'Kyohou' "

Prof. Noriah Bidin, Ph.D
Department of Physics, Faculty of Science, Universiti Teknologi Malaysia
"Thermal Effects In Diode Pumped Vanadate Laser"

Dr. Xavier DANIEL, Ph.D
Lab. Cell and Molecular Biology, UNINE Neuchatel, Switzerland

Invited Speakers

Prof. Dr. Mohd. Marsin Sanagi
Department of Chemistry, Faculty of Science, Universiti Teknologi Malaysia
"Determination of Triazine Herbicides in Sugarcane Samples by Dispersive Liquid-Liquid Microextraction-Gas Chromatography–Mass Spectrometry"

Dr. rer.nat. Ganden Supriyanto, M.Sc
Department of Chemistry, Faculty of Science and Technology, Airlangga University
"(Bio)Sensor For Disease Detection And Food Quality Testing"

Prof. Dr. Suhariningsih
Department of Physics, Faculty of Science and Technology, Airlangga University
"Electric Field Optimization On Diabetes Mellitus Therapy Type II and Hypertension"

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"Plant Hairy Root Culture: A Promising System To Produce Secondary Metabolite"

Dr. Eko Prasetyo Kuncoro, ST, DEA
Department of Biology, Faculty of Science and Technology, Airlangga University
"Biosorption: A Sustainable Technology for Heavy Metals Removal"

Contents

Effect of Exogenous ALA to Increase the Production of Endogenous Porphyrin Staphylococcus aureus Bacteria For Applying Photodynamic Inactivation of Bacteria

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Abstract

Aminolevulinic acid (ALA) is a naturally occurring metabolite in the synthesis pathway of cellular heme production. In bacteria, addition of ALA may induce porphyrin synthesis. ALA induction leads to an increase in the synthesis of uroporphyrin, coproporphyrin and protoporphyrin IX, which are the immediate precursors of heme. In this research, the suspension of Gram positive *Staphylococcus aureus* bacteria ATCC 6538P was added with exogenous ALA (Sigma) in order to increase the production of bacteria endogenous porphyrin, with varying concentration (0, 1.5 mM, 2.5 mM, 3.5 mM, and 5 mM). Bacteria suspension was incubated in the dark at 37°C for 3 h for induction and production of endogenous porphyrin. Each extract, containing the produced endogenous porphyrins, characterized by fluorescence, UV-Visible spectroscopy and HPLC using reverse phase chromatography. Porphyrin production was demonstrated by the fluorescence emission peaks that appeared in the extracts of the ALA treated bacteria. The result showed that the excitation of the *Staphylococcus aureus* endogenous porphyrin at 405 nm yielded a fluorescence peak at 623 nm. The increasing of bacteria porphyrin absorbance comparable with increasing of ALA concentration. A significantly absorbance increased in the incubation of 3.5 mM ALA. The HPLC analyzed showed that the amount of porphyrin produced increased comparable with incubation of ALA concentration. A significantly endogenous porphyrin production increased in the incubation of 3.5 mM ALA. So the incubation of 3.5 mM ALA concentration significantly increased the bacteria endogenous porhyrin production and the absorption of porphyrin for applying the effectiveness photoinactivation of pathogen bacteria.

Keywords: Staphylococcus aureus, exogenous ALA, endogenous porphyrin production and absorption

1 Introduction

Staphylococcus aureus is a Gram-positive spherical and aerobic facultative bacteria which grows with aerobic respiration or fermentation that yields principally lactic acid. These bacteria are linked by lesion to the acne skin condition and causing skin infection [21]. Hospital strains of *Staphylococus aureus* are usually resistant to a variety of different antibiotics, a few strains are resistant to all clinically useful antibiotics [6]. Since bacteria resistance to antibiotics is becoming an increasing problem, this research is being directed towards photodynamic therapy as an alternative methods for killing bacteria [10]. Photoinactivation of various Gram positive bacteria, including *Staphylococcus aureus* has been demonstrated using exogenous photosensitizer [16].

Staphylococcus aureus glow when exposed to Wood's light, broad-band near UV-light (in this study by dyes laser exposure), be due bacteria produce several endogenous porphyrins [15; 7]. The porphyrins make the bacteria light-sensitive. It has also been shown that when illuminated with blue light, porphyrins damage the cells very efficiently [1; 5]. The emission peak of the best

light source is at 407-420 nm, which is the most efficient porphyrin photodestructive wavelength range [12]. In contradistinction to ultraviolet light, the visible blue light irradiation is not phototoxic to human cells.

Several investigations pointed to light destruction mechanism that is mediated via excited porphyrins and subsequent singlet oxygen reactions [4]. Illumination of the bacteria will excite the porphyrins in the cells into higher singlet states, which will rise to both fluorescence emissions [8]. Another and very important possible fate of the fluorescent state is its spontaneous conversion to a triplet state via of electron spin direction changing, due to *intersystem crossing* (isc).

Porphyrin is a metastable state, which can either donate the excited state energy to a ground state of oxygen molecule, whereby a singlet oxygen ($^1O_2^*$) molecule is created, or abstract an electron from a neighbouring molecule, whereby radical species are formed. The first type of photosensitization reaction is called a type II reaction. In reaction of type I the excited photosensitizer acts as an oxidant and the resulting effect is transfer of either a hydrogen atom or an electron [4], yielding active radicals or radical ions that may cause damage to neighbouring biomolecules. Reactions superoxide radical O_2^- may be formed. Subsequently stronger oxidisers like peroxide OH^- and hydroxyl HO^- radicals are formed [18].

A new method for photosensitizing cells is by enhancing endogenous porphyrin production with δ-aminolevulinic acid (ALA), which is a naturally occurring metabolite in the synthesis pathway of cellular heme production [5]. In bacteria, addition of ALA may also induce porphyrin synthesis. ALA induction leads to an increase in the synthesis of uroporphyrin, coproporphyrin and protoporphyrin IX, which are the immediate precursors of heme [9].

The purpose of this study was to determine effect of exogenous ALA to increase the production of endogenous porphyrin *Staphylococcus aureus* bacteria for applying photodynamic inactivation of bacteria.

2 Methodology

2.1 Bacterial strain

The strain used in this study was Staphylococcus aureus ATCC 6538, which was obtained from the American Type Culture Collection USA

2.2 Bacterial Growth

The bacteria were grown for 18 h on *Staphylococcus* Agar plates. Colonies were transferred into Nutrient Broth. The initial optical density of the culture was between 0.1-0.13 at 660 nm. Cultures were allowed to grow aerobically at 37°C with aeration until they reached an optical density of 0.3-0.35 at 660 nm. The bacterial cells were now harvested by centrifugation, washed twice with 0.1 M phosphate buffer saline (PBS) at pH 6.5 and brought back to the initial volume of 50 ml with PBS. ALA (Sigma) with varying concentration (0, 1.5 mM, 2.5 mM, 3.5 mM, and 5 mM) were added. Bacterial suspension was incubated at 37°C for 3 h for induction and production of endogenous porphyrins. The bacterial cells were now harvested by centrifugation, washed twice with 0.1 M phosphate buffer saline (PBS) at pH 6.5 and brought back to the initial volume of 50 ml with PBS. 10 ml samples were diluted (10^{-6} for control and 1.5 mM ALA, 10^{-3} for 2.5 mM, 3.5 mM and 5 mM ALA), and transferred 4 ml samples into small sterile 2.5 cm diameter plastic dishes and were illuminated. The rest of the culture (40 ml) was centrifuge and the precipitate was further processed for HPLC and spectroscopy UV-Visible analysis.

2.3 ALA solution

Stock solution of ALA was prepared by dissolving ALA to a concentration of 5 mg ml^{-1} in sterile distilled water. The stock solution was kept in the dark at 4°C for maximum of 24 h [16].

2.4 Porphyrin Spectrum analysis

2.4.1 HPLC analysis

Porphyrin extraction used Fotinos et al (2008) method [3]. Bacteria suspension with ALA and non ALA (control) were centrifuged at 2,500 x g for 10 min. The supernatant was discarded and the pellet was extracted by 2 ml of extraction solvent (ethanol, dimethyl sulfoxide, acetic acid, 80:20:1; vol/vol/vol) and stored at -80°C until anlysis. For the extraction of porphyrin, the bacterial wall was disrupted by five sonication cycles of 5 s each at 0°C with a sonicator probe. After centrifugation at 13,500 x g for 4 min, the supernatant was collected and injected into the High Pressure Liquid Chromatography (HPLC) instrument. Porphyrin were separated by reverse-phase chromatography with LiChro CART@250-4 column. The method of Schoenfeld et al (1994) was used [20]. The system is equipped with a Fluorescence detector with an excitation wavelength of 407 nm and emission wavelength of 620 nm. Elution is performed using a gradient of 10% v/v acetonitrile in methanol (solvent A) and 10% v/v acetonitrile in 1 M ammonium acetate pH 5.1 (solvent B). The elution plan was: 30 min linear gradient from 100% B to 10% B followed by 12 min linear gradient from 35% B to 10% B, 5 min isocratic elution and an additional 5 min for returning to 100% B, at a flow rate of 1 ml/min. Porphyrin chromatographic marker kit (Porphyrin product, Logan UT, USA)

was used as standard kit for evaluating the porphyrin produced.

2.4.2 Fluorescence and UV-visible spectroscopy measurement

Cultures were extracted as above and underwent fluorescence spectral determination using spectrofluorometer and spectrophotometer interface. The excitation wavelength is 405 nm and emission is recorded in 550-750 nm range. Cultures extraction at zero time were used as control [1; 12].

3 Result

Direct production of endogenous porphyrins can be achieved by the induction of this pathway by 5-aminolevulinic acid (ALA). The induction was performed by incubation in the dark at 37°C in 0.1 M PBS at pH 6.5. Incubation of *Staphylococcus aureus* bacterial with varying ALA concentration (0, 1.5 mM, 2.5 mM, 3.5 mM and 5 mM) for 3 h resulted in endogenous production of porphyrins. In this context, the sole induction (in the dark) of bacterial culture in PBS for 3 h at 37°C in the presence or absence of ALA didn't cause any decrease in the viability of the culture (pic.1).

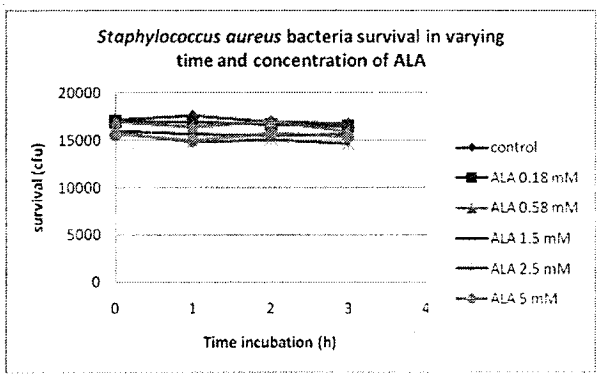

Figure 1: Effect of varying ALA concentration in varying time incubation

An accurate identification of the endogenous porphyrins was performed in an attempt to identify the cause of the photoinactivation sensitivity of *Staphylococcus aureus* bacteria strain. The culture underwent extraction after 3 h of incubation in PBS with varying of ALA concentration. Each extract, containing the produced endogenous porphyrins, characterized by fluorescence, UV-Visible spectroscopy and HPLC using reverse phase chromatography. Coproporphyrin III tetra methyl ester and porphyrin chromatographic marker kit (Logan UT USA) was used as standard of porphyrin.

Porphyrin production was demonstrated by the fluorescence emission peaks that appeared in the extracts of the ALA treated bacteria. Excitation of the *Staphylococcus aureus* at 405 nm yielded a fluorescence peak at 623 nm.The absorbance of the endogenous porphyrins of bacteria were showed in table 1.

Table 1. The absorbance of endogenous porphyrin Staphylococcus aureus bacteria with varying incubation of ALA concentration

ALA concentration (mM)	Absorbance
0	0,6356
1,5	0,642
2,5	0,6959
3,5	0,7368
5	0,7742

Table 1 showed the increasing of absorbance comparable with increasing of ALA concentration. In the lower ALA concentration (1.5 mM) showed the absorbance was similarity with control group (absence of ALA). A significantly absorbance increased in the incubation of 2.5 mM ALA concentration. Pic.2 showed graphic the absorbance of endogenous porphyrin *Staphylococcus aureus* bacteria with varying incubation of ALA concentration.

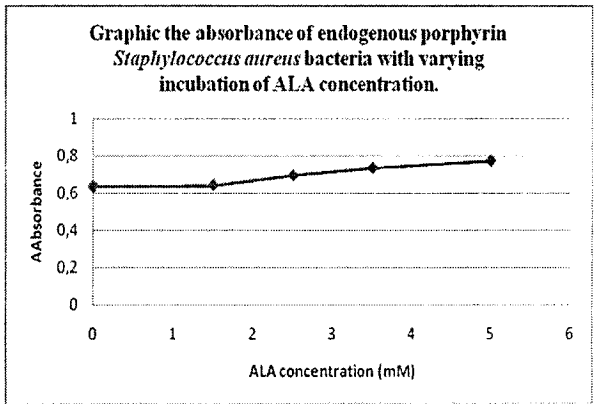

Figure .2: Graphic the absorbance of endogenous porphyrin *Staphylococcus aureus* bacteria versus the varying incubation of ALA concentration

The elution HPLC profile of *Staphylococcus aureus* bacteria culture were summarized in table 2. The HPLC profile indicate that the major porphyrin produced the bacteria was coproporphyrin III . In order to quantity the amount of endogenous porphyrin produced in *Staphylococcus aureus* bacteria upon induction by ALA, the area of peaks were compared to the standard. Using Lambert Beers law [19]:

$$Abs = l\, c\, \varepsilon$$

With l was length (1 cm), c was concentration and ε was absorptivity.
Concentration of the porphyrin was comparable with peak area of HPLC chromatogram. There are 2 high peaks in this coproporphyrin III standard, in the retention time 43 and 47.

Table 2: The elution HPLC profile of *Staphylococcus aureus* bacteria culture

Ret. time	Area control	1.5 mM ALA	2.5 mM ALA	3.5 mM ALA	5.0 mM ALA
	0	1,5	2,5	3,5	5
43	142.338	151.277	167.188	183.111	190.154
47	49.645	42.383	54.477	66.166	65.358

Picture 3 demonstrated the graphic of HPLC peak area versus the varying incubation of ALA concentration.

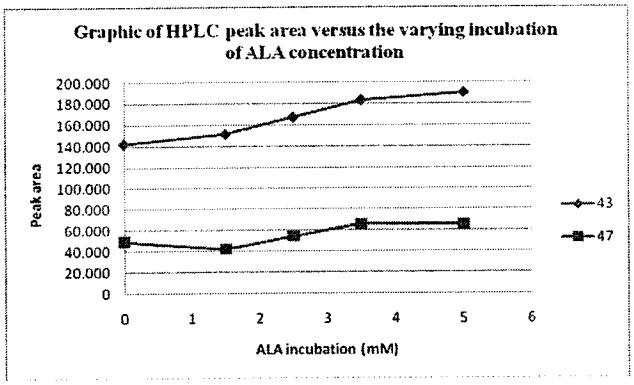

Figure .3: Graphic of HPLC peak area versus the varying incubation of ALA concentration.

Pic.2 and 3 showed that the amount of porphyrin produced increase comparable with incubation of ALA concentration. Incubation 1.5 mM ALA concentration demonstrated small differences compared with control (absence of ALA) in the porphyrin produced.

Photoinactivation of bacteria by endogenously produced porphyrin is of growing interest in the field of photodynamic therapy [15; 1] as well as the field of acne treatment [10;17]. *Staphylococcus aureus* plays as a central role in the infection and toxinoses in human [21]. Photoinactivation of *Staphylococcus aureus* by its endogenous porphyrin can be caused by coproporphyrin and uroporphyrin, the predominant porphyrin found to be produced by this bacterium [16]. In this study, a high increase of coproporphyrin was found after 3 h of ALA incubation in varying concentration.

The endogenous porphyrins of bacteria cause light sensitive. Porphyrin absorbed light in specific spectra. Efficiently of bacterial photokilling be caused the amount of endogenous porphyrin and suitability the light wavelength with absorbed spectra of porphyrin produced, cause photochemical reaction. In photochemical interactions, light can induce chemical effects and reactions within macromolecules or tissues. Photochemical interactions take place at very low power densities (less than $1W/cm^2$) and long exposure times ranging from seconds to continuous wave [11].

In this study, the kind of porphyrin had intense absorption band in wavelength peak 390-430 nm in blue light, it was called Soret band [13]. The amount of porphyrin increases in the bacteria as a function of ALA concentration, and the photokilling increases as a function of the amount of porphyrin and energy dose of blue laser exposure. For example, the viability of of cultures incubated with 2.5 to 5 mM ALA for 3 h and illuminated with 10 J/cm^2 is decreased less than 1.5 mM with the same light intensity. In biological tissues, absorption is mainly caused by either water molecules or macromolecules such as proteins and pigments [2]. Proteins as well as pigments mainly absorb in the UV and visible range of the spectrum [14].

4 Conclusion

The incubation of 3.5 mM ALA concentration significantly increased the bacteria endogenous porhyrin production and the absorption of porphyrin for applying the effectiveness photoinactivation of pathogen bacteria.

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