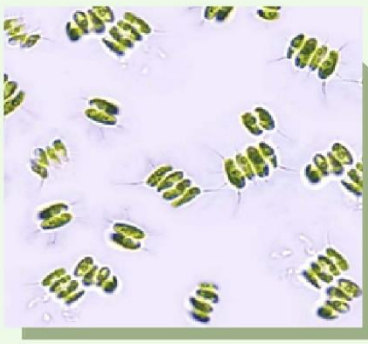
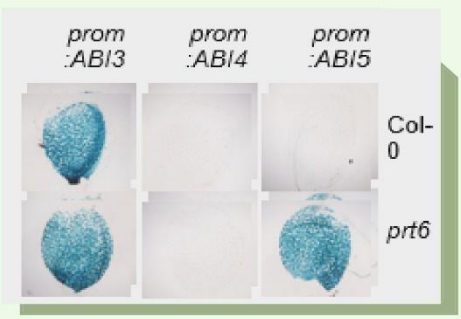
Proceeding of

JOINT SYMPOSIUM ON PLANT SCIENCES AND PRODUCTS

1st From Basic Research to Bioindustry
2nd Plant and Microalgae Bioindustry for Ensuring Food Security and Clean Environment





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• Multipurpose Hall CRCS ITB, Bandung, August 1st-2nd 2019
• Virtual Symposium (Webinar), November 5th- 6th 2020

Organized by

SITH
Plant Sciences and Biotechnology Research Group

Universiti Kebangsaan Malaysia
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PROCEEDING OF

JOINT SYMPOSIUM ON PLANT SCIENCES AND PRODUCTS

1ST FROM BASIC RESEARCH TO BIOINDUSTRY

2ND PLANT AND MICROALGAE BIOINDUSTRY FOR ENSURING

FOOD SECURITY AND CLEAN ENVIRONMENT

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- **Virtual Symposium (Webinar), 5–6 November 2020**

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Kelompok Keilmuan Sains dan Bioteknologi Tumbuhan

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Preface

We are very pleased to present the Proceeding of the first and second Joint Symposium in Plant Sciences and Products. The first Joint Symposium (2019) in plant sciences and products with the theme of 'From basic sciences to bioindustry' linked four topics: 1) Plant Sciences, 2) Plant Biotechnology, 3) Renewable Energy, and 4) Natural Products. Whilst the Second Symposium in 2020 covered the theme 'Plant and Microalgae Bioindustry for Ensuring Food Security and Clean Environment'. Both symposia provided information from basic to applied aspects of plant-based resources. The symposia also provided a setting for discussing recent developments in a wide variety of topics. We believe that plant and algal products, mostly related to food security is an important issue, especially in this pandemic situation where people activities around the world have been very much restricted for safety reason. Besides, clean environment where people can breathe fresh air, drink clean water and not being worried with toxic materials in the environment as an impact for industrial activities, need our commitment scientifically and technologically to reduce the level of environmental pollutions and combat degradation of our precious forest, lands, and environment.

The 1st and 2nd Joint Symposium between Faculty of Science and Technology, Universiti Kebangsaan Malaysia and Plant Sciences and Biotechnology Research Group, School of Life Sciences and Technology, Institut Teknologi Bandung were organized to pursue and to strengthen the academic collaboration between FST-UKM and SITH-ITB which was initiated several years ago. In term of biodiversity resources and climate, Malaysia and Indonesia have many aspects in common. Therefore, good collaboration in education and research can bring mutual benefits for both parties by sharing and exchanging knowledges, expertise, and experiences from each other. Both countries are also rich in natural biological resources which can be explored and utilized for increasing human welfare of both nations.

The Proceeding provides partial record of what was presented, since some papers are published elsewhere. However, they indicate the state of research and all aspects of these important topics and will become a valuable insight to all participants. The papers were accepted for publication based on the relevance and innovation to the plant-algal sciences and applications.

Sincere thanks to the following keynote speakers:
Keynote speakers and invited speakers in the 1st symposium with theme of 'From basic sciences to bioindustry'

1) Prof. Dr. Ismanizan Ismail: Plant miRNAs: A small molecule with huge potential in plant growth and metabolism
2) Prof. Toshiya Muranaka, Ph.D.: Gene editing to improve plant secondary metabolite pathway
3) Assoc. Prof. Dr. Roohaida: Molecular biology and functional genomics in plants
4) Prof. Dr. Sri Nanan B. Widiyanto: Teak biotechnology
5) Prof. Young Hae Choi: Plant metabolomics: Is application and requirement?
6) Dr. Rizkita Rachmi Esyanti: In vitro secondary metabolites production
7) Dr. Robert Manurung: Development of sustainable bioenergy and biochemical products
8) Prof. Dr. Yana Maolana Syah: Secondary metabolite in plants

Keynote speakers and invited speakers in the 2st Symposium with theme of theme 'Plant and Microalgae Bioindustry for Ensuring Food Security and Clean Environment'

1) Prof. Dr. Ir. Siti Rozaimah Sheikh Abdullah: Bioindustrial engineering for clean environment
2) Prof. Dr. J. Theo M. Elzenga: Using soil and endophytic micro-organism to increase productivity, health, and stress resistance of plants
3) Dr. Eko Agus Suyono, S.Si., M.App.Sc.: Microalgae biorefinery
4) Dr. Taufikurahman: Microalgae for clean environment
5) Assoc. Prof. Dr. Roohaida: Genetic engineering application in phytoremediation
6) Dr. Nurulhikma MD Isa: Genetic engineering: from model plant to high value crops
7) Dr. Erly Marwani: Variability of sugar in 'honey taste' sweet potato (*Ipomoea batatas*)
8) Dr. Ima Mulyama Zainuddin: Cassava for ensuring food security in Indonesia
9) Dr. Ir. Agung Hendriadi, M.Eng.: Indonesian's food security in pandemic era

We are also indebted to those who served as the Steering, Scientific, and Organizing committees, as well to supporting teams in organizing the symposia and preparing this proceeding. Without their support, the Symposium would not be successful as it was. We would also like to thank all participants for their contributions to both Symposia programs and for their contributions to the Proceeding. The success of this conference means that we are looking forward to the next event to be organized in 2021.

Best regards,

Dr. Rizkita Rachmi Esyanti
Head of Plant Sciences and Biotechnology Research Group
School of Life Sciences and Technology
Institut Teknologi Bandung

Welcoming Remarks from Dean of

School of Life Sciences and Technology (SITH)

The honorable,
Representative from Faculty of Science and Technology, Universiti Kebangsaan Malaysia, Prof. Dr. Mohammad Kassim, Dean of Faculty of Science and Technology and Group leader of Plant Science and Biotechology Expertise Group, SITH, Dr. Rizkita Rahmi Esyanti, keynote speakers from overseas, distinguished guests and participants, my dear friends and colleagues.

Assalamu'alaikum warahmatullahi wa barakatuh,
God Bless everyone. It is my great pleasure, and on behalf of the School of Life Sciences and Technology, to welcome all of you in The 2nd Joint Symposium SITH-ITB and UKM, Plant Sciences and Product 2020: Plant and Microalgae Bioindustry for Ensuring Food Security and Clean Environment. This is the second event jointly organised by School of Life Sciences & Technology, ITB and Faculty of Science and Technology, UKM. Last year, the same event was also held at ITB here in Bandung, but due to Covid19 pandemic, this second series would need to be organized virtually.

Please allow me to also convey my gratitude to a few key people that play a crucial role in initiating this good cooperation between the School of Life Sciences and Technology at ITB and Faculty of Science and Technology at UKM. From UKM, I would like to thank Assoc Prof Roohaida Othman, Prof. Dr. Shukor Md. Nor and Prof. Dr. Ir. Siti Rozaimah Sheikh Abdullah. From ITB, our dear colleagues: Dr. Taufikurahman, Dr. Rizkita R. Esyanti, Prof. Dr. Sri Nanan Widiyanto. I am also delighted to see that participants of this symposium come from various institutions, not only from Indonesia, but also from Malaysia and India.

Ladies and Gentlemen,
We all have fully comprehended that the world is facing a pressing challenge to provide food for the growing populations, under limited arable lands, through the ever expanding land degradation, and exacerbated by the effect of climate change that to some extent influences crop failures and yield loss. These circumstances require us to explore novel ways of producing food, efficiently utilizing limited spaces, increasing productivity and devising food production systems that are able to adapt to climate change. The current Covid19 pandemic warns us of the urgency and importance of building strategies to grow the appropriate food to ensure that we achieve food security. On top of that, we are also faced with a growing challenge of tackling environmental problems such as water pollutions, that will further lead to the declining water quality and its risks on human health.

This two-day symposium will specifically discuss intensively one of the recent developments on plant biotechnology research including the use of microalgae for bioremediation and biorefinery. This scientific meeting will explore some of the potentials of microalgae as a source of food, whilst providing a solution to ensure food security and clean environment in the context of bio-industry, as we will listen further from the keynote speakers and other presenters in the symposium. I truly hope that the exchanges of ideas will give a contribution to the advancement of new and effective ways to increase food production and tackle environmental issues.

Distinguished guests and participants,
The topic that is raised in this symposium has become one of the central aspects that the School of Life Sciences and Technology delves into for decades. I would therefore like to use this opportunity to quickly introduce to you about our institution. With over 1000 students and 100 lecturers, the School of Life Sciences and Technology runs 10 study programs in two separate campuses, in Ganesha in Bandung and in Jatinangor, Sumedang. This includes undergraduate programs in Biology, Microbiology, Bioengineering, Forestry Engineering, Agricultural Engineering, and Post-harvest Technology – as well as three masters' programs in Biology, Biotechnology and Biomanagement, and one doctoral program in Biology. Our lecturers are grouped into what we call research groups, ranging from Genetics and Molecular Biology, Microbial Biotechnology, Plant Science and Biotechnology – the group that organizes this event, Animal Physiology and Biomedics, Agrotechnology, Forestry Technology, Ecology and Management of Bioresources. We are thriving to be the leading faculty in life sciences and we are open to collaboration and engagements with various parties – other universities, government, private sectors and civil society - to solve life science-related problems.

Lastly, I wish everyone a very successful, enjoyable and productive two day seminar and I would personally like to express my utmost gratitude to the organizing committee and all of the distinguished speakers who are willing to share their knowledge for the betterment of our society, and particularly to address the triple bottom line of sustainable food production.

Wassalamu'alaikum warahmatullahi wa barakatuh.

Sincerely,

Dr. Endah Sulistyawati
Dean of School of Life Sciences and Technology (SITH),
Institut Teknologi Bandung (ITB)
Indonesia

Welcoming Remarks from Dean of Faculty of Science and Technology (UKM)

The honorable,
Dean of School of life Sciences and Technology, Institut Teknologi Bandung, Dr. Endah Sulistyawati, Chief of Plant Science and Biotechology Expertise Group, Dr. Rizkita Rahmi Esyanti, Chairman of Organizing Committee, Dr. Taufikurahman, distinguished speakers and all participants of this symposium.

Assalamu'alaikum warahmatullahi wa barakatuh,
A very good morning to all of you. First of all, it is a great pleasure to me to give a welcoming remark for the second Symposium on Plant Sciences and Product 2020, which is a collaboration between School of life Sciences and Technology, Institut Teknologi Bandung and Faculty of Science and Technology, Universiti Kebangsaan Malaysia.

I would like to congratulate the organizing committee for organizing this symposium after a successfull first symposium in August last year. I understand that this symposium has to be conducted to the online platform due to the covid 19 pandemic and all over the world affected by this pandemic and I hope everyone stay safe and be protected from this virus.

Ladies and Gentlemen,
The collaboration between Universiti Kebangsaan Malaysia and Institut Tekonologi Bandung has been long and wist history, in daily days of the establishment, nearly 50 years ago, UKM, in particular Faculty of Sciences and Technology was indebted to ITB for helping us to recruit the faculty which collabs from ITB starting from our temporary campus In Kuala Lumpur and then in Bani campus two years after our establishment in 1970. As you know this year in 2020, we celebrate our golden jubli, and we proudly declare that we have produced more than 2000 graduated and if we take around 500 student each year for the faculty and by know the faculty has around more than 25000 alumni that graduated from this faculty and I am sure quit number of them come from Indonesia.

Ladies and Gentlemen,
The theme of this symposium this morning. Plant and Microalgae Bioindustry for Ensuring Food Security and Clean Environment is quit fitting to the current development, both Malaysia and Indonesia is moving toward to the knowlegde based technology, a lot of researcher has been carried out on plant and algae and these are renewable bioresources and I'm sure the research finding at risk maturity and it can be translated to the benefit for our society. Perhaps our industry players, in particular, bioindustry to take out this challenge and apply the finding to secure the food supply and adopt technology that contribute to the earth quality. Food Security and Clean Environment are wo main areas under Sustainable Development Goals (SDG) that needs the expert attention. Therefore, this Symposium is the right platform for discussion and I hope participants will benefit from this meeting.

I think about innovation, our faculty, Faculty of Sciences and Technology, UKM, has been able to produce two new varieties of high quality white rice through cross breeding of wild rice species and local high quality white rice species. Two years ago in 2018, we have

launch two new varieties for the farmers we plant and this new variety as come out from researchers and has been conducted at the faculty for nearly 16 years and the new variety can produce more yield about 20-25% than the current production of the variety and it also will be more robust to the plant disease. These are the example of research that has been translated to benefit the society and I hope this symposium will also discuss about the finding. So that the society can also benefit from our research work. Quick glance, I saw there are scholars from across the world, coming from Europe and mostly from Asian region and I hope they can deliver and count on this topic in the end of discussion and I wish all of you have a woderfull session.

Ladies and Gentlemen,
I believe that this close relationshiop between ITB and UKM and the faculty members would extent and expand to other departments. It would be a great idea if we can so that our researcher from other faculty can also participate in this symposiurm in the future. With that note, once again thank you for organizing committee and wish you all purposefull symposium.

Thank you very much

Prof. Dr. Mohammad Kasim
Dean of Faculty Science and Technology,
Universiti Kebangsaaan Malaysia (UKM)
Malaysia

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The 2nd Symposium: Plant and Microalgae Biodunstry for Ensuring Food Security and Clean Environment 2020

The 1st Symposium
2019

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Biomass and Phycocyanin Production from *Spirulina platensis* cultivated in *Anaerobically Digested Dairy Manure Waste* (ADDMW) with Sodium Bicarbonate Addition

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Abstract

Spirulina platensis can be cultivated by using various effluent medium such as *Anaerobically Digested Dairy Manure Waste* (ADDMW). Large production of ADDMW makes it potential to be used as large-scale *S. platensis* cultivation medium. However, one of the obstacles in large scale cultivation is aeration for carbon source supply. Sodium bicarbonate additions in ADDMW as a carbon source can increase *S. platensis* biomass production. This study was conducted to compare the results of *S. platensis* cultivation in Zarrouk medium and ADDMW medium, also to find the effects of bicarbonate addition in ADDMW medium. The results indicated that there is no significant difference of specific growth between *S. platensis* cultivated in Zarrouk medium and ADDMW medium. Also, the highest productivity was obtained in ADDMW with 8.4 g/L sodium bicarbonate addition which reached 0.9831 g/L/day. Addition of sodium bicarbonate mostly affects *S. platensis'* biomass composition. *S. platensis* cultivated in ADDMW with 8.4 g/L sodium bicarbonate addition had the highest carbohydrate content of 41.8%. However, the highest phycocyanin concentration of 3.48% was obtained from ADDMW with 16.8 g/L sodium bicarbonate addition. These results indicate that addition of sodium bicarbonate can increase biomass productivity and phycocyanin content. This study might be a consideration for large-scale cultivation of *S. platensis* in ADDMW medium.

Keywords: Anaerobically Digested Dairy Manure Waste, *Spirulina platensis*, Phycocyanin, Sodium Bicarbonate

1. Introduction

Spirulina platensis is a microalgal cyanobacteria that has been widely used because of its high content of proteins, amino acids, vitamins, and essential fatty acids[1]. Spirulina is also clinically proven to improve the immunity system because it contains a unique antioxidant called phycocyanin[2]. It is a blue pigment found in cyanobacteria[3]. This compound has been commercialized into various products such as food coloring, cosmetics, and biomarker[4].

High synthesis medium cost renders it difficult to produce microalgal biomass. Several studies have found that some effluent can be an alternative for microalgae cultivation[5][6].

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One of them is anaerobically digested dairy waste (ADDMW) or waste that has gone through digestion process of anaerobic bacteria. ADDMW contains 506 mg/L nitrogen and 88 mg/L phosphate compound[7]. Nitrogen and phosphate are important components in growth of microorganisms such as microalgae.

Another obstacle in large-scale cultivation process is aeration for carbon source supply. In a large scale cultivation, aeration cannot be optimal, thus dissolved CO2 in culture medium becomes limited[8]. Therefore, additions of other carbon source such as $HCO3^-$ is needed. It is known that sodium bicarbonate can be used as a carbon source for cyanobacteria[9][3]. Thus, studies on the concentration of addition in ADDMW medium for *S. platensis* cultivation is needed.

The aims of this study were to compare growth quality of *Spirulina* growth in Zarrouk synthesis medium and ADDMW medium, also to determine the effect of sodium bicarbonate addition on the growth, biomass composition, and phycocyanin concentration in *S. platensis* cultivated using ADDMW.

2. Materials and Methods

Materials used include the material for analysis and Zarrouk medium with its composition presented in Table

2.1 Materials used was obtained from Gudang Alat dan Bahan SITH ITB Kampus Ganesha and Jatinangor.

Table 1. Zarrouk medium composition

Component	Concentration
Macronutrient	
NaNO3	2,5 g/L
NaCl	1 g/L
CaCl2	0,04 g/L
K2SO4	1 g/L
MgSO4	0,2 g/L
K2HPO4	0,5 g/L
NaHCO3	18,6 g/L
Na2CO3	7,6 g/L
NaEDTA	0,08 g/L
FESO4	0,01 g/L
Micronutrient	
Trace metals	1 mL
Vitamin B12	10-6 g/L

2.2 Spirulina platensis Culture

S. platensis culture used was obtained from Balai Besar Perikanan Budidaya Air Payau Jepara cultivated using Walne medium. Then, the Zarrouk medium was used for culture inoculation in 30 days.

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2.3 Anaerobically Digested Dairy Manure Waste (ADDMW)

ADDMW used in this study was obtained from biodigester located at Fakultas Peternakan Unpad, Jatinangor, Kabupaten Sumedang.

2.4 Spirulina platensis cultivation

This study took place in Laboratorium Rekayasa Sel dan Kultur Jaringan, Gedung Laboratorium Teknik 1A Institut Teknologi Bandung, Kampus Jatinangor. The room temperature was 21-25 ˚C. Cultivation was conducted using 1 L culture bottle with working volume of 800 mL. Aeration came from aeration pump with the rate of 3 mL/s. LED with light intensity of 4000 lux was used as the light source. The cultivations are presented in Figure 1. Photoperiodism employed was 18 hours of darkness and 6 hours of light. Concentration varieties of NaHCO3 is presented in Table 2.

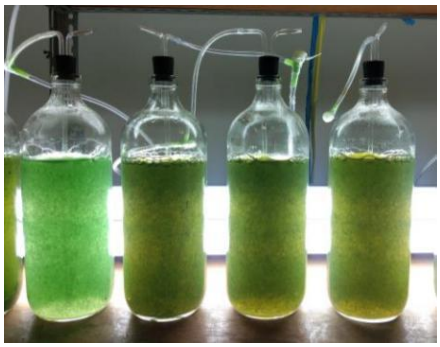

Figure 1. *S. platensis* cultivations

Table 2. Concentration varieties of sodium bicarbonate

Variety	Composition	NaHCO3 (g/L)
Control	ADDMW	0
1	ADDMW	+8,4
2	ADDMW	+16,8

Biomass growth curve was measured using *optical density* parameter at λ 560 nm. Then, the biomass was dried using *freeze dry* method. Dry mass was measured by following equation.

$$Dry\ mass\ \left({}^{a}/_{L}\right) = \frac{A560-1,419}{3,29} x \frac{Vp}{VT} \qquad (1)$$

Vp is the harvest volume for absorbency calculation (mL) and VT is the total volume of the culture's *working volume* (mL).

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2.5 Substrate Analysis Method

Medium's parameters analyzed were nitrate, ammonium, orthophosphate, bicarbonate and sugar. Nitrate concentration analysis was conducted by using H2SO4 salicylate reagent[10]. Ammonium parameter was analyzed using Nessler reagent. Orthophosphate in the medium was analyzed using stannous chloride (SnCl2) and ammonium molybdate reagent. Bicarbonate substrate was analyzed using HCL 2 N titration method with phenolphthalein and methyl orange. Lastly, sugar in the medium was analyzed using Nelson-Somogyi method.

2.6 Chlorophyll Pigment Analysis

Chlorophyll analysis employed extraction method with acetone solution[11]. 10 mg of dry biomass was dissolved with 1 mL of acetone. Then, it was incubated for 5 minutes. After the incubation, the chlorophyll concentration was measured by the following equation.

$$Chlorophyll\left({}^{mg}/_{mL}\right) = 11.24A662 - 2.04A645 \qquad (2)$$

2.7 Phycocyanin Analysis

Phycocyanin of Spirulina's dry biomass was extracted using cold maceration method at 4° C temperature. Solution used was 0.05 M pH 6,9 phosphate buffer[12]. Maceration took place for 24 hours in darkness. The sample was then centrifuged at 5100 rpm for 2 minutes. Supernatant was measured using spectrophotometer at λ 615 and 652 nm. Phycocyanin concentration was measured by the following equation.

$$C - phycocyanin\left({}^{\mu g}/_{mL}\right) = \frac{A615 - 0.4/4A652}{5.34} \qquad (3)$$

2.8 Protein Analysis

Dry biomass was diluted in lysis buffer and sonicated by water bath sonication for 1 hour. The biomass protein in supernatan was quantified by Bradford method using Bovine Serum Albumine as protein standard

3. Results and Discussions

Based on the characterization result (Table 3) of ADDMW used in this study, it is shown that it contained ammonium nitrate and orthophosphate of 59.7 mg/L and 6.53 mg/L. 1.89 mg/L. of ammonium concentration found in ADDMW was very small compared to the reference[16] which was 800-1,400 mg/L. Meanwhile, in other study[7], nitrate and ammonium of 412 mg/L and 97 mg/L concentration was found. Difference in nitrogen content is influenced by the condition of biodigester operation, which includes temperature, retention time, and cattle feed[17] [18].

Some microalgae species has ammonium tolerance of ± 70 ppm[6]. This is because high concentration of ammonium can be toxic. However, because the characterization results showed that ammonium was below its toxicity range, ADDMW obtained did not need to be diluted.

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Table 3. *S. platensis* cultivated varieties

Parameter	Value
Ammonium (mg/L)	59.70 ± 10
Nitrate (mg/L)	6.53 ±1.43
Ortophosphate (mg/L)	1.89 ± 0.21
Sugar (mg/L)	0
pH	9.33

The initial pH of ADDMW used was 9.2. It was more basic than the biodigester *effluent* pH of referenced pig wastewater which was pH 8[6]. This pH increase may be caused by carbonate and bicarbonate ions content. The presence of carbonate and bicarbonate ions in ADDMW can be caused by the addition of calcium carbonate in cattle feed[19]. Calcium carbonate that cannot be digested in the rumen is excreted through urine and feces. Other study [20] has shown that cattle waste contains bicarbonate concentration of 0.3 g/L.

Furthermore, ADDMW used also did not contain sugar. Therefore, microalgal cultivation process occurred in photoautotroph system. Based on dry mass growth curve (Figure 2), *S. platensis* culture was in lag phase in the first 24 hours. It was marked by insignificant growth. In lag phase, adaptation process happens by RNA, enzyme and other molecules synthesis. The result obtained is in line with other study[21]. *S. platensis* with initial nitrogen concentration of ±150 ppm was in lag phase during the first 24 hours. This condition occurred with low nitrogen concentration range, which corresponded with the culture condition of this study. Other studies have shown that with sodium bicarbonate addition of 5.5 g/L and 11 g/L, lag phase occurs for 3.6-3.7 days[1]. In other studies, lag phase happened rapidly at 2-5 hours with high nitrogen source of 1,000-3,000 ppm[22]. These differences may be influenced by the initial condition of the medium which includes nutrition availability, pH, salinity, aeration and light intensity. There are two types of acclimatization in microalgae. The first type is change in yield, while the second type is change in specific growth rate [9].

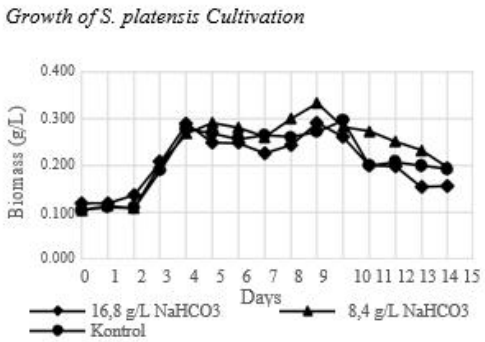

Figure 2. Dry mass growth curve of *S. platensis*

Furthermore, in day 1 and day 2, exponential phase happened in each of the variation of treatments. It was marked with the increase of *S. platensis* total mass. In this phase, cell division happens. In day 3 and 4, linear growth occurred as a result of constant doubling time. Then, specific growth rate value was calculated from linearization of logarithmic equation. Based on ANOVA statistic test (P value 0,05), there was no significant difference in

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specific growth rate between bicarbonate variations (Table 4). This was possible because the nitrate and ammonium were almost depleted in day 5. The availability of nitrogen source is a determining factor in microalgae cell growth. [23]. Specific growth rate is influenced by the availability of nitrogen because it is the main component of protein formation. In Spirulina, harvesting of short light wave is done by phycobiliprotein located in thylakoid membrane[9]. The optimal formation of phycobiliprotein happens in nitrogen 18 g/L and 2,23 g/L[24]. Thus, *S. platensis* cannot form more phycobilin because of nitrogen deficiency[23] which resulted in the decline of photosynthesis and cell division rate[24]. However, by comparing the specific growth rate, it is shown that the control had higher value than the two varieties. This may be caused by the lower initial pH of the medium. The higher the pH of the medium, the lower the solubility of CO2[25]. As the result, *S. platensis* needs to have enzymatic process to change HCO^- and $CO3^-$ ions into source of CO2. Extracellular enzyme used by cyanobacteria to convert $HCO3^-$ and $CO3^-$ is carbonic anhydrase [26]. Enzymatic process requires more energy than direct use of CO2 gas.

Table 4. Kinetic Parameter of *S. platensis* cultivation

Medium	μ (day-1)	dt (day)	Productivity (g L^{-1} day^{-1})	Reference
10 % Zarrouk	0,245[ab]	3,01	0,0468 ± 0,0017	This study
ADDMW + 16,8 g NaHCO3	0,205[a]	3,43	0,0586 ± 0,009	This study
ADDMW + 8,4 g NaHCO3	0,190[a]	3,65	0,0607 ± 0,001	This study
Control	0,228[a]	3,05	0,0400 ± 0,004	This study
16,6% Zarrouk	0,146	4,73	0,067	[21]
Modified Zarrouk medium	0,81	0,85	0,791	[22]

[abcd]ANOVA *Post Hoc* Duncan *Test* (P value 0,05)

In day 5 till day 10, stationary phase of *S. platensis* began. This was marked by insignificant increase of biomass. In day 11 till 14, death phase happened as shown by the decrease of the culture's dry mass. This is also proven by the slight increase in ammonium and nitrate. (Figure 3). *S. platensis* could not start N2 fixation because it did not have *heterocyst*,[27] thus, cell death may cause the increase of nitrate and ammonium concentration. Cell death may happen because of pH change to 11 in day 11-14.

After the 14th day, *S. platensis* culture was harvested, then its dry mass was weighed. The highest increment of dry mass, which was 0,666 g/L, was obtained from the addition of 8,4 g/L NaHCO3, followed by 0,645 g/L obtained from the addition of 16,8 g/L NaHCO3 and control with 0,476 g/L (Figure 4). Although the growth curve of the two varieties and control did not show significant values, there was significant change in productivity value because change in dry mass increase was found. This was the result of the addition of 8,4 g/L NaHCO3 to every cells of *S. platensis* that was higher compared to the other varieties, resulting in larger final dry mass. Increase in cell mass is caused by higher carbohydrate contents. Carbohydrate accumulation is inversely proportional to protein concentration. Culture with 8,4 g/L NaHCO3 addition had the highest concentration of carbohydrate, but the lowest concentration of protein (Figure 5).

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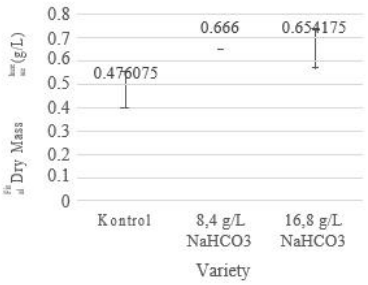

Figure 4. Dry mass of *S. platensis* with harvesting method in day 14

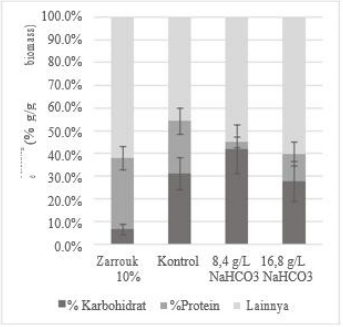

Figure 5. Percentage of protein and carbohydrate in the biomass

In *S. platensis*., carbohydrate is found in a form of glycoge. 1 mol of glycogen has a mass of 6 x10^{23} kDa, while phycobiliprotein, as one of the main proteins in *S. platensis*, consisting of 6 chromophore, has a mass of 240-260 kDa[28] Carbohydrate content may increase as a result of high carbon fixation by *S. platensis*. This is proven by the increase of pH in the culture. Similar pH trend happened in microalgae culture, *Tetraselmis suecica* with NaHCO3 supplementation[17] pH increase in the culture may be an indication of the availability of sufficient carbon source for *S. platensis* activity[9]. In basic pH, bicarbonate ion is converted into CO2 by the following equation

$$HCO_3^- \leftrightarrow CO_2 + OH^- \qquad (4)$$

In pH above 10.2., carbonate ion may also be converted into CO2 gas by the following equation

$$CO_3^{2-} + H_2O \leftrightarrow CO_2 + 2OH^- \qquad (5)$$

Production of OH- from the absorption process of carbon source resulted in the pH culture becoming more basic. If it was assumed that all the carbonates and bicarbonates were converted into CO2, then the pH of the culture could reach 14 [9]. Because of that, the pH of the culture needs to be maintained at optimal condition (pH 9.8-10.2)[23]. Increase of the culture's pH during cultivation is presented in Figure 6.

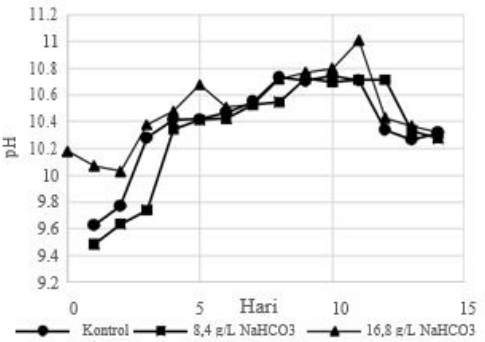

Figure 6. pH changes of the medium graph

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Table 5. Phycocyanin Concentration of *S. platensis* in cultivation condition varieties

Medium	Sodium Bicarbonate (g/L)	Nitrogen (g/L)	Phycocyanin (mg /g biomass)	System
10% Zarrouk	1,68	0,25	10,49%^{d}	*Batch*
ADDMW + 16,8 g/L $NaHCO_3$	16,8	0,05	3,48%^{bc}	*Batch*
ADDMW + 8,4 g/L $NaHCO_3$	8,4	0,05	0,77%^{a}	*Batch*
Control	0,3	0,05	1,91%^{b}	*Batch Fed*
Pig wastewater biodigester output [6]	2,5	0,25	19,5%	*Batch*
Resin production waste [5]	8,5	1,5	17%	*Batch*

^{abcd}ANOVA *Post Hoc* Duncan *Test* (P value : 0,05)

Phycocyanin Production of *S. platensis*

The highest concentration of phycocyanin was found in 16,8 g/L NaHCO3 addition, which was 3,48% followed by 8,4 g/L NaHCO3 addition, which was 1,91% and the lowest was found in control, which was 0,77% (Table 5). Difference in concentration could be represented by the blue color from the crude phycocyanin extract (Figure 7).

The higher the phycocyanin concentration is, the bluer it becomes. In 8,4 g/L NaHCO3 addition, very low concentration of phycocyanin is proven by the accumulation of carbohydrates which has been explained in previous section. However, the addition of 16,8 g/L of NaHCO3 resulted in higher concentration of phycocyanin than other varieties. This may be caused by the pH medium reaching 10,7 – 11,01. In that pH range, carbon source is available in carbonate ion form. Carbonate and bicarbonate ions are known for its unique role in photosystem II. Carbonate and bicarbonate ions can be an alternative glutamate ligand in non-heme Fe[29] which cause an increase in electron transport efficiency from Qb to Qa[30]. Electron transport efficiency resulted in ATP and NADPH accumulation, which is used for synthesis of substances in other cycles[31]. Several studies have shown the increase of pigments with carbonate[17] and bicarbonate[32] additions, but the influence of carbonate and bicarbonate ions in phycobiliprotein production is yet to be fully known.

Figure 7. Phycocyanin crude extract (Left to right: Zarrouk medium 10%, ADDMW + 16,8 g/L NaHCO3, ADDMW + 8,4 g/L NaHCO3, Control)

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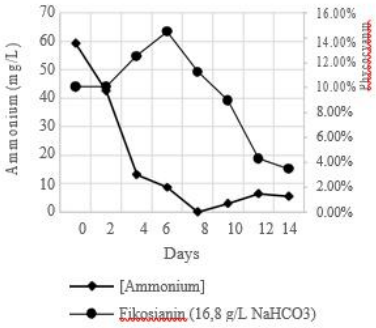

Figure 8. Ammonium concentration in the medium relative to phycocyanin graph

There was an increase in concentration of phycocyanin in day 1 till day 3. This increase occurred in logarithmic phase with sufficient nitrogen availability. In day 5-14, there was a significant decrease in phycocyanin concentration. It decreased when the ammonium concentration in the medium was almost depleted (Figure 8). Phycobilin is the source storage of N for cyanobacteria[13], in nitrogen dificient condition, cells will use phycobiliprotein as its source of nitrogen [24]. That being said, *S. platensis* is capable of photosynthesis by using chlorophyll a, but when the concentration of phycocyanin is below 50% of the optimal concentration, photosynthesis process will decline significantly and stop [24]. In ADDMW medium, concentration of phycocyanin was much lower than concentration of phycocyanin found in cultivation in Zarrouk medium that reached 10,49%. The output of phycocyanin concentration is also lower than *S.platensis* cultivation in pig wastewater biodigester output medium[6] which is caused by the use of *fed batch* system in the reference. *Fed batch* system maintains the nitrogen concentration of the medium to prevent phycocyanin decay.

4. Conclusion

Based on the results of this study, there is no significant difference between the specific growth rate of *S. platensis* cultivated in Zarrouk medium and ADDMW medium. Furthermore, sodium bicarbonate addition does not significantly affect the specific growth rate of *S. platensis* in ADDMW medium. This is possible because sodium bicarbonate's effect will only appear in a nitrogen-rich condition. However, sodium bicarbonate addition has significant effects on the composition of *S. platensis* biomass. Also, sodium bicarbonate concentration have a

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Biomass Production of *Chlorella vulgaris* and *Chlorella pyrenoidosa* Cultivated in Bioslurry and Treated Using Different LED Types

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Abstract

Microalgae are photosynthetic organisms that have potential as an alternative source of protein. Anaerobic digested dairy manure wastewater (ADDMW) or bioslurry constitutes a great opportunity for microalgae growth medium since it is low-cost and eliminates negative environmental impact. *Chlorella vulgaris* and *Chlorella pyrenoidosa* have the ability in lowering excessive nutrients in wastewater and simultaneously produce protein-rich biomass. In this study, biomass production of microalgae was enhanced using LEDs (red, blue, and white). We also measured orthophosphate and ammonia reduction in bioslurry. The result showed that red LED gave the best result for biomass production. Best biomass productivity was given by *C. pyrenoidosa* i.e. 0.114 ± 0.021 $g.L^{-1}day^{-1}$, while the productivity *of C. vulgaris* was 0.098 ± 0.014 $g.L^{-1}day^{-1}$. Various LED types, however, did not affect orthophosphate and ammonia reduction and also microalga's protein content. Orthophosphate and ammonia were reduced by 52-64% and 88-90% by *C. vulgaris* and 42-57% and 89-91% by *C. pyrenoidosa*, respectively. Protein content in *C. vulgaris* was slightly higher than in *C. pyrenoidosa.*

Keywords: bioslurry; Chlorella pyrenoidosa; Chlorella vulgaris; LED; protein

1. Introduction

Protein is one of primary metabolite that has increasing in demand as global population grows. Therefore, it is needed to develop alternative sources of protein to supplement the existing conventional sources in order to meet the global demand. Microalgae have been identified as one of the most reliable sources of protein because of its high protein content and valuable nutritional profile. Some microalgal sources present a protein content higher than conventional animal or plant sources [1]. Microalgae have higher protein yield per unit area (4-15 tons/Ha/year) compared to terrestrial crops, such as soybean, pulse legumes, and wheat (0.6-1.2 ton/Ha/year, 1-2 ton/Ha/year, and 1.1 ton/Ha/year, respectively) [2]. In addition, microalgae are important as a health promoting factor [3], improving external appearance [4], and natural antioxidants for human and animal [5]. *Chlorella* is being widely used in the healthy food market as well as for animal feed and aquaculture [6], because it is abundant in protein (up tp 68%) and contains all the essential amino acids [7]. Reference [7] also stated that *Chlorella* has proven abilities of removing nutrients (N and P), making it a good candidate for wastewater bioremediation.

To scale up microalgae cultivation into industrial implementation, it is needed to design a

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system production that is both economically viable and high productivity. The exploitation of nutrient-rich and low-cost dairy manure wastewater for algal biomass production and phycoremediation is an option for enhancing the sustainability of dairy industry as well as controlling the adverse impacts of wastewater to the environment and to public health [8]. However, considerable difficulties exist for fully exploiting the use of dairy manure wastewater due to its complex nature (i.e. relatively high level of solids and fibers) [9]. By anaerobic digestion process, organic materials in manure wastewater is reduced. This treatment generates biogas as an environmentally sustainable energy and bioslurry as an effluent that can be utilized as microalgae growth medium.

Cultivation of microalgae that carried out outdoors might be under conditions that are not optimal due to uncertain weather. It can affect the biomass productivity since sunlight is the most critical parameter in microalgae photosynthesis. Therefore, utilization of artificial lighting system could optimize productivity of microalgae biomass. However, to effectively exploit the commercial potential of microalgae, a cheap, durable, reliable, and highly efficient artificial light source is needed. Light-emitting diodes (LEDs) offer one such option as they have the characteristics of narrow-band wavelength and cost- effective power consumption [10, 11].

Study about the performance of microalgae species growing in bioslurry and the wavelength characteristics of LED are therefore a prerequisite for the proper utilization of microalgae in food/feed industry. In this study, emphasis will be placed on effect of different types of LEDs (red, blue, and white) on *Chlorella vulgaris* and *Chlorella pyrenoidosa* biomass production cultivated in bioslurry, biomass growth parameter, protein concentration, and efficiency of ammonia and orthophosphate reduction in bioslurry will be presented and discussed in this paper.

2. Materials and Methods

2.1 Materials

1) Microalgae

Chlorella vulgaris and *Chlorella pyrenoidosa* were got from Balai Besar Perikanan Budidaya Air Payau (BBPBAP) Jepara. Both microalgae species were sub- cultured in Bold Basal Medium (BBM) for inoculum supplementation.

2) Chemical substances

Chemical substances used in this study consist of substances for: making Bold Basal Medium ($NaNO_3$, $MgSO_4 \cdot 7H_2O$, NaCl, K_2HPO_4, KH_2PO_4, $CaCl_2 \cdot 2H_2O$, H_3BO_3, $ZnSO_4 \cdot 7H_2O$, $MnCl_2 \cdot 4H_2O$, MoO_3, $CuSO_4 \cdot 5H_2O$, $Co(NO_3)_2 \cdot 6H_2O$, $EDTANa_2$, KOH, $FeSO_4 \cdot 7H_2O$, H_2SO_4); analyzing ammonia concentration (Nessler, Na·K Tartrat, NH_4Cl); analyzing orthophosphate concentration (Amonium molibdat-H_2SO_4, Gliserol, $SnCl_2$, KH_2PO_4); and analyzing protein concentration (TCA 24% (w/v), *Comassie Brilliant Blue*, Etanol 95%, H_3PO_4 85%, BSA (*Bovine Serum Albumin*)). Chemical substances were provided by School of Life Sciences and Technology, Bandung Institute of Technology.

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3) **Bioslurry**

Growth medium used for microalgae cultivation is anaerobic digested flushed dairy manure wastewater, or called bioslurry. This bioslurry was collected from plant biogas in dairy farm located in Faculty of Animal Husbandry, Padjajaran University, Jatinangor. Bioslurry was first filtrated using cotton, and then second using double filter paper. Filtrated bioslurry was diluted until the concentration of ammonium and orthophosphate were up to 60 ppm and 20 ppm, respectively. The aim of the dilution was to avoid restricted microalgae growth due to colored bioslurry hinder the light penetration.

2.2 Methods

1) Microalgae Cultivation

Initial inoculum concentration was $1.5x10^7$ cells/mL Both *C. vulgaris* and *C. pyrenoidosa* was inoculated in bioslurry into 1 L photobioreactor with working volume of 700 mL. Ratio between inoculum : medium is 3 : 7. The cultures were grown under different type of LEDs (red LED, blue LED, white LED) in quadruplicates. The intensity of the LEDs were 5000 lux. The experiment was conducted in 9 h light and 15 h dark cycle. The light and dark condition were controlled using an electric timer. The aerators were used to provide air for the cultures with the velocity of 1 L/minute. Cultures condition was maintained at pH of 6,7-8,7 and temperature of 27-30°C. The growth of microalgae in bioslurry was assessed for 10 days. Table 1 provides characteristics of LED types used in this study.

Table 1. Characteristics of LED Types

LED Types	Power	Vacuum wavelength
T5 red LED*	22 W	622-720 nm
T5 white LED*	16 W	-
T5 blue LED*	16 W	455-492 nm

*Tube lamp with diameter of 5/8 inch (1,59 cm) and length of 1,2 m

2) Determination of Biomass Microalgae Concentration

The growth of *C. vulgaris* and *C. pyrenoidosa* was monitored daily by measuring the Optical Density (OD) using spectrophotometry at a wavelength of 680 nm. Cells concentration in OD were converted to concentration in dry weight (g/L) using equation in standard curve of *C. vulgaris* and *C. pyrenoidosa.*

3) Nutrients Concentration Analysis

Nutrients concentration in bioslurry were measured on day 0, 3, 5, 7, and 10 of cultivation. A ±13 mL sample culture was centrifuged at 5300 RPM for 5 minutes. The supernatant was used to ammonia and orthophosphate analysis.

a) Orthophosphate Concentration Analysis

Orthophosphate in bioslurry were analyzed based on $SnCl_2$ method. Firstly, a 12,5 medium sample and aquades as a blank were prepared. Sample was added by 0,5 mL

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ammonium molibdat solution, then added by 1 drop $SnCl_2$. After 10 minutes, the absorbance of sample was measured with spectrophotometry at a wavelength of 660 nm. Absorbance values were converted into orthophosphate concentration using equation in standard curve of KH_2PO_4.

b) Ammonia Concentration Analysis

Ammonia in bioslurry were analyzed based on Nessler method. Firstly, a 12,5 medium sample and aquades as a blank were prepared. Sample was added by 1 drop of seignette salt, then added by 0,25 mL of Nessler reagent. After 10 minutes, the absorbance of sample was measured with spectrophotometry at a wavelength of 420 nm. Absorbance values were converted into ammonia concentration using equation in standard curve of NH_4Cl.

4) Microalgae Harvesting

Microalgae were harvested in day 10 of cultivation. A

±400 mL culture sample were moved into several ±15 mL falcon tube. Culture sample in falcon tube were centrifuged at 5300 RPM for 5 min. The pellets of centrifuged microalgae moved into a microtube to be centrifuged at 15000 RPM for 10 minutes at 4ºC. Then, the harvested cells were freeze dried.

5) Protein Extraction

Microalgae protein extraction was conducted based on hot-TCA (trichloroacetic acid) method [13] with a slight change. For each microalgae species, 3 mg of freeze-dried microalgae biomass was weighed out and put into microtube. Samples were resuspended by vortexing in 250 µL (6% (w/v)) TCA. Homogenates were incubated in a water bath at 95ºC for 15 min. After being allowed to cool to RT, homogenates were centrifuged at 12300 RPM for 20 min at 4ºC. After supernatans were discarded, the pellets were resuspended in 0,5 mL NaOH 0,1 N by vortexing. Homogenates were incubated in a water bath at 55ºC for 3

h. Samples were then cooled to RT, centrifuged at 12300 RPM for 20 min at RT. Supernatant retained for further analysis.

6) Protein Quantification

Protein quantification followed the method of Bradford as explained in Kruger [14]. Extracted supernatant in a volume up to 0,1 mL was pipetted to microtubes. Five mL of Bradford reagent was added to the microtubes and the contents mixed by vortexing. After incubated for 20 min in RT, the absorbance of samples at 595 nm were measured against a reagent blank prepared from 0,1 mL and 5 mL Bradford reagent. The weight of protein was plotted against the corresponding absorbance resulting in a BSA (Bovine Serum Albumine) standard curve used to determine the protein in unknown sampled.

7) Data Analysys

Parameters that were analyzed in microalgae growth kinetics were specific growth rate (μ (day^{-1})), doubling time (dt (day)), and productivity (r ($g\ L^{-1}\ day^{-1}$)). Equation (1), (2), and (3) were used to determine three of the parameters [15, 16],

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$$u = \frac{ln\,(N_2/N_1)}{t_2 - t_1} \quad (1)$$

$$dt = \frac{ln\,2}{\mu} \quad (2)$$

$$r = \frac{N_2 - N_1}{t_2 - t_1} \quad (3)$$

where N_2 and N_1 are biomass concentration (g/L) at t_2 and t_1 (day).

Parameters that were analyzed in nutrition reduction in bioslurry were ammonia and orthophosphate reduction rate and efficiency. Equation (4) was used to determine ammonia/orthophosphate reduction rate (R_i (ppm/day)), and (5) was used to determine ammonia/orthophosphate reduction efficiency (η (%)) [17, 18],

$$R_i = \frac{S_0 - S_f}{t_f} \quad (4)$$

$$\eta = \frac{S_0 - S_f}{S_0} \times 100\% \quad (5)$$

8) Data Statistical Analysis and Interpretation

This research was experimented in quadruplicate which three of the best data were selected to be statistically analyzed. Statistical analysis for this research were mean and standard deviation calculation using Microsoft Excel 2016, and also Two-way Analysis of Variance (ANOVA) using IBM SPSS Statistics 22. Post-hoc analysis for LED types was Tukey analysis. Value p<0,05 in one of the factor indicates that there are significant effect to the dependent variable. Value p<0,05 in factor 1*factor 2 indicates that there are interaction between factors.

3. Results and Discussion

A. Growth Kinetics of C. vulgaris and C. pyrenoidosa

Growth curve of both species can be seen in Fig. 1 and 2. Based on Fig. 1, exponential phase occured in *C. vulgaris* right after the first day of cultivation. Based on Fig. 2, *C. pyrenoidosa* directly entered exponential phase, without lag phase occured. This were due to the high initial inoculum concentration (0,28 g/L) so there were no notable changes in environmental conditions surrounding microalgae compared to previous cultivation condition. Lau et al. (1995) stated that the higher initial inoculum density, the nutrition reduction could be higher [19], it is because this condition could avoid self-shading and auto- inhibitor accumulation [20].

This result indicated that red LED is the optimum light for enhancing biomass production of *C. vulgaris* and *C. pyrenoidosa*. The LED types order for both microalgae production in terms of dry weight was red > white > blue. Research by Xu et al. [21] showed that red LED generated highest *C. vulgaris* biomass compared to use of yellow, purple, blue, green, and white LED. Red LED (674 nm) has the highest efficiency because it only takes a small amount of photon flux to stimulate microalgae maximum growth [22]. Moreover, Matthijs et al. [23] stated that lights with red wavelengths are the optimum lights for *C. pyrenoidosa* culture. Microalgae biomass production is strongly affected by the light wavelengths received by microalgae. Chlorophyll in microalgae plays a vital role in photosynthesis since chlorophyll absorbs energy from light. Specifically, the green pigment chlorophyll in microalgae could efficiently absorb red

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wavelength [23]. Meanwhile, blue LED could lead to larger cells [24, 25, 26], as a result of delayed cell division [27].

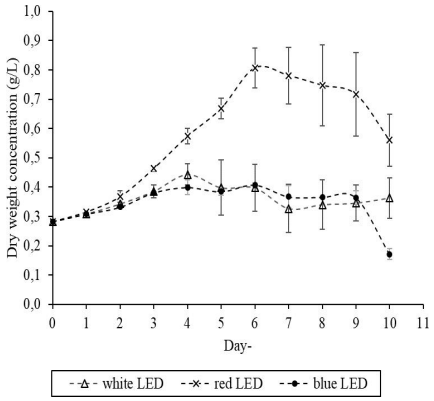

Figure. 1 Growth curve of C. vulgaris cultivated in bioslurry with variation of LED types

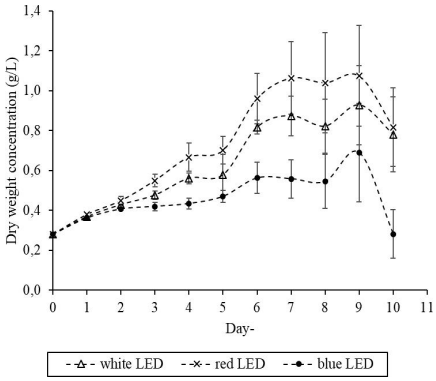

Figure. 2 Growth curve of *C. pyrenoidosa* cultivated in *bioslurry* with variation of LED types

The effect of red, blue and white LEDs on *C. vulgaris* and *C. pyrenoidosa* productivity can be seen in Fig. 3. There is significant productivity result among LEDs and interspecies factor ($p < 0.05$). However, there was no relationship between the combination of LED and species, which meant that the combination of two factors gave the same pattern on the dependent variable. *C. pyrenoidosa* was found to produce higher biomass than *C. vulgaris,* which indicated that *C. pyreniodosa* had better adaptation ability grown in b*ioslurry* medium. Doubling time and specific growth rate also resulted in similar pattern (Table 2).

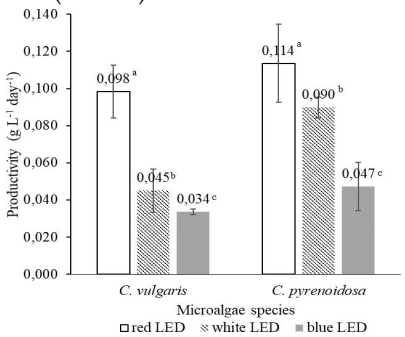

Figure. 3 Graph of *C. vulgaris* and *C. pyrenoidosa* productivity (Species p<0,05; LED p<0,05; Species*LED p> 0,05; Two-way ANOVA; LED *post-hoc* Tukey)

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Table 2. *C. vulgaris* and *C. Pyrenoidosa* Specific Growth Rate and Doubling Time

LED Types	Specific growth rate (μ)		Doubling time	
	C. vulgaris	*C. pyrenoidosa*	*C. vulgaris*	*C. pyrenoidosa*
	day^{-1} ± std		day ± std	
Red LED	0,187 ± 0,019 ^{a}	0,204 ± 0,022 ^{a}	3,738 ± 0,367 ^{b}	3,434 ± 0,401 ^{b}
White LED	0,120 ± 0,026 ^{b}	0,179 ± 0,007 ^{b}	6,085 ± 1,476 ^{ab}	3,870 ± 0,152 ^{ab}
Blue LED	0,102 ± 0,005 ^{c}	0,115 ± 0,023 ^{c}	6,806 ± 0,321 ^{a}	6,264 ± 1,273 ^{a}

B. Orthophosphate Reduction in Bioslurry

Fig. 4 shows that phosphate concentration was increasing in the 7th day. This might be caused by cells that were in a stress state then lead to lysis cells. Cells lysed phospate contents to the bioslurry medium causing the phosphate concentration increased. Based on Fig. 6, ammonia have been reduced to almost 90% on day 5, while microalgae cells began to enter stationary phase after day 6 (Fig. 1 and 2), this was saying that lack of ammonia sources triggered the cells became stress. Based on statistical analysis, there were no significant difference given by the 3 types of LEDs and both of the species to the reduction of nutrients (Fig. 5 and Table 3). However, it can be seen that white LED gave higher result in reducing the nutrients (ammonia and orthophosphate), although this was not statistically significant. This indicated that white LED spectrum was better in trigger signaling on nutrient uptake by microalgae, compared to red LED that have specific wavelength for photosynthesis.

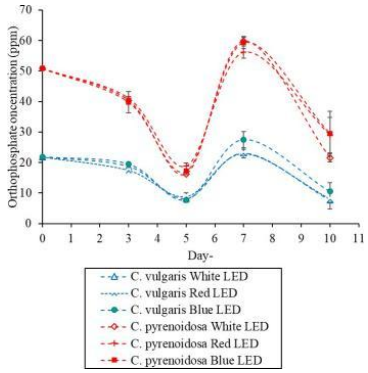

Figure. 4 Curve of orthophosphate reduction in bioslurry by *C. vulgaris* and *pyrenoidosa* cultivated with various types of LED

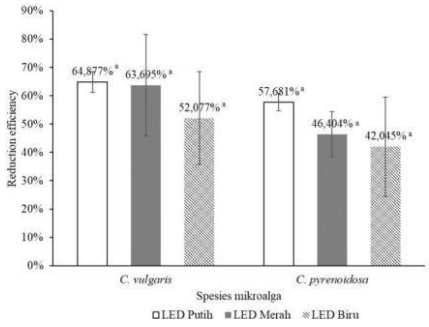

Figure. 5 Graph of orthophosphate reduction efficiency in bioslurry by *C. vulgaris* and *C. pyrenoidosa* (Species p>0,05; LED p>0,05; Spesies*LED p>0,05; Two-way ANOVA; LED *post-hoc* Tukey

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Table 3. Orthophospate Reduction Rate by *C. vulgaris* and *C. pyrenoidosa*

LED Types	Orthophosphate reduction rate	
	C. vulgaris	*C. pyrenoidosa*
	ppm/day ± std	
White LED	1,42 ± 0,080 [a]	2,934 ± 0,151 [a]
Red LED	1,39 ± 0,391 [a]	2,358 ± 0,404 [a]
Blue LED	1,14 ± 0,358 [a]	2,139 ± 0,893 [a]

C. Ammonia Reduction in Bioslurry

Neither types of LEDs nor both species gave a tremendous difference in terms of reducing ammonia level. Ammonia reduction efficiency by *C. vulgaris* and *C. pyrenoidosa* cultivated with three types of LED was ranging from 88-91% (Fig. 7). Moreover, this result indicated that both species have the same ability in remediation of ammonia and orthophosphate in bioslurry medium. However, based on the statistic, there were a significant difference ($p < 0.05$) in the LED types on ammonia reduction efficiency. This happened because the standard deviation was very small, causing the statistics said significant, eventhough the differences weresmall.

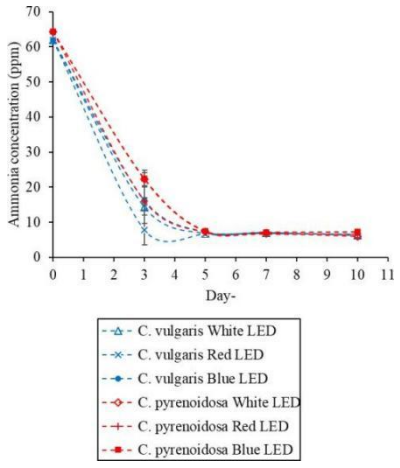

Figure. 6 Curve of ammonia reduction in bioslurry by *C. vulgaris* and *C. pyrenoidosa* cultivated with various types of LED

TABLE IV. ORTHOPHOSPHATE REDUCTION RATE BY *C. VULGARIS* AND *C. PYRENOIDOSA*

LED Types	Ammonia reduction rate	
	C. vulgaris	*C. pyrenoidosa*
	ppm/day ± std	
White LED	5,62 ± 0,068 [a]	5,835 ± 0,095 [a]
Red LED	5,59 ± 0,169 [a]	5,785 ± 0,042 [a]
Blue LED	5,47 ± 0,083 [a]	5,740 ± 0,105 [a]

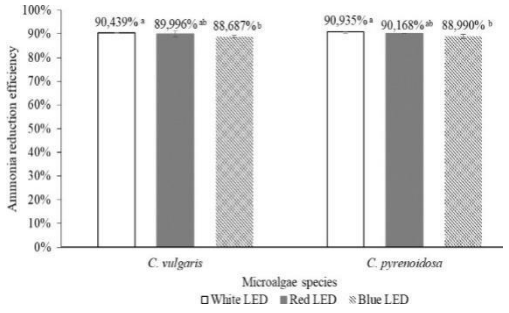

Figure. 7 Graph of ammonia reduction efficiency in bioslurry by *C. vulgaris* and *C. pyrenoidosa* cultivated with various types of LED (Species $p>0,05$; LED $p<0,05$; Spesies*LED $p>0,05$; Two-way ANOVA; LED *post-hoc* Tukey)

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D. Protein Content Analysis in C. vulgaris and C. pyrenoidosa

The result on protein content indicated that LED types did not affect microalgae protein content. The light wavelength radiated by LED affects the biomass synthesis, but does not have a direct effect on protein production. Protein content could be increased by optimizing light intensity and photoperiodism [28], but not by varying the wavelength. Therefore, further research should be analyzed about the effect of light intensity and photoperiodism on microalgae protein production.

Based on Fig. 8, microalgae species has a significant difference ($p<0,05$) on protein content, with *C. vulgaris* having a slightly higher protein content than *C. pyrenoidosa*. Based on the literature by Becker [29], *C. vulgaris* has a protein content up to 58%, slightly higher than *C. pyrenoidosa* protein content with 57%. However, protein content obtained from this research is considerably different from the literature. This is due to the hot-TCA method limitation in extracting protein from microalgae. In addition, the absence of de-oiling or lipids extraction prior to protein extraction also causes the protein quantification to be inaccurate.

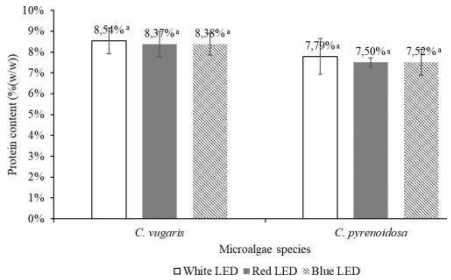

Figure 8. Graph of protein content in *C. vulgaris* and *C. pyrenoidosa* cultivated in *bioslurry* with various LED types (Spesies $p<0,05$; LED $p>0,05$; Spesies*LED $p>0,05$; Two-way ANOVA; LED *post-hoc* Tukey)

4. Conclusions

This study showed that red LED gave the best result for enhancing biomass production. Best biomass productivity was given by *C. pyrenoidosa,* which valued 0,114 ± 0,021 $gL^{-1}day^{-1}$, while the productivity of *C. vulgaris* was 0,098 ± 0,014 $gL^{-1}day^{-1}$. Whereas, type of LEDs did not give results much differently in orthophosphate and ammonia reduction and also in protein content. Orthophosphate and ammonia are reduced by 52-64% and 88-90% by *C. vulgaris* and 42-57% and 89-91% by *C. pyrenoidosa,* respectively. Protein content was 8,3-8,5% (w/v) in *C. vulgaris*—slightly higher than in *C. pyrenoidosa* which ranged 7,5-7,8% (w/v).

This study found that anaerobic digested dairy manure wastewater has the potential to be a cost-effective culture medium for commercial rich-protein microalgae, *C. vulgaris*, and *C. pyrenoidosa*, which could in turn produce value-added by-products. Furthermore, it is required to analyze more about the effect of light intensity and photoperiodism in order to achieve light sources that require less energy but having advantages lighting effects for microalgae cultivation.

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Vegetation Community Structure and Ecological Status of Riparian in Upstream Citarik, Taman Buru Masigit Kareumbi (TBMK) Watershed, West Java

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Abstract

This study was conducted in Taman Buru Masigit Kareumbi (TBMK), West Java, specifically at the riparian zone of the upstream area of Citarik watershed. TBMK serves as a water source and as a part of the river continuum at Citarum River. The function of this area has been decreased due to land conversion to agriculture system. This study aims to determine the vegetation community structure and ecological status of the upstream riparian zone of Citarik watershed. It was conducted at five sites of riparian zone, i.e Site 1, vegetable fields, Site 2, bamboo forest, Sites 3 and 4, an area of deer cages of 28 and 8 hectares, and Site 5, tourism area. The vegetation community structure was analyzed based on the Important Value Index, Shannon-Wiener Diversity Index, and Simpson Domination Index. Riparian ecological status was determined by the QBR Index, based on the structure and quality of vegetation cover and river channel alteration. The plant communities in riparian zones consist of eight trees species, 11 shrubs species and 15 herbs species. Based on its QBR Index, the riparian ecological status is as follows: Sites 1, 2, and 4 possessed moderate quality with moderate disturbances; while Sites 3 and 5 had poor qualities, with high disturbances.

Keywords: Citarik watershed, Community structure, Riparian, Riparian quality, QBR

1. Introduction

Watersheds integrate land areas with rivers and their tributaries as a unit. The watershed holds rainwater, in which the rainwater flows through the tributaries to the main river, called the sub-watershed [1]. Riparian zones act as buffers between terrestrial ecosystems and river ecosystems. The area plays an important role in both of ecology and economy sectors. As part of the watershed, there are only 1.4% riparian zones left of the earth's total landscape, due to land degradations and human activities [2]. However, the riparian zone is important as it possesses several benefits, such as preventing flooding and erosion, maintaining the hydrological cycle, and maintaining the diversity of flora and fauna around the river area [3–5].

The Citarik watershed is one of the rivers located in the TBMK (Taman Buru Masigit Kareumbi) conservation area, West Java. The Citarik river is a source of water for people within the area and has an important value as part of the upstream river continuum of the Citarum River [6]. The pressure for agricultural land has triggered the community to expand it across the boundaries of the TBMK conservation area, which changes the riparian regions

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of the upper watershed Citarik area [7]. This affects vegetation structure in the area, which in the long term can deplete the ecological function within the area. In order to conserve the Citarik watershed as the upstream part of the Citarum River, data on community structure and riparian ecological status are needed for the area's long-term restoration plan.

This study aims to determine the vegetation community structure and riparian ecological status in the upstream riparian area of the Citarik watershed. Vegetation community structure was analyzed based on Important Value Index (IVI), Shannon-Wiener diversity Index, and Simpson Domination Index. The Riparian ecological status is determined by the QBR Index [8] based on the sum of scores from four aspects of assessment. includes total vegetation cover, structure of vegetation cover, quality of vegetation cover, and river channel alteration.

2. Materials and Methods

2.1 Materials

2.2 Methods

The study area is in the upstream Citarik Watershed, TBMK, Sumedang - Bandung Regency. This research was conducted for two months, from December 2018 to January 2019. The coordinates of the site research located at 06 ° 56'58,6 "LS 107° 55'26,2" BT - 06° 57'16,2 "LS 107° 54'42,6" BT. Based on Schmidt Ferguson's climate classification, the site has the type of climate C (medium climate) with an average rainfall of 1874 mm /year. Air humidity ranges from 60-90%, and the average temperature is 23ºC [6, 7]. Figure 1 illustrates the location of the study.

Figure 1. Research location (*google earth*, 2019)

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Five sites have been chosen, located along the upper stream of Citarik consisting of diverse human activities. These areas include riparian residing in the Cigumentong area with vegetable fields, bamboo forest areas, the area of 28 hectares of deer cages, the area of 8 hectares of deer cages, and the area of tourist area entrances.

2.2.1 Research Design

Vegetation analysis of riparian was done at five different sites. A series of vegetation analysis was carried out, through sampling in a multilevel plot based on life stage of vegetation. Riparian ecological status were determined by using the QBR Index [8]. An analysis of riparian vegetation was carried out through sampling in multilevel plots. The size of plot was 10x10 meters for tree and pole analysis, 5x5 meters for analysis of shrubs and stakes, and 2x2 meters for analysis of herbs and seedlings. Sampling was taken from the left and right sides of the river body and was done three times. The distance between the plots was three meters and was drawn in a straight line. The research plot scheme used in each research site is shown in Figure 2.

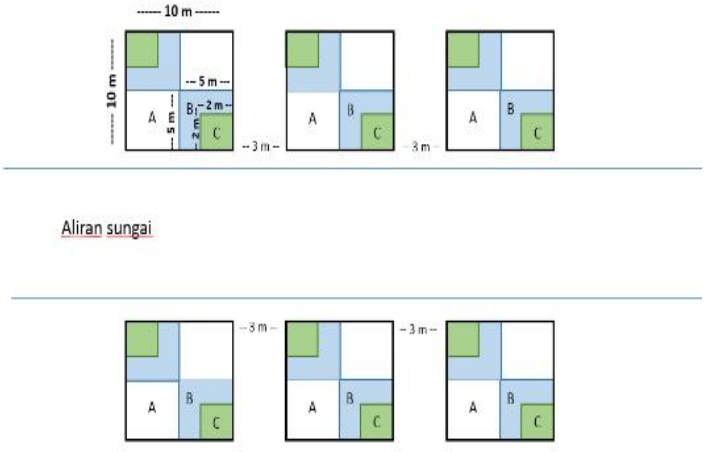

Figure 2. The diagram of nesting plot in the reasearch

2.2.2 Microclimate and Soil Condition

Water temperature and air humidity was recorded using a data logger. Edhaphic conditions, such as soil temperature, moisture, and pH was measured by a Weksler thermometer soil and pH tester. Data was taken three times. All data were analysed using SPSS *software for statistical factors*.

2.2.3 Identification and Riparian Vegetation Analysis

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Plant samples taken from the field were grouped according to their life forms (trees, shrubs, or herbs). Then, each species was identified. The community structure at the five sites was determined based on Important Value Index (IVI) and named by the two highest values of IVI.

2.2.4 QBR Index Method

The QBR index is determined based on four parameters of vegetation coverage (total vegetation cover, structure of vegetation cover and quality of vegetation cover), and state of river flow. Each parameter score is ranged between 0-100. It is further divided into five categories of riparian ecological status, symbolized by five different colors. Details of riparian ecological status use the QBR index are as follows.

Table 1. Quality classes according to the QBR index [8]

Riaprian Habitat Quality Class	QBR	Colour
Riparian habitat in natural condition	>95	Blue
Some disturbance, good quality	75-90	Green
Disturbance important, fair quality	55-70	Yellow
Strong alteration, poor quality	30-50	Orange
Extreme degradation, bad quality	<25	Red

Table 2. Total riparian cover

Score	Description
25	>80% of riparian cover
10	50-80% of riparian cover
5	10-50% of riparian cover
0	<10% of riparian cover

Table 3. Cover structure

Score	Description
25	>75% of tree cover
10	50-75% of tree cover or 20-50% tree cover but 25% covered by shrub
5	Tree cover lower than 50% but shrub cover at least between 10% and 25%
0	<10% of either tree or shrub cover

Table 4. Cover quality

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Score	Description	Type		
		1	2	3
25	Number of native tree species	>1	>2	>3
10	Number of native tree species	1	2	3
5	Number of native tree species	0	1	1-2
0	Absence of native trees	0	0	0

Table 5. Channel alteration

Score	Desciption
25	Unmodified river channel
10	Fluvial terraces modified and constraining the river channel
5	Channel modified by rigid structure along the margins
0	Channelized river

3. Result and Discussion

3.1 Comunity Structures

The result of the vegetation analysis on five sites along the upstream riparian of the Citarik watershed, uncovered several findings. The number of species in the area consisted of eight species of trees, 11 species of shrubs, and 16 herbaceous species, as shown in Figure 3.

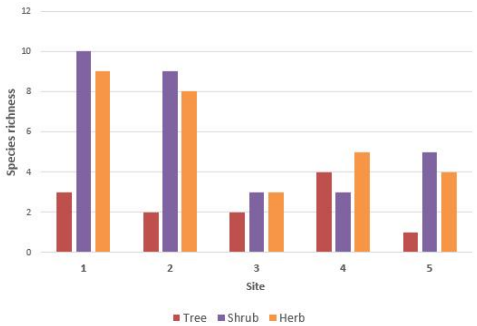

Figure 3. Species richness at 5 sites in upstream Citarik watershed

Figure 3 illustrates how most of the tree species were grown on site 4 (four species), whereas most shrubs were found on site 1 (10 species), while most herbs were found on sites 1 (nine species) of herbs. Based on vegetation analysis, the five sites were formed by different plant communities, as shown in Table 6.

Most of the shrub communities were structured by *Chromolaena* and *Clidemia*, while the herbacious communities were formed by *Ageratina*, along with other species. Site 4 had the highest number of species, while Site 5 had the least amount, with only one species

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Table 6. The highest Important Value Index (IVI) at Sites 1-5

Site	Highest Important Value Index		
	Tree	Shrub	Herb
1	*Pinus-Coffea*	*Clidemia-Chromolaena*	*Ageratina-Agerotum*
2	*Gigantochloa-Ficus*	*Clidemia-Chromolaena*	*Molineria-Sida*
3	*Pinus-Gigantochloa*	*Chromolaena-Clidemia*	*Ageratina-Physalis*
4	*Schima-Agathis*	*Chromolaena-Clidemia*	*Ageratina-Sida*
5	*Altingia excelsa*	*Calliandra-Chromolaena*	*Ageratina-Crossocephalum*

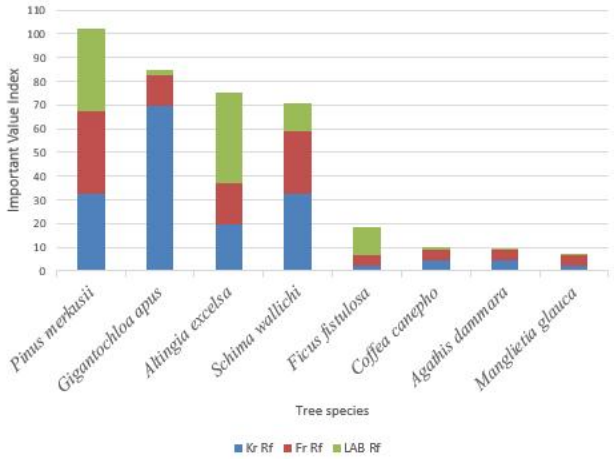

Figure 4. IVI of tree in research area

Figure 4 shows that there are eight tree species growing in the upstream Citarik sub-watershed with different IVI. *Pinus merkusii* (pine) had the highest important value index (102%), followed by *Gigantochloa apus* (bamboo) (84.62%). Pine and bamboo are species planted previously by the West Java Forest Service and Perum Perhutani. The upstream of Citarik watershed is located at an altitude of 1175- 1181 m above sea level, with an average rainfall of 1874 mm per year. This condition is suited for pines to grow. As stated by Bharali [9], pine trees will grow well in ecosystems with a height of 400-2000 meters above sea level, with rainfall of 1200 -3000 mm per year. Bamboos, which are often found grown in tropical Asia, is often found on riverbanks [10]. This species can grow well on sandy or clay soils, in lowland or highlands, which is 1500 meters above sea level. Bamboos have a strong root on the ground as it has branched rhizomes. This causes it to be widely planted in areas with high slopes and riparian areas [11].

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In shrub life forms, Site 1 and Site 2 has 10 and eight herbaceous species, respectively, with the name *Clidemia-Chromolaena* community. On Site 3 and Site 4, there were three herbacieous species with the community name *Chromolaena-Clidemia*. On Site 5 there were five species of shrubs with the community name *Calliandra-Chromolaena* (Table 6).

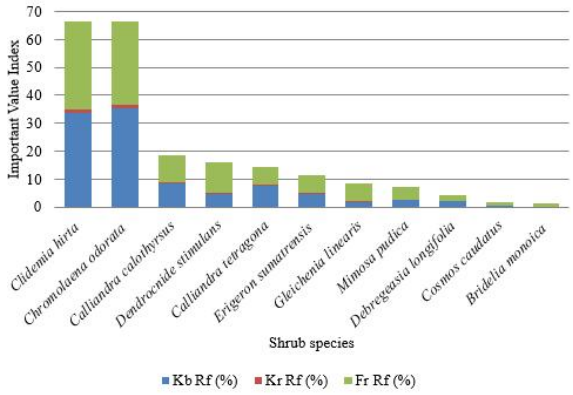

Figure 5. IVI of shrub in research area

Based on Figure 5, there were 10 species of shrubs grown around the upstream Citarik watershed. The highest IVI score was found in *Clidemia* (65.12%). The next highest IVI was *Chromolaena odorata* (64.97%). *Clidemia hirta* (harendong bulu) is an invasive weed that can grow and spread rapidly, while defeating native species. *C.hirta* also grows in tropical climates with high humidity and can be found in open forests, planting forests, roadside areas, and riparian zones [12]. *Chromolaena odorata* and *Clidemia hirta* are invasive species. *Chromolaena odorata* (kirinyuh) is an invasive species growing on curbs, grasslands and riparian zones. *C. odorata* can also inhibit the growth of other species by producing terpenoid from leaves and stems. Meanwhile, the seeds can be spread by wind and riparian fauna [13]. In herbaceous living forms, site 1 consisted of nine herbaceous species of the *Ageratina-Ageratum* community. In Site 2, there were eight herb species with the community name *Molineria-Sida*. On Site 3 there were three herbaceous species with the community name *Ageratina-Physalis.* On Site 4 there were five herb species with the community name *Ageratina-Sida*. Finally, on Site 5 there were four herbaceous species with the community name *Ageratina-Crossocephalum* (Table 6).

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Based on Figure 6, there were 16 herb species at the upstream of Citarik watershed. The species with the highest IVI was *Ageratina riparia* (39.39%), followed by *Molineria capitulata* (26.20%). *Ageratina riparia* (teklan) is an invasive species that grows in agricultural areas, natural forests, plantations, and riparian zones. The species can grow in sheltered conditions and be exposed to sunlight, while the seeds can be spread by water, wind and by human activities [14]. It is mostly found in upstream riparian Citarik watershed, as the area has undergone land conversion to as agriculture or vegetable fields.

Molineria capitulata (congkok) grows well in damp and warm areas, as well as in conditions with less shade [15]. *M. capitulata* mostly covered Site 2 area. The Shannon-Wiener diversity index at each site varies (Figure 7).

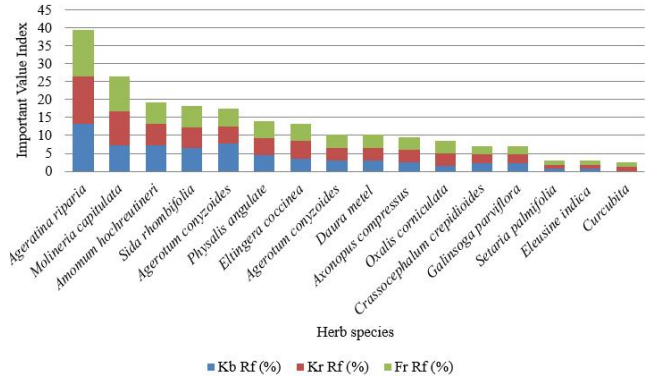

Figure 6. INP of herb in research area

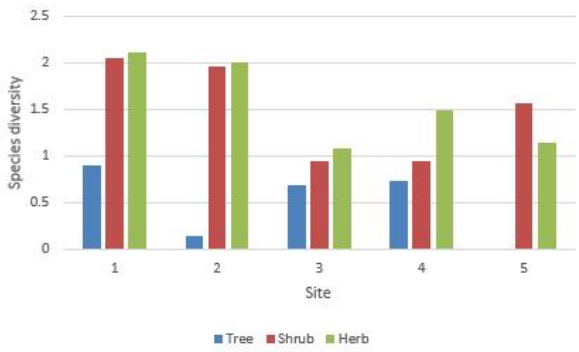

Figure 7. Diversity index in upstream Citarik watershed

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The results of the Shannon-Wiener diversity index (H ') indicate that tree shrubs and herbs species on Site 1 have the highest diversity index compared to Site 2 to Site 5. According to Molles [16], disturbances that are too high or too low in an ecosystem, reduce diversity in an ecosystem. In this study, Site 1 was a riparian that area close to fields planted with chickpeas, cauliflower, eggplants. This condition made the disturbances in the area greater than the disturbances found on Site 2 (bamboo forest area), Site 3 (deer cage area of 28 hectares) and Site 4 (deer cage area of 8 hectares). However, the disturbances were of smaller intensity compared to Site 5 (area of tourist and field entrance). This caused the diversity index of trees, shrubs, herbs on Site 1 higher than the other four sites (Figure 7).

Based on the Simpson Dominance Index, there were no species dominated in the form of shrubs or herbs in all five sites. However, two species of tree dominated the areas of Site 2 and Site 5. In Site 2 it was dominated by *Pinus merkusii* and *Gigantochloa apus*, while in Site 5 it was dominated by *Altingia excelsa.*

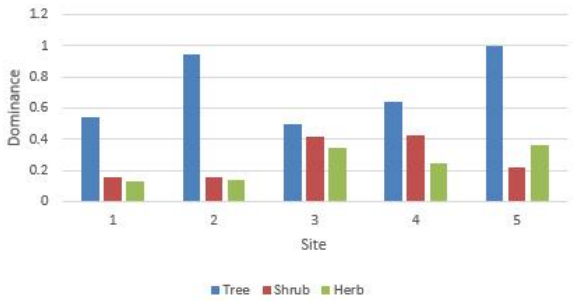

Figure 8. Dominance vegetation in upstream Citarik watershed

3.2 Microclimate and Soil Condition

Microclimate factors, such as air temperature, air humidity, light intensity, and edaphic (soil temperature, soil moisture, and soil pH) has an effect to the structure of vegetation. Table 7 and Table 8 show the results of microclimate and edaphic measurements in each study site.

Table 7. Microclimate

Parameter	Site 1	Site 2	Site 3	Site 4	Site 5
Temperature (°C)	23,09	22,86	23,57	23,38	22,28
Humidity (%)	93,49	93,93	92,97	92,60	93,28
Light intensity (Lux)	6838,33	2461,67	6185,00	7355,00	10996,67

Microclimates in all sites of this study did not show any differences. This result is supported by statistical analysis, which showed no significant differences in temperature, and air humidity (p value> 0.05) in all sites studied. Site 5, which is close to vegetable fields and the entrance of tourist areas, has the highest light intensity. The site also has low vegetation cover. Table 8 shows edaphic condition for each site.

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Table 8. Edaphic condition at five sites

Parameter	Site 1	Site 2	Site 3	Site 4	Site 5
Temperature (°C)	21,39	20,44	21,20	21,39	21,94
Moisture (%)	57,50	65,00	61,67	65,33	60,50
Soil pH	6.77	6.73	6.70	6.77	6.73

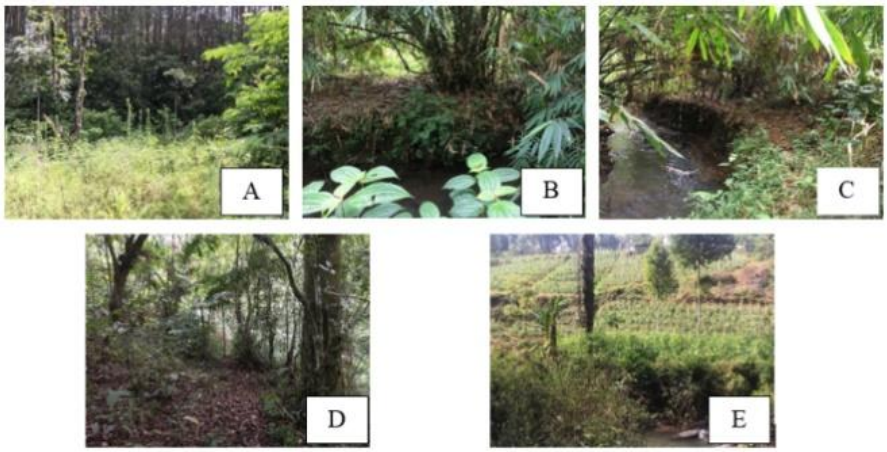

Figure 8. Environmental condition at the surrounding of research sites A) Site 1 B) Site 2 C) Site 3 D) Site 4 E) Site 5

3.3 Ecological Status of Riparian

QBR index varied between sites. On Sites 1, 2 and 4, the QBR index was 60-65, with means the status ecology of the sites were moderate and the quality was moderate too. On Sites 3 and 5, the QBR index was 50. The QBR index in each site is illustrated in Figure 9.

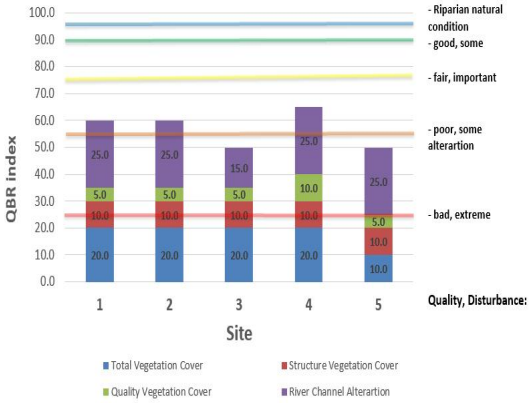

Figure 9. Ecological status of riparian in upstream Citarik watershed

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Figure 9 shows parameters of total vegetation cover consider the percentage of overall cover of both trees, shrubs, and herbs [8]. The total vegetation cover score of the five sites ranged between 10 - 20. Site 5 showed the lowest total vegetation cover of 10, compared to the other four sites with a score of 20 (Figure 9). Site 5 consisted of vegetable fields and was close to the entrance of tourist areas, therefore had the lowest total vegetation cover compared to the others.

Riparian zones that are fully covered by vegetation will improve riparian quality. The leaves that fall from the tree have several functions, such as becoming organic matter in food webs, providing habitat for riparian fauna, reducing erosion and stabilizing temperatures [17].

The tree cover on each site was dominated by different species. Species dominance in each site are as follows: Site 1 and Site 3 were dominated by *Pinus merkusii*, Site 2 was dominated by *Gigantochloa apus*, Site 4 was dominated by *Schima wallichi*, and finally Site 5 was dominated by *Altingia excelsa*. The strong and long root system within the trees are more efficient at extracting nutrients from the soil, compared to that of understory vegetation. This ensures tree cover structures can maintain river stability and prevent flooding [18].

Vegetation cover quality scores from the five sites ranges from 5 to 10. Site 3 had the highest vegetation cover quality (Figure 9). The area had native tree species i.e *Schima wallichi* and *Agathis dammara* [19] to Agroforestry Database book, riparian degradation can be caused by the reduction or replacement of native species with non-native species. At the local scale, riparian vegetation is degraded due to land clearing or human activities such as planting, grazing livestock, and the construction of settlements. In addition, the main cause of riparian degradation is the inclusion of non-native species and invasive species [8].

Flow water condition is a parameter to consider modifications to the river flow made by humans, or by the presence of building structures in riparian areas. Site 3 had the lowest score of 20 (Figure 9). Within the area was a dam which flowed to Sumedang Regency as a source of water for the community. Changing channels in the river can reduce the quality level of riparian areas. The existence of deliberate changes made by human activities can reduce shading, increase the level of erosion, reduce the stability of buffer temperature, and reduce the remnants of wood and leaves from riparian vegetation that enters the stream as organic matter [18, 20].

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3.4 Water Quality of Riparian

An analysis of water quality status in the Citarik sub-watershed was also carried out by Tim Kembara Citarik Wanadri at 14 research stations in 2018. The status of water quality of the Citarik watershed was determined using the STORET index, which was then compared with class II water quality standards (Republic of Indonesia Government Regulation No. 82/2001). Based on TSS, BOD and Total coliform, the quality of water in Citarik river were moderate with polluted in fair category. This result is similar with the results found on the riparian quality from Site 5, which also showed poor quality with large disturbances. Site 5 was an area consisting of various land conversions, in the form of vegetable fields and tourist area entrances. Reduced vegetation cover, or canopy, in riparian areas can lead to erosion and river sedimentation by chemicals and organic matter. This can further inhibit river flow, thereby reducing river quality [21].

Table 9. Water Quality of Citarik river according STORET Index

Stasiun/ Site	Water quality				Ecological status of riparian
	Class	Quality category	Polluted category	The value does not meet standard parameter	
1	C	Moderate	Fair polluted	BOD, Total coliform	Disturbance important, fair quality
2	C	Moderate	Fair polluted	TSS, BOD, Total Coliform	Disturbance important, fair quality
3	C	Moderate	Fair polluted	BOD, Total Coliform	Strong alteration, poor quality
4	C	Moderate	Fair polluted	pH, Total Coliform	Disturbance important, fair quality

4. Conclusion

Riparian areas in Upstream Citarik, Taman Buru Masigit Kareumbi (TBMK) Watershed, consisted of trees (eight species), shrub (11 species) and herbaceus (16 species). Site 1 had the highest diversity of tree species. The riparian ecological status in the upstream Citarik watershed at Taman Buru Masigit Kareumbi ranged between moderate to poor quality, with moderate to severe disturbances. This condition indicates that the area need to be rehabilitated to keep functioning and to make Citarum river in better condition.

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Acknowledgement

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Inventory of Invasive Plant at Ganesha Campus of ITB

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Abstract

Ganesha Campus of ITB with an area of about 286,830 m^2 has diverse vegetation. The aim of the study was to evaluate the possible presence of invasive species on the Ganesha Campus of ITB. The research was conducted as a first step in controlling the population of invasive species at the Ganesha Campus of ITB. Data retrieval was carried out by roaming as far as 3.6 km along the road corridor on the ITB campus. The 3.6 km long observation line was divided into 36 plots with a length of 100 m/plot. Observations showed that on the Ganesha Campus of ITB there were 165 species belonging to 60 families. Of the 165 species, 76% species are alien species. This number is much higher when compared to local species which is only 24%. The species with the highest frequency found on the Ganesha Campus of ITB was *Kylinga nemoralis* with a frequency value of 0.86, but it was not classified as an invasive plant. There are 18 species of alien plants that are considered invasive. From the results obtained, it can be concluded that the Ganesha Campus of ITB has higher foreign plants than local plants but not all of these foreign plants are invasive plants.

Keywords: inventory, introduction, invasive, alien plant, frequency.

1. Introduction

Invasive plants can successfully occupy and disperse in new habitats and have the potential to cause economic, environmental and health disturbances. The presence of invasive alien species is closely related to introduction activities. Factors that cause this introduction include agriculture, animal husbandry, transportation, and biological control [1]. In general, invasive alien plant species have several criteria, namely fast growth, fast reproduction, high spreading ability, wide tolerance to environmental conditions, and association with humans. The presence of invasive alien species can cause economic and ecological losses. For example, the species *Euphorbia esula*, native to Europe, has invaded Canada, causing an estimated $40.2 million in losses in 2010, or nearly double the last estimate made in 1999. The losses include $10.2 million in direct losses. Based on the value of lost land for grazing, $5.8 million for the cost of roadside application of the herbicide *Euphorbia esula*, and an indirect cost of $24.1 million [2]. Ecologically, invasive alien species can compete with local species and can suppress growth and change community structures in the environment. In Indonesia, the presence of invasive alien species has been reported to disturb several national parks which are conservation areas in Indonesia. An example is the presence of *Acacia nilotica* in Baluran National Park, *Lantana camara* in Meru Betiri National Park and *Chromolaena odorata* in Ujung Kulon National Park. In Baluran National Park, *Acacia nilotica* has been the main cause of savanna decline of around 50% in 2004, thus disturbing the balance of the entire ecosystem [3]. Ganesha Campus of ITB with an area of about 286,830 m^2 has quite a variety of plant species. This is supported by the many gardens created in each Faculty/School as well as

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wild plants that grow by themselves. The purpose of the study was to evaluate the possible presence of invasive alien plants on the Ganesha Campus of ITB. This research needs to be done as a first step towards controlling invasive alien plant species.

2. Method

2.1 Research Time and Place

This research was conducted from January to February 2017 at the Ganesha Campus of ITB.

2.2 Data Retrieval

Figure 1. Plot pick-up path

Data retrieval is done by roaming method [4]. This method is carried out by walking along a 3.6 km path along the corridor of the Ganesha Campus of ITB road with the division of plots each of 100 m so that from the 3.6 km route, 36 plots are obtained. The picture of the research path can be seen in Figure 1. Inventory is carried out by recording the plants found. Recording is also done in the form of location, photo, scientific name, local name, and habitus. The frequency of each plant species found is calculated based on the formula [5]

$$\boldsymbol{Frequency\ (F)} = \frac{\boldsymbol{number\ of\ plots\ found\ species}}{\boldsymbol{total\ number\ of\ plots}}.$$

The identification of invasive species is then carried out by cross-checking the Global Invasive Species Database site belonging to the Invasive Species Specialist Group (ISSG).

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3. Result and Discussion

3.1 Plant Composition

There are 165 species of plants found in the Ganesha Campus of ITB. The plots with the highest number of species are plot 17 which is located in the eastern part of East GKU and plot 13 which is around the SITH ITB botanical garden which is located in the western part of East GKU. While the plot with the lowest number of species is plot 35 which is around the West Campus Center building.

3.2 Plant Species Frequency

The results of the observations can be seen in Figure 2. Based on observations, the species with the highest frequency was *Kylinga nemoralis*. Of the 10 species, ISSG categorizes 2 of them into invasive species, namely *Oxalis corniculata* and *Ageratum conyzoides*.

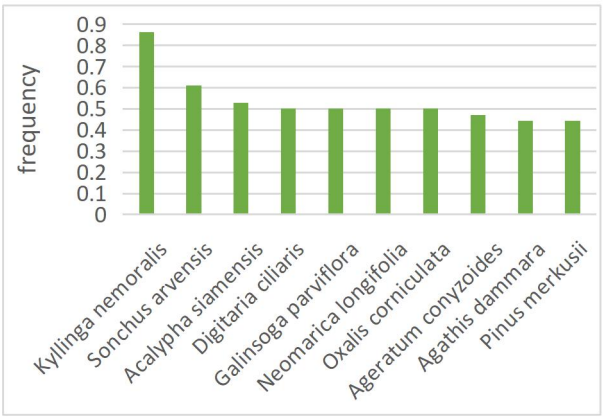

Figure 2. The ten species with the highest frequency found on the ITB Ganesha campus

3.3 Percentage of Local and Alien Plants

Local plants on the Ganesha Campus of ITB have a percentage of 24% (40 species) while alien species reach 76% (125 species). This number is partly due to the introduction of alien plants in the form of ornamental plants and road protection plants. Based on observations, of the 125 foreign species found 64% (80 species) of which are ornamental plants and road protection plants.

3.4 Invasive Plants

Based on cross-checks conducted on the ISSG site, 18 invasive species were found on the Ganesha Campus of ITB (Table 1). Of the 18 invasive species, 4 of them were ornamental plants, namely *Sanseviera trifasciata, Furcraea foetida, Lantana camara and Agave americana*. Based on the frequency, the ornamental plant *Sanseviera trifasciata* has a fairly high frequency among other plants, which is 0.417. However, this plant is not expected to pose a threat. This

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is because the distribution space is limited by both other plants and humans. *S. trifasciata* which is located in the Ganesha Campus of ITB is generally planted close to various other ornamental plants so that it is estimated that there will be quite a high competition in obtaining nutrition. In addition, pruning is also done by gardeners if the *S. trifasciata* is considered too dense.

Table 1. List of invasive plants according to ISSG found in Ganesha Campus of ITB

No	Latin Name	Frequency	Alien/Local	Family
1	*Oxalis corniculata*	0.500	Alien	Oxalidaceae
2	*Ageratum conyzoides*	0.472	Alien	Asteraceae
3	*Sansevieria trifasciata*	0.417	Alien	Liliaceae
4	*Furcraea foetida*	0.250	Alien	Agavaceae
5	*Impatiens walleriana Hook. f.*	0.167	Alien	Balsaminaceae
6	*Mikania micrantha*	0.167	Alien	Asteraceae
7	*Psidium guajava*	0.167	Alien	Myrtaceae
8	*Elephantopus mollis*	0.083	Alien	Asteraceae
9	*Lantana camara L*	0.083	Alien	Vernanaceae
10	*Cyperus rotundus*	0.056	Alien	Cyperaceae
11	*Elaeis guineensis*	0.056	Alien	Arecaceae
12	*Imperata cylindrica*	0.056	Local	Poaceae
13	*Leucaena leucocephala*	0.056	Alien	Fabaceae
14	*Agave americana*	0.028	Alien	Asparagaceae
15	*Bambusa vulgaris*	0.028	Alien	Poaceae
16	*Mimosa pudica*	0.028	Alien	Fabaceae
17	*Paspalum scrobiculatum L.*	0.028	Local	Poaceae
18	*Passiflora edulis Sims*	0.028	Alien	Passifloraceae

3.4.1 Controlled Invasive Plants

In the Ganesha Campus of ITB area, several plant species listed as invasive species have a low frequency or in other words, these species are rarely found in this area. The low frequency can be caused by routine maintenance and pruning by gardener so that indirectly the spread of some invasive species is controlled. Some of these controlled invasive species are as follows.

a. *Sanseviera trifasciata.* At the Ganesha Campus of ITB this plant is a plant that is planted and controlled mechanically by trimming the leaves or dismantling the clumps so that although it is often found in several parks within the Ganesha Campus of ITB, this species has a controlled distribution.

b. *Furcraea foetida.* This species is a monocarpic plant or flowering only once. However, it takes 7-10 years to form flowers [6]. Due to its long life cycle *Furcraea foetida* is not expected to pose a threat.

c. *Psidium guajava* (guava). The guava plant is a plant that is easy to breed, however, the frequency of this plant in the Ganesha Campus of ITB is relatively low. This is because the fallen seeds will be cleaned up immediately by the gardeners.

d. *Elaesis guineensis* (palm tree). Palm tree at Ganesha Campus of ITB is not expected to spread and disturb ecosystem. Palm tree seeds will be difficult to germinate because they require a temperature of 35^{o}C [7]. The average temperature in the city of Bandung is in the range of 23.4^{o}C. In addition, fruit formation in palm tree itself is influenced by several factors, including the success of pollination and the availability of nutrients. For pollination, palm tree has a different maturation time between male flowers and female flowers so that self-

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pollination is very rare [7]. Therefore, it is unlikely that the oil palm planted at the Ganesha Campus of ITB will bear fruit because it is planted at a considerable distance.

e. *Agave americana. Agave americana* is a plant that can reproduce both sexually and asexually. However, the sexual development of this monocarpic plant takes a long time, which is about 10-30 years [8]. Vegetatively, Agave reproduces by rhizome. However, due to environmental conditions under the shade of large trees and college buildings, its growth is not optimal. *Agave americana* requires at least 6 hours of direct sunlight to grow optimally [9].

f. *Bambusa vulgaris* (yellow bamboo). *Bambusa vulgaris* more often reproduces by budding, but because of the narrow growing space and limited by paving blocks this plant does not have the potential to become an invasive plant.

g. *Lantana camara.* This plant includes flowering ornamental plants that flower throughout the year [9]. The spread of *Lantana camara* is very easy because the seeds are favored by birds. This species can also produce adventitious shoots on the roots so that it can form a fairly dense clump. In addition, this plant is also able to grow on less fertile soil [10].

h. *Leucaena leucocephala.* At the Ganesha Campus of ITB, *Leucaena leucocephala* grows in areas that have been occupied by other plants such as grass and other ornamental plants. It is possible that this is why *Leucaena leucocephala* is not widely spread on the Ganesha campus. The growth of lamtoro will be disturbed if in its new habitat there are other plants that grow tightly.

i. *Paspalum scrobiculatum*. This plant belongs to the Poaceae family. At the Ganesha ITB Campus, *Paspalum scrobiculatum* has a relatively low frequency so that although it is included in the list of invasive plants according to ISSG, this plant is not classified as a species with a high level of invasiveness.

j. *Passiflora edulis*. *Passiflora edulis* thrives in both the tropics and subtropics. This plant is quite resistant to drought stress. When the environment is dry, passion fruit plants will drop their leaves to reduce evaporation. Seed dispersal for this species is generally carried out by humans or other primates who eat the fruit. The absence of a suitable dispersal agent causes the spread of this species is not in the category of disrupting.

3.4.1 Invasive plants that have not been optimally controlled

a. *Oxalis corniculata.* This plant is able to produce seeds in abundance and easy to grow in an open place and get sunlight. The spread of which is carried out by humans, either intentionally or not. In addition, birds also play a role in the dispersal of seeds of this species. *Oxalis corniculata* also has seeds that are viable for up to 1 year, and are pioneers in disturbed land. To control this species can be done by pulling directly by hand or burying this plant with soil so that the plant dies and the seeds cannot germinate because they do not get light.

b. *Ageratum conyzoides*. *Ageratum conyzoides* belongs to the Asteraceae family, has an achene-shaped fruit and is very easy to spread with the wind. This plant is capable of producing 40,000 seeds per individual [11] so it is easy to find. Handling for this species can be done by pulling it mechanically or by burying it until it is completely buried. *Ageratum conyzoides* seeds need direct sunlight to germinate, therefore if they are completely buried by the soil it will inhibit the germination process.

c. *Impatiens walleriana*. This species reproduces mainly by seeds. When they are ripe, the seeds wrapped in capsules will explode when touched or dropped. The seeds then grow very close to each other. To control it can be done by trimming the plant or pulling it down to the roots.

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d. *Mikania micrantha*. This species is a vine that can grow quickly and cover other plants until their growth becomes stunted. This plant is also able to produce seeds in large quantities and is able to regenerate from the stem segment. Dispersal is carried out by wind. An effective control for this species is to cut the part closest to the ground periodically because it can reduce the *M. micrantha* population by up to 90% [12].
e. *Elephantropus mollis.* This species is a member of the Asteraceae family whose leaf cover is able to inhibit the germination and growth of ornamental grasses. This species is able to grow on less fertile soil. To control it can be done by hoeing it, especially before flowering.
f. *Cyperus rotundus. Cyperus rotundus* have tubers with a high degree of viability so controlling this grass requires extra effort. The formation of new tubers can occur within 17 days after the emergence of roots. Bulbs are formed in response to the abundance of carbohydrates, photoperiod, and temperature. One individual *Cyperus rotundus* can produce 100 tubers in 12 weeks [13]. Although it has many tubers and is easy to grow, *Cyperus rotundus* does not grow well in the shade. Control of this species by cutting regularly 1 to 3 times a week can reduce the population and inhibit the formation of new shoots.
g. *Imperata cylindrica. Imperata cylindrica* is one of the most annoying weeds. This plant is able to inhibit the growth of other plants in the vicinity because in addition to effectively competing with other plants for water and nutrients, this plant also has allelopathic compounds that can inhibit the growth of other plants. In addition, *Imperata cylindrica* is also capable of producing seeds and roots that are resistant to various environmental conditions. Therefore the reeds if left alone can dominate a large area. To control this species, it can be done by hoeing until the stems are broken and piling up the stems and leaves while the rhizomes are at the top of the soil surface. Rhizomes exposed to direct sunlight will then die.
h. *Mimosa pudica. Mimosa pudica* can flower throughout the year and is able to produce up to 675 seeds/plant/year. Pollination of *Mimosa pudica* seeds is usually done by wind. At the Ganesha Campus of ITB, although this plant currently has a low frequency, it must be watched out for. This is because this plant is able to produce about 700 seeds per individual per year. In addition, the seeds of this plant are generally spread by attaching to human clothing or mammalian hair [14].

3.5 Control with Herbicide

In addition to controlling invasive species mechanically, it can also be done chemically with herbicides. Herbicides that are commonly used to control broadleaf plants are herbicides with an active ingredient of 2,4 D. 2,4 D is an artificial plant auxin hormone applied to dicotyledonous plants that will cause the plant to produce the hormone ethylene [16]. In contrast to natural auxins which can be easily degraded by plants, 2,4 D lasts a long time in plants so that plants produce excessive production. Excessive ethylene production in plants will cause effects such as wilting and death. This active ingredient only affects broadleaf weeds, presumably because the distribution structure of auxin in broadleaf (dicot) and narrowleaf (monocot) plants is different. However, the details of the mechanism have not been fully achieved [15]. In addition to herbicides containing the active ingredient 2.4D, herbicides containing the active ingredient glyphosate can also be used. Glyphosate is a herbicide capable of killing both broadleaf and narrowleaf plants. Glyphosate kills target plants by inhibiting the synthesis of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS). Plants exposed to glyphosate will generally experience chlorosis and shortening of the internodes [16].

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4. Conclusion

Based on the research results, local plants have a percentage of 24%, much smaller than alien plants which reach 76%. There were 18 species of plants classified as invasive species, namely *Oxalis corniculata* (Oxalidaceae), *Ageratum conyzoides* (Asteraceae), *Sanseviera trifasciata* (Liliaceae), *Furcraea foetida* (Agavaceae), *Impatiens walleriana Hook. f.* (Balsaminaceae), *Mikania micrantha* (Asteraceae), *Psidium guajava* (Myrtaceae), *Elephantopus mollis* (Asteraceae), *Lantana camara L.* (Vernanaceae), *Cyperus rotndus* (Poaceae), *Elaesis guineensis* (Arecaceae), *Leucaena leucocephala* (Fabaceae), *Agave americana* (Asparagaceae), *Bambusa vulgaris* (Poaceae), *Mimosa pudica* (Fabaceae), *Passiflora edulis* (Passifloraceae), *Imperata cylindrica* (Poaceae), and *Paspalum scrobiculatum* (Poaceae). Invasive alien plants that have the highest frequency in the Ganesha Campus of ITB is *Oxalis corniculata*, followed by *Ageratum conyzoides*.

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Micropropagation of Antelope Orchid (*Dendrobium antennatum* Lindl.)

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Abstract

Dendrobium antennatum Lindl. is a fragrant and long-lasting flower orchid. As a commercial plant, a micropropagation method is needed to produce many plantlets. This study aims to observe the effects of 6-benzylaminopurine (BAP) concentration and coconut water on *D. antennatum* shoot multiplication and plantlet formation. This study was divided into two steps: initiation in liquid medium and formation of shoot and root in semi-solid medium. Shoot bud with 0.5 – 1 cm length were used as explant. First, explant was cultured in liquid VW medium supplemented with 20 g/L sucrose, 5 mg/L BAP, and 15% (v/v) coconut water. Then explant was transferred to semi solid VW medium supplemented with 20 g/L sucrose, 0.6% gelrite, BAP (0.5, or 10 mg/L), 15% (v/v) coconut water, and 250 mg/L active carbon. Explants were then subcultured every 30 days. This study showed that explant only swelled in liquid medium. Explant in semi-solid medium explants formed new shoots which further developed into plantle. New shoots in explant derived from axillary bud or protocorm-like bodies (Plb). Formation of axillary shoot was first observed on 30th day in VW0, VW0CW, and VW5CW. Formation of Plb was first observed on 15th day in all medium. Protocorm-like bodies differentiation into shoot was first observed on 30th day of culture in almost all medium, except VW0. Following subcultured, explant in VW5CW medium produced most shoots with average of 4.474 ± 4.018 shoots per explant. Plantlet formation was first observed on the 30th day after subcutured in VW0CW and VW5CW medium. In this experiment, we found VW0CW medium produce more plantlet per explants. Based on that result we conclude that VW5CW is the best medium for shoot multiplication and VW0CW is the best medium for plantlet regeneration.

Key words: 6-benzylaminopurine, micropropagation, coconut water, *Dendrobium antennatum*, shoot, protocorm-like bodies

1. Introduction

Dendrobium antennatum Lindl. is one of orchid that has unique shape which resemble antelope horns, hence the name antelope orchid. This orchid also has a fragrant and long-lasting flower that can be used as cut flower [1].

There are many things that needs to be considered when cultivating *Dendrobium antennatum* Lindl. One of them is the propagation method. Vegetative propagation is commonly used in commercial scale cultivation because the time needed is relatively shorter than using

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generative method (seeds) [2]. Vegetative propagation can be done traditionally or with micropropagation both methods produce offspring with uniform quality [3].

Micropropagation is more commonly used than traditional method in commercial scale because it can be scaled up. [3]. Micropropagation is an asexual propagation method that is used aseptic technique to produce plantlet from organ, tissue, or cell [4].

There are many factors that affects plant growth and development in micropropagation like basal medium, plant growth regulator, organic additive, temperature, and light intensity [5]. Plant growth regulator and organic additive are two factors that is commonly used to get the optimum result for orchid micropropagation.

An artificial cytokinin, 6-Benzylaminopurine (BAP) is plant growth regulator that commonly used in orchid micropropagation. [6]. Sharma and Tandon [7] showed that the of BAP could support protocorm-like bodies (Plb) developmentin Dendrobium wardianum further developed into shoot. Coconut water is commonly used as organic additive because it contains many beneficial substances for plant growth like vitamin, mineral, plant hormone, and sugar [8].

Micropropagation is an important method for orchid cultivation. In orchids micropropagation, different kind of orchid has different response to the effect of basal medium, plant growth regulator, and organic additive [5]. BAP and coconut water are substances that is often used for *Dendrobium* orchid micropropagation. Therefore, the combination of these substances could probably increase plantlet formation for *Dendrobium antennatum* Lindl. micropropagation.

This study aimeds to observe the effect of 6-benzylaminopurine (BAP) concentrations and coconut water addition on *D. antennatum* shoot multiplication and plantlet formation

2. Materials and Methods

2.1 Explant source

Dendrobium antennatum Lindl. explants that were used in this study were the collection of Laboratorium Mikroteknik, School of Life Science and Technology, Bandung Institute of Technology. Explants sized 0.5 - 1 cm with one to three tiny leaves that came from seeds were used in this study.

2.2 Meristem Activation in Initation Medium

Explants about 15 – 20 pieces were put inside 100 mL erlenmeyer flask containing 40 mL sterile liquid Vacin & Went (VW) medium supplemented with 20 g/L sucrose, 5 mg/L BAP, and 15% (v/v) coconut water. The explant in erlenmeyer flask were incubated in rotary shaker agitated at 75 rpm with temperature of 23-25°C and 16/8 hours photoperiod for ±9 weeks with three times of subculture.

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2.3 Shoot Multiplication and Plantlet Regeneration in Regeneration Medium

Explant was then transferred to six different types semi-solid medium which were composed of VW medium (VW0), VW medium supplemented with 5 mg/L BAP (VW5), 10 mg/L BAP (VW10), VW basal medium without/with coconut water (VW0CW, 5%VW5CW), and 10 % (VW10CW). All media contained 20 g/L sucrose and 250 mg/L activated charcoal, also gelrite 0.6% as medium solidifier. Each bottle of medium contains around 4-6 explants. Explants were cultured in semi-solid medium for two months. Sub culture was done every month so that explant didn't experience nutrition deficiency.

2.4 Observation

Observation were done when explants were transferred to semi-solid medium and every week when the plant had been transferred to semi-solid medium. Qualitative observation was conducted when explants were transferred. Qualitative and quantitative observations were conducted every week after explants were transferred. Data that were taken are the number of protocorm-like bodies (Plb), shoot, root, and plantlet.

3. Results and Discussions

3.1 Explants Growth Response in Initiation Medium

Explants that were activated in initiation medium after ±9 weeks were observed to have an increase in volume (swelling) as shown in figure 1. Swelling in pseudobulb and leaf was probably happened because of cell proliferation that was induced by coconut water and BAP that existed in liquid medium. Coconut water is known to contain cytokinin that can increase cell proliferation [9]. Absorption of nutrition was increased because agitation in liquid medium. Agitation also increase the aeration of liquid medium. Both of them affect the swelling in explant [10]. Aeration was used to circulate oxygen that was needed by organism when in liquid medium [11]. Swelling on the pseudobulb showed a function as storage organ. The stored nutrition would probably be used for flowering and shoot formation [12].

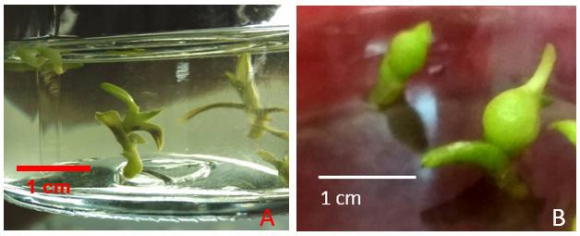

Figure 1. Explant in liquid medium (A). Explant from liquid medium that were then transferred to semi-solid medium (B)

Micropropagation studies of *Dendrobium* conducted by Puchooa (2004) and Nge *et al*. (2006) showed that VW liquid medium produce more Plb than its semi-solid counterparts. These studied showed that agitated liquid medium increased the explant proliferation in micropropagation [12][15]. Agitated liquid medium probably affect growth polarity,

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inhibiting root and shoot formation that meant this medium wasn't suitable for explant differentiation [5]. There were no formation of new roots or shoots observed in liquid medium. Because of this regeneration process needed to be done in semi-solid medium in order to work.

3.2 Explant Growth Response in Regeneration Medium

3.2.1 Explant Morphogenesis

Explant that had been transferred from liquid medium to semi-solid medium produced Plb. Figure 2 showed that Plb is formed on the tip of the leave and the bottom of explant.

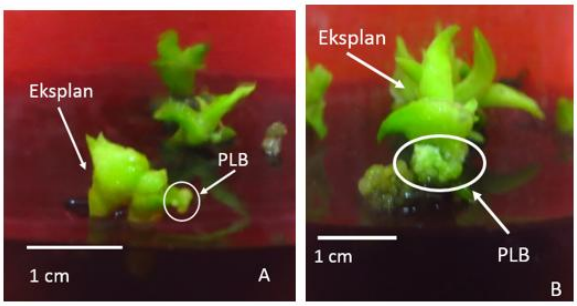

Figure 2. Plb that were formed on the tip of young leaf (A) and the bottom of the explant (B).

Protocorm-like bodies (Plb) is a rounded shape structure that is similar with protocorm, a structure that formed when orchid seed germinates. [5]. Plb is orchid somatic embryo that has the same growth characteristic with protocorm [14]. Protocorm-like body was known could be form from cells in the apical [15].

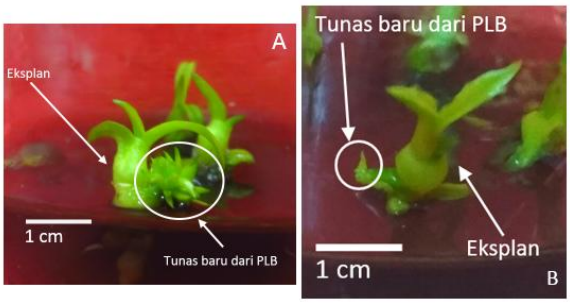

Figure 3. Shoots that developed from Plb on the tip of the leaf (A) and pseudobulb (B)

Protocorm-like bodies could regenerate into new shoot. The part that would develop into new shoot is called shoot apical meristem (SAM). Shoot apical meristem (SAM) aimed to be new shoot. The process of becoming new shoot is marked by bulge that later would develop to leaf primordium [15]. Figure 3 showed shoot that developed from Plb on the tip of the leaf and the pseudobulb.

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Other than through PLB, shoot can also directly formed from stem nodes (axillary bud) (figure 4A). Study that was conducted by Dohling *et al*. (2012) showed that development of axillary bud could be induce in vitro with addition of plant growth regulator like Naphthaleneacetic acid (NAA) or BAP [16].

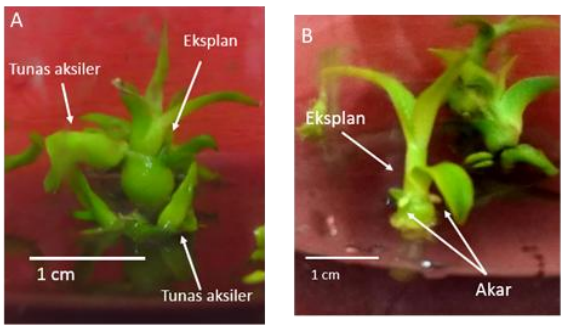

Figure 4. Axillary buds that were formed in explant (A) and roots that were formed in explant (B)

Some explants were also observed to develop adventitious roots (Figure 4B). Roots were formed only from the original explant, axillary shoot has not developed root up to 60th day of culture. Endogenous auxin in the explant could probably stimulate root formation. This result was different from the study of Dilshad *et al.* (2008), root development from original explant in *Dendrobium* could be observe on 30th day using Murashige-Skoog basal medium with addition of Indole-3-acetic acid (IAA) and BAP. This different performance was probably due to the different in media and growth regulator used. The addition of exofenous auxin could stimulate root formation, so root formation could occur faster. Meanwhile, our media lack of exogenous auxin, therefore root formation rely only on the endogenous levels of auxin in the explants.

3.2.2 Effects of Different Medium to The Formation of PLB, Shoot, and Root

The result showed that variation in medium used resulted different growth rate of explant. Growth here were defined as Plb, shoot, and root formation. Shoot and root formation is a sign that explant has regenerated into plantlet. Plantlet is explant that is completely regenerated its shoot and root. Table 1 showed the effect of different regeneration medium (semi-solid medium) on the PIb and roots formation.

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Table 1. *Dendrobium antennatum* Lindl. growth in regeneration medium

PLB						Akar					
Medium/Hari	0	15	30	45	60	Medium/Hari	0	15	30	45	60
VW0	-	+	+	+	+	VW0	-	-	-	+	+
VW5	-	+	+	+	+	VW5	-	-	-	+	+
VW10	-	+	+	+	+	VW10	-	-	-	-	-
VW0CW	-	+	+	+	+	VW0CW	-	-	+	+	+
VW5CW	-	+	+	+	+	VW5CW	-	-	+	+	+
VW10CW	-	+	+	+	+	VW10CW	-	-	-	+	+
Tunas Baru dari PLB						Tunas Aksiler					
VW0	-	-	-	-	-	VW0	-	-	+	+	+
VW5	-	-	-	+	+	VW5	-	-	-	-	-
VW10	-	-	+	+	+	VW10	-	-	-	-	-
VW0CW	-	-	+	+	+	VW0CW	-	-	+	+	+
VW5CW	-	-	+	+	+	VW5CW	-	-	+	+	+
VW10CW	-	-	+	+	+	VW10CW	-	-	-	+	+
+ : Ada - : Tidak ada											

Table 1 showed that PIb had developed in all media used. It seemed that medium composition could affect PIb formation, as indicated by PIb development in all media used, with or without supplementation of plant growth regulator/ coconut water. In addition, PIb induction was also probably cultured in initiation medium (VW liquid medium). Liquid medium contained BAP and coconut water that could induce cell division and morphogenesis that affected Plb formation positively [10]. Medium in liquid state also commonly used to induce Plb because it could increase explant proliferation [7]. Plant growth regulator used could stimulate higher number of PIb.

Root formation was observed in almost all medium except in VW10. Absence of root growth was probably du to high concentration of cytokinin, BAP (10 ppm) in the VW10 medium. This result is similar with the result found in the study conducted by Kukulczanka and Wojciechowska (1983), which indicated that high concentration of cytokinin could inhibit root formation in *D. phalaenopsis* [18]. Study conducted by Yang *et al*. using mutat *Dendrobium* explant which lack of cytokinin oxidase, showed that mutant had an increase of endogen cytokinin concentration compared to normal explant. This increase was also marked by the increase of shoot formation and decrease in root formation [20]. This study shows that high concentration of cytokinin could inhibit root formation in *Dendrobium*.

Even though VW10CW had a high concentration of cytokinin, explant still could regenerate root. But, compared to the others with lower BAP concentration, regeneration was slower. This could probably due to chemical composition of coconut water. It has been known that coconut water contains many beneficial substances like amino acids, vitamin, sugar, mineral, and plant hormone like auxin and cytokinin [9]. Coconut water also known could trigger root formation in *D. lasianthera* better than other organic additive like pepton or banana [20].

Direct shoot formation through axillary bud formation was occurred in every medium except in VW5 and VW10 medium. Shoot formation in these media started with formation of Plb first. Shoot formation through Plb was not developed on the explant in VW0 medium. VW0 medium only consisted basal medium with no addition of plant growth regulator or organic additive. This absence of substances made this medium couldn't induce the formation of shoot in explant directly or through Plb. BAP in one of important growth substance for shoot formation. Study conducted by Goswami *et al*. showed that BAP could increase the regeneration of Plb into plantlet in *Dendrobium* sp. [21].

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Coconut water also known to increase vegetative growth in explant. The absence of coconut water probably led to slower shoot formation in VW5 and VW10 explants compared to other medium that contained coconut water [22]. Coconut water also known to contain many plant hormones, one of them is auxin [10]. Combination of auxin and cytokinin was suitable to induce shoot formation in orchid [23]. The average amount of Plb, shoot, and plantlet per explant at the end of observation was shown in Figure 5, 6, and 7.

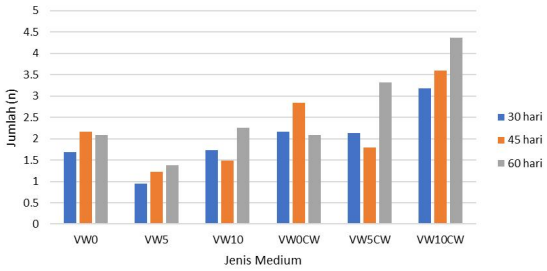

Figure 5. Average number of Plb per explant

According to figure 5, on the 60th day, explants cultured in VW10CW formed more PIb than in the other media. Coconut water was known to contain many kinds of plant hormones, one of them was cytokinin that could induce Plb formation [10]. Sharma and Tandon [7] showed that the use of BAP could increase Plb formation that later developed into shoot in *D. wardianum* [9].

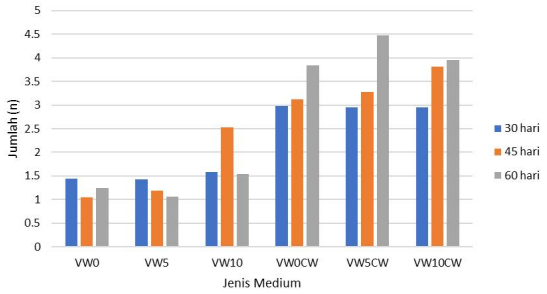

Figure 6. Average number of shoot per explant

Figure. 6, showed that Vacin-Went medium supplemented with 5% coconut water could induced higher number of shoot formation, than the other used media. It is assumed that cytokinin concentration in coconut water applied through the growing media used could induced cell division, especially for shoot formation. Higher concentration of BAP could not produce many small shoots, however these shoots were usually failed to developed [24].

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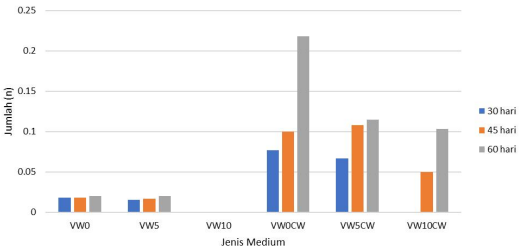

Figure 7. Average number of plantlet per explant

Figure 7 showed that more plantlets were formed in VW basal medium on the 60th day. More plantlets developed in the medium containing coconut water compare to the medium containing BAP. Absence of BAP in medium and addition of coconut water, made a better result for the root formation in explant.

Kukulczanka dan Wojciechowska study in 1983 indicated that high concentration of cytokinin could inhibit root development in *Dendrobium phalaenopsis* [19]. Coconut water also known to induce better root formation in *Dendrobium lasianthera* compared to others organic additive like peptone or banana [20].

4. Conclusion

VW medium supplemented with 5% coconut water is the best medium for shooting multiplication, meanwhile VW basal medium without coconut water supplementation is the best medium for plantlet regeneration in *Dendrobium antennatum* Lindl.

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Micropropagation of Banana cv. Mas and cv. Kepok with Different Concentrations of Benzyl Amino Purine

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Abstract

Banana is one of the main commodities in Indonesia. There are various kinds of banana cultivars that are cultivated in Indonesia, two of which are banana cultivars, cv. mas and cv. kepok. The demand for bananas must be balanced with their production, included its seedlings. One method that can be used to cultivate bananas is through *in vitro* propagation (tissue culture). The objective of this experiment was to evaluate the addition of synthetic BAP hormones in various concentrations to induce shoots multiplication of banana explants. Explants used were derived from primordial shoots in the 'sucker'. Explants were grown in instant Murashige and Skoog (MS) medium containing vitamins with the addition of BAP. The BAP concentration used in this study was 2 ppm and 4 ppm. The results showed that multiplication of shoots in banana cultivar Mas was better in MS medium with 4 ppm BAP concentrations, whereas that in banana cultivar Kepok was better in 2 ppm of BAP. The BAP concentration in the medium also influence the morphological conditions of the culture. It can be concluded that mas and kepok cultivars need different concentration of growth regulator requirements.

Keywords: Banana cv. Mas, Banana cv. Kepok, Micropropagation, Benzyl Amino Purine, Concentration

1. Introduction

Banana plant belongs to the class of Liliopsida, Zingiberales and family Musaceae [1]. In general, plants included in this group come from tropical and sub-tropical regions such as Southeast Asia, Africa and Papua New Guinea [2].

Banana cultivars naturally develop from the species of *Musa acuminata* (genome A) and *Musa balbisiana* (genome B). Diploid cultivars (AA and AB groups), triploids (AAA, AAB, ABB groups) and tetraploid (AAAB groups) derived from hybrids among *M.acuminata* subspecies (9 subspecies). This cross between cultivars produces various types of new cultivars that are consumed today. However, the results of these crosses cause difficulties in propagating bananas generatively.

Banana cultivar Mas is a cultivar of the *M.acuminata* species and has a diploid genome, namely AA [3]. This banana is widely cultivated and consumed in Indonesia, it is also used for traditional ceremonies. Banana cultivar kepok belongs to the *M paradisiaca* species with the triploid genome, ABB [3]. For this triploid banana, effective and efficient banana propagation methods are needed to meet the demand of good seedlings.

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Tissue culture is a method of growing tissue, organs or all parts of a plant using *in vitro* method. This method utilizes the totipotent properties of plants. Totipotent is the ability of meristematic cells to differentiate into other cells type and become one whole organism. Factors affecting tissue culture are nutrition, sterile condition and growth regulators such as plant hormones [4]. There are several groups of phytohormones, one of which is cytokinin. Cytokinin are chemical compounds that induce an increase in the rate of DNA synthesis and cell division. Therefore, cytokinin can increase cell division in the shoot system and affect the development of undifferentiated tissue. This study used two different variations of the hormone BAP (6-benzylaminopurine), which is an artificial cytokinin hormone, to find best concentration for the shoot multiplication of banana cultivar mas and kepok. The aim of this study is to evaluate the best additional BAP concentration for banana cultivar mas and kapok multiplication.

2. Methodology

2.1 Explant source

The sucker of Banana that were used in this study were collected from Bali and Subang. Sucker were collected from young banana shoots.

2.2 Explant preparation

First, explants of bananas were peeled, and part of the meristem was collected. They were washed with running water for 2 hours, and then immersed in a 3% fungicide solution. After that, explants were washed using a 10% Clorox solution and two drops of tween 80, followed with rinsing in sterile distilled water 3 times. Sterile explants were transferred into the MS medium supplemented with BAP.

2.3 Optimization of BAP concentration.

Micro-propagation was carried out for 3 months in instant Murashige and Skoog (MS) media containing vitamins with the addition of benzyl amino purine (BAP). The concentration of BAP was optimized through a preliminary test to evaluate the effect of BAP concentration on the number of shoots formation on banana explants. Mas and Kepok cultivars were propagated on MS medium with the addition of BAP concentrations of 2 ppm and 4 ppm. Furthermore, the explants were incubated at room temperature with lighting 16/8 hours of light and dark a day. The parameters measured were the number and the quality of the shoots produced.

3. Result and Discussion

The MS medium composition used in this research is generally the same for various plant, but there are differences in additional hormones to induce shoot growth and explant size. In this study, the use of synthetic BAP hormones with 2 - 4 ppm concentrations was used to induce the multiplication of shoots on banana explants. Benzyl Amino Purine (BAP) is a synthetic hormone that belongs to the cytokinin group. It induces cells in the shoots to proliferate and cells that divide will develop into new shoots, branches, and leaves [5].

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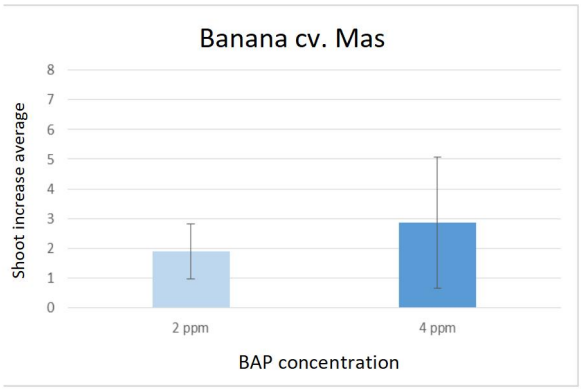

Figure 1. Average Number of cv Mas Shoots after 2 months

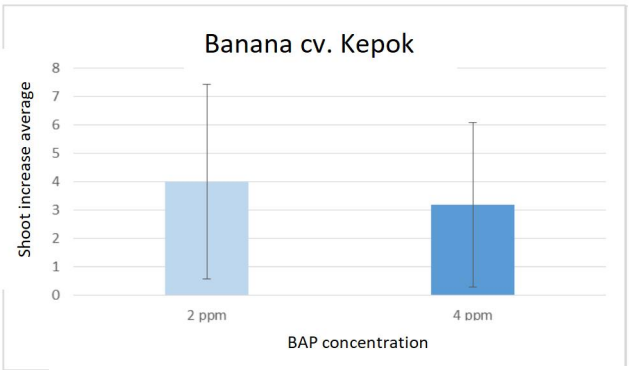

Figure 2. Average Number of cv Kepok Shoots after 2 months

Figure 1 and 2 showed the differences of number in shoot formation at each concentration. Banana cultivar Mas tend to form more shoots in MS medium with 4 ppm BAP concentration , while that in Kepok cultivar was in MS medium with 2 ppm BAP concentration. Concentration of BAP used in the medium also influenced the morphological conditions of culture. This can be seen in Figure 3.

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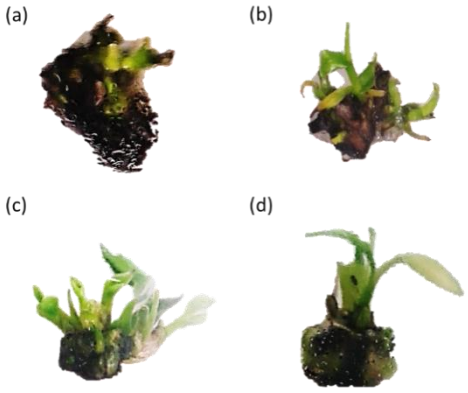

Figure 3. Morphology of banana explant (a) Banana cv. Mas in 2 ppm BAP (b) Banana cv. Mas in 4 ppm BAP (c) Banana cv. Kepok in 2 ppm BAP (d) Banana cv. Kepok in 4 ppm BAP

Optimization results showed that the shoot morphology of Mas cultivars was healthier (indicated by shoot that look greener and fresher) in medium supplemented with 4 ppm BAP hormone, while kepok cultivars need 2 ppm BAP hormone. Research conducted by Arniputri et al. (2018) showed that BAP with a concentration of 2-4 ppm can influence the formation of banana culture shoots [6]. However, there is a difference in growth regulator requirement between mas and kepok cultivars in inducing shoot bud propagation, which can be related to several factors. One of causes might be the explant condition of each cultivar. The explants used was derived from the primordial shoot tissue that is in the sucker. In the primordial tissue, natural plant hormones produced in the cells are different in each explant, so the addition of growth hormones needed to induce shoot growth are also different. For optimal response, plant hormones must be present in sufficient quantities, no less and no more. More hormones in plants can be toxic to plants themselves, while lower hormones concentration may not be able to induce optimal shoot primordial growth [7].

4. Conclusion

Banana cultivar Mas tend to response better in MS medium supplemented with 4 ppm BAP concentrations. Whereas banana cultivars Kepok responded more on MS medium with 2 ppm BAP concentration.

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Protein and Phycocyanin Production from Spirulina (*Arthrospira platensis*) Using Anaerobically Digested Dairy Manure Wastewater (ADDMW) as A Substrate with Addition of Salinity Treatment

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Abstract

Spirulina (*Arthospira platensis*), commonly called as "superfood" due to its high protein content and properties and efficacy to the human body. Unfortunately, Spirulina production is still constrained due to its high cost of cultivation medium and unoptimized cultivation system. We examined the use of anaerobically digested dairy manure wastewater (ADDMW) as a low-cost alternative cultivation medium for Spirulina. In addition, we add salinity to the medium as a supplement. The salinity treatment used in this research was 0 ppt, 15 ppt, 25 ppt, and 35 ppt. Cultivation of *A. platensis* were carried out for 14 days using culture bottles. The optimum salinity for biomass yield and productivity was 35 ppt. The optimum salinity for producing protein and phycocyanin are 35 ppt and 25 ppt respectively with protein yield reaching 3,144 g protein/g substrate and phycocyanin yield reaching 30,79 g phycocyanin/g substrate. Beside producing protein and phycocyanin, the growth of *A. platensis* in ADDMW could also be used as a phycoremediation process for orthophosphate, ammonium and nitrate in the medium. Orthophosphate removal efficiency is directly proportional to medium salinity however, it does not give a significant difference on ammonium removal efficiency. Nitrate removal efficiency was disturbed by nitrification process that occurred.

Keywords: *Arthrospira platensis,* ADDMW, salinity, phyccyanin, protein

1. Introduction

Ever since the industrial revolution in the 19th century, mankind has invented various technologies to help with their daily lives. However, technological development is usually not accompanied by adjustments to the surrounding environment. This causes environmental damage which later becomes the cause of various immunity related disease in the human body. As an example, the damage to the earth's ozone layer causes an increase in the UV radiation level which instigated skin cancer, cataract, and degradation of the human body's immune system. In addition to these problems, the increasing human population worldwide raises a very serious problem, namely global food crisis. Overall, these problems have led to an increasing demand of food supplement around the world.

According to [1], food supplements are defined as food that contain concentrated nutrients or other substances that have physiological effects on the human body in addition as a

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complement to the normal diet. One of the most known and most consumed food supplements is Spirulina. Spirulina is the common name of the green-blue microalgae (cyanobacteria) *Arthrospira.* Spirulina contains high nutritional components and has many benefits for the human body compared to other food sources. Protein content in spirulina can reach up to 70% of its dry weight, much higher than other vegetable protein sources hence why spirulina is often referred to "superfood" or "food of the future" [2]. In addition, spirulina also contains many compounds beneficial to the human body, such as phycocyanin.

Phycocyanin is a chromophore pigment found in many cyanobacteria and red algae with diverse uses. Phycocyanin has antioxidant, antiviral, and antimutative and other beneficial properties [2]. This compound is often used as natural food colorant, cosmetics ingredient, as well as fluorescent markers for medical purposes [3]. Although it has been commercialized massively since the 19th century, the phycocyanin industry still faces many problems. The biggest problem faced by this industry is the high cost of production caused by the expensive cultivation substrate. As an alternative substrate, ADDMW (Anaerobically Digested Dairy Manure Wastewater) could be used.

ADDMW is the by-product of the anaerobic digestion of organic waste such as cow manure using biodigester. This process utilizes the natural bacteria found in cow manure to reduce contaminant levels in the wastewater through anaerobic degradation. The main product produced in this method is biogas which can be used to generate electricity and ADDMW as the by-product. ADDMW contains useful nutrients (nitrogen and phosphor source), hence why it can be used as an alternative substrate for cultivating autotrophic organisms such as spirulina [4]. Spirulina cultivation using ADDMW medium has two main advantages, lowering the production cost also phycoremediation of the ADDMW by spirulina culture.

Cultivation of spirulina using ADDMW medium has not been studied intensively until now, so this process is not well optimized. Optimization of the cultivation process can be done by manipulating the environmental factors of the culture. On an industrial scale, one of the easiest environmental factors to be manipulated is the salinity level by addition of salt. Salinity stress can affect photosynthesis process, increase respiration, and influence the synthesis and metabolism of proteins and carbohydrates in cells [5], hence at the optimum salinity level, the highest content of protein and phycocyanin in the biomass can be obtained. In this research, the effect of salinity treatment on the growth, productivity and production of phycocyanin and protein from spirulina (*Arthrospira platensis*) cultivated using ADDMW medium and the effect of medium salinity on the efficiency of nutrient reduction is studied.

2. Materials and Methods

2.1 Microorganism (*A. platensis*)

A. platensis strain used in this study was obtained from Balai Besar Perikanan Budidaya Air Payau (BBPBAP) Jepara, Central Java. The culture was propagated using 50% concentration Zarrouk medium using 1 litre capacity bottles (80% working volume) at room temperature. The light source used in this study is white fluorescent lamp with intensity of 2300 lux and

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16:8 photoperiodism. Aeration using aquarium air pump with flow of 0,3 Litres per Minute (LPM). Propagation was done for three weeks.

2.2 Anaerobically Digested Dairy Manure Wastewater (ADDMW) Medium

ADDMW used in this study was obtained from Fakultas Peternakan, Universitas Padjajaran, Jatinangor, West Java. Before being used as the substrate for *A. platensis* cultivation, ADDMW medium was separated from its solid content by filtration and sterilized using autoclave. $NaHCO_3$ was added as additional carbon source and to elevate the medium's pH. Concentration of $NaHCO_3$ in the medium is 8.5 g/L.

2.3 Cultivation Condition

Cultivation was done for 14 days using 1 litre capacity culture bottles with 80% working volume. Each salinity treatments are replicated 7 times. The prepared ADDMW medium was added with NaCl salt according to the salinity treatments (0 ppt as control, 15 ppt, 25 ppt, and 35 ppt). *A. platensis* culture was homogenized manually before being added to the culture bottles with a 4:1 ratio of culture to ADDMW medium. Light intensity is conditioned at 2300 lux with 16:8 photoperiodism, and aeration at 0,3 LPM at all time. Sampling of culture was done every two days by taking ± 7 mL of culture sample from every bottle.

2.4 Kinetic Parameters

Growth of culture was monitored every two days by measuring the culture's optical density (OD) at 560 nm using a spectrophotometer [6]. The OD values were converted to its dry weight concentration (g biomass DW/L) equivalent using previously prepared standard calibration curves. Specific growth rate (day^{-1}) and doubling time (day) were calculated from the dry weight concentration data using Eq. (1, 2) respectively.

$$\mu = \frac{\ln(Xf) - \ln(Xi)}{t} \quad (1)$$

$$dt = \frac{\ln 2}{\mu} \quad (2)$$

2.5 Biomass Harvesting

Biomass harvesting was done using filtration screen (150 mesh). The harvested biomass was kept in a centrifuge tube to be dried by freeze drying at Laboratorium Biokimia ITB. The dried biomass' weight was calculated to estimate the productivity using Eq. (3) and kept for further analysis.

$$P = \frac{Xt - Xo}{\Delta t} \quad (3)$$

2.6 Pigment Extraction and Quantification

Chlorophyll-a, total chlorophyll, and total carotenoid content in the biomass was estimated using acetone according to the method reported by [7]. Dried *A. platensis* biomass was extracted using acetone with a ratio of 10:1. Sample was incubated for 24 hours in the

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refrigerator (4°C) and filtrated using filtration paper to separate the extract from the biomass. Optical density of extract at 470 nm, 645 nm, and 662 nm were measured using spectrophotometer. Pigment quantification were calculated using Eq. (4-7) in µg/mL unit which was converted to g/mg biomass.

$$Cla = 11{,}24 \times A662 - 2{,}04 \times A645 \quad (4)$$

$$Clb = 20{,}13 \times A645 - 4{,}19 \times A662 \quad (5)$$

$$Total\ Cl = 7{,}05 \times A662 + 18{,}09 \times A645 \quad (6)$$

$$Total\ Ct = \frac{1000 \times A470 - 1{,}09 \times Cla - 63{,}14 \times Clb}{214} \quad (7)$$

2.7 Phycocyanin Extraction and Quantification

Phycocyanin content in *A. platensis* biomass was extracted and quantified according to the method described in [8]. 40 mg of dried *A. platensis* biomass was added with 10 mL of 0,1 M phosphate buffer (pH 7). The sample was incubated for 24 hours in the refrigerator (4°C) and filtrated using filtration paper to separate the extract from the biomass. Optical density at 620 nm was measured using spectrophotometer and phycocyanin content was quantifed using Eq. (8). Phycocyanin productivity was calculated to determine optimum salinity to produce phycocyanin using Eq. (9).

$$\%Fikosianin = \frac{A620 \times V \times 100}{3{,}39 \times S} \quad (8)$$

$$P = \frac{(Xt \times Pt) - (Xo \times Po)}{\Delta t} \quad (9)$$

2.8 Protein Extraction and Quantification

Protein content was extracted according to method described by [9]. Extraction was done by adding 3 mL of 0,5 N NaOH to 50 mg of dry biomass and heated at 80°C for 10 minutes. Sample was then cooled to room temperature then centrifugated at 5000 RPM to separate extract from its biomass. Extraction was repeated for three times, where the third extraction was done at 100°C. The resulting extract from three extraction was combined to quantify the crude protein content using Bradford method. To determine the optimum salinity for producing protein from *A. platensis* in ADDMW, protein productivity was calculated using Eq. (9).

2.9 Nutrient Medium Content Analysis

Orthophosphate content in the medium was calculated every two days as a phycoremediation parameter of *A. platensis* in ADDMW. Orthophosphate content in the medium was calculated using $SnCl_2$ method. The resulting nutrient content was then used to calculate nutrient content removal efficiency of *A. platensis* culture using Eq. (10).

$$R = \frac{C_i - C_t}{C_i} \times 100\% \quad (10)$$

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2.10 Statistical Analysis

Data presented in this paper are the mean of seven replication and analyzed by calculating its standard deviation and One Way ANOVA. Significant levels for all analysis were set to $p<0,05$. All statistical analysis was done using Microsoft Excel 2016.

3. Results and Discussion

3.1 Effect of Medium Salinity on A. platensis Growth

A. platensis culture at 15, 25, and 35 ppt salinity treatments was still in the lag phase until the second day, marked with a decline in biomass from day 0, however in the control treatment (0 ppt) *A. platensis* culture did not go through lag phase at all, marked by the increasing of its biomass since day 0 as seen in Figure 1. Until the 14th day of cultivation, biomass at all treatments are still inclining and have not reached the stationary phase. The highest biomass was seen at 35 ppt salinity treatment and the lowest biomass at 15 ppt.

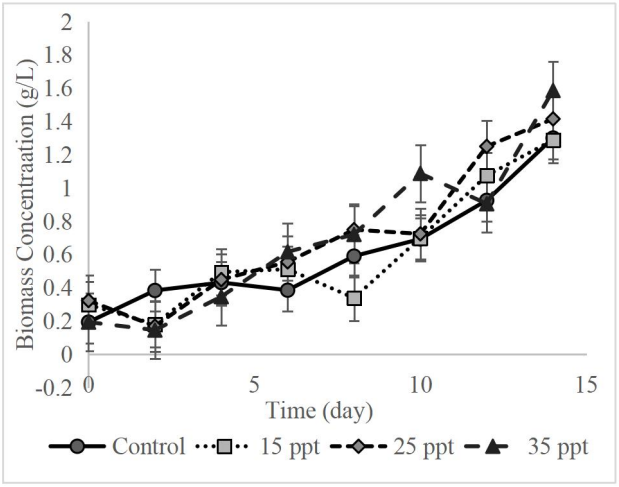

Figure 3 *A. platensis* Growth Curve

Salinity stress usually causes a decline in *A. platensis* biomass with an exception at its optimal salinity. Optimal salinity level to produce biomass diverse from one strain to the other, but generally ranges from 9-12 ppt [6, 10-12]. According to [6], the difference in this optimum salinity may be caused by the different cultivation condition used in each study. In [6] study, the medium used was a mixture of sea water and Zarrouk medium and the optimum salinity for producing biomass was seen at 11,67 ppt, meanwhile in [11] study, the medium used was a mixture of grey wastewater and sea water and the optimum salinity was seen at 9,3 ppt [6][11].

In this study, optimum salinity to produce *A. platensis* biomass is at 35 ppt. This result is also supported by the growth kinetic parameters from all variation as seen in Table 1, where highest specific growth rate was seen at 35 ppt salinity, followed by control, 25 ppt, and last

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15 ppt. At 35 ppt salinity, *A. platensis* only needed 4,6 days to double its biomass. Differences in optimum salinity may also because of the genetic difference between each *A. platensis* strain used in each study [6]. Difference in strain may result in different tolerance mechanism as the cell will utilize its available components from genetic expression. In this study, salinity tolerance mechanism used by *A. platensis* is by using luxury uptake mechanism.

Table 1 Growth Kinetic Parameters of *A. Platensis*

Treatment	μ (day^{-1})	*dt* (day)
Control	0,136	5,1
15 ppt	0,105	6,6
25 ppt	0,108	6,4
35 ppt	0,150	4,6

Luxury uptake mechanism is a mechanism in which cells assimilate phosphor exceeding its immediate need for growth and store it as polyphosphates [13]. Polyphosphate is a linear polymer made of many orthophosphate monomers and can be found in many organisms, such as cyanobacteria. Cyanobacteria is known to store polyphosphates excessively, as polyphosphate has important physiological function in bacteria's adaptation mechanism to new environment and stressful environments (e.g. low level of nutrient, high salinity) [14]. Studies conducted by [13] and [15] found that at zero level nutrient, *A. platensis* is still able to increase its biomass. In this study, high assimilation of orthophosphate at 35 ppt as seen in Table 2, may be the reason of increasing biomass until the 14th day of cultivation. Table 3 also shows an increasing trend of the biomass productivity of *A. platensis*. This result shows that orthophosphate assimilation is one of the defense mechanisms used by *A. platensis* to tolerate salinity stress, although further study needs to be conducted to see how *A. platensis* utilizes polyphosphate as a defense mechanism to salinity stress

Table 2. Orthophosphate Removal Efficiency

Treatment	Orthophosphate %removal Efficiency
Control	21%
15 ppt	41%
25 ppt	65%
35 ppt	75%

Biomass productivity in ADDMW medium shows an inclining trend as seen in Table 3 where biomass productivity increases as the medium's salinity is increased ($p<0,05$). According to

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these results, it can be concluded that the optimum salinity to produce *A. platensis* biomass is at 35 ppt.

Table 3 *A. platensis* Biomass Productivity

Treatment	Productivity (g biomass/day)
Control	0,0140
15 ppt	0,0118
25 ppt	0,0183
35 ppt	0,0379

3.2 Phycocyanin Content in *A. platensis*

Phycocyanin is an accessory pigment in cyanobacteria used for photosynthesis and alternative protein source at low nitrogen level [16]. In this study, highest phycocyanin content was seen at 25 ppt salinity and lowest at 35 ppt as seen in Figure 2. Low phycocyanin content at 35 ppt salinity may be caused by detachment of phycocyanin from tilakoid membrane due to high level of salinity. The same phenomenon was seen by [11] where they detected phycocyanin in the *A. platensis* cultivation medium at 30-35 ppt salinity, indicating detachment of phycocyanin from PS-II.

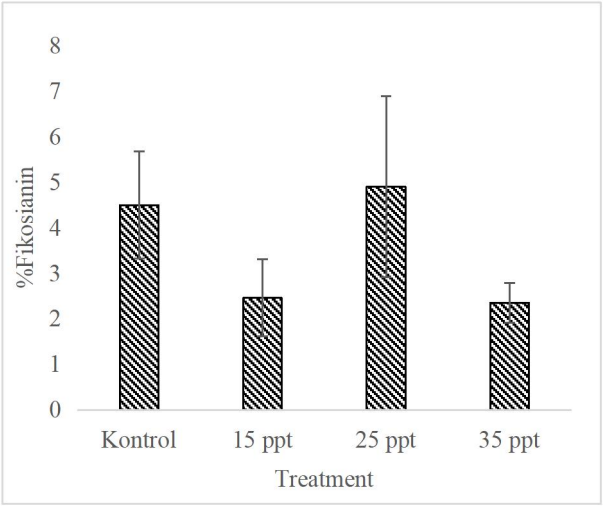

Figure 2 Phycocyanin Content in *A. platensis*

Phycocyanin productivity as seen in Table 4, is highest at 25 ppt with the lowest productivity at 15 ppt, reaching below zero. The sub-zero phycocyanin productivity seen at 15 ppt salinity is due to its low biomass production and low phycocyanin content, lower than the initial

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phycocyanin content which reached 8% of its dry weight. These results are similar to study conducted by [14] where optimum salinity to produce phycocyanin is at 23 ppt with 6,01% content, also at salinity level above 23 ppt, phycocyanin content declines. Differences in the phycocyanin content values are mainly due to the difference in strain used in each study and difference in cultivation condition. In [14] study, the medium used is Zarrouk medium at 50 mL scale, whereas in this study ADDMW is used as the medium at 800 mL scale.

Table 4 Phycocyanin Productivity

Treatment	Phycocyanin Productivity (g phycocyanin/day)
Control	0.0132
15 ppt	-0.0933
25 ppt	0.0142
35 ppt	0.0133

Figure 3 shows that total chlorophyll and total carotenoid content in *A. platensis* are not statistically different (p>0,05), indicating that medium salinity does not influence the synthesis of the pigments in *A. platensis* at all. Carotenoid as a secondary metabolite is usually synthesized as a defense mechanism towards stress condition in the environment [10]. Total carotenoid content in this study is not affected by the medium's salinity (p>0,05), indicating that the *A. platensis* strain used in this study does not use carotenoid as its defense mechanism against salinity stress.

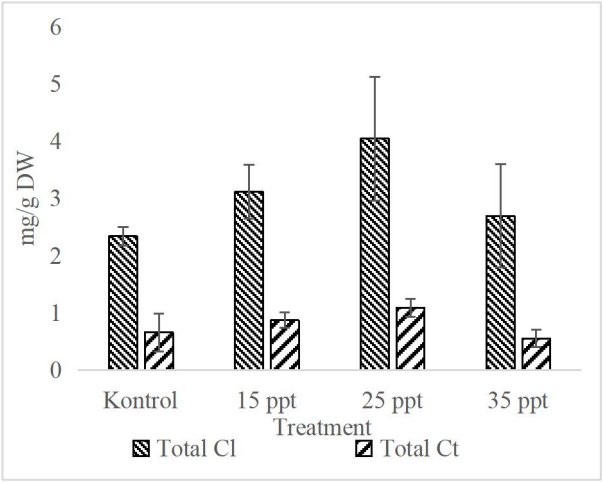

Figure 3 Total Chlorophyll and Total Carotenoid Content in *A. platensis*

Although total chlorophyll and total carotenoid content in the cell are not statistically different, ratio of phycocyanin to chlorophyll-a in *A. platensis* showed a significant difference (p<0,05). Phycocyanin to chlorophyll-a ratio shows the same trend as the phycocyanin

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content in *A. platensis* as seen in Figure 4, generally shows a declining trend, however peaks at 25 ppt. Similar results were seen in studies conducted by [17-18] in which phycocyanin content declines but chlorophyll-a content is constant with the increasing of medium salinity. Nevertheless, these results are contradictive with results by [6, 11] in which chlorophyll-a content is inversely proportional to medium salinity level, resulting in an increasing trend of phycocyanin to chlorophyll-a ratio. According to [6], high phycocyanin to chlorophyll-a ratio indicated a salt absorbing mechanism by phycobilisome complex in the PS-II system in comparison to chlorophyll-a. Salinity stress causes inhibition in electron transfer from PS-II, causing damage in phycobilin complex of *A. platensis.* This damage results in excitation energy shifting from PS-II to PS-I which contains chlorophyll-a so that cells can still produce energy needed to extrude Na^+ ions to keep the osmotic balance in the cell [17]. This may be the cause of different results from this study with studies conducted by [6, 11, 17-18], which is due to the difference in defense mechanisms against medium salinity used by *A. platensis* strain. In [6, 11] studies, the *A. platensis* strain used utilizes this defense mechanism, indicated by the increasing phycocyanin to chlorophyll-a ratio as opposed to phosphor luxury uptake utilized by the strain in this study.

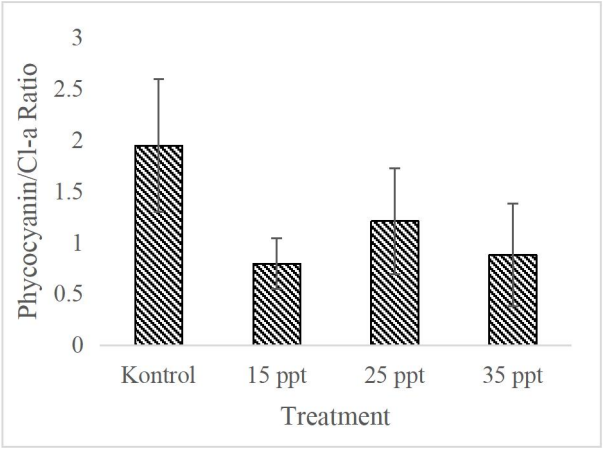

Figure 4 Phycocyanin to Chlorophyll-a Ratio in *A. Platensis*

3.3 Protein Content in *A. platensis*

Total protein content in *A. platensis* is inversely proportional to medium salinity level as shown in Figure 5 with protein content ranging from 13-20%. These results are very low compared to normal protein content in *A. platensis* which can reach up to 70% of its dry weight [6]. Differences in results are mainly caused by different protein extraction method used. In this study, protein extraction method used is based on Rausch (1981) study, using NaOH at high temperature condition. This method should be accompanied by a cell rupture mechanism, either by mechanical grinding or sonication to ensure that all protein contents in

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cell are extracted by NaOH. Nevertheless, protein content trend results in this study are similar to [6, 10] results.

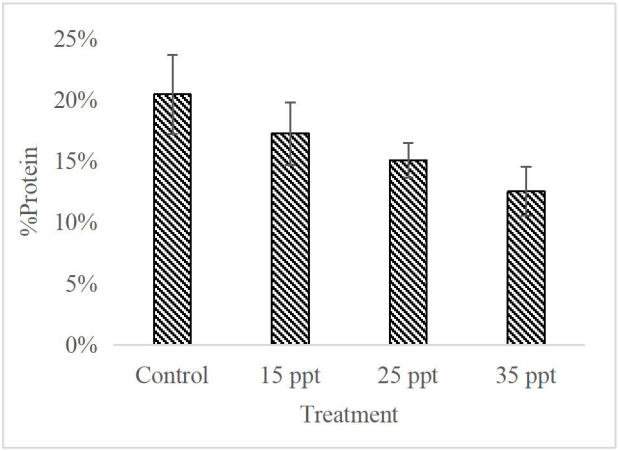

Figure 5 Protein Content in *A. platensis*

According to [19], salinity stress will cause a difference in biochemical composition of *A. platensis* biomass, usually a decline in protein content. Difference in biomass composition is caused by shifting in energy utilization by *A. platensis* to accommodate osmotic imbalance due to the entering of cations (usually Na^+) [11]. This shift in energy usually causes a blockage in protein synthesis path. Although the lowest protein content was found in 35 ppt as shown in Table 4, the highest protein productivity is at 35 ppt. This result is caused by the abundance of biomass produced in 35 ppt can compensate the low protein content. To conclude, the optimum salinity to produce protein from *A. platensis* cultivated in ADDMW medium is 35 ppt.

Table 5 Protein Productivity

Treatment	Protein Productivity (mg protein/day)
Control	1.954
15 ppt	$8{,}7x10^{-3}$
25 ppt	0.306
35 ppt	3.237

3.4 Phycoremediation Potential of *A. platensis* in ADDMW

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ADDMW as the by-product of cow manure anaerobic degradation process still contains high level of orthophosphate which may harm the environment if it is not treated. The usage of ADDMW as cultivation medium for *A. platensis* has two main advantage, lowering the production cost of spirulina powder also as the further step of ADDMW remediation. In this study, *A. platensis* potential in lowering orthophosphate level in ADDMW is studied.

Figure 6 shows the orthophosphate level in ADDMW. It shows a declining trend for all salinity treatment. Microalgae and cyanobacteria have luxury uptake mechanism for phosphor where cells assimilate phosphor excessively and store it as polyphosphate as phosphor reserve [20]. Increase of orthopshophate content in ADDMW at twelfth day of cultivation may be caused by excretion of phosphate due to the maximum capacity of polyphosphate storage in *A. platensis* cells [21].

Removal efficiency of orthophosphate is directly proportional with medium salinity ($p<0,05$) with highest orthophosphate assimilation at 35 ppt as shown in Table 2. This is mainly caused by salinity stress due to high salinity level, causing *A. platensis* cells needing more energy to regulate their osmotic balance and to store it as polyphosphate as salinity stress defense mechanism [14, 20].

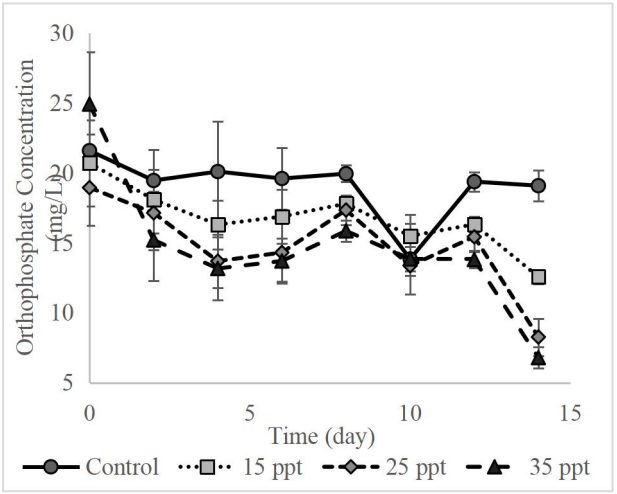

Figure 6 Orthophosphate Level in ADDMW Medium

A. platensis culture in ADDMW medium shows a passable potential for phycoremediation process of orthophosphate level with removal efficiency ranging between 21-75%. In comparison to study conducted by [22], which utilized microalgae *Chlorella vulgaris* to treat ADDMW medium, result in this study is lower. Removal efficiency of orthophosphate reached 94,48% in [22] study. High removal efficiency in [22] study may be caused by the natural bacteria living in ADDMW, because ADDMW medium was not sterilized before being used, whereas in this study ADDMW was sterilized before usage. This theory is also

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supported by [23] result in which ADDMW medium without addition of any other organism has a removal efficiency of 78,66% during 14 days of observation. This comparison shows that *A. platensis* culture has a good phycoremediation potential for lowering orthophosphate level in ADDMW.

4. Conclusion

A. platensis growth in ADDMW medium is directly proportional to medium salinity up to 35 ppt. Optimum salinity for producing *A. platensis* biomass is at 35 ppt medium salinity with a productivity of 0,0379 g biomass/day. Optimum salinity to produce phycocyanin from *A. platensis* cultivated in ADDMW medium is 25 ppt, with phycocyanin productivity reaching 0,0142 g phycocyanin/day. Optimum salinity to produce protein is at 35 ppt medium salinity with protein productivity of 3,273 mg protein/day. *A. platensis* culture has a great phycoremediation potential for lowering orthophosphate level in ADDMW with removal efficiency ranging between 21-75%.

Acknowledgments

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Temporary Immersion Bioreactor: A Solution in Pasak Bumi (Eurycoma longifolia) Somatic Embryo Production

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Abstract

Pasak bumi is an important herbal medicine in Soultheast Asia. However, it is becoming an endangered species due to over exploitation. Somatic embryogenesis should provide solution to the problem, but conventional technique results in abnormal morphology embryo and time consuming. The aim of study is to investigate immersion interval time of pasak bumi somatic embryo production in temporary immersion bioreactor. The somatic embryo is immersed temporary in medium for a minute at interval of 3, 6, and 12 hour and were incubated for 8 week, at room temperature, and 16 h photoperiod. At harvesting, the cultures were weighted aseptically and the number of embryo at every stages was counted using inverted microscope. The longer the immersion time interval, the more somatic embryos produced. The normal embryos was also produced in bioreactor with time interval immersion every 12 hours for a minute. In this interval time, the average number of globular is 165 embryos, the heart is 42 embryos, and the torpedo is 15 embryos. Normal embryos succesfully grew and developed to later stage of embryos.

Keywords: interval time immersion, pasak bumi, propagation, somatic embryo, temporary immersion bioreactor.

1. Introduction

One of bioreactor that is usually used to produce somatic embryo is temporary immersion bioreactor. This bioreactor has successfully been used to increase production of several plants at different interval times, such as: *Theobroma cacao* [9], *Coffea arabica* [4], *Citrus deliciosa* [2], and *Hevea brasiliensis* [3]. Beside that, the production of pasak bumi embryo somatic in conventional method has been done in previous research [10], but it was only grow at early stages and time consuming [10].

Pasak bumi is a important plant that used for herbal medicine in Soulth-East Asia, such as: Indonesia, Malaysia, China, Philippines, and Thailand. The plant part that usually used in industry is root, and when root of pasak bumi is harvested directly, without cultivation, it will threaten preservation of pasak bumi in Indonesia [12]. Due to many uses of pasak bumi, the demand of pasak bumi has been increased. However, the pasak bumi seedling grows slowly and start to produce seed when plant is 3-4 years old.

One of the method to produce plantlet in a short time and large quantities is by in vitro culture, i.e through somatic embryogenesis. Somatic embryogenesis is the process of embryogenesis in somatic cells (both haploid and diploid) through similar process with zygotic embryo development, and finally produce new plant without gamet fusion [13]. The

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steps of somatic embryogenesis are pro-embriogenic mass induction, followed by somatic embryo development, maturation, dessication, and plant regeneration [7].

We investigated bioreactor-based somatic embryogenesis propagation that can produce, maintain, and grow pasak bumi in large quantities and shorter time. This research used modification of temporary immersion bioreactor to determine appropriate time interval of embryo immersion in the medium.

2. Materials and Methods

2.1 Plant Materials

Seed-derived callus was used as explant in this research. This callus was maintained to proliferate in the MS basal medium [8] supplemented with 2.25 mg/L 2,4-D (2,4-Dichlorophenoxyacetic acid), 2.0 mg/L kinetin, 0.8% agar, and 3% sucrose. Culture was then incubated at 25o±2oC with 12 h photoperiod. Culture was subcultured every four week into fresh medium.

2.2 Pasak Bumi Somatic Embryos Induction

Callus stock from proliferation medium were weighed 0.5 g aseptically and transferred into the liquid medium to induce somatic embryos. Induction medium consisted of Murashige-Skoog basal medium, 1 mg/L 2,4-D, 2 mg/L BAP, 3% sucrose, and 0,1 g/L activated charcoal [10]. These suspension culture were incubated on a shaker, agitated at 120 rpm, at room temperature, and 16 h light photoperiod for four week. These somatic embryo suspension was cultured for two week and then was used to the next step for somatic embryo development in bioreactor.

2.3 Somatic Embryo Development in Bioreactor with Different Time Interval of Immersion

Embriogenic suspension culture were weighed 3 g aseptically and transfererred into the upperside compartment of bioreactor. Bioreactor used in this research was modified Temporary Immersion Bioreactor (Figure 1). The bottom side of bioreactor contained 300 mL medium of somatic embryo development. These medium was consisted of Murashige-Skoog basal medium, 1 mg/L 2,4-D, 2 mg/L BAP, 3% sucrose, and 0,1 g/L activated charcoal. Embryo was immersed in medium for a minute temporarily with interval time every 3 hours, 6 hours, and 12 hours in different bioreactor [1,3]. The control embryo was cultured in 100 mL erlenmeyer and was agitated at 120 rpm. The air was lifted using a pump at almost the same pressure with atmosferic pressure, so that the medium could ascended into upper compartemen. Embryo culture were incubated in bioreactor for 8 week, at room temperature, and 16 h photoperiod for four week. At harvesting, the cultures were weighted aseptically and the number of embryo at every stages was counted using inverted microscope.

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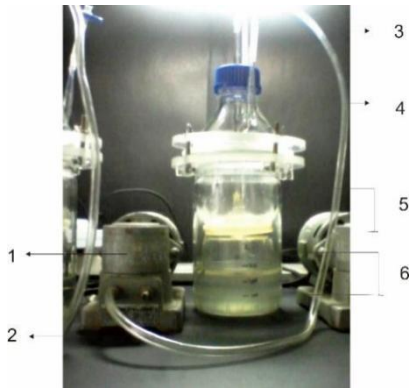

Figure 1. The immersion bioreactor. The part of bioreactor are 1. Air pump, 2. Rubber tube, 3. Air inlet, 4. Air outlet, 5. Up compartemen to place the cell/tissue 6. Base compartemen to place the medium. When the pump is on, air enter through the base compartement to up compartemen, and air exit to outlet. When the air flow to upper compartmen, the medium in base compartemen flow to up compartemen simultaneuosly.

3. Resuls and Discussion

The number of embryos at every stages during 2 months in bioreactor is different when treated with different immersion interval time (Figure 2). The highest number of embryos is produced in bioreactor with immersion time interval every 12 hours. In these bioreactors, the average number of globular is 165 embryos, the heart is 42 embryos, and the torpedo is 15 embryos. The lowest number of embryos is produced in control which cultured in 100 mL Erlenmeryer with continuos immersion. In control treatement, the average number of globular was 71 embryos, the heart was 16 embryos, and the torpedo is 1 embryo.

Embryos produced with treatment every 6 hours is higher than embryos produced with treatment every 3 hours (Figure 2). In bioreactor with immersion time interval every 6 hours, the average number of globular is 144 embryos, the heart is 27 embryos, and the torpedo is 7 embryos. In bioreactor with immersion time interval every 3 hours, the average number of globular is 82 embryos, the heart is 27 embryos, and the torpedo is 1 embryo. The more normal embryos is produced, the longer interval time of embryo.

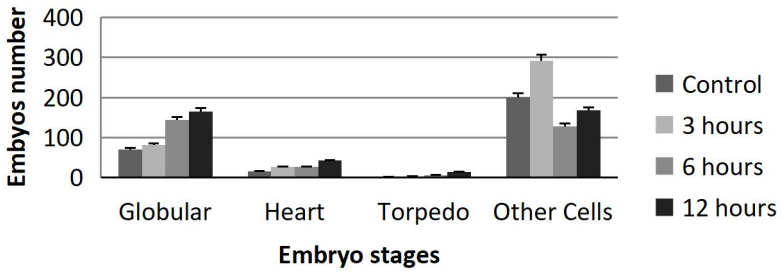

Figure 2. Embryo number at different interval time immersion. The embryos stage are globular, heart, and torpedo. Other cells mean callus cell. The interval time of immersion are at 3 hours (diagonal), 6 hour (horizontal), and 12 hours.

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The highest number of somatic embryos is produced in bioreactor with immersion time interval every 12 hours for a minutes, while the lowest number of somatic embryos is produced in control that immersed contionusly. The longer the immersion time interval in medium, the more embryos is produced in bioreactor. The somatic embryos of *Coffea arabica* and *Hevea brasiliensis* in RITA bioreactor produced similar results [3-4]. The highest number of *C.arabica and H.brasiliensis* somatic embryos was produced in bioreactor with immersion time interval every 12 hours for a minutes.

The time interval of immersion could influence growth and development of somatic embryos, due to optimal nutrition and plant growth hormone uptake from medium to embryos [6]. The longer the time interval of immersion in medium, the more normal morphology embryo were produced. Normal embryo morphology would influence embryo development into the later stages, since deformation of somatic embryos caused the embryos to develop difficultly.

The comparison of somatic embryo morphology at various stages of developments observed with various interval times of medium immersion (Figure 3). The treatment in interval time every 3 hours for a minute and every 6 hours for a minute produce similar morphology of embryos. The somatic embryos at globular and heart stage are deformed in these treatment. Cell protrusions are generated at several sides of embryos. In addition, the embryo in torpedo stage are produced imperfectly. Somatic embryos in interval time every 12 hours for a minute produce normal morphology, no cell protrusions and develop into torpedo stage. Visually, these torpedo is more compact than torpedos in other treatment.

The somatic embryos in bioreactor with immersion time interval every 3 hours for a minute and every 6 hours for a minute have a deformed morphology at globular and heart stages, and undeveloped perfectly at torpedo stage. These is predicted due to hyperhydricity. Embryos constantly in direct contact with liquid medium (either part or whole part of tissues) usually are sustained hyperhydricity [5]. These are characterized by damage morphology and physiology, i.e. shoot system damage and waterlogged tissues [5].

In contrast, somatic embryos produced in bioreactor with immersion time interval every 12 hours for a minute have normal morphology and developed into torpedo. The treatment every 12 hours for a minute has minimal exposure in liquid medium, avoided hyperhidricity. The shorter exposure in liquid medium cause normal morphology of pasak bumi somatic embryos that affecting somatic embryos development into torpedo stage.

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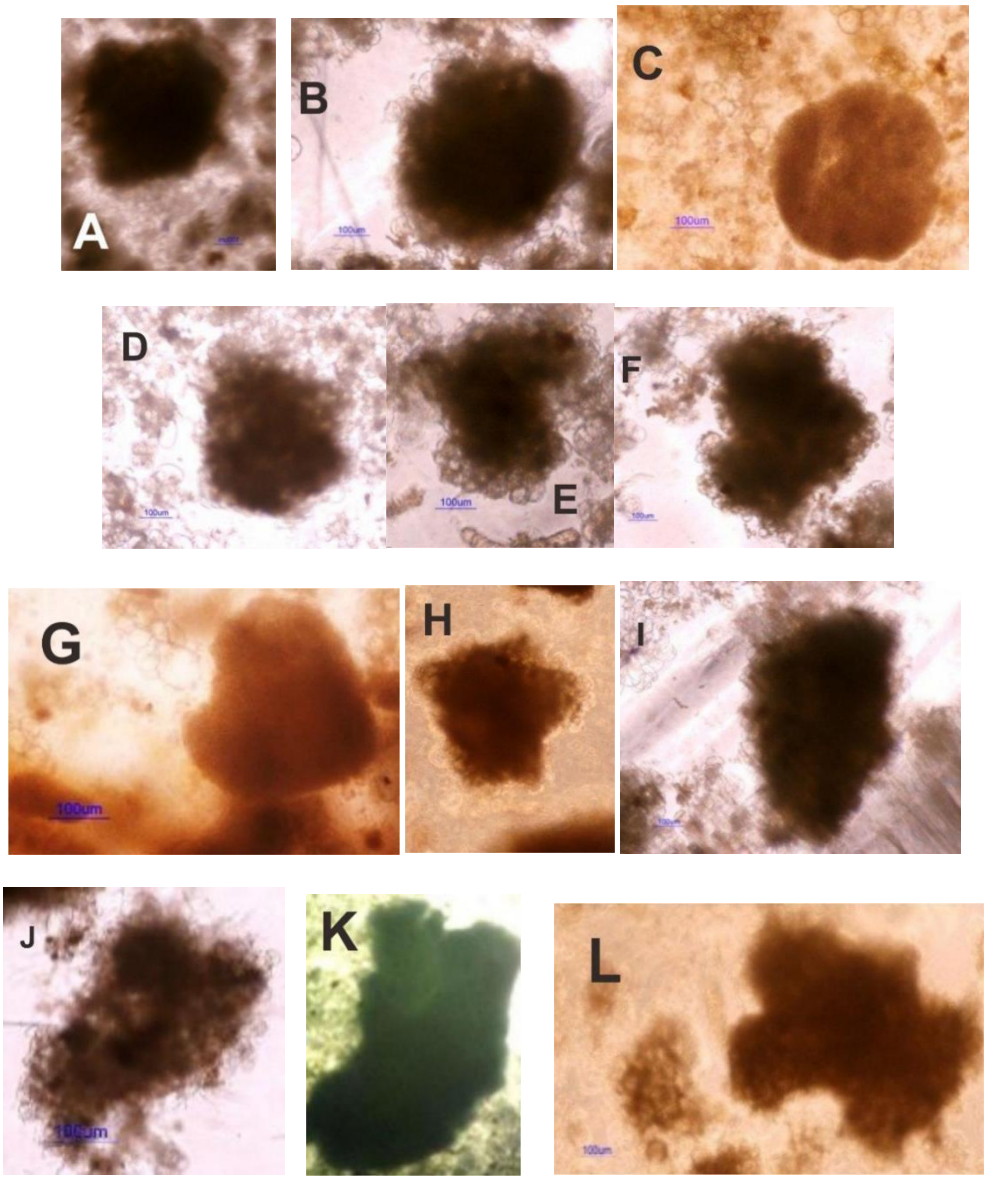

Figure 3. The morphology of somatic embryo (globular, heart, and torpedo) in different treatment of bioreactor. The globular embryo immersed for a minute at 3 hours (A), 6 hours (B), 12 hours (C), and control (D). The heart embryo immersed for a minute at 3 hours (E), 6 hours (F), 12 hours (G), and control (H). The torpedo embryo immersed for a minute at 3 hours (I), 6 hours (J), 12 hours (K), and control (L)

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4. Conclusion

In conclusion, the highest number of normal pasak bumi somatic embryos formation was produced in bioreactor with time interval of immersion every 12 hours for a minute. Using this treatment, embryos was avoided from hyperhydricity. So that, embryos succesfully grew and developed into torpedo stage more than other treatment.

Acknoledgements

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The Effect of Plant Age on the Content of Essential Oil from 'Nilam' (*Pogostemon cablin* Benth.) Shoot Culture on Thin Layer Culture Batch System

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Abstract

Pogostemon cablin Benth. or better known as patchouli is one of the leading varieties in Indonesia with the highest content of patchouli alcohol compounds compared with other varieties. Some literature show that the age of the plants affects the process of patchouli oil synthesis to be able to produce patchouli oil with patchouli alcohol content at least 30%. The study was conducted to determine the effect of harvest time on the essential oil content of plant shoots culture and to evaluate the bioconversion of medium substrates into shoot biomass. In this study, patchouli shoots were grown on Murashige and Skoog (MS) media without the addition of growth regulators. This shoot culture was cultivated for 28 days with harvest time variation every 7 days. The growth rate of biomass was 0.054 mg DW/day with doubling time of 12.78 days. The value of the sucrose content and the conductivity of the medium were correlated with the rate of increase in biomass. The yield of patchouli oil obtained ranged from 2.34 – 3.23%. The oil content found on Gas Chromatography-Mass Spectrometry (GC-MS) analysis was farnesane (0,18% - 0,28%), eicosane (1,17% - 4,24%), squalene (0,35% - 0,46%), and most groups of alkanes and fatty acids. However, no patchouli alcohol compound was found in the tested oil sample.

Keywords: harvest age, *Pogostemon cablin* Benth., thin layer culture (TLC), rate of growth, essential oil yield, shoot culture.

1. Introduction

The world's demand for essential oils and the aromatic compounds used in drug synthesis, flavors, fragrances, perfumes, and cosmetics continues is increasing. Most essential oils have therapeutic activity as antiseptic, stimulant, analgesic, antirheumatic, and diuretic [1]. In Indonesia, there are about 40 species of plants producing essential oils, 12 of which have already been used widely and 9 of the 12 species are well known in the export market, one of them was the patchouli [2]. Patchouli plant (*Pogostemon cablin* Benth.) or better known as patchouli belongs to the family Lamiaceae. The main product extracted from this plant is patchouli oil. The quality and price of patchouli oil was determined from the percentage of patchouli alcohol (PA) content [3]. Patchouli oil character were listed in Table 1.

World market's demand for patchouli oil reaches 2,000 tons per year with the largest quantity of patchouli oil produced in Indonesia [4]. According to the Direktorat Jenderal Bea dan Cukai (2013), the production of patchouli oil in Indonesia in 2011 reached 1,567 tons. Patchouli oil demand is high due to the fixative properties of patchouli oil, so the aroma was

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lasting. Patchouli oil was generally obtained from oil distillation contained in the leaves and stems of plants using the method of steam distillation.

Patchouli flowers rarely so it becomes an obstacle to develop good quality plants from seeds. Patchouli cultivation was done through stem cuttings, which was limited by the lack of availability of superior parent plants and the large variety of population. Plants propagated from stem cuttings are small, diverse, and susceptible toward various diseases caused by *Meloidogyne* spp. and *Pratylenchus brachyurus* nematodes [5]. Therefore, an alternative method for propagation of patchouli biomass using in vitro culture was needed so that the need of patchouli oil with the appropriate PA standard can be fulfilled.

Table 1. Patchouli oil quality standard (SNI 06-2385-2006)

No.	Parameter	Unit	Requirements
1	Color	-	Light Yellow – Red Brown
2	Density 25°C/25°C	-	0.950-0.975
3	Refractive index 20°C (nD^{20})	-	1.507-1.515
4	Solubility in Ethanol 90% at temperature 20°C ± 3°C	-	Clear solution or light opalescence in the ratio of 1:10 volume
5	Acid numbers	-	Max. 8
6	Ester numbers	-	Max. 20
7	Optical rotation	-	(-)48° – (-)65°
8	Patchouli alcohol ($C_{15}H_{26}O$)	%	Min. 30
9	Alpha copaene ($C_{15}H_{24}$)	%	Max. 0.5
10	Iron content (Fe)	mg/kg	Max. 25

In vitro culture or also known as tissue culture is a method of plant propagation using a semi-solid or liquid medium in a glass container in an aseptic state. The use of liquid medium for propagation of *in vitro* explants provides an economical advantage in terms of time and production costs since media are easily absorbed compared to that in solid media[6]. *In vitro* cultures using this liquid medium can be performed on thin layer systems.

One of the factors influencing the production of essential oil and PA content was the age of the plant. The accumulation of essential oil in the leaves was directly proportional to the age of the plant, since some genes related to the enzymes in the pathway are expressed after plants are more than one month [4, 7]. Therefore, this study was conducted to determine the effect of harvest time on the yield and patchouli oil content, patchouli growth curve, and the rate of nutrient absorption by patchouli biomass during cultivation mass in thin layer culture system.

2. Materials and Methods

2.1 Sterile Culture of *P. cablin*

The sterile patchouli culture used in this study was obtained from Esha Flora, Bogor. Propagation of patchouli shoot was done on semi-solid and liquid medium. Patchouli shoots

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were acclimatized from semi-solid medium to liquid medium for 1 week with agitation rate of 60 RPM. The lighting time during acclimatization was 16:8 h (light:dark), light intensity???.

2.2 Medium Culture Preparation

The medium used in this study was the Murashige and Skoog (MS) medium without the addition of growth regulators. The pH of the medium was set in the range of 5.6 to 5.8 before it was sterilized using an autoclave at 121ºC and 1.5 kg/cm^2 for 15 min.

2.3 Shoot Culture on Thin Layer System

Patchouli shoots that had been acclimatized and measured by weight of 1 g, were cultivated in 5 mL of liquid MS medium in Erlenmeyer 100 mL and given agitation of 60 RPM. The culture was incubated for 28 days (harvesting was done every 7 days) at 25ºC with a lighting time of 16:8 h (light:dark),. The final data that was taken each time harvesting such as medium sucrose content, medium conductivity, final weight of explant, and residual volume of medium.

2.4 Medium Sucrose Content Analysis

Analysis of the initial and final sucrose content of the medium was carried out using the Milwaukee MA871 Refractometer. The Brix index shown by the refractometer was converted using a standard curve of sucrose to show the mass of sucrose in the medium.

2.5 Medium Conductivity Analysis

Analysis of the initial and final conductivity of the medium was carried out using the Conductivity Meter 4303 Lutron Conductivity Meter.

2.6 Growth Rate Analysis

The growth rate was analyzed based on the dry weight data plotted into the graph. The abscissa axis represents time, whereas the ordinate axis represents ln biomass. Calculation of growth rate was done by using equation [8]:

$$\mu = \frac{\ln(X) - \ln(X_0)}{t} \quad (1)$$

where X is the cell concentration at time t (g/L) and X0 is the cell concentration at time t = 0 (g/L). The time required to double the biomass of the cell (biomass was directly proportional to concentration) can be calculated using equation [8]:

$$\mu = \frac{\ln(2X_0) - \ln(X_0)}{\tau} \quad (2)$$

$$\mu\tau = \ln(2) \quad (3)$$

$$\tau = \frac{\ln(2)}{\mu} \quad (4)$$

where was the time it takes to double the biomass of the cell.

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2.7 Extraction and Purification of Patchouli Oil

Patchouli shoot biomass was dried using oven at 50°C for 20 h, then extracted through steam distillation process. The temperature and pressure for the distillation process were 100°C and 1 atm [9]. The extraction process was carried out for 6 h [10]. The separation of water molecules was carried out by addition of sodium sulfate anhydrous and then centrifuged at a rate of 3,000 RPM for 5 min.

2.8 Analysis of Patchouli Oil Content

Testing of patchouli oil content was done by using Gas Chromatography and Mass Spectrometry (GC-MS) Agilent Technologies 5975C VL MSD equipped with DB 5 MS detector. The initial temperature of 41ºC was increased to 240ºC at a rate of 10ºC/min and then held for 5 minutes. The identification of the patchouli oil component is based on the ratio of the mass spectra obtained in gas chromatography to that obtained from the GC-MS library [10].

2.9 Statistical Analysis

The weight data of the formed biomass was analyzed using ANOVA test. The trust interval used for statistical tests using IBM SPSS 20 software was 95%.

2.10 Mass Balance Analysis

The data of sucrose content was used as the basis for making the following biomass forming equation [11]:

$$aC_{12}H_{22}O_{11} + bNH_4NO_3 + cO_2 \rightarrow CH_{1,27}O_{0,43}N_{0,45} + dH_2O + eCO_2$$

3. Results and Discussion

Propagation of patchouli culture *in vitro* began with the initiation and subculture stage. The results showed that MS medium without the addition of growth regulator can be used for patchouli stem elongation, whereas MS medium with the addition of 0.5 ppm BAP can be used for patchouli shoot propagation. Thereafter, explants were used in the stage of acclimatization and treatment. The results showed that the roots began to form on a 21-day patchouli shoot culture. Shoots that have roots were acclimatized to the soil medium. The results showed that medium ½ MS without the addition of growth regulation can be used for propagation of patchouli seedlings. The process of biomass propagation of patchouli shoot culture can be seen in Figure 1.

The results of *in vitro* cultivation for 1 month showed that the shoot and patchouli leaf color was a bit dark green and began to appear yellowing leaves on the culture of 21 days. Patchouli roots began to form in the culture of 21 days which my be related to the synthesis

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of stress hormone ethylene. The decrease of growth rate and morphology condition of patchouli shoot culture after 14 days old culture was thought to be caused by the limited of sucrose content in culture medium. The final condition of fresh of patchouli shoots of cultivation in thin layer culture system is shown in figures 2. Growth of patchouli biomass on harvest age yields a growth rate value of 0.054 mg dry weight per day with a doubling time of 12.78 days (Figure 3).

Sucrose in the medium served as a carbon source for the growth of patchouli shoot biomass. Another factor that may cause a decrease in growth rate and morphology of patchouli shoot culture was the sensitivity to shear stress. Sensitivity to shear stress which arises from aeration and/or agitation can cause cell damage. In this research, agitation using a shaker with a speed of 60 RPM was the cause of shear stress and can cause cell damage. The response of plant cells to shear stress varies with the age of cultivation which was also related to cell size or aggregate, shown from the height and mass of the plantlet.

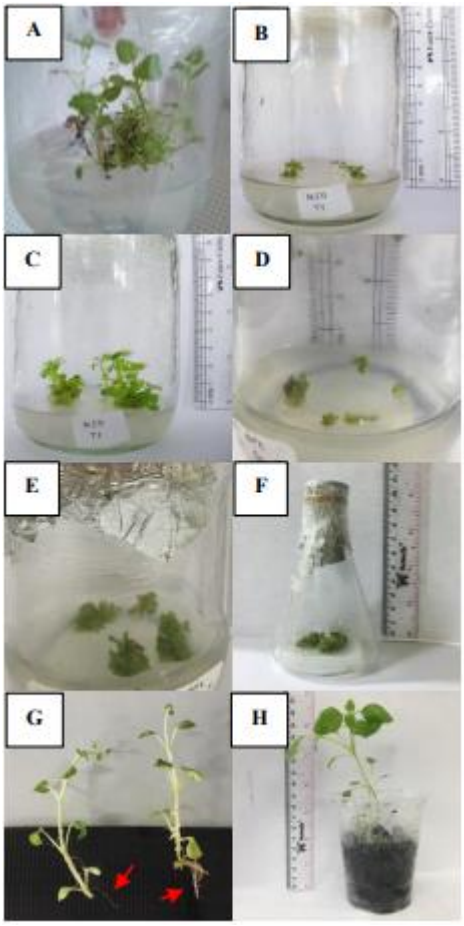

Figure 1. Biomass propagation process of patchouli shoots culture. Sterile patchouli culture from Esha Flora (A), initiation of patchouli shoot culture on MS0 medium (B), subculture of patchouli shoot on MS0 medium (C), initiation of patchouli shoot culture on MS + BAP 0.5 ppm (D), subculture of patchouli shoot on MS + BAP 0.5 ppm (E), acclimatization and

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treatment on medium ½ MS of thin layer system (F), root formation at 21 days old patchouli shoot culture (G), result of acclimatization of patchouli shoot on soil medium for 1 month (H)

Statistical analysis showed that there were differences, both for fresh weight and dry weight of patchouli shoot culture, which was significant due to differences in harvest age. The growth rate of the patchouli shoot culture takes place directly into the exponential phase without going through the lag phase. The lag phase was assumed to occur during the acclimatization of explant from semisolid medium to liquid medium for 1 week.

The rate of sucrose consumption during the cultivation period was 0.0023 g/mL/day (Figure 4). The decrease in sucrose concentration showed that sucrose was consumed very high until day 14, after which it decreases because of the limitation of the content of sucrose. One study of hairy root culture and cell suspension Symphytum officinale showed that sugar was all consumed by day 14 [13].

The value of sucrose concentration was also supported by data on the growth rate of patchouli biomass which continued to increase until day 14, but began to decrease after the 14th day. Growth of inoculum culture in liquid medium approximately started at day 6 and started to stop at day 14, because on day 0 until 5th day might be in lag phase as to prepare for cell division where at this stage medium sucrose concentration decrease quickly [12]. The phenomenon occurred according to the tendency that high sucrose consumption rates produce more efficient bioconversion [14].

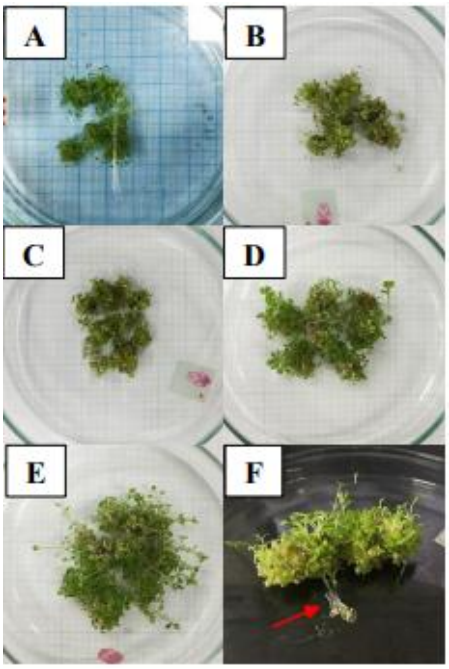

Figure 2. Fresh conditions of patchouli shoots cultivation results in the thin layer system at age 0 day (A), 7 days (B), 14 days (C), 21 days (D), 28 days (E), roots start to form at the culture of patchouli shoot 21 day (F)

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The conductivity value decreased with increasing of plant age. This showed that there was absorption of ions from the medium by plant cells. The ion absorption rate was 0.206 mS/day (Figure 5). The absorption of this ion was supported by the difference of concentration of ion content between in the medium and culture, so the amount of ion absorbed increasing. Decrease in the rate of ion absorption occurred after day 14 which also showed decreasing biomass growth rate as the age of explant increases.

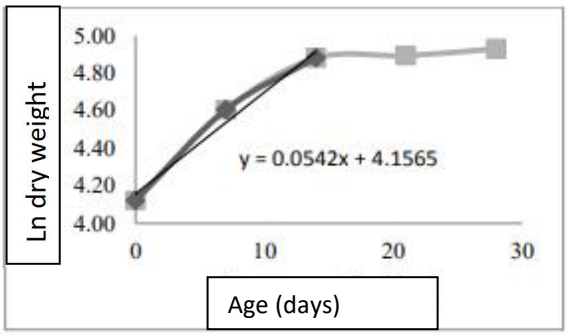

Figure 3. The growth kinetics curve of patchouli shoot culture

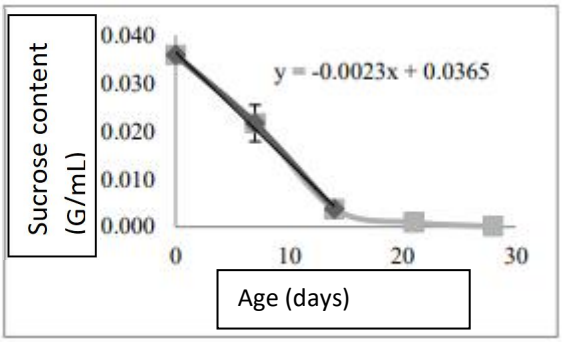

Figure 4. The curve of the sucrose consumption rate of patchouli shoots culture on the thin layer system

Harvest age was closely related to the plant growth phase that reflects the physiological maturity of the plant and had a strong relationship with the production and content present in the plant [15]. This was related to gene (PatPTS) expression in the biosynthetic pathway of secondary metabolites in plants that increase along with increasing plant age [16]. Other studies have shown that harvest age also had a significant effect on growth components such as plant height, number of branches, and number of leaves, and on patchouli crops such as fresh weight, dry weight, and oil yield [15].

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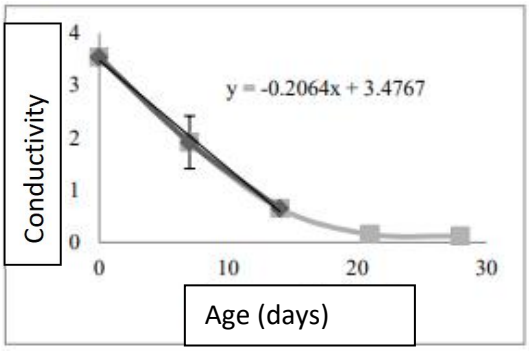

Figure 6. The curve of the conductivity value reduction rate of patchouli shoot culture on the thin layer system

The yield of patchouli oil obtained ranged from 2.34% to 3.23%. The sample of patchouli oil was then tested using gas chromatography-mass spectrometry (GCMS) method. The results obtained from the GC-MS test showed that there were farnesane (0.18% - 0.28%), eicosane (1.17% - 4.24%), squalene (0.35% - 0.46%), and most groups of alkanes and fatty acids, but no PA compounds found in patchouli oil for any variation in harvest age (Table 2).

Table 2. The component of patchouli oil analysis using GC-MS

Compound name	Age of harvest (days to-)							
	7		14		21		28	
	t (min.)	L (%)	t (min.)	L (%)	t (min.)	L (%)	t (min.)	L (%)
Farnesane	5.26	0.21	5.26	0.28	5.27	0.18	5.26	0.23
Eicosane	12.8	1.18	12.7	4.24	12.7	2.08	12.8	1.17
Squalene	19.9	0.35	-	-	19.9	0.46	-	-

t = retention time (min.); L = peak area (%)

Table 3. Mass balance of culture with harvest age of 7th day

	$C_{12}H_{22}O_{11}$	NH_4NO_3	O_2	$CH_{1,27}O_{0,43}N_{0,45}$	H_2O	CO_2
Reaction coefficient	0.39	0.23	3.43	1.00	4.07	3.64
MR (g/mol)	342	80	32	26.45	18	44
Reaction (mol)	0.00027	0.00016	0.0024	0.0007	0.0028	0.0025
Dry weight of hypothetical biomass (g)				0.080		
Dry weight of actual biomass (g)				0.100		

Table 4. Mass balance of culture with harvest age of 14th day

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	$C_{12}H_{22}O_{11}$	NH_4NO_3	O_2	$CH_{1,27}O_{0,43}N_{0,45}$	H_2O	CO_2
Reaction coefficient	0.39	0.23	3.43	1.00	4.07	3.64
MR (g/mol)	342	80	32	26.45	18	44
Reaction (mol)	0.00050	0.00029	0.0044	0.00127	0.0052	0.0046
Dry weight of hypothetical biomass (g)				0.096		
Dry weight of actual biomass (g)				0.132		

Table 5. Mass balance of culture with harvest age of 21st day

	$C_{12}H_{22}O_{11}$	NH_4NO_3	O_2	$CH_{1,27}O_{0,43}N_{0,45}$	H_2O	CO_2
Reaction coefficient	0.39	0.23	3.43	1.00	4.07	3.64
MR (g/mol)	342	80	32	26.45	18	44
Reaction (mol)	0.00052	0.0003	0.0046	0.00133	0.0054	0.0048
Dry weight of hypothetical biomass (g)				0.097		
Dry weight of actual biomass (g)				0.133		

Table 6. Mass balance of culture with harvest age of 28th day

	$C_{12}H_{22}O_{11}$	NH_4NO_3	O_2	$CH_{1,27}O_{0,43}N_{0,45}$	H_2O	CO_2
Reaction coefficient	0.39	0.23	3.43	1.00	4.07	3.64
MR (g/mol)	342	80	32	26.45	18	44
Reaction (mol)	0.00052	0.0003	0.0046	0.00134	0.0055	0.0049
Dry weight of hypothetical biomass (g)				0.098		
Dry weight of actual biomass (g)				0.138		

Research conducted by Yu et al. (2015) states that the patchouli alcohol content in patchouli oil was directly proportional to increasing plant age [16]. This was demonstrated by the absence of the patchouli alcohol compound in patchouli oil extracted from patchouli leaves at the early juvenile growth stage (1-2 weeks of age), but the compound was found in patchouli oil extracted from the leaves of the patchouli plant at 4 weeks old, despite its concentration still very low. The patchouli alcohol compound was synthesized by the role of patchoulol synthase (PatPTS) enzyme which converts farnesyl pyrophosphate (FPP) to patchouli alcohol. PatPTS gene expression increases with increasing plant age [16] leading to increased accumulation of patchouli alcohol compounds.

Trichome is a form of differentiation of epidermal cells and is the site of biosynthesis, secretion, and the accumulation of essential oils [17]. The increased number of trichome glands in the leaves was thought to increase the patchouli oil content [18]. The number of trichome glands has a positive correlation with sesquiterpenes which is a patchouli oil component [17]. Patchouli alcohol was the main sesquiterpene formed during the maturation process of plants, while harvesting was done in early juvenile growth stage, so patchouli

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plants have not started to synthesize patchouli alcohol compounds and other terpene compound groups [4]. In this study, it was suspected that the epidermal cells present in the leaves had not yet differentiated into trichome glands.

The calculation of mass balance was done by using approach to the reaction of root biomass formation of Atropa belladonna [11]:

$0{,}39C_{12}H_{22}O_{11} + 0{,}23NH_4NO_3 + 3{,}43O_2 \rightarrow CH_{1{,}27}O_{0{,}43}N_{0{,}45} + 4{,}07H_2O + 3{,}64CO_2$

The reaction of substrate conversion into biomass was based on the respiratory reaction occurring in the culture system. The calculation base used was sucrose as a carbon source.

Based on table 3 - 6, we found that the actual dry weight of patchouli biomass resulting from in vitro shoot culture in thin layer system obtained slightly higher than the dry weight of hypothetical patchouli biomass. This showed that the process bioconversion of the medium substrate into biomass in the thin layer culture system took place efficiently due to the suitable environmental conditions for the growth of patchouli shoot culture. The disadvantage of the mass balance calculation was that it did not know which substrate should be a limiting factor, thus affecting the growth rate of biomass [19]. In addition, shoot culture had different morphologically and physiologically components with roots, so that the structure and metabolism that occur in it must have a difference.

4. Conclusion

The influence of plant age of *P. cablin* Benth. to the growth of patchouli shoot biomass yields a growth rate of 0.054 mg dry weight per day with a doubling time of 12.78 days. The yield of patchouli oil obtained was 2.34% - 3.23%. The GC-MS test results show the presence of farnesane (0.18% - 0.28%), eicosane (1.17% - 4.24%), squalene (0.35% - 0.46%), and most alkanes and fatty acids, but did not show any patchouli alcohol content in the tested oil sample. Mass balance of shoot culture of *P. cablin* Benth. on the thin layer system for harvest age variation gives the result of a hypothetical biomass calculation similar to the actual biomass yield. It showed that the bioconversion of medium substrate into biomass in the shoot culture system of *P. cablin* Benth. on thin layer system occurred efficiently.

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The Impact of Herbicide Paraquat Dichloride Application on Weed Suppression and The Growth of Corn on Dry Land

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Abstract

The use of herbicides in agriculture is increasing with the availability of herbicides in the market. Herbicides are the second most used type of pesticide. Increased use of herbicides in agriculture affects non-target organisms, such as soil arthropods. Soil arthropods are important organisms in the breakdown of organic matter, mineralization of plant nutrients, increase soil aggregation, alter the physical properties of soil, stimulate succession, control pests as well as indicators of contamination. The purpose of this study was to test the impact of application of paraquat dichloride herbicides on corn growth and yield. The application of paraquat herbicides to corn growth has no significant effect on the diversity and abundance of arthropods and soil microorganisms (bacteria and fungi). The application of paraquat dichloride herbicides and carbosulfan insecticides to maize farms did not have a significant effect on the physical characteristics of the soil. The application of paraquat dichloride herbicide suppressed the growth of several types of weeds but does not affect the corn on dry land.

Keywords: Soil Arthropods, Paraquat Dioxide Herbicides, Species Dominance Index, Species Diversity Index

1. Introduction

The use of pesticides in agriculture continues to increase. In the year 2005 there were 1082 type of pesticides in Indonesia. In 2012, the number of pesticide types increased 2475. Based on the target of pesticide widely used is insecticide, herbicide, fungicide, Moluskisida, Rodentisida, Fumigan, Bactericide, Nematisida and so forth. Herbicide is one of the most widely used pesticide types [1]. Paraquat Dichloride is a group of Bipiridilium herbicides such as 1.1 '-dimethyl-4.4 '-bipyridinium dichloride or N, N '-dimethyl-4.4 '-bipyridinium dichloride. The empirical formula of Paraquat dichloride is C12H14N2Cl2, used as herbicide to control the growth of weed and grass in agriculture/plantation areas. [2].

Various number of brands of paraquat dichloride are being sold in Indonesia. This liquid entity is green colored and easily soluble with water. Paraquat is a contacts herbicide which

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activate in leaves, specifically in chloroplast. The plant that absorbs paraquat will impede the process of photosynthesis, specifically in photosystem I, which cause the reactive oxygen species (ROS) such as radical superoxide (O^{2-}) or peroxide acid (H_2O_2) that affects the membrane cell [3]. Lipid peroxidation is one of damage parameter which caused by the increasing number of ROS. This lipid degradation is caused by the reaction process between unsaturated fatty acid in the membrane cell and reactive oxygen species (ROS), which forming lipid hydroperoxides [4]. Accumulation of lipid hydroperoxide in the membrane cell could harm the function of membrane cell and cause the loss of cell permeability and leakage occur [5].

The negative effect of the use of pesticide is frequently higher than the level of target accuracy. The excessive number of pesticide usage in agriculture can potentially induce the pollution of agricultural land. More than 95% of herbicide use induced to water, plants, and non-target organisms such as soil arthropods [1]. Soil arthropods are an important organism that play role in the process of organic material decomposition, mineralization of plant nutrition, increasing the soil aggregation, changing soil's physical state, stimulate succession, and controlling pest as an indicator if there are any pollution [6]. Therefore, testing of impact application of herbicide paraquat dichloride towards the individual numbers, means of species, index of species domination, index of species variation, similarity index of Sorensen soil arthropods in corn field of Haurngombong's village, Sumedang, was conducted in this research.

2. Materials and Methods

This experiment was conducted at the ITB Experimental Field in Haurngombong Pamulihan Sumedang, from July to December 2018. Consisting of 25 plots in the dimensions of 5m x 4.5m/plot. The herbicide application was performed at day 35 after planting using different concentrations of paraquat dichloride, .33 mL/L water, 4.00 mL/L water, 4.66 mL/L water and 5.33 mL/L water, while control was treated with water only. The parameters observed were plant height, number of leaves, stem diameter and weed growth. For every plot of treatments was held on 5m x 4,5m and each of the plot was treated 5 times each repeatedly. The layout plan of treatment of corn planting and research sites are shown in Picture 1.

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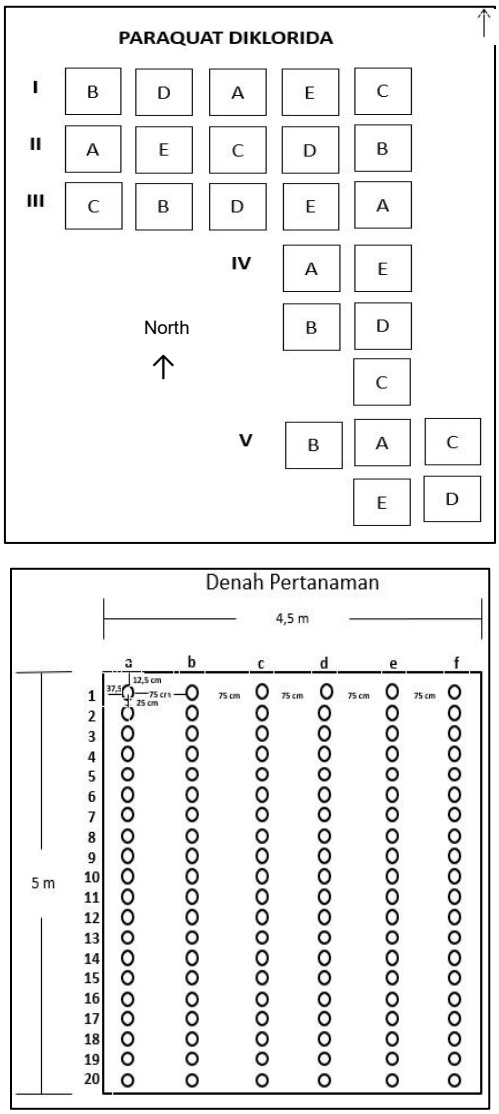

(a) (b)

Figure 1. Design layout (a) and The Corn Planting's Layout (b)

Note:

1. Treatment : A, B, C, D, and E
2. Repetitions : I, II, III, V, and V
3. Size of Partition : 5 m x 4,5 m
4. Soil's Distance : 25 cm x 75 cm
5. Partition's Distance : 1 m
6. Number of Population each Partition : 100 plant

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3. Result and Discussion

The results showed that the treatment of different concentrations of paraquat dichloride reduced plant height, number of leaves, and diameter of corn stems at the age of 60 and 90 days after planting are shown in Picture 2, Picture 3 and Picture 4. Similar effect was also observed on the growth of weeds. The treatment of 4.00 mL / L of water showed an average plant height of 84.10 cm. The treatment of 5.33 mL / L of water showed the highest average number of leaves (6.54), while that of 4.66 mL/L of water induced the largest average stem diameter (1.77cm).

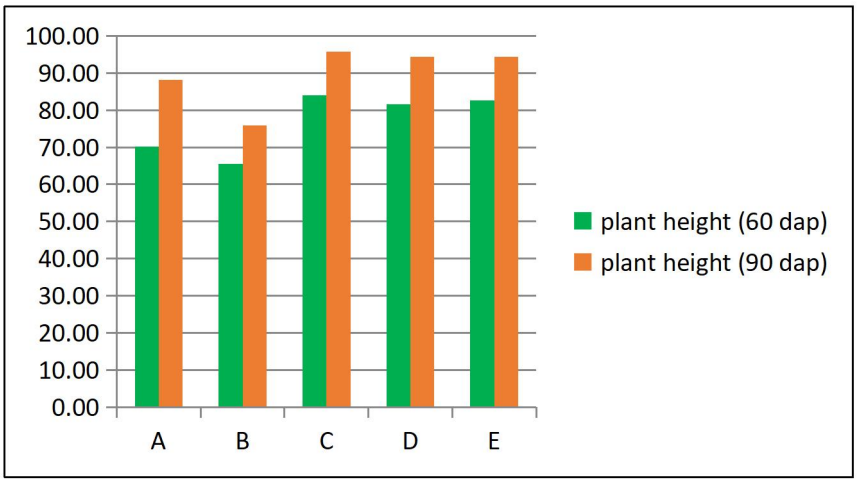

Figure 2. The effect effect concentration of paraquat dichloride on plant height on 60 dap and 90 dap

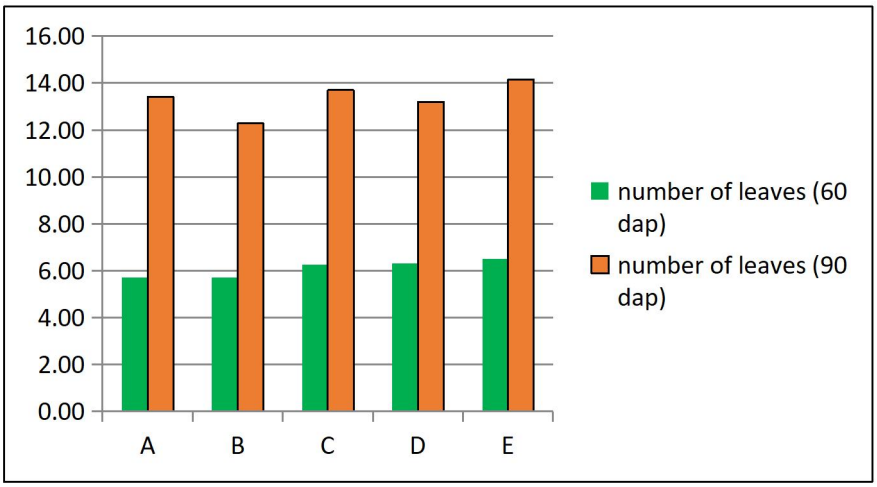

Figure 3. The effect effect concentration of paraquat dichloride on number of leaves on 60 dap and 90 dap

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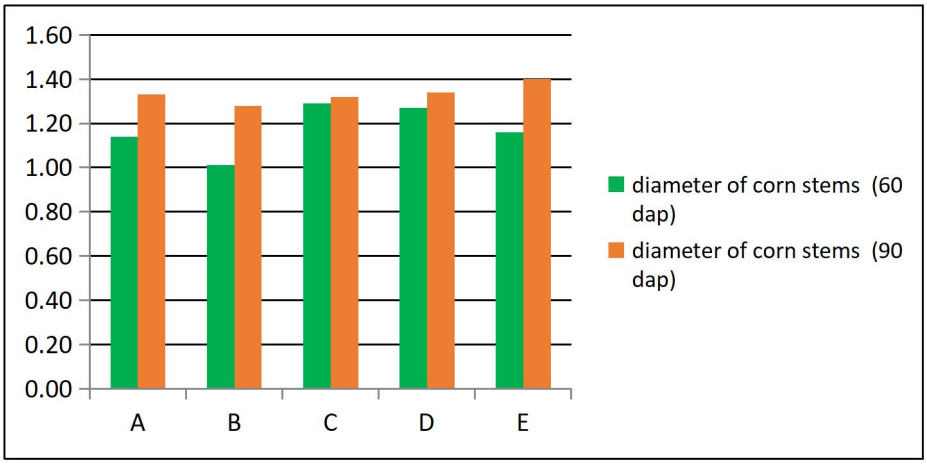

Figure 4. The effect concentration of paraquat dichloride on diameters of corn stem on 60 dap and 90 dap

The condition of the field of the research site that located in Haurgombong, Sumedang in the dry season was extremely hot. Moreover, the water availability was very limited. In this dry season, rain rarely falls, which causing drought in the research site. When the time of collecting T3's data (before the harvest) rain was more often to fall. The pH and relative humidity test at the research sites shown by Table 1. From the result of the research, the soil's pH is relatively neutral. Then the relative humidity of the soil is significantly increasing at the end of the research, because at the end of the research the rainy season begin to starts.

Table 1. Soil's pH and relative humidity of Paraquat Dichloride

	At the Beginning		At the End	
	Soil's pH	Relative Humidity (%)	Soil's pH	Relative Humidity (%)
Avg.	6,9	15,4	6,8	66,9
Max. Value	6,9	37,5	7	90
Min. Value	6,9	5,0	6,4	50

The results of the research in shown that the overall average of bulk density is 0,960 ± 0,273 g/cm^3. The result of the research, which shown by Table 4.25, average of soil's permeability is 5,074 ± 0,4 cm/hour, that can be categorized as moderate/ medium permeability (2-6,25 cm/hour). The data of the texture of the soil can be seen in Table 2 and the whole data of soil's chemical analysis can be seen in Table 3.

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Table 2. Result of Soil's Texture

Soil's Texture (%)	Paraquat dichloride					
	Before			After		
	Avg.	Max. value	Min. Value	Avg.	Max. value	Min. Value
Sand	9,333±2,517	12,000	7,000	7,000±1,000	8	6
Dust/Grit	27,333±2,887	29,000	24,000	26,000±1,000	27	25
Clay	63,333±1,155	64,000	62,000	67,0000,000	67	67

Tabel 3. Result of Soil's Chemical Analysis

PARA METER	Paraquat diklorida					
	Before			After		
	Avg.	Max. value	Min. Value	Avg.	Max. value	Min. Value
pH(H_2O)	5,283±0,133	5,370	5,130	6,067±0,623	6,65	5,41
C-organic (%)	1,333±0,202	1,450	1,100	1,510±0,157	1,65	1,34
N-total (%)	0,177±0,029	0,210	0,160	0,167±0,021	0,19	0,15
C/N	7,667±1,155	9,000	7,000	9,333±1,528	11	8
P_2O_5 HCl 25% (mg/100g)	250,340±13,536	263,790	236,720	225,570±48,611	280,84	189,45
P_2O_5 (Bray) (ppm P)	53,417±27,878	84,190	29,850	22,087±11,488	30,19	8,94
K_2O HCl 25% (mg/100 g)	55,000±26,133	77,290	26,240	35,933±29,291	63,39	5,1
KTK (cmol/kg)	18,370±0,686	19,160	17,920	17,510±0,468	17,92	17

The growth of corn plant results shown after the spray of pesticide with paraquat dichloride active in several different concentrate affects the height of the plants, number of leaves growth, and the diameter of stalk of the corn plant. In case of parameter of height of the plants, the highest average of plants height occurs in the C treatment area, with the plants average of height of 84,10 cm. But the C treatment area does not make any concrete/significant results compared to other treatment area. For the highest number of leaves parameter, the E treatment area has the most number of leaves among any other treatment area, with the average of 6,54. But, this also does not concretely afflicts to the matter of number of leaves. The result of C treatment area show the average number of leave

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of 6,26. In the parameter of widest stalk diameter, the D treatment area shown to have the widest average of stalk diameter, with 1,77cm. But this also does not make any concrete different to the stalk diameter of the plant. In this research, the corn plant with carbosulfan treatment does not grow, so there is no data for this type of plant with that treatment. Before the research is conducted, an observation of dominant weeds at the research sites was held, there are several dominant types of weeds in the paraquat dichloride research plot: *Mimosa pudica* L., Asystasia gangetica (L.) T. Anderson., *Alternanthera bettzickiana* (Regel) G. Nicholson, *Cynodon dactylon (L.) Pers.* Other than that, there also a number of weeds that grow only at the plot of carbosulfan treatment area, which are: *Eleusine indica* (L.) Gaertn., and *Panicum repens* L.

Table 4. Average growth of the corm plant from 60 days to 90 days

Treatment	60 days after plant			
Paraquat Dichloride	Plant's Height (cm)	Number of leaves		Stalk's diameter (cm)
A	70,22^{ab}	5,72^{a}		1,14^{a}
B	65,50^{a}	5,72^{a}		1,01^{a}
C	84,10^{b}	6,26^{ab}		1,29^{a}
D	81,58^{ab}	6,30^{ab}		1,77^{a}
E	82,60^{b}	6,54^{b}		1,16^{b}
	90 hst			
Paraquat Dichloride	Plant's Height (cm)	Number of leaves	Stalk's diameter	Number of Corncob
A	88,08^{ab}	13,42^{ab}	1,14^{a}	1,33^{a}
B	75,88^{a}	12,28^{a}	1,01^{a}	1,28^{a}
C	95,84^{b}	13,70^{ab}	1,29^{a}	1,32^{a}
D	94,30^{ab}	13,20^{a}	1,77^{a}	1,34^{a}
E	94,42^{b}	14,16^{b}	1,16^{b}	1,40^{a}

4. Conclusion

The application of herbicide paraquat dichloride and carbosulfan insecticide to the corn plant does not affects significantly towards the variety and excessive amounts of arthropods and micro-organism (bacteria and fungi). The application of herbicide paraquat dichloride and carbosulfan insecticide to the corn plant does not affects to the physical and chemical charateristics/condition of the soil of the plantation. Land tenure before the plantation and rain intensity is more effective to affects the number of variety and population of arthropods and soil's micro-organism compare to the pesticide spraying method. The results of the research, can be concluded that herbicide paraquat dichloride does not affects ($p>0,05$) towards the individual numbers, means of species, index of species domination, index of species variation, similarity index of Sorensen arthropods shown that the composition of soil

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arthropods with the control and treatment of highest concentrate has the high similarity because the index of Sorensen indicating over 50%. The application of paraquat dichloride herbicide suppressed the growth of several types of weeds but does not affect the corn on dry land.

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2. Dean of School of Life Sciences and Technology, Institut Teknologi Bandung

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Characteristic Identification of The Chalcone Synthase Gene in Banana (*Musa acuminata* cv. Barangan) Affected by Drought Stress using Bioinformatics Approach

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Abstract

Banana which planted on marginal lands often susceptible to drought stress due to a lack of capillary water in the soil, making it difficult for plants to get water. Mechanisme of plant defense against drought stress still has to be studied further. One of the defensive mechanisms of banana plants against drought stress is by expressing genes that will produce chalcone synthase enzyme related to flavonoid biosynthesis. Chalcone synthase enzyme plays a role in the formation of naringenin which will be used as a substrate for the synthesis of specific flavonoids. Analysis of the gene forming the chalcone synthase enzyme needs to be done to determine the phylogenetics, structural characteristics and pathways of their formation. The analysis showed that chalcone synthase in Musa acuminata was included in the same clade as Arabidopsis thaliana and Vitis vinefera. The differences in chalcone synthase structure are at the ends of sequences 1-83 and 278 - 395. The physicochemichal characteristics of the two genes have the same characteristics. The biosynthetic pathway for the chalcone synthase enzyme is derived from flavonoid biosynthesis by converting p-Coumaroyl CoA to naringenin chalcone through the addition of H atoms, malonyl CoA, and CoA leaching.

Keywords: chalcone synthase, drought stress, flavonoid biosynthesis, naringenin

1. Introduction

Banana (*Musa acuminata* cv. Barangan) is a tropical plant that is favored by the people of Indonesia. Apart from its delicious and sweet taste, this fruit is also easy to obtain and cultivate. Farmers tend to ignore the optimization of banana cultivation because this plant is easy to grow so they underestimate the potential of this fruit in business. Farmers plant bananas as a by-crop on marginal lands so that banana production is less than optimal. Marginal land is land that has the characteristics of clay and dry soil, making it difficult for cultivated plants to obtain water sources [1]. This has resulted in poor banana production among farmers, low production and unsold markets. The stress experienced by banana plants, especially on marginal land, is the lack of available capillary water. The narrow soil pores and the small size of soil particle making it difficult to store water. Lack of water in the soil will result in difficult ion exchange in the roots and changes in osmotic pressure between cells and the environment. This condition will encourage plant cells to release water into the environment by transpiration so that plants will experience drought [2]. One of the actions taken by plants in dealing with drought stress is by changing the metabolic pathway through gene expression.

Profiling transcriptome in *M. acuminata* Colla cv. Barangan Merah has been carried out previously by the Plant Science and Biotechnology scientific group, SITH ITB. Total RNA sources were obtained from plantlets induced by drought stress with PEG concentrations of 2.5%, 7.5%, and 10%. Total RNA was produced from all parts of the banana plant that were tolerant according to the procedure [3]. Sequencing was carried out with Illumina MiSeq 2000. The results of the sequencing showed that there were 104,118,407 nucleotide bases that were identified and successfully assembled into 147,811 contigs. The shortest contig has 199 base pairs while the longest contig has 7393 base pairs. The contig data were then reconstructed using the de novo assembly method on the Pahang DH banana genome available at CIRAD (https://www.cirad.fr/) and DEG analysis was performed to obtain 929 genes with altered expression compared to controls. Based on the results of gene ontology analysis, it was found that five major biological processes were affected, namely: photosynthesis, response to stress, cellular respiration, morphogenesis and organ development, and biosynthesis of secondary metabolites (manuscript in preparation).

In secondary metabolite biosynthesis, flavonoids are one of the compounds capable of acting as antioxidants and signaling molecules used to respond to stress. Oxidation in cells can result in the formation of excess reactive oxygen species (ROS) in cells so that they are toxic and can kill cells [4]. The presence of flavonoids will regulate the ROS degradation enzymes present in chloroplasts, mitochondria and peroxisomes so that the number of ROS in cells can reach the point of homeostasis [5]. One of the genes related to flavonoid biosynthesis that has high expression is chalcone synthase. Chalcone synthase is an enzyme that converts p-Coumaroyl CoA substrate into naringenin chalcone by adding H atoms, malonyl CoA, and CoA leaching [6]. Chalcone synthase is an enzyme that is very important in the formation of flavonoids because this enzyme functions in building flavonoid construction which consists of three carbon rings (C6-C3-C6) [7]. Analysis of chalcone synthase in *M. acuminata* cv. Barangan needs to be done to determine its phylogenetics, characteristics, and biosynthetic pathways as a study in understanding the defensive mechanism of banana plants against drought stress.

2. Materials and Methods

2.1 Materials

Transcriptome data obtained from Plant Science and Biotechnology research group SITH ITB. The transcriptome data obtained from banana (*M. acuminata* cv. Barangan Merah) plantlet that were induced by drought stress. Drought stress induced by giving PEG 6000 solution (2,5%, 7,5% and 10%) which is equivalent to drought conditions with a potential water of -0.19 bar, -0,93 bar and -1.48 bar [8].

2.2 Methods

2.2.1 Phylogenetic Analysis

Phylogenetic analysis was carried out on the amino acid sequence of chalcone synthase proteins from various types of plants with algae as outgroup. The amino acid sequence of the chalcone synthase protein was obtained from the NCBI website (https://www.ncbi.nlm.nih.gov/). The amino acid sequence used came from the plants

Nicotiana tabacum (NP_001312634.1), Capsicum annuum (AFL47798.1), Solanum lycopersicum (AEK99072.1), Solanum tuberosum (AGU70032.1), Vitis vinifera (NP_001268064.1), Zea Mays (NP_001149508.1), and Glycine max (NP_001304585.2). Sequence of the chalcone synthase protein from *M. acuminata* obtained from transcriptome data and will be compared with the model plant Arabidopsis thaliana (NP_196897.1), while Galdieria sulphuraria (EME29910.1) is an outgroup in phylogenetic analysis. Alignment was carried out on all amino acid sequences using MEGA7 software to create phylogenetic trees based on the neighbor joining method with bootstrap 1000 replications. The distance in the phylogenetic tree is represented by a scale indicating evolutionary distance and the substitution number indicates the degree of confidence of the formed phylogenetic tree.

2.2.2 Protein Structure Analysis

Protein structure analysis was carried out by comparing the structure of the chalcone synthase protein in Musa acuminata plant with Arabidopsis thaliana model plants. Analysis was performed by predicting secondary protein structures with PredictProtein (https://open.predictprotein.org/) and predicting the tertiary structure of proteins with SwissModel (http://swissmodel.expasy.org/interactive). Analysis of physicochemichal properties consists of hydrophobicity, solubility, isoelectric point and protein molecular weight. Protein hydrophobicity was analyzed by Kyte-Dollylte Hydropathy Plot (https://fasta.bioch.virginia.edu/fasta_www2/fasta_www.cgi?rm=misc1), protein solubility was analyzed by Protein Sol (https: //protein-sol.manchester. ac.uk/), and the isoelectric point and molecular weight were analyzed by Compute pi / Mw (http://web.expasy.org/compute_pi/).

2.2.3 Pathway Analysis

Biosynthetic pathway analysis was carried out to see the process of formation of naringenin chalcone. KEGG was used to observe the chalcone synthase biosynthetic pathway in Arabidopsis thaliana model plants. Gene ontology refers to Uniprot (https://uniprot.org/) and TAIR (https://arabidopsis.org/).

3. Results and Discussion

3.1 Phylogenetic Analysis

The chalcone synthase from plants compared to banana plants produced a phylogenetic tree showing the proximity of chalcone synthase from Musa acuminata to Vitis vinivera and Arabidopsis thaliana (Figure 1). Other shrubs gathered in one clade, while Zea Mays has quite a difference with other clades. Algae are used as an outgroup to see the differences in chalcone synthase produced by plants and algae. Flavonoids are generally produced in the endoplasmic reticulum of cells and transported to ROS-producing organelles such as chloroplasts and miticondria as substances that are oxidized by ROS so that cells can survive drought stress [9].

Phylogenetic analysis was used using neighbor joining method with bootsrap 1000 times. Neighbor joining is an analysis based on the distance method where this method compares

the distance of gene changes by positioning all taxa on one node, then progressively comparing taxa based on changes in gene distance [10]. The phylogenetic tree shows that plants other than Zea Mays have a fairly close range of gene changes with the smallest confidence score. This condition indicates that chalcone synthase has evolved which separates Zea Mays from other plants. The evolution of chalcone synthase in Musa acuminata and Arabidopsis thaliana as model plants has a close distance so that phylogenetically, the chalcone synthase produced by the two plants is not much different.

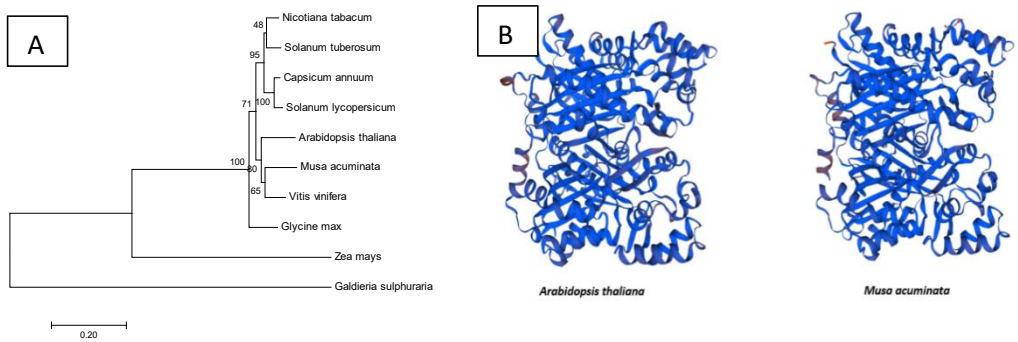


Figure 1. Phylogenetic analysis (A) and the structure of the enzyme chalcone synthase (B) . Phylogenetic trees were arranged by neighbor joining method using algae as outgroups and bootstrap 1000 times. The differences in protein structure exist in the initial and rear ends of chalcone synthase.

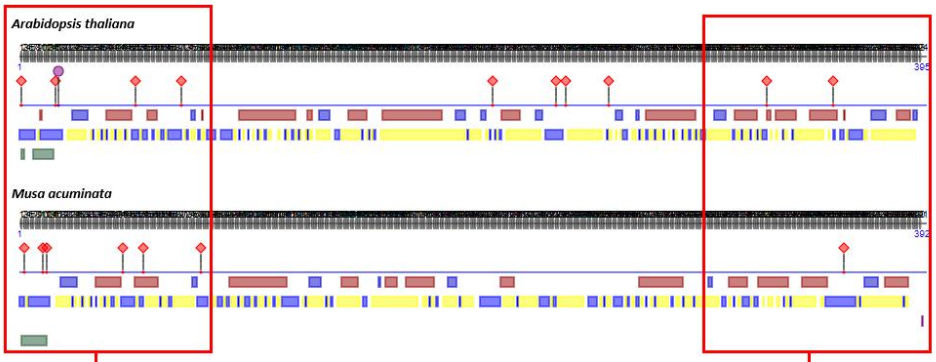
3.2 Protein Structure Analysis

The results of the chalcone synthase protein analysis showed the prediction of differences in the active side of the protein in changing the substrate into the targeted compound. Based on its structure, the chalcone synthase protein in the model plant has differences in the initial and rear ends when compared to banana plants. This is also supported by the differences in the sequences that have been cut with trypsin to see differences in structure. The method of cutting sequences with trypsin is generally carried out to see the integrity of the secondary structure of a protein [11]. The results of cutting the sequences with trypsin showed different pieces of sequences at ends 1-83 and 278 - 395, while the sequences 84-277 produced similar cut sequences (Figure 2). Analysis of physicochemichal properties between the two proteins showed hydrophobicity which was both hydrophobic (Table 1). The solubility level and the isoelectric point are higher in Musa acuminata chalcone synthase, while the higher molecular weight is chalcone synthase Arabidopsis thaliana.

Table 1. Comparison of the physicochemical properties of chalcone synthase between Arabidopsis thaliana and Musa acuminata model plants.

Characteristics	*Arabidopsis thaliana*	*Musa Acuminata*
Hydrophobicity	Hydrophobic	Hydrophobic
Solubility	0,371	0,382
Isoelectric point (Pi)	6,08	6,11
Molecular mass (mw)	43115,72	42892,51

Chalcone synthase is an enzyme that plays a role in the biosynthesis of flavonoids. The protein structure consisting of 4 amino acids (Cys164, Phe215, His303, and Asn336) located in the binding region of CoA and coumaroyl has a role in the decarboxylation of malonyl CoA and the formation of chalcone [6]. This area is an area that has similar sequences in both Musa acuminata and Arabidopsis thaliana, so that functionally, both plants have enzymes with the same role in flavonoid biosynthesis.



Arabidopsis thaliana		*Musa acuminata*	
Location	Amino Acid Sequence	Location	Amino Acid Sequence
1-13	MVMAGASSLDEIR	1-8	MADLQEIR
14-17	QAQR	13-42	AEGPAAVLAIGTATPANVL YQADYPDYYFR
18-47	ADGPAGILAIGTANPENHV LQAEYPDYYFR	46-53	SDHLTELK
48-58	ITNSEHMTDLK	63-66	SMIR
74-83	HMHLTEEFLK	69-78	YMHLNEEILK

Arabidopsis thaliana		*Musa acuminata*	
Location	Amino Acid Sequence	Location	Amino Acid Sequence
288-322	SLDEAFKPLGISDWNS LFWIAHPGGPAILDQV EIK	278-281	NIEK
335-356	HVLSEYGNMSSACVL FILDEMR	282-316	SLVEAFKPLGIDDW NSIFWIAHPGGPAIL DQVEAK
362-395	DGVATTGEGLEWGVL FGFGPGLTVETVVLHS VPL	317-321	IGLQK
		324-328	MQATR
		330-350	VLSEYGNMSSACVL FILDEMR
		353-358	SAADGK
		359-392	ATTGEGLEWGVLFG FGPGLTVETVVLHS MPIVAN

Figure 2. Prediction of protein structure and active site of the chalcone synthase enzyme. The difference in the sequences at the beginning and the end shows the difference in the enzyme structure between Musa acuminata and Arabidopsis thaliana.

3.3 Pathway Analysis

Flavonoid biosynthesis was initiated from the change of cinnamoyl CoA as a substrate to p-Coumaroyl CoA (Figure 3). The conversion of cinnamoyl CoA to p-Coumaroyl CoA is carried out by the enzyme Cinnamate-4-hydroxylase (C4H) which acts to oxidize NADPH-hemoprotein oxidase (FMNH2) [12]. This reaction releases the H atom on FMNH2 to form trans 4 coumarate as a backbone in the formation of flavonoids. p-Coumaroyl CoA will be converted into naringenin chalcone by the chalcone synthase enzyme through the addition of H atoms, malonyl CoA, and CoA leaching [6]. The naringenin chalcone will be converted into naringenin by the chalcone isomerase enzyme. Naringenin will then become the backbone for the formation of more specific flavonoid compounds. The formation of specific flavonoids depends on cytochrom P450 which functions to add H groups to the flavonoid ring [13].

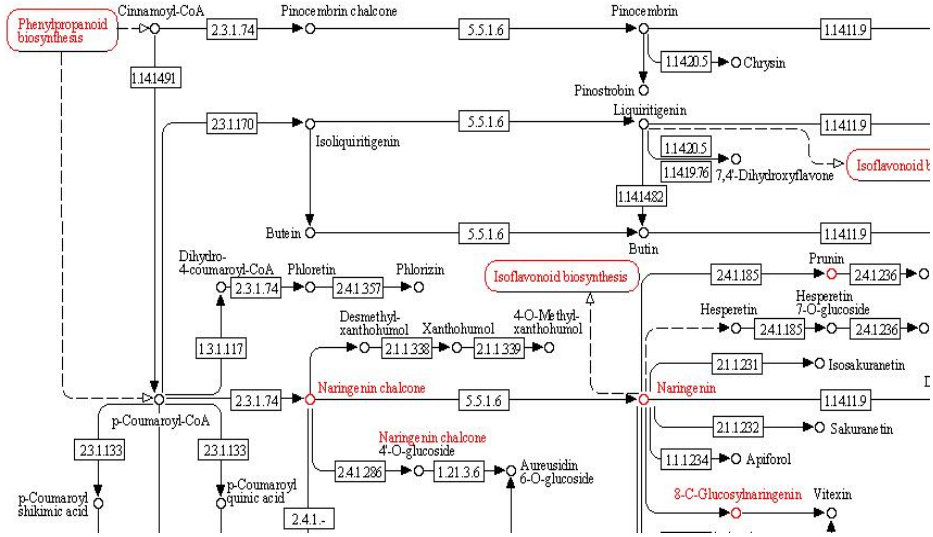


Figure 3. Flavonoid biosynthetic pathway involves the chalcone synthase enzyme (enzyme code KEGG 2.3.1.74) in several reactions. Naringenin is the main compound used to make specific flavonoids according to their roles and functions.

4. Conclusion

The chalcone synthase in Musa acuminata belongs to the same clade as Arabidopsis thaliana and Vitis vinefera. Differences in the structure of chalcone synthase are at the ends of sequences 1-83 and 278 - 395. Different physicochemichal characteristics exist in all aspects of the analysis, but have the same characteristics. The biosynthetic pathway for the chalcone synthase enzyme is derived from flavonoid biosynthesis by converting p-Coumaroyl CoA to naringenin chalcone by adding H atoms, malonyl CoA, and releasing CoA.

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Biomass Production of *Chlorella vulgaris* in Monoculture and Mixed Culture Systems with *Scenedesmus sp.* Using a Vertical Tank Reactor and Ability to Reduce Nitrate and Ammonium Levels from ADDMW and Walne Medium

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Abstract

In this study, the cultivation of *Chlorella vulgaris* was carried out in monoculture and mixed culture with *Scenedesmus* sp. in a 20 L scale Vertical Tank Reactor on anaerobically digested dairy manure waste (ADDMW) as a comparison with Walne medium. In the mixed culture treatment, the culture volume of *C. vulgaris* was three times as much as the volume of the *Scenedesmus* sp. The Culture was observed for 7 days. The results obtained in this study were the highest specific growth rate produced in the treatment of *C. vulgaris* with Walne medium of 0.407 $days^{-1}$. The highest percentage and reduction rate of nitrate reduction was produced in mixed culture with Walne medium of 70.3% and *C. vulgaris* treatment with ADDMW medium of 0.601 ppm day^{-1}. The highest percentage and reduction rate of ammonium was produced in mixed culture with Walne medium of 67.9% and mixed culture with Walne medium of 0.181 ppm day^{-1}. From the results obtained, optimization is needed for the application of mixed culture and administration of ADDMW medium. Such as mixed culture is done with the ratio of the number of cells between species and pre-treatment on ADDMW medium is done more optimally.

Keywords: *Chlorella vulgaris, Scenedesmus sp.,* ADDMW, vertical tank reactor, mixed culture

1. Introduction

Microalgae are types of phytoplankton that have relatively fast growth. Microalgae can live in freshwater and seawater environments, so they can be found in open space. Microalgae tend to have chlorophyll so they can produce their food (autotrophs) [5]. The nutrients needed by microalgae are almost similar to plants, these nutrients are nitrogen and phosphate. These nutrients can be found in cow manure which can be found in livestock areas [6].

The primary metabolites produced are proteins, lipids, and carbohydrates. Meanwhile, the secondary metabolites produced are chlorophyll, and other compounds [7]. The use of mixed cultures of microalgae can increase the production of bioproducts due to the symbiosis between species [1].

The scale-up process needs careful consideration to optimize the growth of microalgae, one form of optimization that can be done by the use of a controlled bioreactor system tailored to biological processes that occur in microalgae to run effectively and efficiently [8]. In this study, the scale-up process of microalgae cultivation was carried out in a 20 L Vertical Tank Reactor (VTR) with Walne medium and ADDMW medium for cultivation medium with variation ratio between species with *C.vulgaris* as monoculture and mixed cultures with *Scenedesmus* sp.

2. Materials and Methods

2.1 Materials

C.vulgaris and *Scenedemus* sp. are obtained from the Indonesian Institute of Sciences (LIPI). Walne medium is obtained from phytoplankton fertilizer supplier from Semarang, anaerobically digested dairy manure waste (ADDMW) is obtained from biogas production installation at Padjadjaran University, West Java

Bioreactors used for microalgae cultivation are vertical tank reactor (VTR) (Figure 1) with 25 cm diameter and 50 cm height and total volume is 20 L. The base material used is acrylic with a thickness of 5 mm. Light exposure is given through two LED lights with a light intensity of 25 µmol m^{-2} s^{-1}. Aeration is administered at a rate 2 L/min in each bioreactor. Aeration is passed DURAN sparger® Filter Discs with a diameter of 50 mm and porosity of 3 (150-250 microns) before entering the microalgae culture.

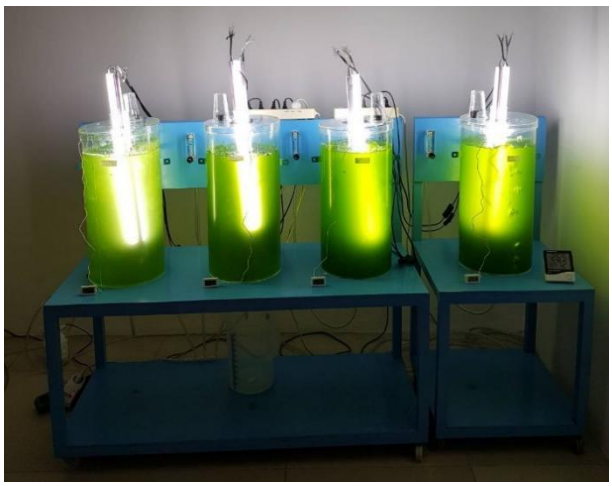

Figure 1 Vertical tank reactor (VTR)

2.2 Methods

2.2.1 Preparation of Medium Cultivation

The cultivation medium used in this study is ADDMW medium, with comparison (control) in the form of Walne medium. ADDMW medium is first inserted into a 1 L glass bottle and then sterilized using autoclave at 121°C and pressure 1.5 bar for 10 minutes. The sterilized ADDMW medium is then filtered using fat cotton so that the solid and liquid fractions are separated. The liquid fraction is used as a cultivation medium, with dilution 4 times.

Walne medium made with Walne medium stock (100%) mixed with aquadest. Walne medium stock is taken as much as 1 mL and mixed with aquadest until reached volume 1 L. Walne medium stock and aquadest medium comparison is 1 mL:1 L.

2.2.2 Microalgae Cultivation for Multiplication

The cultivation of microalgae multiplication is carried out with monoculture at two stages of cultivation. Microalgae inoculum is cultivated as much as 20% of the total volume of culture with 80% cultivation medium. Light exposure is given with a light-dark ratio (photoperiodism) of 16h:8h.

The first stage is performed at a volume of 800 mL with a glass bottle of 1 L volume, the amount of volume produced in the first stage as much as 2 L for each species. The second stage is performed on a vertical tank reactor (VTR), with the volume produced in the second stage as much as 10L for each species.

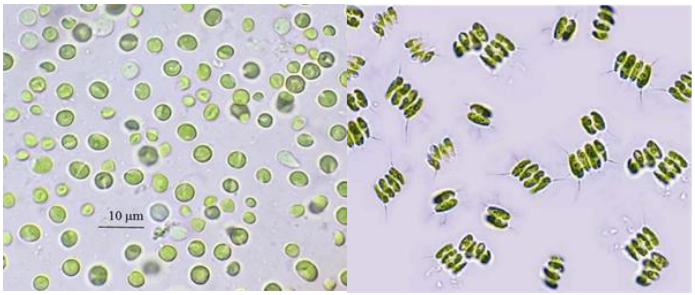

Figure 2 *Chlorella vulgaris* (left) [23] and *Scenedesmus* sp. (right) [24]

2.2.3 Microalgae Cultivation with Treatment

Microalgae cultivation is divided into 2 treatments, with cultural conditions in the form of *C.vulgaris* monoculture and mixed culture *C.vulgaris:Scenedesmus* sp. (3:1), by comparison of volume ratio.

Cultivation is carried out in Vertical Tank Reactor (VTR) with 25 cm diameter and height of 50 cm, and culture volume as much as 20 L. Microalgae inoculum is cultivated as much as 20% of the total volume of culture. Aeration is administered by an air pump at an aeration rate of 3 L/min. Cultivation is carried out over for 7 days.

2.2.4 pH Measurement

Measurement of cultural conditions is carried out at pH with a time span of 12 hours/ triplo measurement. pH measurement is done using pH meter.

2.2.5 Cell Number Measurement

Measurement of the cell number is carried out using a Hemacytometer. Biomass samples are sampled as much as 10 mL on each measurement. The sample is dripped on hemacytometer so that it fills the space on the hemacytometer. Then, the hemacytometer is closed with a glass cover. Sample-filled hemacytometer are observed in light microscopes. The number of microalgae cells is found on the four main grids of the hemacytometer. After the measurement of the number of microalgae cells on the four main grids, determined the average value. The average number of cells is multiplied by 10^4. The calculation of the number of cells is done triplo and carried out in 7 days with an interval of 12 hours

/measurement. The specific growth rate is determined based on the gradient on the growth curve.

2.2.6 Specific Growth Rate Determination

The specific growth rate (day^{-1}) is obtained by the equation:

$$\mu = \frac{1}{t} \ln \left(\frac{Nt}{No}\right) \quad [1]$$

with Nt and No is the number of cells at the end and the number of cells at the beginning in the exponential phase range, gradually. While t is an exponential time (day)

2.2.7 Inorganic Compound Measurement on Cultivation Medium

Measurement of inorganic compounds in the cultivation medium is intended for the measurement of nitrate and ammonium levels. Measurements are carried out at the beginning and end of microalgae cultivation with triplo. Measurement of nitrate and ammonium levels is performed with Vernier© Nitrate and Ammonium Ion-Selective Electrode. Calibration is performed before measurement using a solution of nitrate and ammonium stock at concentrations of 1 ppm and 100 ppm.

2.2.8 Rate and Percentage Reduction of Inorganic Compound in Cultivation Medium

The rate of reduction of inorganic compounds (ppm day^{-1}) in the cultivation medium is calculated by the equation:

$$v = \frac{Co - Ct}{t} \quad [2]$$

with Co and Ct are the initial and final concentrations (ppm) of inorganic compounds. While t is the time of cultivation (days).

Percentage reduction of inorganic compounds (%) in the cultivation medium is calculated by equation:

$$\%E = \frac{Co - Ct}{t} x100\% \quad [3]$$

with Co and Ct are the initial and final concentrations (ppm) of inorganic compounds. While t is the time of cultivation (days).

3. Results and Discussions

3.1 Elaboration of Vertical Tank Reactor

The bioreactor used for microalgae cultivation is a vertical tank reactor (VTR) with batch treatment for medium administration. This bioreactor is a closed system to prevent contamination from other microorganisms by minimizing direct contact with atmospheric air [9]. This bioreactor design has a lighting system inside the bioreactor, as in Figure 3. The advantage of this bioreactor design is that it can increase the contact of the microalgae culture with the light source and the contact between gas and liquid phases so the residence time of the air given to the culture is getting bigger [8].

The aeration system given to this bioreactor comes from the bottom of the bioreactor, with the air passed sparger to produce small, uniformly sized bubbles. The advantage of this aeration system is the evenly mixing of the cultures, which prevents sedimentation of microalgae at the bottom of the bioreactor [10]. The sparger is used to reduce shear stress to microalgae and increase gas hold up and volumetric mass transfer in microalgae cultures [11].

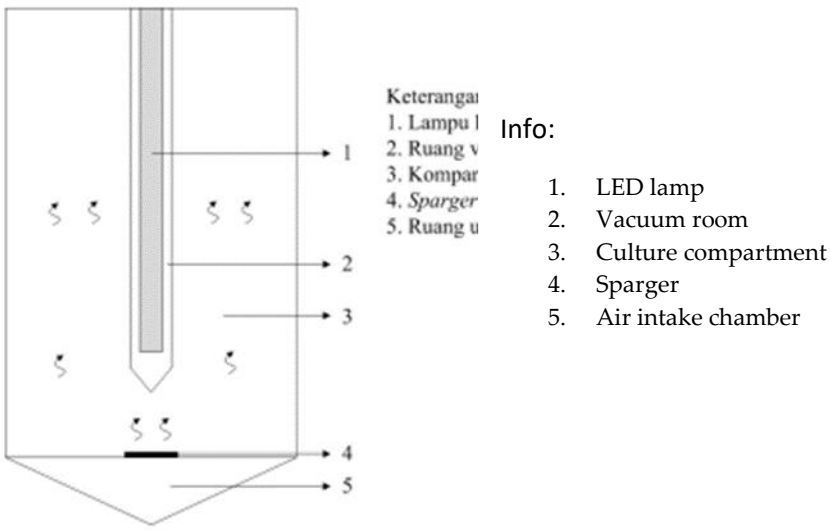

Figure 3 Vertical tank reactor design

3.2 Effect of Mixed Culture on Microalgae Growth

Cultivation of microalgae with culture conditions in monoculture and mixed culture can produce different specific growth rates. In Table 1, it can be seen that different culture conditions can affect the specific growth rate. The highest specific growth rate value in cultivation carried out with Walne medium was obtained in monoculture with a specific growth rate of 0.407 $days^{-1}$. When cultivation was carried out on ADDMW medium, the highest biomass growth rate was obtained in mixed culture with a specific growth rate of 0.344 $days^{-1}$.

The highest specific growth rate was obtained in monoculture with Walne medium (0.407 days-1), while the lowest specific growth rate was obtained in mixed culture treatment with Walne medium (0.308 days-1). The growth rate with ADDMW medium in monoculture and mixed culture was relatively the same (0.321 and 0.344 day-1).

The growth of monoculture and mixed culture with Walne medium showed a difference (Figure 4). In monoculture, microalgae experienced a relatively higher growth compared to mixed cultures. The lag phase of monoculture was shown in the initial cultivation range to t = 22 hours, then entered the exponential stage until t = 106 hours, then entered the stationary stage and decreased at the end of cultivation. Meanwhile, the mixed culture showed a lag phase that was relatively similar to monoculture, at t = 22 hours. The exponential phase is shown in the range t = 22 hours to t = 130 hours and the next phase enters the stationary phase, then decreases until the end of cultivation.

The growth in monocultures and mixed cultures with ADDMW medium showed relatively similar growth between treatments (Figure 5). In mixed culture, the exponential phase began to occur at t = 34 hours, faster than in monoculture that began at t = 48 hours. Meanwhile, the two treatments entered the stationary phase at relatively the same time, namely at t = 149 hours.

Table 1 Specific Growth Rate (day^{-1}) from each culture condition and medium

Culture condition	Walne	ADDMW
Monoculture	0,407	0,321
Mixed culture	0,308	0,344

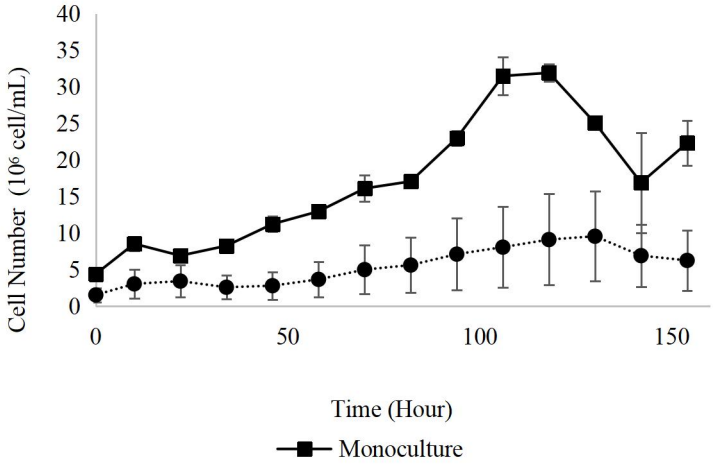

Figure 4 Growth curve microalgae in Walne medium

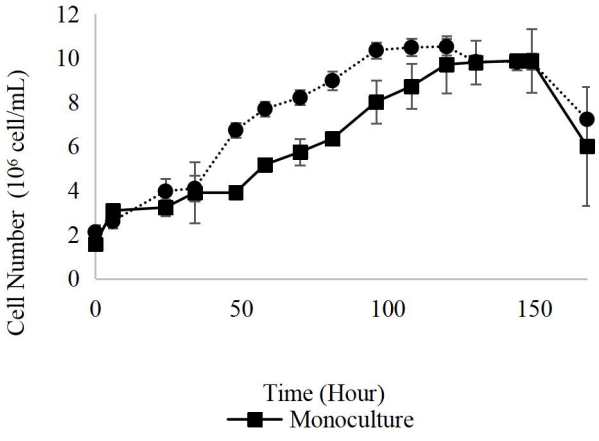

Figure 5 Growth curve microalgae in ADDMW medium

Microalgae growth in monocultures and mixed cultures can be influenced by several reasons. The type of species and the mixed culture ratio used in mixed culture can affect the growth of microalgae. In one of the studies that have been conducted, with monoculture and mixed culture of *Desmodesmus communis*, the productivity of growth was higher in monoculture treatment compared to mixed culture treatment [12]. An unbalanced ratio between species can cause competition so that biomass productivity can decrease [22].

Another condition that can affect monoculture and mixed culture is the difference in the cultivation medium used, especially the content contained in each medium. Based on the results of research that has been done, biomass productivity is influenced by the ratio of N:P ratio in the cultivation medium. The higher the N: P ratio, the lower the biomass productivity [2]. Also, the use of a new medium in microalgae requires acclimatization for microalgae to adapt to the medium [16]. The use of Walne medium in *C.vulgaris* has often resulted in relatively high biomass productivity [21].

In the ADDMW medium, the acclimatization process is needed so that the microalgae can adapt well. Low growth in ADDMW medium can be caused by changes in nutrient composition in ADDMW medium due to pre-treatment given, especially in the autoclave process [17]. Also, pre-treatment of ADDMW medium in the form of dilutions is necessary so the nutrition of microalgae can be given in a composition suitable for microalgae growth [18].

The comparison of microalgae culture growth in each culture condition (monoculture and mixed culture) is shown in Figure 6. Overall, the greatest growth was produced by monoculture on Walne medium with the highest cell count reaching $30x10^6$ cells at t = 118 hours. Meanwhile, in other cultures, the maximum number of cells ranged from $8\text{-}10x10^6$ cells, with relatively the same growth patterns.

The percentage of cells in *C.vulgaris* and *Scenedsmus* sp. in monocultures and mixed cultures in Walne medium (Figure 7) and ADDMW medium (Figure 8). Overall, the two treatments showed relatively the same results, the percentage of *C.vulgaris* cells was more dominant than *Scenedesmus* sp. until the end of cultivation.

When *C.vulgaris* dominates the number of cells at the beginning of cultivation, this dominance will persist until it enters the stationary phase. This can be caused by the morphological structure of the cell walls of *C.vulgaris* which are more complex so that they are resistant to contamination [2, 13]. The absorption of nutrients in the mixed culture treatment also affects the growth of microalgae. In mixed culture, the number of *C.vulgaris* cells was higher than *Scenedemus* sp. so it will absorb more nutrients [13].

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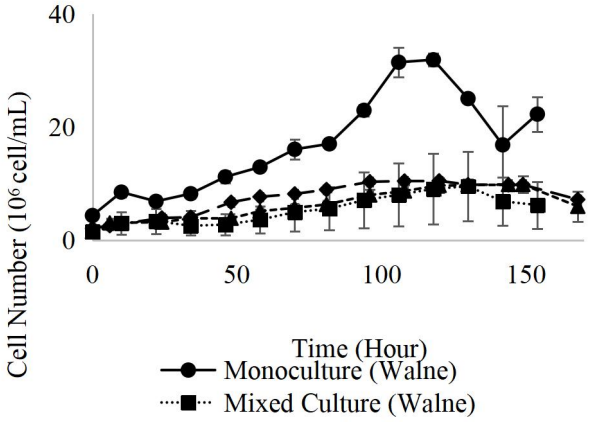

Figure 6 Growth curve microalge in each culture condition

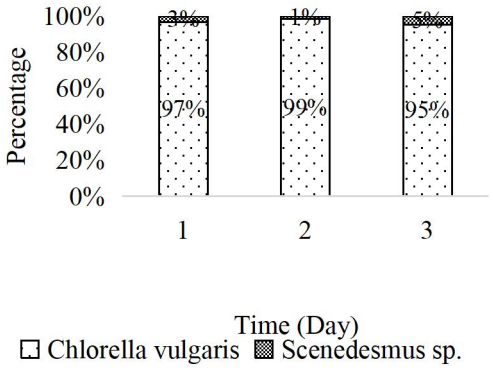

Figure 7 Percentage *Chlorella vulgaris* and *Scenedesmus* sp. in mixed culture Walne medium

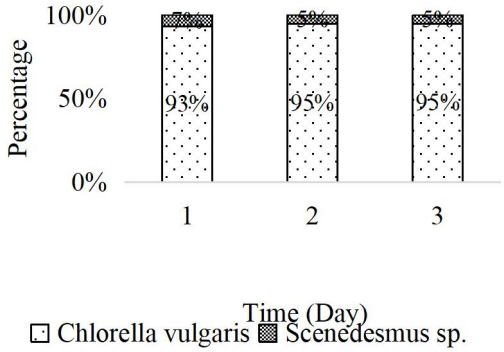

Figure 8 Percentage *Chlorella vulgaris* and *Scenedesmus* sp. in mixed culture ADDMW medium

3.3 Decreased Nitrate and Ammonium Levels in Microalgae Cultures

The content of nitrate and ammonium in the microalgae cultivation medium is needed to fulfill the nitrogen element which plays a role in microalgae growth. The utilization of nitrate and ammonium from waste can be used for microalgae cultivation medium and waste remediation [14]. In each condition, both culture conditions and different medium, there were differences in the percentage reduction and reduction rate.

The highest percentage of nitrate reduction in the Walne medium treatment was obtained in mixed culture with a reduction percentage of 70.3%. While in ADDMW medium, the highest value was obtained in monoculture with a reduction percentage of 68.5%. In this case, the percentage reduction is directly proportional to the reduction rate, so a high percentage reduction will result in a high reduction rate.

The highest percentage of nitrate reduction in all culture conditions was obtained in mixed culture with Walne medium with a percentage of 70.3%. Whereas the highest growth rate of nitrate reduction was obtained in monoculture with ADDMW medium (0.601 ppm day^{-1}), as shown in Table 2.

The highest percentage of ammonium reduction in Walne medium was obtained in mixed culture with reduction percentage of 67.9%. While in ADDMW medium treatment, the highest value was obtained in monoculture with a reduction percentage of 28.9%. In this case, the percentage reduction is directly proportional to the reduction rate, so a high percentage reduction will result in a high reduction rate.

The highest percentage of ammonium reduction was obtained in mixed culture in the Walne medium with percentage of 67.9%. Whereas the highest growth rate of ammonium reduction was obtained in mixed culture in Walne medium (0.181 ppm day^{-1}), as shown in Table 3.

The percentage of nitrate reduction in monoculture with Walne medium showed the lowest percentage (27.7%). For other treatments, the percentage value of nitrate reduction was not too different with a range of 63.9% -70.3%. The reduction percentage of ammonium in Walne medium is greater than that in ADDMW medium. The percentage range for ammonium reduction in the Walne medium was 60.7% -67.9%, while the ADDMW medium was 17.7% - 28.9%. From the data obtained, it can be seen that in the Walne medium, microalgae tend to absorb more ammonium than in the ADDMW medium.

In theory, the nitrogen source that tends to be used in microalgae is in the form of ammonium compared to nitrate, this is due to the use of nitrate which requires enzymes to be used by microalgae, so it tends to use energy in the process. Meanwhile, the use of ammonium can be directly used by microalgae [19]. However, this does not apply to all microalgae species. Based on the research that has been done, the use of different nitrogen sources (ammonium and nitrate) in several types of microalgae, it is found that the amount of reduction tends not to differ between ammonium and nitrate reductions [20].

The pH conditions in culture can also affect the absorption of nutrients by microalgae [3]. The fluctuation in culture pH for different mediums is shown in Figure 9 for Walne medium and Figure 10 for ADDMW medium. In Walne medium, there was a fluctuation in pH at 6.7-8.2. Meanwhile, in the ADDMW medium, the pH fluctuation occurred in the range 7.4-8.4.

Both variations of the medium show fluctuations in pH values between observation points. This fluctuating value can be caused by the aeration of microalgae cultures. Air in the

form of CO2 will dissolve in water in the form of HCO^{3-} so that it affects the pH value of the culture [14]. Overall, the two mediums did not have a big effect on the pH of the culture, only the highest pH value in the Walne medium was lower than that in the ADDMW medium, this could be due to the presence of buffer compounds in the Walne medium [4].

Table 2 Percentage and reduction rate of nitrate from each culture condition and medium

Culture condition	Walne		ADDMW	
	Reduction (%)	Reduction rate (ppm day^{-1})	Reduction (%)	Reduction rate (ppm day^{-1})
Monoculture	27.7	0.133	68.5	0.601
Mixed culture	70.3	0.338	63.9	0.5336

Table 3 Percentage and reduction rate of ammonium from each culture condition and medium

Culture condition	Walne		ADDMW	
	Reduction (%)	Reduction rate (ppm day^{-1})	Reduction (%)	Reduction rate (ppm day^{-1})
Monoculture	60.7	0.162	28.9	0.097
Mixed culture	67.9	0.181	17.7	0.07

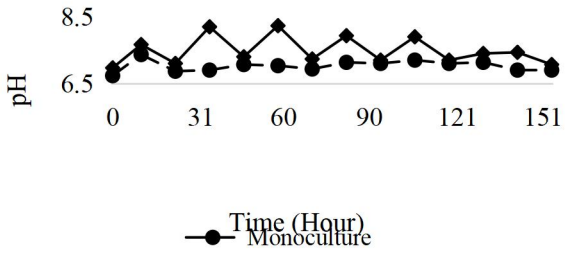

Figure 9 pH condition in Walne Medium

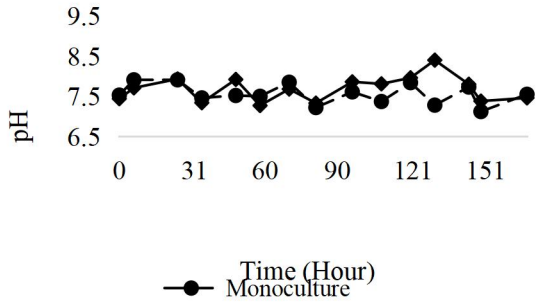

Figure 10 pH condition in ADDMW Medium

4. Conclusions

The highest specific growth rate was produced in monoculture with Walne medium with a rate of 0.407 $days^{-1}$. The largest percentage of nitrate reduction was produced in mixed culture with Walne medium (70.3%) and the largest nitrate reduction rate was produced in monocultures with ADDMW medium with a rate of 0.601 ppm day^{-1}. The largest percentage reduction of ammonium was produced in mixed culture with Walne medium (67.9%) and the reduction rate of ammonium was produced in mixed culture in Walne medium with a rate of 0.181 ppm day^{-1}. From the results obtained, optimization is needed for the application of mixed culture and ADDMW. Such as mixed culture is done with the ratio of the number of cells between species and pre-treatment on ADDMW medium is done more optimally.

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Biomass Production *Scenedesmus* sp. in Monoculture and Mixed Culture Systems with *Chlorella vulgaris*, and Ability to Reduce Nitrate and Ammonium Levels from ADDMW (Anaerobically Digested Dairy Manure Wastewater) Medium Using a Vertical Tank Reactor

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Abstract

In this study, cultivation of *Scenedesmus* sp. was carried out in monoculture and mixed-culture with *Chlorella vulgaris* in a 20 Liter scale Vertical Tank Reactor on ADDMW medium with Walne's comparison medium. In the mixed-culture treatment, the culture volume of *Scenedesmus* sp. three times as much as the culture volume of C. *vulgaris* (3S: 1C). The culture was observed for 7 days. The highest microalgae biomass growth rate (0.395 / hour) was obtained from mixed-culture using the Walne medium, followed by the growth rate in monoculture in the ADDMW medium (0.342 / hour). The highest reduction percentage of nitrate (73%) was obtained in monoculture in the Walne medium, while the highest reduction rate of nitrate (0.53 ppm/day) was obtained from monoculture in ADDMW and mixed-culture in ADDMW (0.52 ppm/day). The highest percentage of ammonium reduction (66%) was obtained from monoculture on Walne medium, and mixed-culture also on the Walne medium (63%). The highest ammonium reduction rate (0.18 ppm/day) was also obtained from monoculture in the Walne medium, followed by mixed culture in the Walne medium (0.17 ppm/day). *Scenedesmus* sp. with moderate Walne at 73.3% with a reduction rate of 0.352 ppm/hour. It can also shown that microalgae has good enough ability to grow in mixed-culture and reduce nitrate and ammonium in the ADDMW medium.

Keywords: ADDMW, *Chlorella vulgaris*, *Scenedesmus* sp., Mixed-culture

1. Introduction

Microalgae have attracted worldwide attention as a biological agent that has the potential to produce biodiesel and high-value bioproducts [1]. Microalgae such as Chlorella *vulgaris* and Scedenedesmus sp. is a type of microalgae species capable of producing high amounts of lipids and protein [2]. *Scenedesmus* is a genus of 70 cylindrical green algae species and generally forms colonies consisting of 2, 4, 8, and 16 cells [3]. C. *vulgaris* is a microalgae habitat for fresh, marine, and brackish water which is included in the green algae

(chlorophyta) group. C. *vulgaris* cells have an ellipsoidal shape 3-8 micrometers in diameter [4].

Researches are currently starting to accelerate the growth of microalgae by culturing several types of microalgae which are commonly called mixed culture [5]. The mixed culture is carried out to facilitate the culture process so that there is no need to separate each microalgae species in an ecosystem, maintain ecosystem stability and productivity and reduce the potential for contamination that commonly occurs in microalgae monocultures.

Microalgae culture for bioproduct production becomes economically feasible if it can utilize wastewater as a source of water and nutrients [6]. One of the wastewaters that can be used as a microalgae growth medium is cow dung or commonly called ADDMW (Anaerobically, Digested Dairy Manure Wastewater). ADDMW can be used as a source of nutrition and a substitute for chemical compounds to produce microalgae biomass because it contains very high concentrations of nitrogen, phosphorus, and other nutrients.

Besides, the compounds contained in ADDMW waste such as nitrogen and ammonia generally have an impact on environmental pollution. So that the pollution effect can be reduced through the use of microalgae. Therefore, this study aims to determine the effect of mixed cultures between C. *vulgaris* and *Scenedesmus* sp. to decrease the concentration of ammonia/ammonium and nitrate in ADDMW medium which has the potential as a bioproduct producer.

2. Materials and Methods

2.1 Materials

Inoculum C. *vulgaris* was obtained from BBPBAP Jepara and *Scenedesmus* sp was obtained from InaCC-LIPI Cibinong. Walne medium is obtained commercially from a supplier of phytoplankton fertilizer in Jakarta. Anaerobically digested dairy manure wastewater (ADDMW) was obtained from Dairy Farm, Faculty of Animal Husbandry, Padjajaran University, Jatinangor. The chemicals used in this study came from the Chemical Materials Warehouse of the School of Life Sciences and Technology (SITH) at the ITB Ganesha campus and the ITB Jatinangor campus.

The bioreactor used in this research is the vertical tank reactor (VTR). The VTR bioreactor used is a tubular photobioreactor made of acrylic material with a capacity of 25 L with a thickness of 5 mm. Bioreactor parts consist of lamp storage, vacuum chamber, culture compartment, sparger, and intake air chamber. The bioreactor has a height of 50 cm and a diameter of 25 cm. The form and scheme of the bioreactor used are shown in Figures 1 and 2, respectively.

Lighting is carried out using two LED lights with a power of 8 Watts. Air aeration is given through the BOYU®AIR PUMP air pump with a power of 10 Watts with a maximum of 10 L / min of air, air aeration is broken down by a DURAN® Filter Discs sparger with a diameter of 50mm and a porosity of 3 (150-250 microns). The research used a digital microscope (Canon, Japan), a pH meter, a Vernier © Nitrate measuring device, and an Ammonium Ion-Selective Electrode.

Figure 1. Vertical Tank Reactor (VTR)

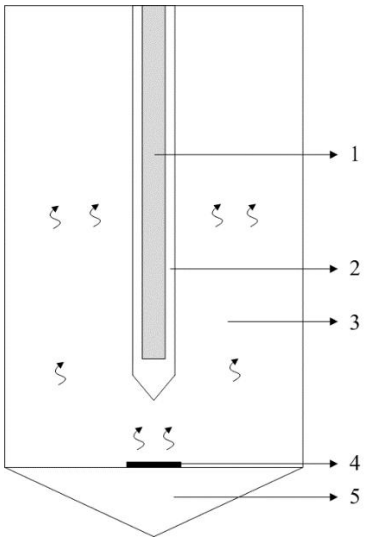

Figure 2. Bioreactor scheme 1. LED Lamp, 2. Vacuum chamber, 3. Culture compartment, 4. Sparger, 5. Air Intake Chamber

2.2 Methods

2.2.1 Preparation of Cultivation Medium

The cultivation medium was made by mixing walne stock medium with distilled water. The walne stock medium was taken as much as 1 ml then mixed with distilled water until the volume reached 1 L. The ratio of the mixture of walne stock medium and distilled water was 1 ml / 1 liter.

The ADDMW medium was put into a 1 L glass bottle and then sterilized using autoclave at a temperature of 121 ° C and a pressure of 1.5 bar for 10 minutes. The sterilized ADDMW medium is then filtered using fat cotton so that the solid and liquid fractions are separated. The liquid fraction was used as a cultivation medium.

2.2.2 Microalgae Inoculum Cultivation

The culture of each microalgae species was carried out using walne medium in a 1 L glass bottle at a working volume of 800 mL. The lighting used is 16 hours/day using white fluorescent. The culture was aerated with a supply of 24% CO2 and gas with a flow rate of 2.08 L / min which was adjusted using a flowmeter. Cultivation was carried out until a volume of approximately 5 liters was obtained with a cell density of at least 105 cells / mL for each microalga.

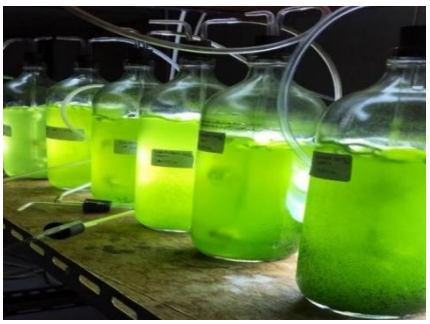

Figure 3. Cultivation of microalgae inoculums in 1 L glass bottles

2.2.3 Acclimatization of Microalgae in ADDMW Medium

The ADDMW medium was first given pre-treatment in the form of sterilization using an autoclave at 121 ° C for 15 minutes and gravimetric reduction of solids. Furthermore, each microalgae C. *vulgaris* and *Scenedesmus* sp were acclimatized on ADDMW medium with 4 times dilution. Exposure was carried out 16 hours/day using white fluorescent. The culture

was aerated with a supply of 24% CO2 and gas with a flow rate of 2.08 L / min which was adjusted using a flowmeter. Cultivation and acclimatization of the ADDMW medium were carried out until a volume of 10 liters was obtained with a cell density of at least 105 cells / mL for each microalga.

2.2.4 VTR Scale Microalgae Culture

Microalgae cultures of C. *vulgaris* and *Scenedesmus* sp were subcultured into a vertical tank reactor (VTR) made from acrylic with a capacity of 25 L with a working volume of 20.

Mixed culture was carried out by mixing the two microalgae with the inoculum volume ratio of *Scenedesmus*: Chlorella (3: 1) with a total culture volume of 20% of the total working volume. Microalgae mixed culture was carried out in walne medium under lighting for 16 hours/day with a flow rate of 2.08 L / minute which was adjusted using a flowmeter. Data were collected twice a day to observe biomass growth.

2.2.5 Sampling and Measurement of Sample pH

A sampling of each treatment was carried out by isolating the culture in 600 ml glass bottles. Sampling was carried out 2 times a day. The pH measurement of the sample was carried out in triple using a pH meter.

2.2.6 Environmental Condition Measurement

Environmental conditions were measured using the temperature and humidity of the cultivation room. Temperature and humidity are measured in a 12 hour/measurement timeframe

2.2.7 Determination of Number of Cells

Count of C. *vulgaris* and *Scenedesmus* sp. performed twice a day, namely day-0,1,2,3,4,5,6, and 7 using a haematometer and microscope. The magnification of the objective lens and ocular lens used are 10x and 40x, respectively. The count was carried out 3 times in the haemacytometer chamber.

2.2.8 Analysis of Nitrate and Ammonia levels in Medium

Measurement of nitrate and ammonium levels was carried out using a Vernier © Nitrate gauge to measure nitrate levels and Ammonium Ion-Selective Electrode to measure ammonium levels. Measurements were made at the beginning and end of microalgae cultivation.

2.2.9 Specific Growth Rate Determination

The specific growth rate (day^{-1}) is obtained by equation:

$$\mu = \frac{1}{t} \ln \left(\frac{Nt}{No}\right)$$

with Nt and No is the number of cells at the end and the number of cells at the beginning in the exponential phase range, gradually. While t is an exponential time (day)

2.2.10 Rate and Percentage Reduction of Inorganic Compound in Cultivation Medium

The rate of reduction of inorganic compounds (ppm day^{-1}) in the cultivation medium is calculated by equation:

$$\boldsymbol{v = \frac{Co - Ct}{t}}$$

with Co and Ct are the initial and final concentrations (ppm) of inorganic compounds. While t is the time of cultivation (days).

Percentage reduction of inorganic compounds (%) in the cultivation medium is calculated by equation:

$$\%\boldsymbol{E = \frac{Co - Ct}{t}\, x100}\%$$

with Co and Ct are the initial and final concentrations (ppm) of inorganic compounds. While t is the time of cultivation (days).

3. Results and Discussions

3.1 Effect of Growth Medium on the Growth of Microalgae Cells

Microalgae cultivation in this study resulted in a growth curve for the number of cells with different results for each treatment. Microalgae curves in monocultures of *Scenedesmus* sp. with walne medium, a monoculture of *Scenedesmus* sp. with ADDMW medium, mixed culture of C. *vulgaris* and *Scenedesmus* sp. with walne medium, and mixed culture of C. *vulgaris* and *Scenedesmus* sp. With the ADDMW medium, it can be seen in Figure 4. Microalgae growth consists of five growth phases, namely the lag phase, the exponential phase, the relative decline phase, the stationary phase, and the death phase [7]

The aeration system given to this bioreactor comes from the bottom of the bioreactor, with the air passed sparger to produce small, uniformly sized bubbles. The advantage of this aeration system is the evenly mixing of the cultures, which prevents sedimentation of microalgae at the bottom of the bioreactor [10]. The use of a sparger as an is reduce shear stress to microalgae and increasing gas hold up and volumetric mass transfer in microalgae cultures [11].

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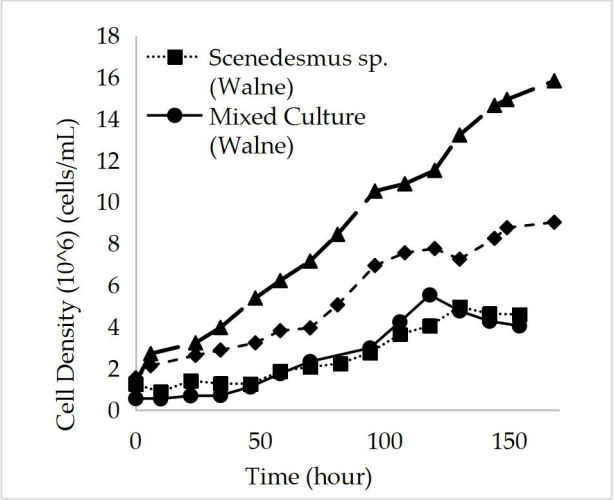

Figure 4. Growth of Monoculture Microalgae Culture of Scenedesmus sp. and mixed culture on walne and ADDMW medium.

Monoculture *Scenedesmus* sp. on walne medium it took 46 hours (2 days) to pass through the lag phase. Another case with monoculture *Scenedesmus* sp. using ADDMW medium which only takes 24 hours (1 day) to get through the lag phase. The difference in lag phase time also occurred in mixed cultures, where mixed culture treatment using walne medium had a lag phase time of 34 hours (1.5 days) while mixed culture using ADDMW medium only took 24 hours (1 day).

The microalgae lag phase is a microalgae adaptation phase that begins after the addition of the inoculum into the culture medium until some time afterward. In this growth phase, organisms undergo metabolic processes but cell division has not occurred so that cell density has not increased significantly [8]. The difference in lag phase time in each treatment is thought to be due to the difference in concentration between the culture media and the microalgae cell body fluids, during the adaptation period the cells recover the enzymes and substrate concentrations to the levels required for growth and the entry of nutrients into microalgae cells occurs through diffusion process as a result of concentration differences between culture media and body fluids [9].

After passing through the lag phase, microalgae growth then enters an exponential phase. The exponential phase is the phase where the cell division process starts and causes the growth rate to increase [8]. Microalgae growth in treatment using ADDMW medium had a longer exponential phase compared to treatment using walne medium. Monoculture treatment of *Scenedesmus* sp. using walne medium through the exponential phase for 84 hours (4 days). Meanwhile, the monoculture treatment of *Scenedesmus* sp. using ADDMW medium goes through the exponential phase for 120 (5 days).

Similar differences also occurred in mixed cultures where the treatment using walne medium went through an exponential phase for 84 hours (4 days) while the treatment using ADDMW medium went through an exponential phase for 120 days (5 days). There was no significant difference between monoculture treatment and mixed culture. Microalgae type *Chlorella* sp. and *Scenedesmus* sp. can generally reach the exponential phase within 4-6 days [10].

This exponential phase time difference can occur due to the difference in nutrient concentration between walne medium and ADDMW medium so that it has an impact on the limited nutrition of microalgae. Although walne is a general fertilizer that is often used for aquatic plants because it has complete nutritional content, walne fertilizer is not the best when compared to other synthetic mediums (BG11, BBM) [11]. This gives the possibility that the nutrient content in the diluted ADDMW medium was 4 times more suitable for microalgae growth in this study than the nutrient content in the walne medium. The higher initial inoculum concentration in treatment with ADDMW medium compared to the initial inoculum concentration in treatment with Walne medium is thought to also be one of the causes of shorter lag phase duration and relatively more cell growth in good microalgae cultivation with monocultures of *Scenedesmus* sp. and mixed cultures [12].

As can be seen in Figure 4, after going through the exponential phase, microalgae cultivation with walne medium treatment enters a phase of decreasing the number of cells or the phase of death. Meanwhile, microalgae cultivation with ADDMW medium treatment began to enter a stationary phase after a decrease in cell growth rate. In general, at the time of cell death, it is suspected that there is a relationship with less nutrient content, both in the cell and in the medium [13].

The removal of nutrients in the medium is associated with an increase in the number of cells in the medium. When the number of microalgae cells increases, the nutrient content in the medium also decreases because the microalgae can utilize nutrients for their growth [14]. The growth that occurred in the walne medium was lower than that in the ADDMW medium. This may be because the amount of nutrients in the walne medium required by the microalgae inoculum to grow is less than in the ADDMW medium.

Before using the ADDMW medium, first, dilute ADDMW 4 times. The use of 4 times dilution is carried out so that the nutrients in ADDMW are not reduced much so that it is sufficient for microalgae growth. The 4 times dilution was obtained through the trial and error method at the time of microalgae acclimatization to ADDMW to obtain the most suitable dilution for microalgae growth. The components contained in the ADDMW medium include nitrogen, phosphate, potassium, lignin, cellulose, hemicellulose, fat, starch, and protein [15].

There is one advantage of walne medium compared to ADDMW, namely the element of boron which functions to retain pigments [11]. This is evidenced by the greener of the microalgae cells cultured in this medium compared to the ADDMW medium as can be seen

in Figures 5 and 6. Boron deficiency can cause microalgae cells to lose pigment. Therefore, the color of cultivation in walne medium is greener than in ADDMW.

Microalgae growth can occur due to the presence of sufficient carbohydrate content to carry out the respiration process in the mitochondria. Scenedesmus and Chlorella vulgaris can produce their supply of carbohydrates through the photosynthesis process that occurs in chlorophyll [16]. A large amount of chlorophyll can increase the growth of microalgae. This study shows the minimal chlorophyll content found in microalgae in the ADDMW medium. However, the microalgae in the medium utilize the number of carbohydrates that are available in the ADDMW medium without the need to be produced through photosynthesis..

Figure 5. Microalgae cultivation on walne medium

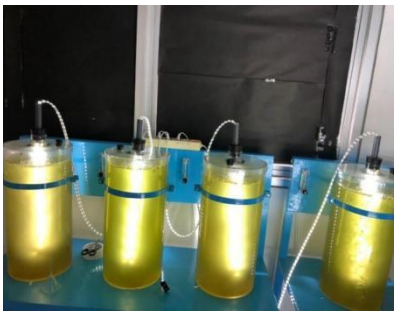

Figure 6. Cultivation of microalgae on ADDMW medium

3.2 Effect of Mixed Culture on Microalgae Growth

The growth curves of microalgae cells for each species on walne and ADDMW medium, respectively, can be seen in Figures 7 and 8. Growth of the microalgae C. *vulgaris* and *Scenedesmus* sp. that was found in mixed culture with walne medium continued to experience an increasing rate with a growth curve pattern that was almost the same for each species. The difference in the growth curve pattern only occurred in the length of the lag phase passed by each species in which *Scenedesmus* sp. those in monocultures and mix cultures experienced a lag phase for 34 hours, C. *vulgaris* contained in mixed cultures experienced a lag phase for 58 hours, while the monocultures of *Scenedesmus* sp. experienced a lag phase for 46 hours.

The duration of the lag phase of each species on the walne medium was different from the duration of the lag phase of each species in the ADDMW medium. Each species both in monocultur and mixed culture experienced a lag phase for 24 hours. The exponential phase that occurs in walne medium has a significant increase in each species in both monoculture and mixed culture compared to the exponential phase that occurs in the ADDMW medium. The cell growth that occurred in each mixed culture with ADDMW medium did not increase

which tended to be significant when compared to the growth of the monoculture cells of *Scenedesmus* sp.

In the ADDMW medium, there were differences in growth curve patterns between C. *vulgaris* and *Scenedesmus* sp. both of which are in mixed culture. Both species experienced an increase in cell numbers in a similar pattern until the 58th hour. Furthermore, C. *vulgaris* experienced an increase in the number of cells until the 81st hour and then decreased until the 130th hour. Meanwhile, *Scenedesmus* sp. had experienced a decrease in the number of cells at the 58th hour to the 70th hour, then continued to experience an increase in the number of cells until the 144th hour.

This phenomenon occurs because of the interaction of each species that is competitive or dominates. In mixed culture, the inoculum volume of *Scenedesmus* sp. higher than C. *vulgaris* supports the growth of *Scenedesmus* sp. a lot more. However, when the number of cells in the initial inoculum was not too much different as shown in Figure 7 using walne medium, C. *vulgaris* had relatively faster growth than *Scenedesmus* sp. Although *Scenedesmus* sp. has a higher number of cells than C. *vulgaris*, the growth curve of C. *vulgaris* cells from the mixed culture treatment found on walne medium and ADDMW medium continues to increase so that it shows the dominance of *Scenedesmus* sp. This is because C. *vulgaris* has a more complex body structure than *Scenedesmus* sp., So that metabolic processes and cell division tend to be faster and resistant to parasites due to the more complex structure of the cell walls [16].

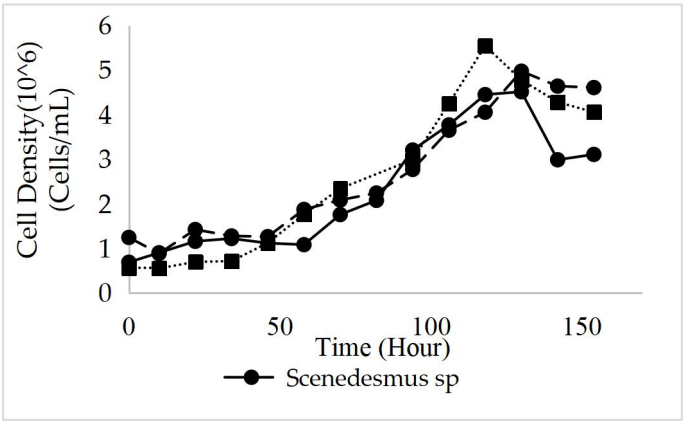

Figure 7. Growth of microalgae culture on walne medium

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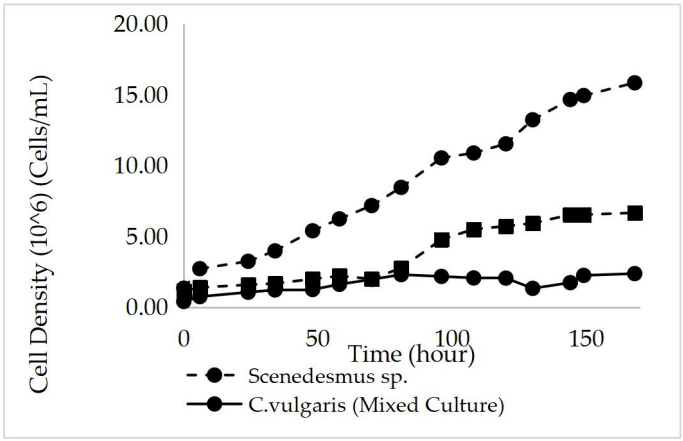

Figure 8. Growth of Microalgae Culture on ADDMW Medium

3.2 Growth Rate in Microalgae Culture

The growth rate in each treatment can be obtained by using the regression formula for the curve. The obtained growth rates can be seen in Tables 1 and 2. The growth rate of Monoculture *Scenedesmus* sp. on walne medium was 0.283 / h, while the growth rate of monoculture *Scenedesmus* sp. on the ADDMW medium is 0.342 / h. The mixed culture growth rate on walne medium was 0.395 / h. Meanwhile, the mixed culture growth rate in the ADDMW medium was 0.232 / h.

The mixed culture biomass growth rate on the medium was higher than the monoculture biomass growth rate of *Scenedesmus* sp. On the other hand, the mixed culture biomass growth rate on the ADDMW medium was lower than the monoculture biomass growth rate of *Scenedesmus* sp. The high growth rate in the walne medium treatment allows the tendency of each species to compete with each other in adapting to the medium. Both species have obtained balanced and appropriate nutrition in the medium so that they utilize nutrients very well which have an impact on the acceleration of biomass growth in each species [11]. In this study, the largest biomass growth rate was produced in mixed culture treatment using Walne medium at a rate of 0.395 / day.

Table 1. Biomass Growth Rate in Each Treatment with Walne Medium

Treatment Variation	Biomass Growth Rate (day-1)
Scenedesmus sp.	0,283
Mixed-Culture	0,395

Table 2. Biomass Growth Rate in Each Treatment with ADDMW Medium

Treatment Variation	Biomass Growth Rate (day-1)
Scenedesmus sp.	0,342

Mixed-culture	0,232

3.2 PH on Microalgae Culture Medium

The pH curves in each treatment with walne medium and ADDMW can be seen in Figures 9 and 10. The pH of the walne and ADDMW medium in each treatment tends to produce a fluctuating curve caused by differences in the time of taking pH data, namely day and night. This happens because, in the absence of light, the cells respire and release CO2, causing the pH of the medium to decrease. Conversely, during the day the culture pH will increase due to the increased photosynthetic activity. As more CO2 is produced as a result of respiration, the reaction moves to the right and gradually releases H + ions which causes the pH of the water to drop [17].

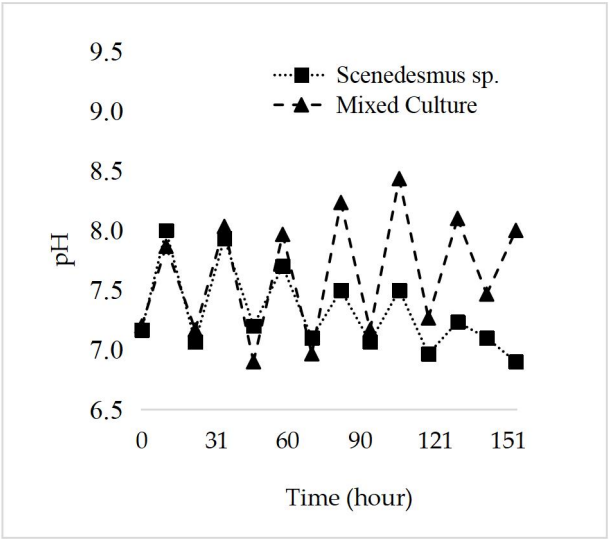

Figure 9. PH curve on walne medium

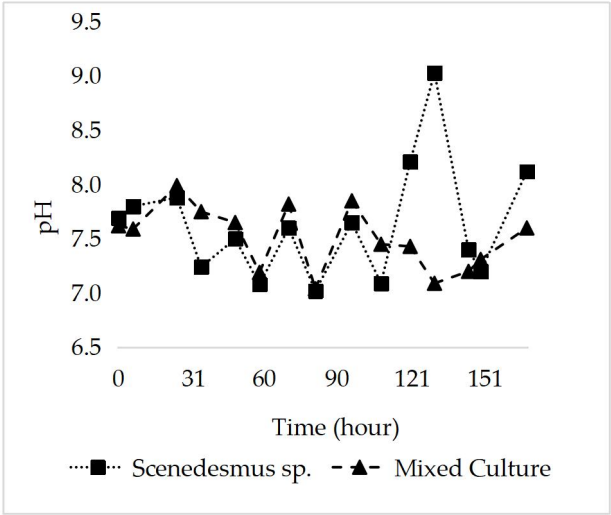

Figure 10. PH curve in ADDMW medium

The pH curves of walne and ADDMW in each species experienced an increase, decrease, and tended to be stable. The increase in pH can be caused by the process of breaking down proteins and utilizing nitrogen by microalgae. According to Horne (1983), the reaction of ammonium formation from ammonia can cause an increase in the pH of the medium, causing the medium to become more alkaline. However, the nitrogen source that can be directly used in the process of protein formation from amino acids is ammonium, so that nitrates and nitrites must first be converted to ammonium according to the following reaction.

$NO_3 + NADPH + NADP \rightarrow NO_2 + H_2O$

$NO_2 + NADPH + NADP \rightarrow NH_2OH + H_2O$

$NH_2OH + NADPH + NADP \rightarrow NH_3 + H_2O$

$NH_3 + H_2O \rightarrow NH_4 + + OH^-$

The pH value does not change significantly because of the dissolved CO2 gas in the medium so that it can be used as a natural buffer. The gas will become carbonic acid which breaks down into carbonate ions and bicarbonate ions. The equilibrium reaction between dissolved CO¬2, carbonic acid, bicarbonate ion, and carbonate ion causes the pH to shift in the range 6-9 and does not increase again [19].

3.2 Percentage and Reduction rate of Nitrate and Ammonia in Microalgae Medium

Microalgae cultivation caused a decrease in nitrat levels in each treatment. As can be seen in Figures 11 and 12, monocultures of *Scenedesmus* sp. In walne and ADDMW medium, the

reduction rates of nitrate were 0.35 ppm/day and 0.53 ppm/day, respectively, with a reduction percentage of 73.3% and 62.3%, respectively. Mixed culture on walne and ADDMW medium obtained nitrate reduction rates of 0.25 ppm/day and 0.52 ppm/day, with a reduction percentage of 51.5% and 55.5%, respectively. The percentage of nitrate reduction in monocultures of *Scenedesmus* sp. higher than the reduction percentage of nitrate in mixed culture.

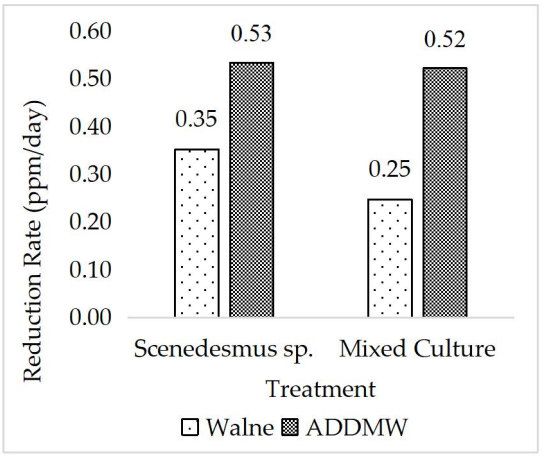

Figure 11. Nitrate Reduction Rate in Each Treatment with Walne Medium and ADDMW

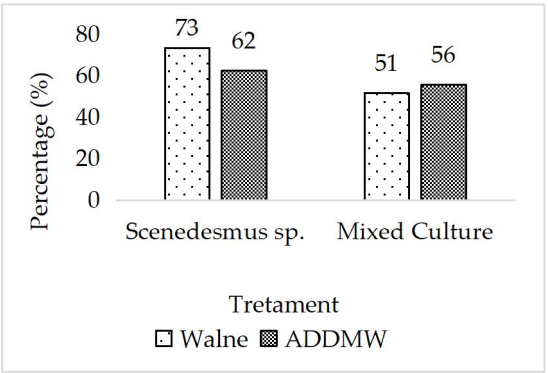

Figure 12. Percentage of Nitrate Reduction in Each Treatment with Walne Medium and ADDMW

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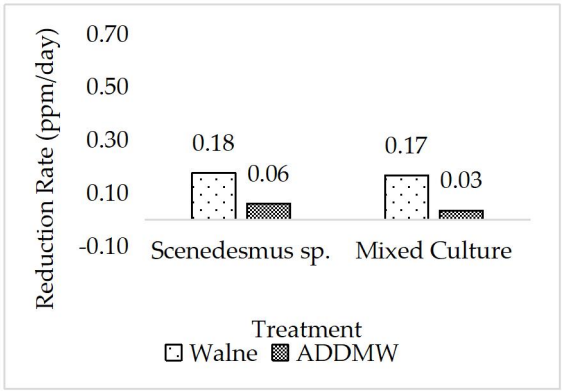

Figure 13. Ammonia Reduction Rate in Each Treatment with Walne Medium and ADDMW

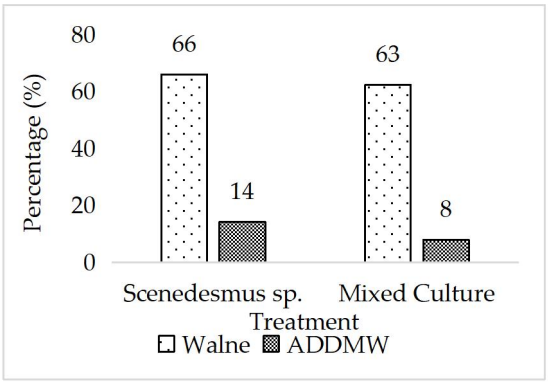

Figure 14. Percentage of ammonia reduction in each treatment with Walne Medium and ADDMW

A decrease in ammonia levels also occurred in each treatment. As shown in Figures 13 and 14, monocultures of *Scenedesmus* sp. on walne and ADDMW medium obtained ammonia reduction rates of 0.18 ppm/day and 0.06 ppm/day, respectively, with a percentage reduction of 66.1% and 14.3%, respectively. Meanwhile, the mixed culture on walne and ADDMW medium obtained ammonia reduction rates of 0.17 ppm/day and 0.03, respectively, with a percentage reduction of 62.5% and 8%, respectively. Percentage and reduction rate of ammonia in monoculture *Scenedesmus* sp. slightly higher than mixed culture on walne medium.

As the percentage of ammonia reduction increases, the pH will increase. These two things are certainly contradictory considering the curve in Figure 9 shows that there is a decrease in pH in walne medium containing the monoculture *Scenedesmus* sp. Compared to the pH of the medium walne contains mixed cultures which tend to be stable and even have increased to be more alkaline. The high nitrate content in the ADDMW medium causes a more significant decrease in nitrate because the nitrate tends to turn into more ammonium. So that the nitrate

concentration is reduced more significantly while ammonium is reduced not so significant due to the effect of the formation of new ammonium at lower pH [20].

The decrease in pH can be caused by ammonia compounds that have changed form into ammonium ions. The relatively high ammonium ion compounds can cause a decrease in pH. Generally, at pH ≈ 8 the ammonium concentration remains constant. Medium conditions with a pH below 8 will cause ammonia to tend to increase (hard to decrease). The inhibition of the decrease in ammonia is supported by a less pH value [20].

Microalgae use inorganic nitrogen for growth, especially nitrate and ammonium. However, some of them can also grow on organic nitrogen. Microalgae can convert various forms of inorganic nitrogen from wastewater to organic nitrogen. Not all of the inorganic nitrogen contained in the medium is absorbed by microalgae. There is a reaction in the medium that can convert inorganic nitrogen into organic nitrogen if there are other compounds in the medium. Although ammonium is the preferred nitrogen source for absorption, microalgae can consume nitrogen from various nitrogen sources, including ammonium, nitrate, nitrite, and urea [21].

4. Conclusions

Microalgae biomass growth rate in monoculture treatment of *Scenedesmus* sp. on walne medium, the monoculture of *Scenedesmus* sp. in ADDMW medium, C1: 3S mixed culture on walne medium and C1: 3S mixed culture on ADDMW medium was 0.283 / h, 0.342 / h, 0.395 / h and 0.232 / h respectively with the largest growth rates produced in the mixed culture treatment. C1: 3S using Walne medium, which is equal to 0.395 / day.

The reduction percentage of nitrate was 73%; 62.3%; 51% and 55.5%. The nitrate reduction rate was 0.35 ppm / day; 0.53 ppm / day; 0.25 ppm / day; and 0.52 ppm / day. The percentage reduction in ammonia / ammonium was 66%, 14%, 63% and 8%. The ammonia / ammonium reduction rate is 0.18 ppm / day; 0.06 ppm / day; 0.17 ppm / day and 0.03 ppm / day.

The largest percentage reduction was produced in the monoculture treatment of *Scenedesmus* sp. with walne medium of 73.3% with a reduction rate of 0.352 ppm / h. The largest percentage of ammonia reduction was produced in the monoculture treatment of *Scenedesmus* sp. with walne medium of 66.1% with a reduction rate of 0.176 ppm/day.

It can be concluded that although the performance of microalgae in monoculture and mixed culture in Walne medium shows the highest, microalgae also shows a good enough ability to grow in mixed culture and reduce nitrate and ammonium.

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Endophytic Bacteria from *Anthocephalus cadamba* Miq, *Albizia chinensis* (Osbeck) Merr. and *Mimusops elengi* L for Controlling *Meloidogyne* sp. and Growth Promoting on Tomato

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Abstract

Root knot nematode *Meloidogyne* sp. is important of plant parasitic nematode which may cause yield loss. Various strategies have been developed to control plant parasitic nematode including including biological control using biocontrol agenst. The objecive of this research was to evaluate the potency of endophytic bacteria isolated from the roots of three forestry plants, Jabon (*Anthocephalus cadamba* Miq.), Sengon (*Albizia chinensis* (Osbeck) Merr.), and Tanjung (*Mimusops elengi* L.) in controlling *Meloidogyne* spp. in tomato. Endophytic bacteria were isolated from the root by surface sterilization method using natrium hypochlorite (NaOCl) 2%, alcohol 70% and Trypsic Soybean Agar (TSA) medium. The results showed that eleven selected endophytic bacteria were observed from these plants roots. Hypersensitive test has shown that all isolates showed negative reaction. Some isolates have found the ability to suppress root galls in tomato up to 80%.

Keywords: hypersensitive, isolate, nematode, plant parasitic, root knot.

1. Introduction

Tomato (*Solanum lycopersicum*) is one of the most widely cultivated vegetable crops in Indonesia. Plant pests and disease in tomato cultivation is often a serious problem. Root knot nematode caused by *Meloidogyne* spp. aare plant parasitic nematodes which are important disease in tomato plants. *Meloidogyne* spp are obligate parasites and can infect almost every higher plant species. *Meloidogyne* spp. infection damaging the root system of plants so as to disrupt the process of transporting water and nutrients. Plants infected with *Meloidogyne* spp. will become dwarfed, yellow and withered. Secondary infection by other pathogens often causes more damage. Attack of Meloidogyne spp. causing the physiological functions of the host plant to be abnormal, thus reducing not only yields but also product quality [1].

Control of *Meloidogyne* spp. quite difficult, because these organisms live in the soil and have a fairly wide range of host plants. Chemical control using fumigant and non-fumigant nematicides is an effective and frequently used control method. The application of the

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chemical compounds contained therein poses a risk to the environment. In addition, as awareness increases about the importance of environmental quality, chemical control needs to be limited. Biological control is the use of organisms or living things as a component of an integrated pest and plant disease control strategy. One of the advantages of biological control is that it is more environmentally friendly when compared to chemical control.

Endophytic bacteria are bacteria that colonize plant tissue without causing disease symptoms [1]. Endophytic bacteria is thought to be able to provide benefits to plants associated with it, either directly or indirectly. Endophytic bacteria generally enter the plant tissue from the soil through the roots and are able to colonize all plant tissues through vascular tissue [2]. This allows endophytic bacteria from forest plants to be used to improve the quality of agricultural crops. Several forest plants, one of which is pine, can survive and grow well in extreme environmental conditions. Several studies on the use of endophytic bacteria for the purpose of controlling plant diseases have been carried out, including effectively controlling Meloidogyne spp. and tomato plant growth promoters [3]. For this reason, research on appropriate methods of exploiting endophytic bacteria from forest plants with the aim of improving the quality of agricultural crops is very valuable in the future. The objective of this study was to isolate endophytic bacteria from Jabon (*Anthocephalus cadamba* Miq.), Sengon (*Albizia chinensis* (Osbeck) Merr.), and Tanjung (*Mimusops elengi* L.) and to evaluate their potential uses in controlling *Meloidogyne* spp. in tomato plants and tomato plant growth.

2. Materials and Methods

2.1 Isolation of endophytic bacteria

Endophytic bacteria were isolated from the roots of three types of forest plants of Jabon (*Anthocephalus cadamba* Miq.), Sengon (*Albizia chinensis* (Osbeck) Merr.), and Tanjung (*Mimusops elengi* L.). The method of isolation of endophytic bacteria was carried out using surface sterilization using sodium hypochlorite NaOCl [1] with several modifications. The roots of jabon, sengon and tanjung plants which are 4-5 months old are taken, then washed and dried for a few minutes on a tissue. After that, the roots were weighed as much as 1 gram and carried out surface sterilization by placing them in 70% alcohol for 3 minutes, 4% NaOCl for 3 minutes and then rinsing with sterile distilled water 3 times. The sterilized roots were then rubbed into a petri dish containing 20% TSA medium which was used as a control. Next, the roots were put into a mortar and 10 ml of sterile distilled water was added, then crushed until smooth. Subsequently, finely crushed roots are diluted 101, 102, 103 and 104 ml. Each level of dilution was taken 0.1 ml, poured on 20% TSA media and leveled with a grader and incubated for 24-48 hours at room temperature. The growing bacteria were purified using sterile toothpicks by scratching them on 100% TSA media and incubated at room temperature for 24-48 hours. The pure grown bacteria were then taken with the aid of a loop needle and transferred to TSB 100% + 30% glycerol media and stored at -4oC.

2.2 Hypersensitivity test

Hypersensitivity test was conducted to determine the potential pathogenicity of endophytic bacterial isolates. The plants used in the hypersensitivity test were 3-4 month old tobacco plants obtained from the Bogor Center for Agricultural Biotechnology & Genetic Resources (ICABIOGRAD). The tested endophytic bacterial isolates were grown in petri dishes containing 100% TSA. After 48 hours of endophytic bacterial isolates were harvested by adding 10 ml of sterile distilled water to the petri dish. The suspension of endophytic bacteria was taken using a syringe to be injected at the bottom of the leaves of a tobacco plant and incubated for 48 hours. Potential pathogenic bacteria showed necrotic symptoms (positive) on tobacco leaves, while non-pathogenic bacteria did not cause necrotic symptoms. Bacteria that did not show necrotic symptoms were used for further testing.

2.3 The Effectiveness of Endophytic Bacteria ageinst *Meloidogyne* spp.

The method of application of endophytic bacteria to tomato plants in this study used two application methods, namely soaking the seeds and soaking the seeds followed by watering them into the soil. Endophytic bacterial suspensions for seed immersion applications were prepared by pouring 10 ml of sterile distilled water into a culture of endophytic bacterial isolates on a petri dish containing 100% TSA media and harvested by shedding it using an inoculation needle. The preparation of endophytic bacterial suspensions for watering the soil is made by means of 1 petri dish containing endophytic bacteria cultures on 100% TSA media, harvested and dissolved in 200 ml of sterile distilled water.

Soaking the seeds. Tomato seeds were immersed in the endophytic bacterial suspension for 2 hours. The seeds are then sown using a tray containing the commercial planting medium "Bio Laksmi". After the emergence of the main leaves, the tomato plants were transplanted into a pot with a diameter of 17 cm containing soil and compost media with a ratio of 1: 1 (v / v). Nematode inoculation was carried out when the plants were about 3 weeks after planting (WAP) by adding 50 grams of soil infested with *Meloidogyne* sp. (equivalent to 3000 larvae) obtained from the experimental garden of IPB Pasir Sarongge Cianjur. Each treatment consisted of 4 replications. Observations of plant height, shoot dry weight, root dry weight and number of root knots were carried out after the plants were 10 week after planting (WAP).

Soaking the seeds and soil drensching. Tomato seeds were immersed in the endophytic bacterial suspension for 2 hours. The seeds are then sown using a tray containing the commercial planting medium "Bio Laksmi". After the emergence of the main leaves, the tomato plants were transplanted into a 17 cm diameter pot containing soil and compost media with a ratio of 1: 1 (v / v). Each plant was given an application of 50 ml of endophytic bacterial suspension when the plants were 1 week old after transplanting. Nematode inoculation was carried out when the plants were around 3 weeks after treatment by adding 50 gram of soil that had been infested with *Meloidogyne* sp. (equivalent to 3000 larvae) obtained from the experimental garden of IPB Pasir Sarongge Cianjur. Each treatment

consisted of 4 replications. Observations on plant height, shoot dry weight, root dry weight and number of root galls were carried out after the plants were 10 weeks after treatment.
The statistical design used in this experiment was a completely randomized design (CRD). The data obtained were then analyzed statistically using the Duncan test with the Statistical Product and Service Solution (SPSS) version 18 program at the 5% real level.

3. Results and Discussion

3.1 Isolation of endophytic bacteria

Endophytic bacteria that were successfully isolated from the roots of jabon (*A. cadamba*), sengon (*A. chinesis*) and tanjung (*M. elengi*) plants consisted of 11 isolates, 4 isolates from the Jabon plant, 3 isolates from the sengon plant and 4 isolates from the cape plant. Research on the diversity of endophytic bacteria originating from forest plants varies depending on the type of plant. The population density of endophytic bacteria from forest plants varies from 10^1–10^6 cfu / g per plant tissue. Endophytic bacteria in the genus *Pseudomonas, Bacillus, Paenibacillus, Erwinia* and *Burkholderia* are endophytic bacteria that are almost frequently found. The gram-positive *Actinobacteria* and gram-negative bacteria in the genus *Acinetobacte*r and *Sphingomonas* have a significant population density in a community of endophytic bacteria in forest plants [4].

Table 1 Results of isolation and hypersensitivity test of endophytic bacteria from roots of jabon, sengon and tanjung plants on tobacco leaves

Code isolate of endophytic bacteria	Origin of plant	Colony colors	Hypersencitive reaction test
S1	*A. chinensis*	Golden yellow	Negative
S2	*A. chinensis*	Murky white	Negative
S3	*A. chinensis*	White	Negative
T1	*M. elengi*	Golden yellow	Negative
T2	*M. elengi*	Red	Negative
T3	*M. elengi*	Yellow	Negative
T4	*M. elengi*	Clear white	Negative
J1	*A. cadamba*	White	Negative
J2	*A. cadamba*	White cloudy	Negative
J3	*A. cadamba*	Grayish white	Negative
J4	*A. cadamba*	Clear white	Negative

Hypersensitive test results on tobacco leaves against 11 endophytic bacterial isolates all showed negative reactions. This is indicated by the absence of necrosis symptoms that arise on tobacco leaves after application of endophytic bacteria for 2 x 24 hours. Teh research [5] states that hypersensitivity testing on tobacco plants is a fast and practical way to determine the pathogenicity of a bacterial culture. Based on the results of hypersensitive reaction tests on tobacco plants, all endophytic bacterial isolates tested were non-pathogenic and potentially as biological agents for control of plant diseases

3.2 Effect of Endophytic Bacteria on the Number of root galls *Meloidogyne* spp

The results showed that endophytic bacterial isolates with the application of soaking the seeds and watering the soil had an effect on reducing the number of tomato plant roots. The method of seed immersion application followed by soil watering is generally better in terms of percentage reduction in the number of root puru compared to the application of seed immersion alone, except isolates S3, J4 and J1. Isolates S3 and J4 with the seed immersion application method alone showed better results than the seed immersion application combined with watering, while the J1 isolates with the seed immersion application method followed by watering the soil (Table 4). A total of 3 isolates, S2, T2, and T3 which were applied by soaking the seeds alone were proven to be able to suppress the number of root knots better than other isolates and significantly different from the control. A total of 7 isolates, S1, S2, S3, T1, T2, T3, and T4 with the application method of seed immersion followed by watering the soil were able to suppress the number of root knots better than other isolates and significantly different from the control, even isolates S1 and T1 were able to suppressing the number of root pots by more than 80%.

Plants infected by nematodes undergo morphological and physiological changes that cause plants to become weak. Juvenile nematode 2 (J2) is the only stage of *Meloidogyne* spp. which can initiate infection. Stage 2 larvae generally infect the plant root tissue behind the root tip, which is an area that is undergoing elongation. Juvenile 2 *Meloidogyne* spp is attracted and moves to the root elongation area looking for a suitable feeding site. Stylet of *Meloidogyne* spp. injecting the surrounding cell walls, releasing protease enzymes derived from secretions of the esophageal glands, encouraging cell division without cell wall division, resulting in the formation of giant cells (giant cells or syncytia) which are food sources for nematodes. At the same time, there is an increase in the number of abnormal cells (hyperplasia) and an increase in the size of the abnormal cells (hypertrophy) of the plant tissue and causes the formation of root pits. [6].

Plant parasitic nematode populations can be controlled by various mechanisms that are active during interactions both within and between different levels or sequences of organisms in the food chain in an ecosystem. These interactions can be mediated by organisms that affect the nematode population by mechanisms that have direct or indirect

effects [7]. Biological control of plant parasitic nematodes can be carried out biologically by introducing antagonistic organisms, environmental manipulation aimed at conservation and enhancement of antagonistic organisms, or a combination of the two strategies. The success of biological control of plant parasitic nematodes is hampered by several factors, including soil properties, microscopic size of nematodes and antagonistic organisms and complex interactions among soil organisms [8; 9]. The bacteria isolated from the roots of forest plants in this study were thought to have the ability as biological agents. This was evidenced by the reduction in the number of root knots in plants treated with endophytic bacteria compared to control plants. The mechanism of endophytic bacteria against the suppression of plant parasitic nematodes is largely unknown and is speculative. Predation, competition, parsitism and antibiosis are included in the specific mechanisms of biological agents [10]. Apart from these mechanisms, the endophytic bacterial isolates in this study are thought to be able to induce the resistance of host plants to pathogens. Induction of host plant resistance can be in the form of local and / or systemic resistance induction [11].

Table 2 Effect of treatment of endophytic bacteria with seed immersion and seed immersion followed by soil drenching on the number of root galls caused by *Meloidogyne* spp.

Isolate endophytic bacteria	Seed immersion			Seed immersion + soil drench		
	Numbers of gall per root system		Percentage effectivity	Number of gall per root system		Percentage effectivity
Control	263.00	bc		615.50	c	
S1	142.00	ab	46.01	114.50	ab	81.40
S2	106.75	a	59.41	145.00	ab	76.44
S3	367.00	c	-39.54	127.75	ab	79.24
T1	133.25	ab	49.33	85.25	a	86.15
T2	107.75	a	59.03	200.00	ab	67.51
T3	88.75	a	66.25	183.25	ab	70.23
T4	175.25	ab	33.37	189.50	ab	69.21
J1	168.25	ab	36.03	634.67	c	-3.11
J2	130.66	ab	50.32	543.00	c	11.78
J3	167.50	ab	36.31	457.50	bc	25.68
J4	273.25	bc	-3.90	452.00	bc	26.57

[a]The numbers on the same treatment and column followed by the same letter are not significantly different at the 5% test level (Duncan's multiple interval test).

Multiple applications seed treatment followed by soil drenching increase the population of growth-inducing endophytic bacteria more than the application of seed immersion alone. These results are consistent with the research of [3] who reported additional application of watering to the soil gave additional bacteria to the roots and increased antagonistic activity, thereby stimulating the growth of tomato plants.

Plant parasitic nematode populations can be controlled by various mechanisms that are active during interactions both within and between different levels or sequences of organisms in the food chain in an ecosystem. These interactions can be mediated by organisms that affect the population of nematodes with mechanisms that provide direct or indirect effects [7]. Biological control of plant parasitic nematodes can be carried out biologically by introducing antagonistic organisms, environmental manipulation aimed at conservation and enhancement of antagonistic organisms, or a combination of the two strategies. The success of biological control of plant parasitic nematodes is hampered by several factors, including soil properties, microscopic size of nematodes and antagonistic organisms and complex interactions among soil organisms [8; 9]. The bacteria isolated from the roots of forest plants in this study were thought to have the ability as biological agents. This was evidenced by the reduction in the number of root knots in plants treated with endophytic bacteria compared to control plants. The mechanism of endophytic bacteria against the suppression of plant parasitic nematodes is largely unknown and is speculative. Predation, competition, parsitism and antibiosis are included in the specific mechanisms of biological agents [10]. Apart from these mechanisms, the endophytic bacterial isolates in this study are thought to be able to induce the resistance of host plants to pathogens. Induction of host plant resistance can be in the form of local and / or systemic resistance induction [11].

Endophytic bacteria play an important role in controlling plant parasitic nematodes [1,12,17]. The application of endophytic bacteria strains JM22 (*Enterobacter asburiae*) and 89B-61 (*Pseudomonas flourescens*) by immersing the seeds was able to suppress the number of eggs and root knots of *Meloidogyne* spp. on cotton and cucumber [1]. [13] reported that endophytic bacteria isolated from tagetes were able to suppress the root lesio nematode population (*Pratylenchus penetrans*) in potato plants. The results of research by [14] reported that the endophytic bacteria isolated from patchouli was able to suppress the root wound nematode population (*Pratylenchus brachyurus*) in patchouli.

3.3 Effect of Endophytic Bacteria on Plant Growth

A total of 11 endophytic bacterial isolates were tested to determine their effect on tomato plant growth. The results showed that some endophytic bacterial isolates with the seed immersion method were able to increase the height, crown dry weight or root dry weight of tomato plants. Isolates S3, T2, J1 and J3 were able to increase plant height and were significantly different than the control, with an increase of 26.39%, 17.47%, 21.93% and 16.36%. Isolates S3, J1 and J3 were able to increase crown dry weight compared to control, with an increase in percentage of 81.86%, 99.47% and 71.65%. Isolate J3 was able to increase root dry weight the highest compared to other isolates (Table 2**).**

The results showed that all endophytic bacterial isolates with the application of seed immersion followed by watering the soil were able to increase the height and dry weight of the canopy, but it did not significantly affect the dry weight of tomato plant roots. Isolate T1 was able to increase plant height better than other isolates and was significantly different

from control, with an increase of 25.86%. Meanwhile, isolate J4 was able to increase shoot dry weight and root dry weight compared other isolates were significantly different from the control for shoot dry weight parameters, with an increase of 48.26% (Table 3).

Table 3 Effect of treatment of endophytic bacteria with the seed immersion method on the growth of tomato plants

Isolate of endophytic bacteria	Plant height (cm)		Dry weight of shoot (g)	Dry weight of root (g)
Control	67.25	ab	5.68 ab	0.98 cd
S1	66.00	a	4.83 a	0.47 a
S2	77.25	bcde	10.33 d	0.55 ab
S3	85.00	e	9.37 bcd	0.80 abcd
T1	72.50	Abcd	6.33 abc	0.60 ab
T2	79.00	Cde	8.45 abcd	0.78 abcd
T3	77.75	bcde	8.75 abcd	0.70 abc
T4	75.25	abcde	8.03 abcd	0.65 abc
J1	82.00	De	11.33 d	0.58 ab
J2	70.66	Abc	9.10 bcd	0.83 abcd
J3	78.25	Cde	9.75ccd	1.08 d
J4	76.00	abcde	7.85 abcd	0.88 bcd

[a]The numbers on the same treatment and column followed by the same letter are not significantly different at the 5% test level (Duncan's multiple interval test).

The results showed that the application method of endophytic bacteria by soaking the seeds and watering the soil had a significant effect on plant height and shoot dry weight, but did not affect on root dry weight of tomato plants (Table 3).

The results of this study indicate that some endophytic bacterial isolates from forest plants are able to stimulate tomato plant growth.[3] reported that the treatment of endophytic bacteria by application of seed immersion, root immersion and ground watering affected the growth of tomato plants. [14] reported that treatment of endophytic bacteria by watering can increase the growth of patchouli plants. According to [2], the ability of endophytic bacteria to produce growth hormones such as auxins, cytokinins and gibberellins is the most common thing in increasing plant growth and development. Other positive effects of endophytic bacteria in enhancing growth and development such as the ability to fix nitrogen are reported in several species of endophytic bacteria including Azospirillum sp., Enterobacter cloacae, Alcaligenes, Acetobacter diazotrophicus, Herbaspirillum seropedicae, Azoarcus sp. and Ideonella dechlorantans. The positive effects of endophytic bacteria on plant growth can also occur by indirect mechanisms, including reduced levels of parasitism, disease severity and incidence, and decreased susceptibility to abiotic stress such as tolerance to drought or cold temperatures [15]. In addition, some endophytic bacteria are also able to stimulate plant

growth through the production of adenine and vitamin B12 derivatives. Some endophytic bacteria are reported to be associated and act as growth promoters in several kinds of vegetable crops including tomatoes. [16] reported tomato seedlings inoculated with the bacterium Pseudomonas sp. WCS417r strain showed increased plant growth accompanied by colonization in internal root tissue which caused suppression of pathogenic bacterial colonization of the Pseudomonas group which was detrimental to the tomato plant.

Table 4 Effect of treatment of endophytic bacteria with the seed immersion method followed by soil drensching application on the growth of tomato plants

Isolate of endophytic bacteria	Plant height (cm)		Shoot dry weight (g)		Root dry weight (g)	
Control	87.00	a	15.48	a	1.48	a
S1	90.00	a	15.93	a	1.30	a
S2	96.50	Ab	16.73	ab	1.20	a
S3	100.25	Ab	16.70	ab	1.18	a
T1	109.50	B	18.63	ab	1.40	a
T2	102.00	ab	18.63	ab	1.28	a
T3	97.25	ab	18.90	ab	1.28	a
T4	98.25	ab	18.70	ab	1.68	a
J1	98.00	ab	22.03	ab	1.33	a
J2	97.25	ab	18.88	ab	1.63	a
J3	97.50	Ab	20.15	ab	1.40	a
J4	101.50	ab	22.95	b	1.68	a

[a]The numbers on the same treatment and column followed by the same letter are not significantly different at the 5% test level (Duncan's multiple interval test).

4. Conclusion

Endophytic bacterial isolated from the roots of Jabon (*Anthocephalus cadamba* Miq.), Sengon (*Albizia chinensis* (Osbeck) Merr.), and Tanjung (*Mimusops elengi* L.) indicated the potentiality for controlling plant parasitic nematodes Meloidogyne spp and for promoting the growth of tomato. Treatment of endophytic bacterial isolates using the application method of soaking the seeds and watering the soil had a significant effect on increasing growth and suppressing the number of tomato plant roots. Isolates S1 and T1 with the application method of soaking the seeds as well as watering them to the soil were able to reduce the number of root knots caused by *Meloidogyne* sp. in tomato plants up to 80%.

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Potential Evaluation of Water Hyacinth (Eichhornia crassipes (Mart.) Solms) as a Growth Media for *Vanda foetida* J.J.Sm.

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Abstract

Despite being common to be used as orchid planting media, the existence of tree ferns is threatened, hence it is necessary to search for alternatives. This study is conducted to evaluate the potential of water hyacinth as a growth media. The measured physiochemical properties of the water hyacinth and tree fern (control) media respectively: bulk density 80 - 130 g/dm^3, 150 - 160 g/dm^3; porosity 28 - 43%, 45- 70%; water absorption 242 - 465%, 250 - 450%; humidity 7 - 13%, 11 - 18%; organic content 76 - 89.5%, 80.5 - 97% and cation exchange capacities of 40 - 46 cmol (+)/kg, 71 - 77 cmol (+)/kg. Growth observations were carried out for 84 days, focusing on the growth of shoot, and the formation of leaf and root. The growth of orchids on the water hyacinth media is not significantly different compared to the tree fern. It can be concluded that the water hyacinth have the potential to be used as a growth media for *Vanda foetida* J.J.Sm., but to determine the long-term effect of the media on the orchid growth, further research would be needed.

Keywords: *Tree fern, water hyacinth, Vanda foetida J.J.Sm., growth, physical and chemical properties.*

1. Introduction

The high demand for orchids is caused by the high aesthetic value of its flower [1]. People's interest in orchids is increasing, as indicated by orchid production in Indonesia which tends to increase from year to year [2]. Orchid production increased by 23%, from 19.7 million stalks in 2014 to 24.7 million stalks in 2018. Meanwhile, the largest production in 2018 occurred in West Java province with 8 million orchids [3].

Various types of growth media are available to support orchid growth [4]. Some of the planting media that are often used include moss media, wood chips, charcoal, brick, wood shavings, tree bark, coconut fibers, and fern-root media [5]. The fern-root media is one of the planting media that is commonly used in orchid cultivation because of the ability to hold water and maintain root moisture [6].

In Indonesia, there are at least two species of tree ferns that are commonly used as a growth medium for orchids, *Cyathea contaminans* and *Cyathea latebrosa* or as the local Sundanese called it "Bagedor" and "Sieur" ferns. These ferns are taken from their natural habitat, then processed into media [7,8]. The existence of the two plants is threatened and grouped into

CITES Appendix II list [9]. The demand for fern media is increasing along with orchid production [10]. It is feared that the demand for ferns media will threaten the existence of the tree ferns. Therefore, an alternative medium for orchid growth is needed to reduce the exploitation of tree ferns.

Water hyacinth (*Eichhornia crassipes*) is one of the most invasive freshwater plants in the world [11]. The rapid growth of the Water hyacinth population damaging the local ecosystem [12, 13]. These problems harm the people's economy, especially farmers and fishermen [14]. Therefore, controlling the population of water hyacinth is necessary to reduce the ecological impact. Population control can be done physically through the direct collection. However, this method can cause other problems such as the accumulation of water hyacinth waste and the need to allocate an area to accommodate this waste [15].

Apart from having a detrimental effect, the existence of water hyacinth can also provide benefits. Water hyacinth is a hyperaccumulator that could absorb heavy metals such as lead and chromium in water through the roots. The heavy metals are then stored in the cell vacuoles of the root, stem, and leaves [16]. Because of these properties, water hyacinth can be used in the phytoremediation process [17].

Water hyacinth can be used as an additional source of animal feed, compost, and biogas [18, 19, 20, 21]. However, the use of contaminated water hyacinth can potentially expose livestock and crop to heavy metal [19, 22]. The consumption of livestock and agricultural products exposed to heavy metals can harm the human health [23, 24]. Therefore, it is necessary to utilize water hyacinth waste safely and does not endanger human health. One of the possible uses of water hyacinth is as a growing medium for ornamental plants. It is hoped that water hyacinth can be utilized as a planting medium for epiphytic orchids, which has a role as a place of attachment, maintaining humidity, and retaining nutrients carried by rainwater and dust [25]. It is expected that the use of water hyacinth as a medium for orchid growth will reduce the exploitation of the protected tree ferns.

2. Materials and Methods

2.1 Materials

Water hyacinths were taken from the following place: Sapan rice fields Area, Bandung City; Cinanggerang river, Sumedang Regency; tanning waste-polluted river in Garut, and Wastewater Treatment Plant (IPAL) at one of the tannery factories in Garut. *Vanda foetida* which is being used came from Pagar Alam, Sumatra. The orchids used are 20-30 cm high, 30-50 cm wide, and 6-12 leaves.

2.2 Methods

2.2.1 Time and Location

The research was conducted from 5 October 2019 to 5 March 2020 on a garden located in Parongpong, West Bandung Regency, West Java. The garden is equipped with a shading net

(shade level of 65-75%). The microclimate conditions of the location during the study were: 24 ± 4°C and 72 ± 21% of moisture.

2.2.2 Research Design

This research is using a completely randomized design (CRD) treatment consisting of 6 groups and 6 repetitions. The six groups consist of the fern media group (P) as a control and 5 groups of water hyacinth media (M1 - M5) which originated from different growing places and conditions: Cinanggerang river, Sumedang Regency (M1); Sapan rice fields, Bandung City (M2); waters polluted with tannery wastewater in Garut City (M3) and two different ponds inside the Garut tanning wastewater treatment plant system (IPAL) (M4 and M5).

The test was carried out by observing the growth of orchids and measurement of the physicochemical parameters of the media. In this research, all of the media were divided into two groups, namely Group A, which consisted of media that had been planted with orchids for 84 days, while group B was not. Group A and B respectively will represent the condition of the media after and before planting.

2.2.3 Growth Media Production

The water hyacinth is washed thoroughly then dried under the sun for 5 days. The water hyacinth is then cut 3 to 5 cm long. 50 g of dried water hyacinths and 2 L of water are then blended with a Philips HR2116 blender. 2 L of the mixture is then poured into a molding tool. The medium is then dried for 5 days. Wire mesh is then attached to add structure to the dried media (Figure 1). The fern root media which is being used have the same size as the water hyacinth media (15 × 20 × 5 cm).

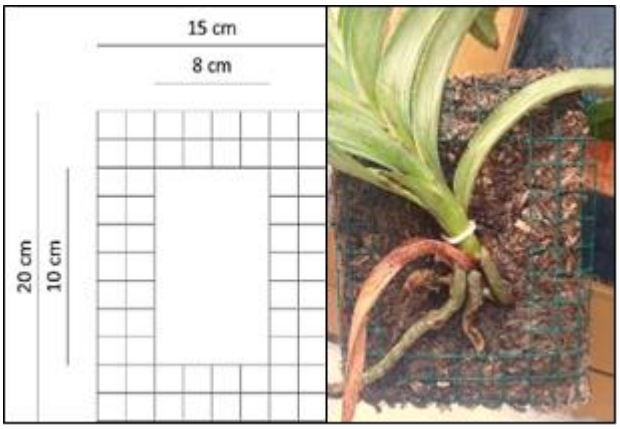

Figure 1. Media frame

2.2.4 Orchids Planting

Orchids are planted by tying the stems together with threads on the medium. All orchids that have been planted on the media are then placed on a wooden frame as shown in Figure 2. The distance between the media is 20 cm. Every day the whole orchids were sprayed with Liquinox vitamin B1 (5 mL / 4 L water) and GrowMore NPK foliar fertilizer (5 g / 4 L water) for 84 days.

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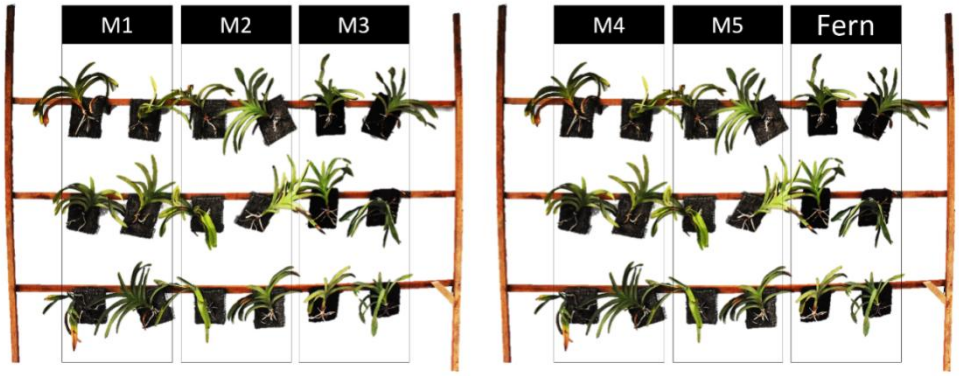

Figure 2. Planting arrangement

2.2.5 Growth Observation

Growth observations were carried out for 84 days, focusing on shoot growth (width and height) and the formation of the leaf and root. Orchid height is measured from the base of the shoot to the tip of the first leaf. Orchid width was measured at the widest part perpendicular to the shoot axis (Figure 3). New leaf formation is noted when new leaflets appeared apically. New root formation on the stem is also noted.

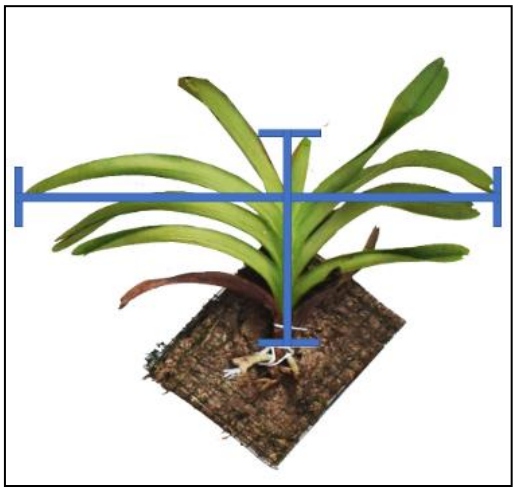

Figure 3. Orchid shoot measurement

2.2.6 The Physical Properties Test

2.2.6.1 Bulk density

Bulk density is the ratio of weight to volume of the media. First, the media is measured to get the total volume (V_t). Then, the media is dried in an oven at 120°C for 12 hours then weighted to get the dry weight (m_0). The Bulk density is calculated by dividing the dry weight (m_0) by the total volume of media (Vt) [26].

$$\text{Bulk density} = \frac{m_o}{V_t}$$

2.2.6.2 Porosity

The porosity of the media was obtained by the water displacement and weighing method. First, the media is measured to get the total volume (V_t). then, the media is dried in an oven at 120°C for 12 hours then weighted to get the dry weight (m_0). After that, the media is immersed underwater for 12 hours. The saturated weight of the media (m_w) is subtracted with its dry weight (m_0). The difference (m_w-m_o) is then divided by the water density to get the total volume of water (V_{water}). The porosity can be defined as the ratio of the total empty space per total volume (V_0/V_t). It is assumed that the total empty space inside the media is equal to the total number of water that the media can hold. Hence, the porosity can be also defined as (V_{water}/V_t) [27, 28].

$$\text{Porosity (\%)} = \frac{V_{Water}}{V_t} \times 100\%$$

2.2.6.3 Water Absorption

The water absorption or water holding capacity is the amount of water that be absorbed per gram of media (% w/w). The water absorption of the media was obtained by the water displacement and weighing method [29]. First, the saturated weight of the media (m_w) is subtracted with its dry weight (m_0). The difference (m_w-m_o) is then divided by the dry weight of the media.

$$\text{Water absorption (\%)} = \frac{m_w - m_o}{m_o} \times 100\%$$

2.2.6.4 Moisture

The moisture of the media was obtained by the weighing method [30]. First, the media is weighted to get the normal weight (m_n). Then, the normal weight of the media (m_n) is subtracted with its dry weight (m_0). The difference (m_n-m_o) is then divided by the normal weight of the media. The obtained value is the humidity which is expressed in %.

$$\text{Moisture (\%)} = \frac{m_n - m_o}{m_n} \times 100\%$$

2.2.7 The Chemical Properties Test

2.2.7.1 Organic Content

Measurement of organic content was carried using the Loss on Ignition (LOI) [31]. Firstly, the whole medium sample is dried in an oven at 110°C for at least four days [32]. Two grams of samples from the six media in the same group were taken and homogenized into one composite sample. Overall, there were 12 composite samples (six each in groups A and B). Two grams of each composite sample were taken and put into the incandescent dish for the combustion process. The sample and incandescent plate were re-weighed using an analytical balance.

Samples were burned in a furnace at a temperature of 650°C for four hours. When finished, the hot sample is placed in a desiccator to lower the temperature until it reaches room temperature. After cooling, the sample was again weighed to obtain the ash weight. Organic compound content is obtained by calculating the difference between the dry weight and ash weight of the sample.

2.2.7.2 Cation Exchange Capacity

The test of cation exchange capacity is carried out by sending samples to the Raksa Buana Laboratory located on JL Phh. Mustopa Gang, Pelita III, No. 29, Cikutra, Cibeunying Kidul, Kota Bandung, West Java 40124. All media samples were tested on this parameter (72 samples, 36 samples in groups A and B respectively).

2.2.8 Data Analysis

First, the normality test is carried out with Shapiro-Wilk. Then, the significance of treatment is carried out using the One-Way ANOVA or Kruskal-Wallis. Differences among treatment means were assessed by Tukey's Post Hoc or paired Mann-Whitney test. All statistical tests were performed at the 5% level.

3. Results and Discussion

3.1 Growth Parameters

Observation of orchid growth was carried out for 84 days. The parameters recorded were the growth in width and height of shoots and the number of individuals growing roots. Based on the observations, the measured growth in height and width of the whole orchid is less than one cm. As seen in Figure 4, the orchid plant in the water hyacinth growing media group from the Cinanggerang (M1) river has the highest growth of shoot height, at 0.63 mm/week. This value was not different from the fern group (control), which was 0.63 mm/week. The lowest growth of shoot height was observed in the water hyacinth growing media group of IPAL (M5), at 0.21 mm/week.

The orchids in the fern media group (control) had the highest growth in shoot width, at 0.63 mm/week. The lowest growth of shoot width was observed in the water hyacinth group of rivers polluted with waste (M3), at 32 mm/week (Figure 4). The rate of increase in height and width between all media groups was not significantly different based on statistical tests ($P<0,05$).

The entire orchid group has a relatively equal number of leaves with 6-12 leaves per individual. Based on observations, there was no increase in the number of leaves. The number of individual leaves between all groups of water hyacinth and fern media was not significantly different based on statistical tests at the 5% level.

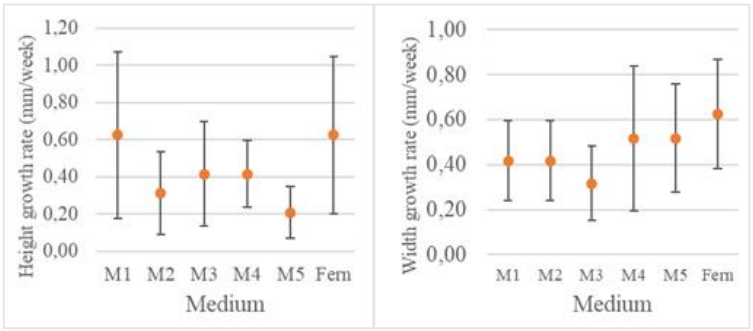

Figure 4. Orchid shoot height and width growth rate.

All orchids grown on water hyacinth media have a higher parameter value (Figure 5). The highest percentage was observed in the orchid group grown on water hyacinth media from the wastewater treatment plant (M4 and M5) which was 50% and the lowest was in the fern group, which was 20%. Even so, the percentage of the number of individuals forming roots was not significantly different.

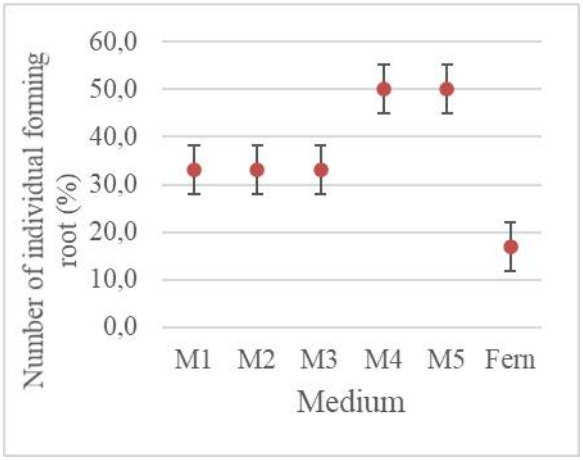

Figure 5. The percentage of orchids forming root per group.

Roots are important organs because they act as a link between orchids and their growing media. Orchids can absorb material from the substrate through root contact with the growth medium [33]. Therefore, the growing medium will affect the orchid growth after the roots have grown and contacted the media. In this study, it was found that most orchids had not yet grown roots so that the growth medium had not yet been able to influence the orchid growth. It is known that there is no difference in the number of leaves. Although it could be observed that there was a difference in the value of the increase in shoot height between the water hyacinth medium and the fern medium (control), this difference was not significant ($p > 0.05$). *Vanda foetida* orchid growth is very slow, just like other epiphytic orchids in general [34]. Normally, planting *Vanda sp.* orchids for three months only showed a change in leaf and root length of less than one cm [35]. The same thing was observed in this study, the increase

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in length and width of orchid shoots in all growing media groups for 84 days did not exceed 1 cm. In another study, planting orchids *Vanda sp.* for 16 weeks on various planting media has not affected the value of the growth parameter in the number of leaves [34]. Therefore, further research is needed with a longer period to determine the long-term effect of water hyacinth media on the growth of *Vanda foetida.*

3.2 Physical Profile of Media

As can be seen in Figure 6, media group A has a lower physical profile than media group B. In group A, the bulk density of the Cinanggerang river water hyacinth media group (M1) has the lowest number, which was 73.80 dm3, while the highest value is owned by the fern media group (control) at 145.93 g/dm3. In group B, the lowest number observed on water hyacinth media group that grew in rivers polluted with tanning waste (M3) at 80,50 g/dm3, while the highest observed on fern media group, at 164,68 g/dm3. The bulk density of fern media has higher and significantly different from most water hyacinth media (P>0,05).

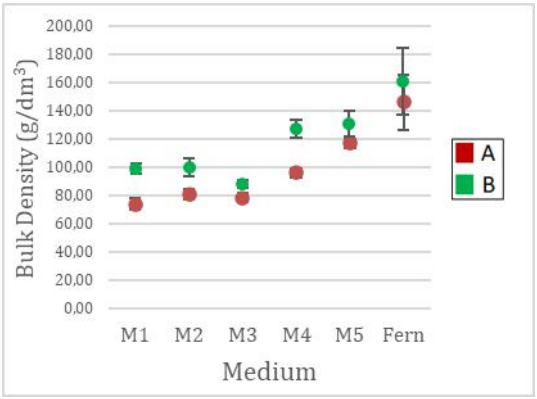

Figure 6. Bulk density.

Bulk density is the ratio of weight to volume of the media [36]. Usually, the value of bulk density is in line with the density of the medium. In general, orchid roots will be easier to penetrate in media with low bulk density. Several types of epiphytic orchids can grow well with bulk density values reaching 1.100 g/dm3 [37]. When compared to charcoal and fern media, water hyacinth medium has a lower bulk density value hence it can be more easily penetrated by orchid roots.

It can be seen in Figure 7, in group A, the porosity value of the water hyacinth media group from the Cinanggerang river (M1) has the lowest value, which was 20.94%, while the highest value belongs to the fern media, which was 37.58%. In group B, the lowest value was owned by the water hyacinth media group that grew in the Sapan rice fields (M2) 28.62%, while the highest number measured on the fern media at 69.69%. It is known that all water hyacinth media have different porosity values from fern media based on statistical tests at the 5% level. The overall porosity value of the water hyacinth medium ranged from 20 - 40%. This value is lower compared to the fern media which has a porosity of 45-70%. The difference in porosity level between fern media and water hyacinth can be caused by various factors such as size, texture, distribution pattern, surface area, and the pore texture of the media [38].

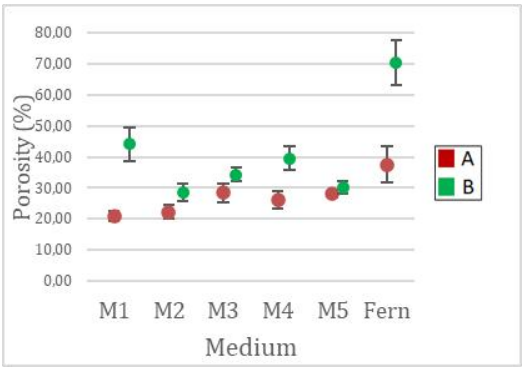

Figure 7. Porosity.

Epiphytic orchids do not require growing media with high porosity values. Epiphytic orchids can even grow on logs and rocks [18]. Orchids grown on media with high porosity (75%) show better growth when compared to orchids grown on media with lower porosity [27]. Media with low porosity can be mixed with other growth media that have a higher porosity to achieve an adequate level of porosity [38]. Media with high porosity allow gas exchange to take place [39]. The fern media has macropores to provide aeration, maintain the moisture and water content so that it can support the orchid root growth [40]. Apart from providing aeration, high porosity also provides drainage which can reduce water content in the media and prevent mold overgrowth [41]. It should be considered that media that is too porous are lacking the ability to hold the water and nutrient [42].

Overall, as can be seen in Figure 8, group A media has a lower water absorption than media group B. In group A, the highest water absorption capacity is owned by the water hyacinth media group that grows in rivers polluted by tanning waste (M3) at 357.35% and the smallest number, the water hyacinth media group grown in the wastewater treatment plant (M5) 241.44%, while the fern media group (control) at 271.72%. Then in group B, the highest number observed in the Cinanggerang river water hyacinth media group (M1) with a value of 465.60%, and the smallest was in the water hyacinth media group grown in the wastewater treatment plant (M5) with a value of 242.84%, while the media group fern (control) had a value of 444.04%.

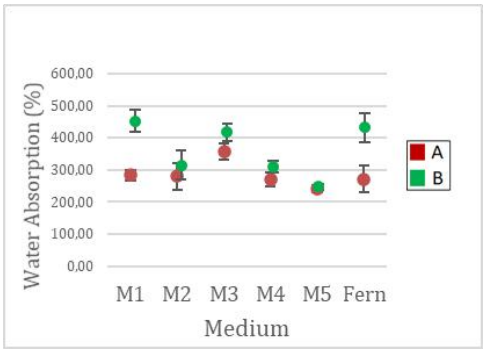

Figure 8. Water absorption.

Water absorption indicates the ability of a medium to hold water. This parameter is important because, without adequate water supply, orchid plants can experience drought stress and die [39]. Overall, the water hyacinth medium had similar values to the fern medium. All water hyacinth media were declared no different from fern media based on statistical tests at the 5% level. It can be said that the water hyacinth media has the best water absorption as well as the fern medium.

The moisture was measured after the watering process was stopped. This is done to get the natural moisture value. In the results of Moisture parameters measurement (Figure 9), media group A has a lower number compared to group B. In group A, the Moisture of the water hyacinth media group that grew on WWTP (M5) had the smallest value, 4.04%, while the highest number measured on the fern media, 11.08%. In group B, the M5 media group had the smallest value, at 7.13%, and the highest measured on fern media, at 21.99%. Both water hyacinth and ferns media have low moisture values. Therefore, to maintain the humidity of the orchid it is necessary to water it regularly.

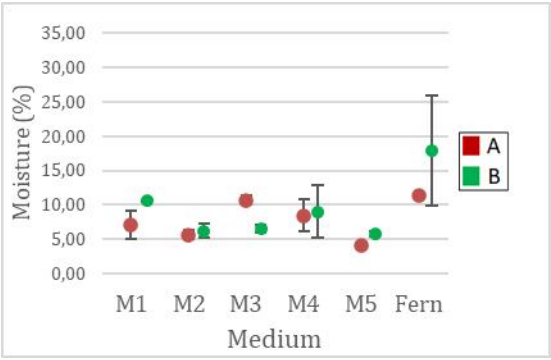

Figure 9. Moisture.

3.3 Chemical Profile of Media

Overall, the group of media that was given the planting treatment (group A) had lower organic matter content than group B (Table 1). The decrease in organic matter content is likely due to the decomposition process [42]. In group A, the highest value of organic content was measured on the water hyacinth media from the Cinanggerang river (M1) with a value of 86%, while the lowest value was in the water hyacinth media group grown in the Sapan rice fields (M2) with a value of 76%.

Table 1. Organic content.

Medium		M1	M2	M3	M4	M5	Fern
Organic Content	A	86%	76%	79%	84%	80%	81%
	B	90%	86%	89%	86%	85%	97%

Epiphytic orchids generally take up most of the macronutrients such as nitrogen and carbon from the surrounding air, either directly or with the help of microorganisms. In orchid media, the substrate with high organic content does not act as the main source of nutrition. Media with high organic content generally acts as a good pH buffer and has a high cation exchange capacity value [39, 43]. Even so, orchids can still obtain additional nutrients from substrates such as woody stems. Mycorrhizae will decompose the substrate and absorb nutrients through hyphae. The orchid roots will then receive nutrients from the mycorrhizae [44]. Therefore, the selection of orchid media can be based on its organic content.

Epiphytic orchid planting media must be able to hold nutrients carried by rainwater and dust [25]. This ability is often represented by the value of cation exchange capacity (CEC). The cation exchange capacity is a parameter that indicates the ability of a medium to hold and release compounds or elements in the form of positive ions. The CEC of water hyacinth has a lower value compared to the tree fern media. It can be seen in Figure 10, in group A, the lowest CEC of media are measured on the water hyacinth group grown in the wastewater treatment plant (M4), at 32,48 cmol(+)/kg, while the highest number measured on the fern media group, at 77,22 cmol(+)/kg. In group B, the lowest CEC measured on the water hyacinth media group from Sapan rice fields (M2), at 40,63 cmol(+)/kg, while the highest CEC value measured on the fern media group with a value of 74,95 cmol(+)/kg. Based on the results of statistical tests, all water hyacinth media had significant differences in CEC values on fern media (P>0,05).

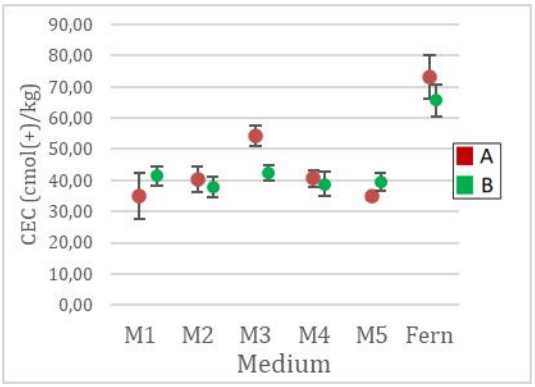

Figure 10. Cation exchange capacity.

Generally, a medium with a high organic content value will also have a high CEC value. Organic compounds have positive and negative charges so they can hold various forms of ions. Examples of nutrients in the form of cations include calcium, magnesium, potassium, and sodium [33]. In general, the greater the CEC value, the more nutrients in the form of cations can be accommodated. Despite having a lower value, the CEC level of water hyacinth media is adequate to hold nutrients for epiphytic orchids [37].

3.3.4 The Relationship Between the Physiochemical Profiles of Media and The Orchid Growth

It is assumed that if a new medium has similar physical and chemical properties to the media that is commonly used, the media has potential as an orchid growth media. Based on statistical analysis, as can be seen in table 2, the water hyacinth media has several similarities to the fern media. The similarity between water hyacinth and fern media is the parameters of porosity, moisture, and water absorption. The difference is in the bulk density and cation exchange capacity. Although different, the CEC value of water hyacinth media is quite high. Water hyacinth media also has a better bulk density value. Because of this, it can be concluded that the water hyacinth media has the potential to be used as an orchid growing medium. Based on the growing location and condition, the media groups M2 (water hyacinth from the rice fields), M3 (water hyacinth from polluted river areas), and M4 (wastewater treatment plant) have the greatest potential to replace fern media because of the low bulk density and similar properties with the fern media on the parameters of porosity, moisture, and water absorption ($P>0.05$).

No differences in growth parameters were observed in orchids grown on water hyacinth and ferns media. This is probably due to the lack of contact between the orchid roots and the media. The growth medium will affect orchid growth through root contact with the media [44]. During the observation period, it was seen that most orchids had not yet formed a new root.

Table 2. The similarity of the five groups of water hyacinth to fern medium. Note: (+) not significantly different from the fern media group ($P< 0.05$), and (-) significantly different from the fern media group ($P>0.05$).

Growth parameter	Medium					
	Water hyacinth					Fern
	M1	M2	M3	M4	M5	
Height Growth	+	+	+	+	+	+
Width Growth	+	+	+	+	+	+
Individual forming root	+	+	+	+	+	+
New Leaf formation	+	+	+	+	+	+
Physiochemical Parameter						
Bulk density	-	-	-	-	+	+
Porosity	-	+	+	+	+	+
Moisture	+	+	+	+	-	+
Water absorption	+	+	+	+	+	+
Cation Exchange Capacity	-	-	-	-	-	+

In the additional observations, after planting for 8 months, differences in root penetration of *Vanda foetida* were observed on water hyacinth medium (Figure 11) and fern medium (Figure 12). In water hyacinth media, orchid roots grow and penetrate the media, while in fern media, most of the orchid roots only grow on the outer surface of the media. According to the

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literature, it is known that media with low bulk density values can be more easily penetrated by orchid roots [37].

For 84 days planting period, the effect of the planting medium on orchid growth cannot be observed. It is known that the physical and chemical profile of the growth medium will affect orchid growth over a longer planting period. In the research that discusses the effect of the physicochemical profile of media on orchid growth, the length of time of the study can reach one year or more, so the effect of media on the orchid growth can be observed [27, 36, 37]. Therefore, to determine the long-term effect of water hyacinth media on the growth of *Vanda foetida*, further research with a longer period is needed.

Figure 11. The root growth on water hyacinth media.

Figure 12. The root growth on fern media.

4. Conclusions

Based on the research it can be concluded that:

(1) no differences in growth parameters were observed in orchids grown on water hyacinth and ferns media. However, due to the low growth rate of orchids, further research is needed;

(2) water hyacinth media has similar physical and chemical profiles to fern media, so it has the potential to be used as a growth media for *Vanda foetida*, however, it is necessary to add structure because of the fragile nature of the medium.

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Production of Lipid and Protein from Mixed Cultivation of *Chlorella vulgaris* and *Scenedesmus* Using Anaerobically Digested Dairy Manure Wastewater (ADDMW) as Cultivation Medium

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Abstract

Microalgae have been cultivated commercially to produce algal biomass and various metabolites beneficial to meet human needs, e.g., lipid as biofuel and protein as a source of essential protein for human health. This research aims to determine the effect of mixed cultivation of *Chlorella vulgaris* and *Scenedesmus* sp. towards the production of primary metabolites, chlorophyll, and inorganic compounds reduction on ADDMW medium. Mixed cultivation showed a dominance of C. *vulgaris* growth compared to that of *Scenedesmus* sp. Mixed cultivation also produced microalgal biomass with a specific growth rate of 0,232–0,344 day^{-1}, biomass yield of 6,147–12,540 g L^{-1}, and biomass productivity of 2,090–3,073 g L-1 day^{-1}. These growth performances were approximately the same as those of monoculture. Mixed cultivation produced lipid with productivity of 0,387–0,514 g L-1 day^{-1}, significantly higher compared to that of monoculture (0,243–0,272 g L^{-1} day^{-1}). Nevertheless, the protein (20,400–21,580 mg (g^{-1} biomass)) and chlorophyll content (4,194–5,174 mg L^{-1}) of the mixed culture were not significantly different compared to those of monoculture.

Keywords: Microalgae, ADDMW, Mixed cultivation, C. *vulgaris*, *Scenedesmus* sp.

1. Introduction

Commercial utilization of microalgae has been developed for the past 30 years. Microalgae are cultivated commercially to produce algal biomass and various metabolites beneficial to meet human needs, both primary and secondary metabolites. Various secondary metabolites that can be used as natural coloring can be extracted from microalgae, e.g., phycocyanin from *Spirulina* sp. [1] and β-carotene from *Dunaliella salina* [2]. Microalgae biomass contains many primary metabolites e.g., lipid that can be used as biofuel to reduce the usage of fossil fuel and the negative effects of global warming [3]. In Indonesia, microalgae are cultivated to produce protein and vitamin as a source of various products e.g., food, pharmaceutical, and cosmetics [4].

The development of mixed cultivation of microalgae has become a prospect to increase the production of microalgal bioproducts. Mixed cultivation is developed because of its potential to increase the adaptability of the microalgae culture to disruption from other microorganisms through metabolic communication mechanism and division of labor [5], therefore increasing the productivity of microalgal bioproduct. Mixed cultivation also has the

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potential to reduce contamination by bacteria, viruses, protozoa, yeast, or mold that may decrease microalgal bioproduct production significantly [6].

Microalgae cultivation using dairy wastewater as a cultivation medium has been immensely developed, including anaerobically digested dairy manure wastewater (ADDMW), which is the liquid fraction from anaerobic digestion of dairy manure [7,8]. The inorganic compounds in dairy manure can be converted to various high-value metabolites.

This research aims to determine the effect of mixed cultivation of *C. vulgaris* and *Scenedesmus* sp. towards the production of primary metabolites, chlorophyll, and inorganic compounds reduction on ADDMW medium. The objective of this research is to determine how mixed cultivation of microalgae is a good prospect to develop better production of microalgal bioproducts. Research on the potential of mixed microalgal cultivation can be used as a basis to design the production of microalgal bioproducts with a larger scale and higher productivity.

2. Materials and Methods

2.1. Materials

The bioreactors used to cultivate the algae for both monoculture and mixed culture were vertical tank reactors (VTR). They were tubular photobioreactors made of acrylic as shown in Figure 1. The bioreactor schematic is shown in Figure 2. The bioreactor had a total capacity of 25 L, while an LED strip lamps with an intensity of 25 μmol m^{-2} s^{-1} were used the lighting system. The sparger used to bubble the gas particles were made of borosilicate.

Figure 1 The bioreactor used in this research

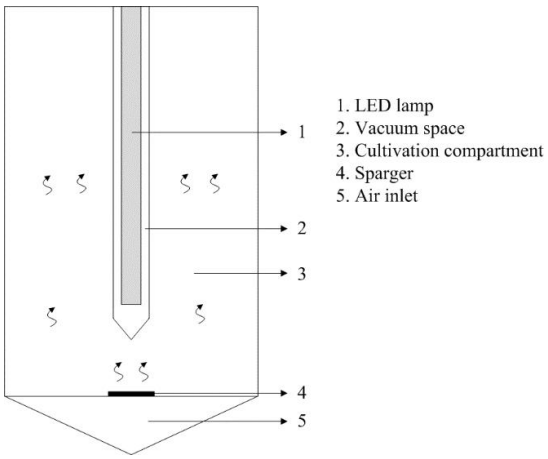

Figure 2 Bioreactor schematic

This research utilized an analytical scale (Precisa, Switzerland), pH meter (Mettler Toledo, Switzerland), UV-VIS spectrophotometer (Shimadzu, Japan), centrifuge (Thermo Fisher Scientific, USA), sonicator (Branson, USA), autoclave (ALP, Japan), digital microscope (Canon, Japan), and rotary vacuum evaporator (Buchi, Switzerland) provided by the School of Life Science and Technology, Bandung Institute of Technology. *Scenedesmus* sp. was acquired from Indonesian Culture Collection, Research Center for Biology, Indonesian Academy of Sciences, Bogor. *C. vulgaris* was acquired from the Center of Freshwater Fish Cultivation, Jepara, Central Java. Anaerobically digested dairy manure wastewater (ADDMW) was acquired from a biogas installation of Faculty of Animal Husbandry, Universitas Padjadjaran, West Java. All chemicals including macronutrients ($NaNO_3$, $MgSO4.7H_2O$, K_2HPO_4, KH_2PO_4, etc.) and trace elements ($ZnSO_4.7H_2O$, $MnCl_2.4H_2O$, vitamins, etc.) used in this research were acquired from School of Life Science and Technology, Bandung Institute of Technology.

2.2. Methods

2.2.1. Cultivation Medium Preparation

Bold's basal medium (BBM) was made by previously creating ten stock solutions as described in Table 1. 10 mL from stock solutions 1–6 and 1 mL from stock solutions 7–10 was mixed for every 1 L of medium. Distilled water was added until the desired volume was reached. The medium was then sterilized using autoclave on the temperature of 121°C and pressure of 1,5 bar for 10 minutes.

AF-6 medium was made by previously creating ten stock solutions as described in Table 2. 10 mL from stock solutions 1–6 and 1 mL from stock solutions 7–10 was mixed for every 1 L of medium. Distilled water was added until the desired volume was reached. The medium was then sterilized using autoclave on the temperature of 121°C and pressure of 1,5 bar for 10 minutes.

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Table 1 Bold's basal medium (BBM) composition

Stock solution number	Stock solution name	Concentration (g L^{-1})
1	$NaNO_3$	25
2	$MgSO_4.7H_2O$	7,5
3	NaCl	2,5
4	K_2HPO_4	7,5
5	KH_2PO_4	17,5
6	$CaCl_2.2H_2O$	2,5
7	Trace elements:	
	$ZnSO_4.7H_2O$	8,82
	$MnCl_2.4H_2O$	1,44
	MoO_3	0,71
	$CuSO_4.5H_2O$	1,57
	$Co(NO_3)_2.6H_2O$	0,49
8	H_3BO_3	11,42
9	EDTA	50
	KOH	31
10	$FeSO_4.7H_2O$	4,98
	H_2SO_4 (conc.)	1 mL L^{-1}

ADDMW was poured inside 1 L glass bottles, then sterilized using autoclave on temperature of 121°C and pressure of 1,5 bar for 10 minutes. The sterilized ADDMW medium was then filtered using cotton pads to separate the solid and liquid fractions. The liquid fraction was used as cultivation medium.

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Table 2 AF-6 medium composition

Stock solution number	Stock solution name	Concentration (g L^{-1})
1	$NaNO_3$	140
2	NH_4NO_3	22
3	$MgSO_4.7H_2O$	30
4	K_2HPO_4	5
5	KH_2PO_4	10
6	$CaCl_2.2H_2O$	10
7	Fe-citrate	2
8	Citric acid	2
9	Trace elements:	
	$Na_2EDTA.2H_2O$	5
	$FeCl_3.6H_2O$	0,98
	$MnCl_2.4H_2O$	0,18
	$ZnSO_4.7H_2O$	0,11
	$CoCl_2.6H_2O$	0,02
	$Na_2MoO_4.2H_2O$	0,0125
10	Vitamin solution:	
	Thiamine	0,01
	Biotin	0,002
	Cyanocobalamin	0,001
	Pyridoxine	0,001

2.2.2. Microalgae Inoculum Cultivation

Cultivation of each microalgae species was performed inside 1 L glass bottles with the working volume of 800 mL on each bottle as shown in Figure 3. The subculture of each species was done with a volumetric ratio of inoculum : medium = 1 : 4. Cultivation of each microalgae species was done for 1 week. In the first week, *C. vulgaris* cultivation was done using Bold's basal medium (BBM), while *Scenedesmus* sp. cultivation was done using AF-6 medium. In the following week, both species were acclimatized in the ADDMW medium by subculturing them in ADDMW which was diluted by a factor of 25.

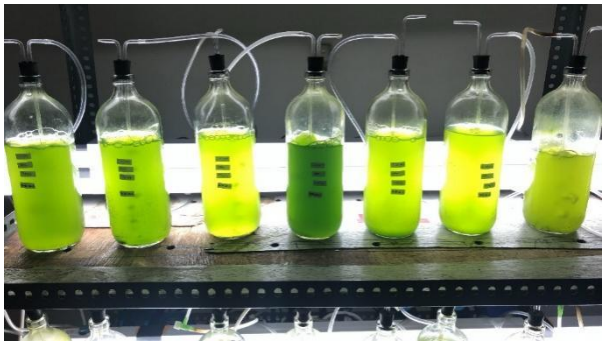

Figure 3 Microalgae cultivation in 1 L glass bottles

2.2.3. Microalgae Cultivation on 20 L Tubular Photobioreactor and Mixed Microalgae Cultivation Variations

Microalgae cultivation was performed in a tubular photobioreactor made of acrylic with a total working volume of 20 L. The cultivation variations used in this research were monoculture and mixed culture. Mixed cultivation was performed with a volumetric ratio of inoculum : medium = 1 : 4. ADDMW diluted by a factor of 4 was used as the cultivation medium. The microalgae were cultivated for 7 days. All cultivation variations were conditioned at the aeration rate of 3 L/minute, room temperature (~25°C), and photoperiodism of 16:8 (L:D).

2.2.4. Sampling and pH Measurement

The sampling of each cultivation variation was done by isolating approximately 600 mL of the culture on glass bottles. Sampling was done twice a day. pH measurement of the samples was done once a day using a pH meter.

2.2.5. Cell Number Determination

The determination of the number of microalgal cells was done using a hemacytometer, sized 1 mm × 1 mm × 0,1 mm. The sample was pipetted on the hemacytometer until it covered the whole chamber. The hemacytometer was then covered with a cover glass. The number of cells was then determined using a digital microscope by determining the average number of cells from the four gridded areas of the hemacytometer, then multiplied by 10^4. Cell number determination was done in triplicate. The cell growth curve was then made by plotting the cell number data against time. The specific growth rate was determined based on the linear gradient of the cell growth curve.

2.2.6. Determination of Microalgal Biomass Yield and Productivity

30 mL of the culture was poured into 3 falcon tubes, 10 mL on each tube. The culture was then centrifuged at the speed of 5.000 rpm for 10 minutes. The supernatant was removed, then the microalgal fresh weight was determined by calculating the difference between the

biomass-containing tube and the empty tube. Biomass yield was determined by dividing the fresh weight by the initial culture volume. Biomass productivity was determined by dividing the highest biomass productivity by the time in which the highest productivity was achieved.

2.2.7. Lipid Extraction and Determination of Microalgal Lipid Productivity

100 mL of culture was poured into 10 falcon tubes, 10 mL on each tube. The culture was then centrifuged at the speed of 5.000 rpm for 10 minutes. The supernatant was removed, then the microalgal fresh weight was determined by calculating the difference between the biomass-containing tube and the empty tube.

Lipid extraction was done using a modified version of the Bligh & Dyer method [9]. The microalgae pellet acquired from the centrifuged tubes was mixed with 100 mL of distilled water, then sonicated using the sonicator. The sonicated biomass was then poured into a separatory funnel and mixed with chloroform and methanol with a volumetric ratio of 1:1 (v/v). The funnel containing the sample and the solvent was mixed for 5 minutes, then positioned downward to allow both solvents to separate. Lipid-containing chloroform fraction was separated from the funnel, then the solvent was evaporated using a rotary vacuum evaporator. The remaining solution was poured on a beaker glass, and the remaining solvent was allowed to evaporate. The weight of the lipid was determined by calculating the difference between the lipid-containing glass and the empty glass. The lipid productivity was determined by dividing the weight of the recovered lipid by the initial culture volume (100 mL).

2.2.8. Microalgal Protein Extraction

Protein extraction was done using a solvent extraction method with NaOH as the solvent [10]. 3 mL of 0,5 N NaOH was added to biomass-containing falcon tubes. The tubes were then incubated in an 80°C water bath for 10 minutes to allow protein extraction. The extracted sample was then centrifuged at the speed of 5.000 rpm for 10 minutes. The supernatant was poured to a reaction tube, then the procedure was repeated one more time.

2.2.9. Determination of Microalgal Protein Content

Protein content determination was done using the Bradford method [11]. 100 mg of Coomassie Blue G250 was solubilized in 50 mL of 96% ethanol. 100 mL of 85% phosphoric acid was then added to the mixture. Distilled water was added until the volume reached 1 L. The mixture was then filtered using Whatman No. 1 filter paper. The filtrate was then used as the reagent to determine the protein content.

The protein content of the extracted samples was determined by mixing 1 mL of the extracted samples' supernatant with 5 mL of Bradford reagent. The absorbance of the sample was then determined on a 595 nm wavelength using the UV-VIS spectrophotometer. The absorbance value was converted to protein content using the equation derived from the standard curve made using bovine serum albumin (BSA) with concentrations at the range of 0 – 1.000 ppm. The protein weight inside the extract was determined by multiplying the

protein content value with the volume of the supernatant. The percentage of protein content was then determined by dividing the protein weight (in mg) by the fresh weight of algal biomass (in g).

2.2.10. Determination of Microalgal Chlorophyll Content

30 mL of the culture was poured into 3 falcon tubes, 10 mL on each tube. The culture was then centrifuged at the speed of 5.000 rpm for 10 minutes. The supernatant was removed, so that microalgal fresh weight remained in the tubes. Extraction was done by adding 5 mL of 96% ethanol to the biomass. The sample was then stored in the refrigerator for at least 24 hours. The sample was then sonicated for 90 seconds using the sonicator. The absorbances of the sample on 649 nm (A_{649}) and 665 nm (A_{665}) wavelengths were determined using a UV-VIS spectrophotometer. The chlorophyll content was then determined using equations (1) and (2) [12]. The chlorophyll a content was determined using the equation

$$Chl_a = 13{,}95A_{665} - 6{,}88A_{649} \qquad (1)$$

with Chl_a chlorophyll a content (mg/L), while the chlorophyll b content was determined using the equation

$$Chl_b = 24{,}96A_{649} - 7{,}32A_{665} \qquad (2)$$

with Chl_b chlorophyll b content (mg/L). The total chlorophyll content was determined by adding the values of chlorophyll a and chlorophyll b content.

2.2.11. Data Analysis and Interpretation

All parameters measured in this research were analyzed using one-way analysis of variance (one-way ANOVA), followed by Duncan's multiple range test to determine whether there was a significant difference between the values of the parameters. A parameter was considered significantly different when the value of $p < 0{,}05$.

3. Results and Discussions

3.1 The Effect of Mixed Microalgae Cultivation on Cell Growth and Microalgal Biomass

In this research, the microalgae cultivation was done using four variations: monoculture of *C. vulgaris* (***Chlorella***), monoculture of *Scenedesmus* sp. (***Scenedesmus***), mixed culture of *C. vulgaris* dan *Scenedesmus* sp. with the ratio of 3 : 1 (v/v) (***C3:S1***), and mixed culture of *C. vulgaris* dan *Scenedesmus* sp. with the ratio of 1 : 3 (v/v) (***C1:S3***). The microalgal cultivation system is shown in Figure 4 and 5. In Figures 4 and 5, the cultivation variations respectively from left to right are monoculture of *C. vulgaris*, monoculture of *Scenedesmus* sp., mixed culture of *C. vulgaris* and *Scenedesmus* sp. with the ratio of 3 : 1 (v/v), and mixed culture *C. vulgaris* and *Scenedesmus* sp. with the ratio of 1 : 3 (v/v).

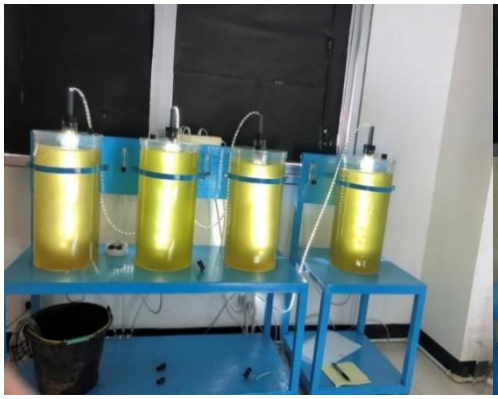

Figure 4 Microalgae cultivation on 0th day

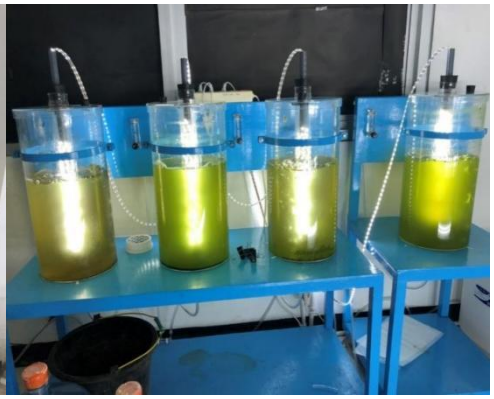

Figure 5 Microalgae cultivation on 7th day

The cultivation growth curves of ***Chlorella***, ***Scenedesmus***, ***C3:S1***, and ***C1:S3*** are shown in Figure 6. The ***Chlorella*** and ***C3:S1*** variations which were dominated by *C. vulgaris* entered exponential phase on t = 48 hours, while the ***Scenedesmus*** and ***C1:S3*** variations which were dominated by *Scenedesmus* sp. entered the exponential phase on t = 24 hours and t = 6 hours, respectively. The ***Chlorella*** and ***C3:S1*** variations entered the death phase after t = 149 hours, while the ***Scenedesmus*** and ***C1:S3*** only just entered the stationary phase at the same period.

The phenomenon observed at the *Chlorella*-dominated variations was like another monoculture of *Chlorella* sp. in ADDMW medium [14]. It entered the lag phase in only 2 days and the death phase in 6 days (144 hours). On the other hand, the *Scenedesmus*-dominated variations entered the exponential phase more quickly than *Chlorella*-dominated variations. Monoculture of *Scenedesmus* sp. grew with a relatively stable rate and entered a shorter and steadier lag phase. Monoculture of *Scenedesmus* sp. in ADDMW as cultivation medium in similar research also entered an early lag phase in only 24 hours and kept growing steadily until it reached stationary phase on the 12th day [15].

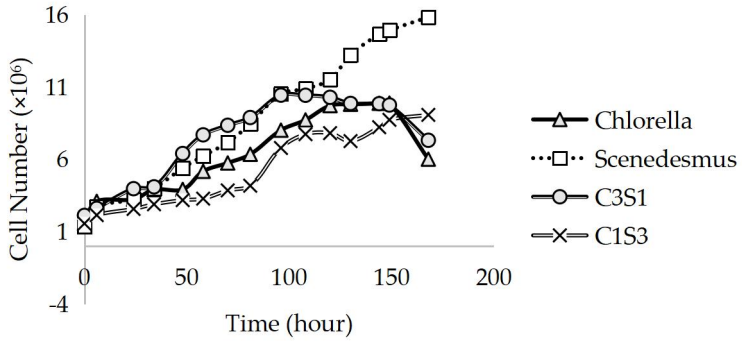

Figure 6 Comparison of all microalgae cultivation variations

The interaction between *C. vulgaris* and *Scenedesmus* sp. on both ***C3:S1*** and ***C1:S3*** are shown respectively in Figures 7 and 8. On the 0th day, the cell number of *C. vulgaris* on ***C3:S1*** culture and *Scenedesmus* sp. on ***C1:S3*** culture comprised approximately 75% of the total cell number (78,61% and 73,85%). Nevertheless, on both variations, it can be implied that *C. vulgaris* had faster growth rate compared to that of *Scenedesmus* sp. This can be implied from the increasing cell number percentage of *C. vulgaris* in both variations as time went by. Both species are Chlorophytes that produces energy source through photosynthesis.

Dominance of *C. vulgaris* over *Scenedesmus* sp. can be explained by its more complex structure compared to that of *Scenedesmus* sp., one of them is the thicker and more complex cell wall structure of *C. vulgaris*, therefore more resistant to diseases [5]. Competition in acquiring nutrients essential for growth can also describe this phenomenon.

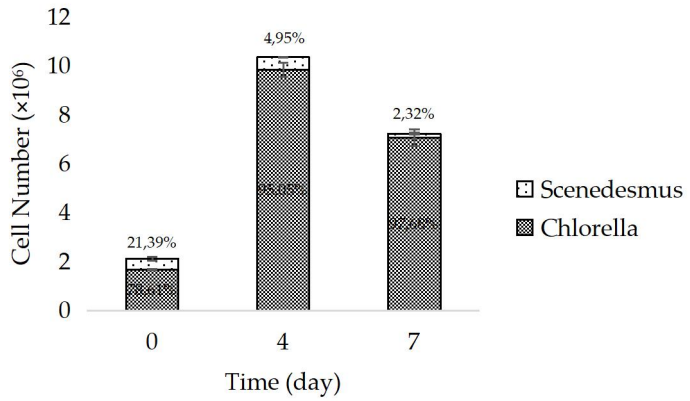

Figure 7 Cell number percentage in mixed culture of *C. vulgaris* and *Scenedesmus* sp. with the ratio of 3 : 1 (v/v)

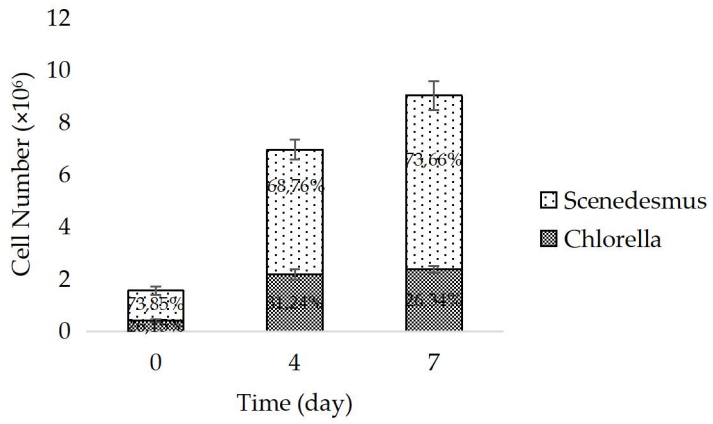

Figure 8 Cell number percentage in a mixed culture of *C. vulgaris* and *Scenedesmus* sp. with the ratio of 1 : 3 (v/v)

The specific growth rate, biomass yield, and biomass productivity of each cultivation variations are shown in Table 3. The variation with the highest specific growth rate was ***C3:S1***, followed by ***Scenedesmus***, ***Chlorella***, and ***C1:S3***. Nevertheless, the specific growth rate values of ***Chlorella***, ***Scenedesmus***, and ***C3:S1*** did not have significant differences ($p > 0,05$), but they were significantly higher than ***C1:S3*** ($p < 0,05$). Based on the biomass yield, ***C3:S1*** had significantly lower biomass yield ($p < 0,05$) compared to ***Chlorella***, ***Scenedesmus***, and ***C1:S3***. All cultivation variations produced biomass with no significant difference in productivity ($p > 0,05$). The change of the culture's pH value is shown in Figure 9. All cultivation variations showed stable value of pH between 7 – 8,5, although ***Scenedesmus*** cultivation once reached a pH value of 9,03.

Table 3 Biomass growth parameters on each cultivation variation

Cultivation variation	Specific growth rate (day^{-1})	Biomass yield ($g\ L^{-1}$)	Biomass productivity ($g\ L^{-1}\ day^{-1}$)
Chlorella	$0,321 \pm 0,023^{a}$	$13,127 \pm 3,350^{A}$	$2,238 \pm 0,403^{\alpha}$
Scenedesmus	$0,342 \pm 0,024^{a}$	$15,810 \pm 2,925^{A}$	$2,259 \pm 0,418^{\alpha}$
C3:S1	$0,344 \pm 0,057^{a}$	$6,147 \pm 1,896^{B}$	$3,073 \pm 0,948^{\alpha}$
C1:S3	$0,232 \pm 0,004^{b}$	$12,540 \pm 0,626^{A}$	$2,090 \pm 0,104^{\alpha}$

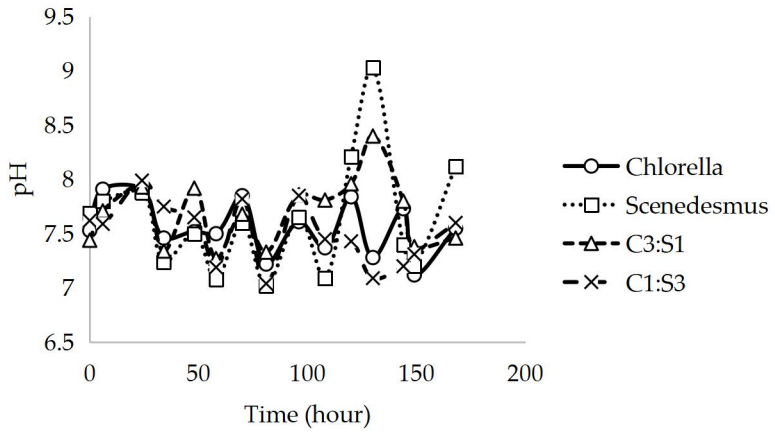

Figure 9 The change of pH values on each cultivation variation

In this research, the mixed cultivation variations produced microalgal biomass with similar performances compared to those of monoculture. This result was contrary to similar cultivation using *Chlorella saccharophila* and *Scenedesmus* sp. which proved that mixed microalgae cultivation was able to increase the performance of microalgal biomass production [3]. Nevertheless, mixed microalgae cultivation which involved more than one species of microorganism could also lead to under yielding compared to monoculture. Several factors that need to be considered to increase mixed culture performance are light, water and nutrition supply, and interaction between the various species. The decreasing

productivity of mixed cultivation may occur if the cultivation system is mainly dominated by fast-growing species but with low productivity [16]. This research showed that the growth of *C. vulgaris* dominated the growth of *Scenedesmus* sp., resulting in a nutrient uptake imbalance by both species. The decreasing growth performance of *Scenedesmus* sp. caused the failure of the mixed cultivation to expectedly grow effectively.

3.2 The Effect of Mixed Microalgae Cultivation on the Production of Microalgal Lipid

The productivity of lipid produced by each cultivation variation is shown in Table 4. All cultivation variations produced lipid with the highest productivity on the 3rd day of cultivation, except *C1:S3* on the 2nd day of cultivation. Information for lipid productivity on the 4th to 7th day were not used because the measured lipid weight data were too small, therefore the values of the lipid productivity were not representative. Nevertheless, the lipid productivity of each cultivation variation was different compared to each other ($p < 0,05$).

Table 4 Lipid productivity on each cultivation variation

Cultivation variation	Lipid productivity ($g\ L^{-1}\ day^{-1}$)
Chlorella	$0,243 \pm 0,002^{a}$
Scenedesmus	$0,272 \pm 0,000^{b}$
C3:S1	$0,514 \pm 0,002^{c}$
C1:S3	$0,387 \pm 0,000^{d}$

Table 5 Lipid productivity on various cultivation variations

Species	Medium	Lipid productivity ($g\ L^{-1}\ day^{-1}$)	References
C. vulgaris and *Scenedesmus* sp.	ADDMW	0,387–0,514	This research
Nannochloropsis gaditana and *Dunaliella salina*	Dairy wastewater	0,383	[17]
C. saccharophila and *Scenedesmus* sp.	Agricultural wastewater	0,978–1,053	[3]
C. saccharophila and *Scenedesmus* sp.	BG-11*	0,854	[3]
Chlorella and *Ettlia*	BG-11*	0,077–0,580	[18]
C. vulgaris and *R. glutinis*	Chu13*	0,283	[19]

Note: asterisks (*) show synthetic medium

C3:S1 produced lipid with the highest productivity, followed by ***C1:S3***, ***Scenedesmus***, and ***Chlorella***. Similar research that co-cultivated *Chlorella saccharophila* and *Scenedesmus* sp. using agricultural wastewater as its medium produced lipid with the productivity 20–33% higher than that of monoculture [3]. This showed that mixed cultivation of microalgae was able to produce lipid with higher productivity. Microalgae cultivation using wastewater as its cultivation medium also has the potential to increase microalgal lipid productivity (Table 5).

Lipid is one of the main bioproducts targeted from the commercial cultivation of microalgae. Mixed cultivation has the potential to increase microalgal lipid productivity. Increasing the diversity of microalgal species has the potential to increase microalgal lipid production even more. Similar research showed that highly-diverse mixed cultivation of microalgae comprised of two species of *Nannochloropsis* and two species of *Dunaliella* produced lipid with higher content and productivity compared to both monocultures and mixed cultures with the lower number of microalgal species [17].

Mixed cultivation performance can also be increased by co-cultivating microalgae with other species of microorganisms, namely yeast e.g., *Rhodotorula glutinis* [19] or nitrifying bacteria [20]. Interaction between microalgae and these two types of microorganism can enhance phycoremediation potential of the algae while increasing microalgal productivity. Microalgae as autotrophic organisms produce oxygen as a by-product that can be used by heterotrophic microorganisms like yeast and bacteria to reduce nitrogen ions concentration. The carbon dioxide produced by these microorganisms can be used by microalgae to carry out photosynthesis, therefore increasing its productivity [21].

3.3 The Effect of Mixed Microalgae Cultivation on the Production of Microalgal Protein

The protein content of each cultivation variation is shown in Table 6. All variations produced their highest protein content on the 3rd day, except *Chlorella* on the 6th day (t = 144 hours). The cultivation variation that produced the highest protein content was *C3:S1*, followed by *C1:S3*, *Scenedesmus*, and *Chlorella*. Nevertheless, the protein content value of each cultivation variation did not have significant differences between them ($p > 0,05$). Therefore, it was indicated that the mixed cultivation variations used in this research were able to produce a protein with similar performances compared to monoculture.

Table 6 Protein content on each cultivation variation

Cultivation variation	Protein content (mg (g^{-1} biomass))
Chlorella	13,936 ± 7,566[a]
Scenedesmus	16,745 ± 5,085[a]
C3:S1	21,580 ± 8,598[a]
C1:S3	20,400 ± 9,787[a]

In an effective cultivation condition, mixed cultivation should have been able to produce a protein with higher content compared to the monoculture (Table 7). Co-cultivation of *Chlorella* sp. and *Ettlia* sp., another freshwater microalgae species that is also a Chlorophyte, in similar research produced 15% higher protein content compared to monoculture of *Chlorella* sp. [18]. In addition, the amount of protein extracted in this research was considerably low because they only ranged in 1,39 – 2,16% of total microalgal biomass, meanwhile protein made up to 30 – 50% of microalgal biomass [6]. This phenomenon was possibly due to the low performance of protein extraction. Moreover, the cells of *C. vulgaris* have a more complex cell wall structure, and the microalgal biomass was not significantly pre-treated.

Table 7 Protein content on various cultivation variations

Species	Medium	Protein content (mg (g^{-1} biomass))	References
C. vulgaris and *Scenedesmus* sp.	ADDMW	20,400–21,580	This research
Freshwater algae consortium and *cyanobacteria*	Municipal wastewater	367	[22]
Freshwater algae consortium and *cyanobacteria*	Municipal wastewater	340–380	[23]
Chlorella,*Scenedesmus*, *Chlamydomonas*, and *Isochrysis*	Swine manure wastewater	80–430	[24]
Chlorella and *Ettlia*	BG-11*	400	[18]
Chlorella sorokiniana	BBM*	146	[25]

Note: asterisks (*) show synthetic medium

In this research, protein extraction was done by adding NaOH without any pre-treatment. Extraction performance could be enhanced by performing pre-treatment methods to effectively disrupt microalgal cell walls, e.g., ultrasonication, the use of high-pressure cell disruptor [26], and the use of enzymes [27].

3.4 The Effect of Mixed Microalgae Cultivation on the Production of Chlorophyll

The chlorophyll content of each cultivation variation is shown in Table 8. *Chlorella* and *C1:S3* had their highest chlorophyll content on the 4th day, while *Scenedesmus* and *C3:S1* had their highest content on the 3rd day. *C1:S3* produced the highest chlorophyll content, followed by *Scenedesmus*, *C3:S1*, and *Chlorella*. Nevertheless, there were no significant chlorophyll content differences between those variations ($p > 0,05$). It showed that mixed cultivation variations in this research produced chlorophyll with similar performances compared to monoculture.

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Table 8 Chlorophyll content on each cultivation variation

Cultivation variation	Chlorophyll content (mg L^{-1})
Chlorella	3,656 ± 2,231[a]
Scenedesmus	4,367 ± 1,529[a]
C3:S1	4,194 ± 1,549[a]
C1:S3	5,174 ± 1,574[a]

Similar research showed that mixed microalgae cultivation on wastewater medium caused a decrease in the production of chlorophyll as the most abundant microalgae pigment, especially in Chlorophytes [18,28]. The decline in chlorophyll production is due to the interaction between microalgal species, causing loss of incident energy that was important for microalgal growth [18]. The decline in chlorophyll production was followed by an increase in microalgal biomass productivity, therefore the chlorophyll production was inversely proportional to biomass production, while in this research chlorophyll production was not proportional to biomass production in any way. Therefore, it can be concluded that the mixed cultivation variations used in this research did not have any effect on the performance of chlorophyll synthesis.

The chlorophyll produced in this research was not remarkably high. This can be explained by the medium used in the cultivation. The use of wastewater as a cultivation medium resulted in the decrease of chlorophyll synthesis compared to the use of synthetic medium (Table 9). Chemical stress that occurred during the cultivation may decrease chlorophyll synthesis and increase the production of other pigments, namely carotenoids [31]. ADDMW used as the cultivation medium had a high value of biochemical oxygen demand (BOD), therefore causing chemical stress to the algae [32]. The existence of chemical stress may induce the algae to produce more secondary pigments like carotenoids and less primary pigments like chlorophyll [33]. In this research, the carotenoid content of the algae was not measured, but the low chlorophyll content strongly indicated that the chemical stress caused by using ADDMW as a cultivation medium triggered the decrease of chlorophyll production. Nevertheless, the utilization of ADDMW as a cultivation medium to produce valuable pigments is very feasible since the use of ADDMW may reduce the production cost, mainly because the use of an expensive synthetic medium as a source of nutrients is not necessarily needed [34].

Table 9 Chlorophyll content on various cultivation variations

Species	Medium	Chlorophyll content (mg L^{-1})	References
C. vulgaris and *Scenedesmus* sp.	ADDMW	4,194–5,174	This research
Freshwater algae consortium	Carpet industry wastewater	2,9	[28]
Chlorella and *Scenedesmus*	Shrimp cultivation wastewater	1,246	[29]
Chlorella and *Ettlia*	BG-11*	35,36–54,91	[18]
Scenedesmus	SC*	79,73	[30]

Note: asterisks (*) show synthetic medium

4. Conclusions

Microalgae were cultivated in anaerobically digested dairy manure wastewater and four variations (***Chlorella***, ***Scenedesmus***, ***C3:S1***, and ***C1:S3***). Their biomass growth, lipid production, protein production, and chlorophyll production were assessed. The specific growth rate of ***C3:S1*** (0,344 day^{-1}) was the highest of all variations, but it was not significantly different to that of ***Chlorella*** and ***Scenedesmus***. ***Scenedesmus*** produced the highest biomass yield (15,810 g L^{-1}), but it was not significantly different to that of ***Chlorella*** and ***C1:S3***. In terms of biomass productivity, all variations produced microalgal biomass with similar productivities (2,090–3,073 g L^{-1} day^{-1}). In general, in both ***C3:S1*** and ***C1:S3***, C. *vulgaris* showed dominance over *Scenedesmus* sp. due to its higher growth rate and more complex cell wall structure.

The ***C3:S1*** variation produced lipid with the highest productivity (0,514 g L^{-1} day^{-1}), compared to other variations. The research showed that high diversity of microalgae species could potentially increase microalgal lipid production. All variations showed similar performances in protein production with 13,9–20,4 mg (g^{-1} biomass) of protein content, despite the low figures, possibly due to ineffective extraction of microalgal protein. All variations also had similar chlorophyll contents (3,656–5,174 mg L^{-1}), which were considerably low due to the use of wastewater medium with a high value of biochemical oxygen demand.

Acknowledgments

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Propagation of *Dendrobium antennatum* Orchid Using Tissue Culture Method (Filter Paper Bridge Bioreactor)

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Abstract

Indonesia is one of the countries that cultivates Dendrobium orchids and controls more than 50% of orchid business. The development of hybrids and new types of orchids increased orchid export volume by 27.92%. Moreover, dendrobium orchids can also be used as edible flowers and as medicines because they contain many antioxidant compounds. The increase in orchid market demand should be offset by an efficient shoot production system. Tissue culture can be used as a solution to multiply orchid shoots that are identical to their parental in a short time. This study was aimed to compare the propagation of *Dendrobium antennatum* orchid shoots from three different systems namely continuous submerged system (liquid system), Filter Paper Bridge bioreactor system (FPB) and Semi-solid Medium system (SSM) system. The culture media were prepared in VW media, added with 5 ppm of BAP hormone, sugar 20 g/L and for SSM system was added by agar 5.5 g/L. Cultivation of orchid shoots was carried out for 21 days. The results showed the best increment in fresh weight and dry weight in FPB systems (37.41%, 12.78%), liquid systems (11.93%, 8.99%), and SSM systems (9.29%, 4.83%) respectively. It was also supported by the best growth value obtained in FPB systems (86.14%), liquid systems (69.77%), and SSM systems (68.67%). Orchid growth rates in FPB, liquid, and SSM systems were 0.0151 g/day, 0.0054 g/day, and 0.0042 g/day with bioconversion efficiency respectively 13.77%, 9.95%, and 5.09%. Mass balance analysis showed the highest gain values were obtained in FPB systems (90.94%), liquid systems (88.33%), and SSM systems (86.21%).

Keywords: *Dendrobium antennatum,* tissue culture, Filter Paper Bridge bioreactor, mass balance analysis, growth

1. Introduction

Dendrobium is the second largest group among the orchid family (Orchidaceae), with approximately 1600 species spread from Japan, Korea, Malaysia, Indonesia, New Guinea, and Australia. The potential of Dendrobium in the field of economics has been utilized and developed in many countries. In tropical countries such as Thailand, Singapore, Malaysia, including Indonesia, Dendrobium is used as a potted ornamental plant and a significant cut flower. In addition to being used as an ornamental plant, orchids are also widely used in traditional medicine systems. One example in China, orchids are believed to be pioneers of medicinal plants, especially as a source of antioxidant compounds [2]. From the results of previous research, several types of Dendrobium orchids are also processed into edible flowers [3].

The world orchid market's potential is expected to increase in the coming year as hybrids and new types of orchids grow. Indonesia is one of the countries that cultivate Dendrobium orchids and controls more than 50% of orchid businesses globally. In 2018, the orchid export volume in Indonesia increased by 27.92% [4]. However, today the population of *Dendrobium antennatum* orchids is thinning to low levels due to various activities such as habitat destruction, degradation, fragmentation and illegal harvesting for trade and consumption [5]. The increasing market needs for orchids and the lack of quality seeds led to the need for proper cultivation methods to meet those markets' needs.

Orchid propagation is generally done in conventional ways, such as cuttings, seed planting, occultation, and grafts. However, the proliferation of orchids in traditional ways has drawbacks, one of which is that it requires large numbers of orchid seedlings. While orchids have a low sprout power, which is less than 1% [6]. This is because the orchid seed size is tiny and does not have an endosperm as a food reserve [7]. This causes conventional orchid propagation to take a long time to grow orchid seedlings. Therefore, a fast and precise method of orchid propagation is needed to meet market demand.

Tissue culture method can be used as a plant propagation solution, which is challenging to breed conventionally. The tissue culture method has advantages, among others, that the seeds produced will have identical properties to their parents and can produce large numbers of seeds in a short time, and do not require a large space [8].

Propagation using tissue culture methods in this study used a bioreactor. Bioreactors are designed in such a way that the implementation of orchid shoot propagation goes well. In this study, a bioreactor was used with the Filter Paper Bridge (FPB) model. This bioreactor serves to provide nutrition periodically over a period of time. The FPB bioreactor system is designed to use filter paper as a support system as well as an absorption medium for *Dendrobium antennatum* orchid explants. Tissue culture can be maximized by controlling optimal environmental conditions for explants, one of which is the culture system and the right medium. Therefore, in this study, the growth of orchid shoots was compared in FPB systems, semi-solid medium, and liquid medium.

2. Research Materials and Methods

2.1 Materials

In this study, the *Dendrobium antennatum* orchid explant was used and obtained from the School of Life Sciences and Technology, Bandung Institute of Technology.

The chemicals used during the study were Vacint & Went medium, BAP growth hormone (Benzyl Amino Purin), distilled water, ethanol, and alcohol from chemical warehouses, Engineering Laboratory XI, Bandung Institute of Technology, Ganesha Campus.

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2.2 Methods

2.2.1 Preparation of Culture Medium

The medium used to initiate the explants was 1.67 g / L Vacint & Went (VW) medium, 8 g / L agar, 20 g / L sugar, and 6-benzylaminopurine (BAP) 5 ppm [9]. In the liquid system and FPB, there is no addition of agar in the medium, while in the semi-solid system, 5 grams of agar is added to the VW medium [10]. The pH is adjusted in the range 5.6-5.8. The culture medium was sterilized using an autoclave with a 1.5 atm pressure at a temperature of 121ºC for 85 minutes. [11].

2.2.2 Subcultures of *Dendrobium antennatum*

Bottle source for orchid seedlings in aseptic conditions is opened slowly. The orchid explants were removed from the bottle using sterilized tweezers. The orchid seeds are then transferred to a sterile agar medium bottle. Seeds are cultivated for 3 weeks in culture bottles at a temperature of 25 ± 2°C under light exposure (bright conditions) for 24 hours. [9].

2.2.3 Cultivation of *Dendrobium antennatum* in Filter Paper Bridge Bioreactor, Semi-solid Medium, and liquid systems

A culture that can grow is characterized by new shoots and roots on a solid medium. Before being put into the bioreactor, the culture was acclimatized in an Erlenmeyer containing sterile liquid VW medium for 3 days. After acclimatization for 3 days, the orchids were weighed as much as 1 gram, and the initial shoots were calculated using sterile aluminum foil. Then the culture was inserted into the Filter Paper Bridge bioreactor containing 10 ml of sterile liquid VW medium. Previously, the Paper Bridge Filter Bioreactor has been designed according to a modification from the research [12], by using an Erlenmeyer flask equipped with filter paper that is made a cross. The treatment control was a continuous submerged system (liquid system) using an Erlenmeyer flask containing 10 ml of sterile liquid VW medium. The culture was transferred to a culture bottle containing 25 ml of VW semi-solid medium aseptically in the semi-solid medium system. The liquid system and the Paper Bridge Filter bioreactor system are agitated by a shaker with 150 rpm speed. Observations were made for 21 days with room temperature 25°C under light exposure (bright conditions) for 24 hours [9].

2.2.4 Measurement of Fresh Weight, Number of Shoots, and Dry Weight

After 21 days, the orchid's fresh weight was determined using an electronic balance. Orchid fresh weight was measured under aseptic conditions using sterile aluminum foil. Weighing the fresh weight and calculating the final shoots of the orchids is done after leaving the bioreactor. After calculating the fresh weight and number of orchid shoots, the samples were oven-dried at 70°C and weighed the dry weight periodically until it was constant.

2.2.5 Medium pH Measurement

The initial and final pH measurements of the medium were carried out with a pH meter. Before analyzing the medium, the pH meter was calibrated with a standard buffer solution (pH 7). The pH meter probe that has been calibrated is immersed in the medium until the number shown on the pH meter display is constant.

2.2.6 Medium Conductivity Measurement

The medium to be tested is prepared. Measurement of the initial and final conductivity of the medium was carried out using a conductivity meter. The conductivity meter is immersed in the medium for a few seconds. The number that appears on the screen is recorded and the measurement is repeated three times.

2.2.7 Measurement of Medium Sucrose Content

Measurement of the initial and final sucrose content was carried out with a calibrated refractometer using distilled water as a solution of 0% sucrose concentration. The medium was dropped on the refractometer until the sucrose content was obtained in Brix (%) units. The calculation of the bioconversion efficiency of sucrose can be calculated by (Eq. 1) [13].

$$\text{Bioconversion Efficiency} = \frac{\text{Initial Dry Weight (g)} - \text{Final Dry Weight (g)}}{\text{Consumption of sucrose (g)}} \times 100\% \quad \text{.....(Eq. 1)}$$

3. Result and Discussion

3.1. Visual Observation of Orchid Shoots during Subculture and in the Culture System

Visual observation of orchid shoots' growth was carried out during subculture, before and after entering the bioreactor. This qualitative data is needed to see the suitability of the culture system that will be used in cultivation in a bioreactor.

Based on the data in Table 1, orchid shoots during the subculture process were fresh green, some had formed other shoots, and some had developed roots. This is supported by the right combination of basic media and growth regulators to increase the activity of cell division in the process of morphogenesis and organogenesis [14]. During the subculture process, some orchid cultures are contaminated. Contamination in the subculture of orchid shoots can be caused by bacteria, which is characterized by the presence of pink clumps around the medium and orchid shoots. Some of the causes that allow contamination include contamination originating from explants, both internally and externally, microorganisms that enter the media, materials, or tools that are less sterile, dirty work environments and culture rooms, and carelessness implementation process. subculture [15].

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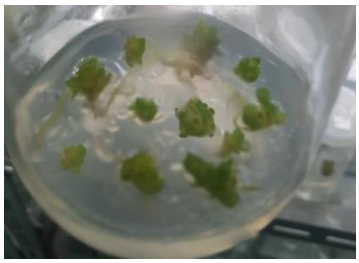
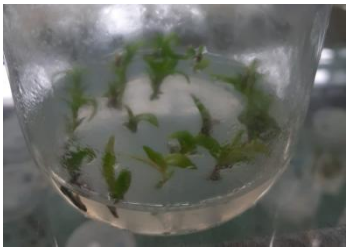
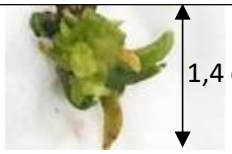
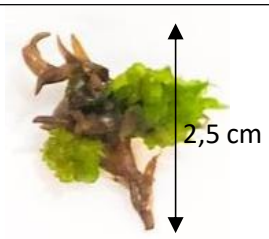
Table 1 Visual comparison of subcultures of *Dendrobium antennatum*

Initial Subculture	Final Subculture
	

In Table 2, it can be seen the visual differences of orchid shoots from each culture system. In general, orchid shoots in the FPB bioreactor system appeared to have better visuals. It was clear that the new shoots were healthy and bright green. There was an increase in orchid shoots in the liquid system and SSM, but some of the shoots changed color to brown (browning).

Table 2 Visual comparison of orchid shoots in liquid, FPB, and SSM systems

Culture System	Initial (d_0)	Final (d_{21})
Liquid		
Filter Paper Bridge (FPB)		

Semi-solid Medium (SSM)		

The browning phenomenon can occur due to the increased production of oxidized phenol compounds caused by injured tissue presence during the initiation process. Phenol has an essential natural function in regulating the oxidation of IAA. When the phenol concentration in the tissue increases, phenol compounds can be toxic to culture [16]. Also, browning is also an indication that an explant has hyperhydricity. Hyperhydricity is a phenomenon in which there are metabolic disorders that involve high water concentrations in plants [17].

Table 3 Percentage of browning orchid shoots in the liquid, FPB, and SSM systems

Culture System	Final Number of Shoot (d_{21})	Number of browning Shoot	
		Average	Percentage (%)
Liquid	37	2	5,4 ± 0,02
FPB	47	1	2,12 ± 0,01
SSM	36	1	2,7 ± 0,03

Based on Table 3, the liquid system's orchid shoot culture experienced a higher browning phenomenon than the FPB bioreactor system. In a liquid system, the orchid shoot culture is in direct contact with the medium. The direct contact of the orchid culture with the medium continuously can cause the explants to experience oxidative stress. High oxidative stress can reduce the quality and yield of the culture [18]. Hyperhydricity can also be influenced by high oxidative stress and the level of reactive oxygen species (ROS) accumulation experienced by explants. The longer the explants are in direct contact with the medium, the higher the accumulation of ROS in plants because the more frequent plant activity oxidizes fatty acids in cells causing damage to membranes and DNA [18]. Submerging the shoots continuously in a liquid medium can also cause low O2 diffusion to cause the tissue in a hypoxic state. Hypoxia in the tissue can induce the formation of excess ROS which can lead to cell death [20].

3.2. Analysis of Biomass Growth and Number of Orchid Shoots

Orchid growth can be seen from the increase in fresh weight, dry weight, and the number of orchid shoots. Based on Figure 1, the three culture systems showed an increase in orchid shoot wet weight. The highest increase in fresh weight of orchid shoots was obtained in the FPB bioreactor, which was 37.41% compared to the liquid system, which was 11.93%, and the SSM system 9.29%. The highest orchid dry weight gain was also obtained in the FPB system, namely 12.78%, while in the liquid system and SSM, it was 8.99% and 4.83%, respectively.

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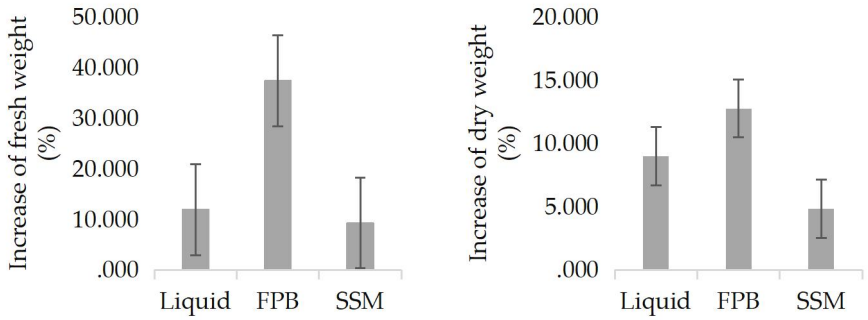

Figure 4 Fresh weight and dry weight increase of orchid shoots in liquid, FPB, and SSM systems

The significant difference in the increase in fresh weight of orchid shoots in the liquid system and FPB bioreactor compared to the SSM system can occur because of differences in the medium's physical properties, especially viscosity. The use of a liquid medium will make it easier for the orchid culture to absorb the nutrients in the medium compared to using a semi-solid medium [21]. The addition of agar in the SSM system increased the viscosity of the culture medium. The higher the viscosity of a liquid, the lower the mass transfer surface area of the gas-liquid, which causes a decrease in the mass transfer coefficient [22]. In addition to the increase in fresh weight, there was also an increase in dry weight in the three culture systems. Based on Figure 2, it can be seen that the highest gain in fresh weight was also obtained in the FPB system, followed by the liquid and SSM systems.

The rise also followed the increase in orchid biomass in the number of orchid shoots. The highest growth in the number of orchid shoots was obtained from the FPB bioreactor system, namely 86.14%, while the liquid system and SSM respectively increased by 69.77% and 68.67%

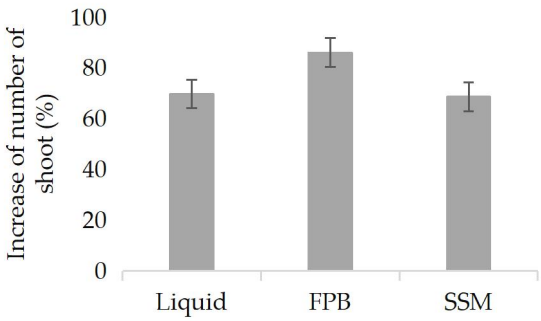

Figure 5 The orchid shoots growth in the liquid system, FPB, and SSM

The increasing of the number of orchid shoots is supported by using a suitable medium, especially because there is the addition of the appropriate growth hormone, namely BAP (benzylaminopurin). The BAP hormone is a chemical compound that belongs to the cytokinin class that plays a role in shoot growth. According to Wareing and Philips (1981), cytokinins can stimulate plant cell division and interact with auxins in determining the direction of cell differentiation [23]. The addition of the right growth hormone will stimulate orchid plants to form new shoots.

3.3. Orchid Growth Kinetics

The growth of orchid shoots in the liquid system, SSM, and FPB bioreactor can also be seen from the growth kinetics. The kinetics of orchid shoot growth can be calculated using the (Eq. 2)

$$\frac{d}{dt}[X] = \mu_{max} X \quad \text{.....(Eq. 2)}$$

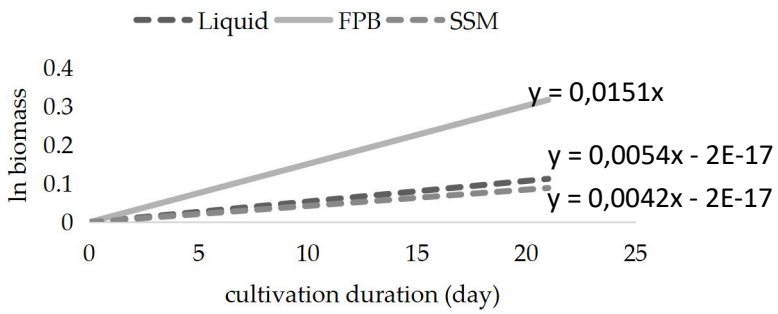

Figure 6 The growth rate of orchid shoots in the liquid system, FPB, and SSM

Based on Figure 3, it can be seen the difference in the maximum growth rate of orchid shoots in each culture system. The best maximum growth rate was obtained in a culture system using an FPB bioreactor, which was 0.0151 g / day. Whereas in the liquid system and SSM, the maximum growth rates of orchid shoots were 0.0054 g / day and 0.0042 g / day, respectively. The growth rate value is following the data on fresh and dry weight gain of orchids where the best growth of orchids is by using a FPB bioreactor system followed by a liquid system and an SSM system.

3.4. Analysis of Culture Medium in Orchid Growth

The propagation of orchids by in vitro culture method is influenced by several factors, one of which is the culture medium's condition. The medium analysis was performed at the beginning and the end of the observation. In this study, the measured medium parameters were the amount of sucrose, pH, and conductivity of the medium.

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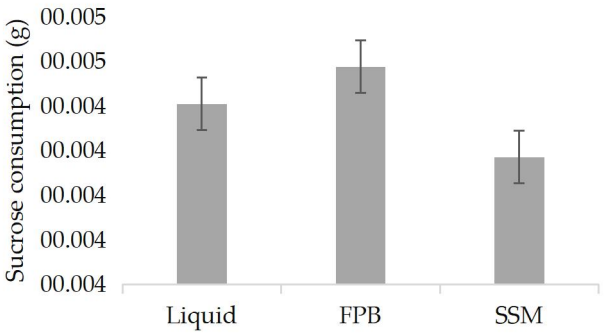

Figure 7 Sucrose consumption from orchid shoot culture in the liquid system, FPB, and SSM

For 21 days, the highest sucrose consumption was obtained in the FPB bioreactor system (0.0045 grams) compared to the liquid system (0.0044 grams) and the SSM system (0.0043 grams) (Figure 4). The change in the sucrose level of the medium in the FPB system was more significant than the other systems due to the possibility of the culture medium being absorbed and left in the filter paper, which functions as a support system. Based on the sucrose consumption data, the efficiency value of sucrose's bioconversion into biomass can be calculated. The sucrose's bioconversion efficiency in the SSM system had the smallest value, namely 5.09%, compared to the liquid system and the FPB bioreactor system, namely 9.95% and 13.77%, respectively. This proves that using a support system in the form of filter paper in the FPB bioreactor system can help optimize the absorption of the medium to increase the biomass accumulation from the bioconversion medium.

In addition to changes in sucrose amount in the medium, analyzes were carried out on changes in pH and conductivity of culture medium in the liquid system, FPB bioreactor, and SSM (Figure 5). The pH and conductivity of the medium are related to the concentration of inorganic ions and can indirectly reflect growth trends and nutritional requirements of explants [24].

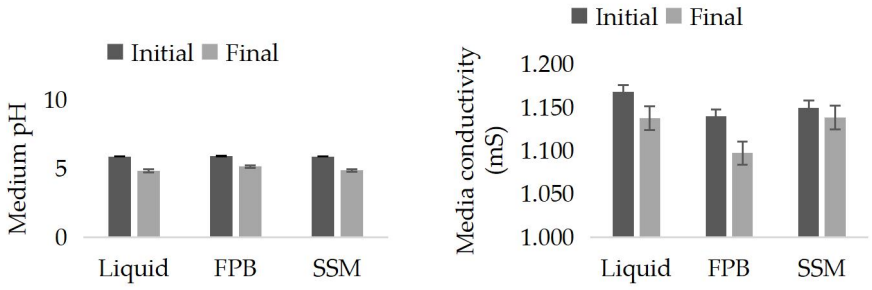

Figure 8 The initial and final pH and conductivity values of the culture medium in liquid system, FPB, and SSM

Based on Figure 5, it can be seen that the decrease in the acidity of the medium in the three culture systems is not statistically significant. The change in the medium's pH value indicates the exchange of free hydrogen (H +) and hydroxyl ions (OH-). According to Chen et al. (2014), pH analysis can be used as a diagnostic parameter for symptoms of abnormal growth in tissue culture, such as browning and necrosis [25]. In addition to a decrease in pH, there was also a decrease in the conductivity of the medium (Figure 5). The conductivity value of the medium will be inversely related to the biomass produced. The decrease in the conductivity of the medium indicates the absorption of inorganic ions from macro and micronutrients by the plant cells [24]. This is following the research results where the final conductivity of the culture medium is the lowest, namely using an FPB bioreactor to produce the most significant biomass.

3.5. Mass balance analysis of orchid shoot culture

The conversion of sucrose to biomass can be analyzed using a mass balance approach. The mass balance calculation for *Dendrobium antennatum* shoot growth is calculated following the growth reaction equation for the *Vanda tricolor* orchid [26]. The basis for calculating the mass balance in this experiment is data on initial and final sucrose consumption measured using a refractometer. Mass balance calculations for each culture system can be seen in Table 4, Table 5, and Table 6.

Table 4 Mass balance calculations in liquid systems

Liquid	0.39 $C_{12}H_{22}O_{11}$	0.23 NH_4NO_3	3.43 O_2	$CH_{1,27}O_{0,43}N_{0,45}$	4.07 H_2O	3.64 CO_2
Moles before reaction	0.0005	0.0062	0.06	0.004	0	0
Moles that react	0.0004	0.0002	0.0031	0.0009	0.004	0.003
Moles after reaction	0.0002	0.0060	0.0599	0.0048	0.004	0.003
Mass after reaction	0.0475	0.4834	1.917	0.1262	0.07	0.145
Actual biomass (g)					0.112	

Table 5 Mass balance calculations in *Filter Paper Bridge*

FPB	0.39 $C_{12}H_{22}O_{11}$	0.23 NH_4NO_3	3.43 O_2	$CH_{1,27}O_{0,43}N_{0,45}$	4.07 H_2O	3.64 CO_2
Moles before reaction	0.001	0.0062	0.0831	0.004	0	0
Moles that react	0.0004	0.0002	0.0032	0.0009	0.004	0.003
Moles after reaction	0.0002	0.006	0.0799	0.0047	0.00	0.003
Mass after reaction	0.0460	0.4831	2.558	0.1256	0.07	0.147
Actual biomass (g)					0.114	

Table 6 Mass balance calculations in Semi solid Medium

SSM	0.39 $C_{12}H_{22}O_{11}$	0.23 NH_4NO_3	3.43 O_2	$CH_{1,27}O_{0,43}N_{0,45}$	4.07 H_2O	3.64 CO_2
Moles before reaction	0.001	0.006	0.063	0.954	0	0
Moles that react	0.000	0.000	0.003	0.0009	0.004	0.003
Moles after reaction	0.0002	0.006	0.060	0.955	0.00	0.003
Mass after reaction	0.057	0.483	1.916	0.1195	0.07	0.146
Actual biomass (g)					0.103	

Based on the mass balance calculation, it can be seen that there are differences in the actual biomass values and theoretical biomass in the liquid system, FPB, and SSM. The theoretical biomass in the three systems is greater than the actual biomass formed. Based on these data, the ratio of the actual weight to the three culture systems' theoretical weight can be calculated. The best ratio of actual and hypothetical biomass (yield value) was obtained by using the FPB bioreactor culture system, which was 90.94%. In the liquid culture and SSM systems, the yield values were 88.83% and 86.21%. This value indicates that the use of FPB bioreactors gives the best results compared to liquid and SSM systems

4. Conclusion

Tissue culture system using Filter Paper Bridge bioreactor on *Dendrobium antennatum* orchid shoots can effectively increase orchids' yield compared to liquid systems (continuous submerged) and semi-solid medium (SSM) systems. This was indicated by the increase in fresh weight and the highest number of orchid shoots obtained from the Filter Paper Bridge bioreactor system (37.41% and 86.14%), followed by the liquid system (11.93% and 69.77%) and the lowest on the SSM system (9.29% and 68.67%). The increase in biomass and number of orchid shoots was followed by a high growth rate and sucrose bioconversion efficiency in the tissue. The highest growth rate and bioconversion efficiency were obtained in the FPB bioreactor system (0.0151 g / day and 13.77%), followed by the liquid system (0.0054 g / day and 9.95%), and the smallest was obtained with the SSM system (0.0042 g / day and 5.09%). Based on the mass balance calculation, the recovery value (ratio of actual biomass to theoretical biomass) from the FPB bioreactor system, liquid system, and SSM system was 90.94%, 88.33%, and 86.21%, respectively. The results of the experiment using the FPB bioreactor system gave the smallest percentage of browning (2.12%) compared to the SSM system (2.7%) and the liquid system (5.4%).

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The Potency of Some Fresh Water Macroalgae as The Source of Biodiesel

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Abstract

Macroalgae has emerged as a potential aquatic species for biodiesel production. This study was conducted to assess the potency of freshwater macroalgae as a source of biodiesel. In this study, five freshwater macroalgae, identified as *Spirogyra* sp., *Hydrodictio*n sp., *Cladophora* sp., *Hara* sp., and *Nitell*a sp., were collected, dried, and extracted with n-hexane. The n-hexane extracts were then fractionated by adding methanol. Following separation from the methanol extract, activated carbon was added into the n-hexane extract to absorb green and yellow pigments. The active carbon was removed by filtration. The extracts were then evaporated to the dryness and the total oil was weighted. The highest oil content was obtained from *Nitell*a sp. i.e. 0.61%, followed by *Cladophora* sp., 0.56%; *Hydrodiction* sp., 0.54%, *Chara* sp., 0.30%, and the lowest content of 0.15% was obtained from *Spirogyra* sp. The characteristics of macroalgae oil were further analyzed based on the value of acid number, saponification and iodine using standard methods of FBI-A01-03, FBI-A03-03 and FBI-A04-03, respectively. Acid number, saponification and iodine value of the five tested samples ranging between 55-143 mg KOH/g, 400-500 mg KOH/g, and 3-28 g I_2/100 g, respectively. These results implied that the assessed samples had low oil content with minimum quality due to the high acid number, low iodine value and high saponification number. These parameters also indicated that the macroalgae oils have short carbon chains resembling butyric acid (C-4) that although do not meet the standard requirement of national biodiesel in Indonesia may well be used as a source for fine chemicals.

Keywords: *Macroalgae, biodiesel, algae oil*

1. Introduction

Most of the energy we used nowadays for various purposes are derived from fossils fuel, which include petroleum, coal and natural gas. It takes several million years for these nonrenewable energies to form and accumulate. The usage of these fuel energy increase year to year as human population increase exponentially. In consequence, demand for the fossils fuel in particularly petroleum, increase rapidly. This followed by the increase of rapid exploration and production, which in turn deplete the reserves of fossils fuel [1].

If such conditions are unconsidered, we will soon face shortages of fossils fuel. Therefore, in the last decade there have been intensive searches for alternative energy sources which can supplement or replace the fossils fuel. One of the prominent alternative energy is biofuel, in particular biodiesel. Biodiesel can be obtained from biomass of recently living organisms, such as plant, animal, and microbes. It is considered as renewable energy since it accumulates much faster, within a year, than fossils fuel. Biodiesel mainly derived from land plant such as palm oil (*Elaeis guineensis*) [2].

Following the development of plant based renewable energy, algae have promising potential as a renewable energy sources for biodiesel production [3], as indicated by the high lipid content in a number of algae. The lipid can be converted into biodiesel by the process of transesterification [4].

Most algae are aquatic species that can be found easily either in marine or freshwater. Algae grow much faster than terrestrial plant, so they are makes available almost all the year. Most research on algae for the biomass production of oil were mainly focused on microalgae, such as Chlorophyceae, Dinophyceae, and Bacillariophyceae which were reported to contain oil (lipid) in high quantity [5]. On the other hand, very few reports are available concerning the use of larger size algae (macroalgae) as source of biodiesel. A study on biodiesel from macroalgae reported *Spirogyra* sp. and *Oedigonium* sp. Contained adequate oil to be converted into biodiesel i.e *Spirogyra* sp. contained 7.3% oil w/w and *Oedigonium* sp. contained 9.2% [4].

Conversion processes of algal oil into biodiesel were claimed to be similar as oil derived from land plants [6]. The difficulties in the productions of biodiesel from alga lie in finding an algal species that accumulate high lipid and in establishing a cultivation system that is suited to the species for rapid growth. Considering that Indonesia has a wide area covered by water (marine and fresh water), where a number of macroalgae can be found easily, we aim to discover freshwater macroalgae species that contain a high oil content and to evaluate their potency to be used as a source of biodiesel through oil quality assessment.

2. Methodology

2.1 Sample collection

In this study, several fresh water macroalgae were evaluated for their potency as a source of biodiesel. The samples were collected from fresh water ecosystem (ponds, rice fields and the upstream of river) in the areas of West Java i.e. Bandung, Bogor, Garut, and Sukabumi. The samples were collected using plankton nets. The collected algae were then placed in a plastic bucket and taken to a laboratory for identification and further processing.

2.2 Drying and extraction

In the laboratory, the samples of macroalgae were immediately washed with tap water to removed dirt. The clean samples were subsequently aerated and sun-dried for 1-2 days and

then re-dried in an oven with a temperature of 60°C for 2x24 hours to obtain a constant dry weight. Total dry weight was recorded for each of algae samples. The dried samples were extracted by soaking in the solvent of n-hexane (1:3, algae: n-hexane) for 2x24 hours.

2.3 Evaporation and discoloration

The hexane extracts of macroalgae were filtered using glass funnel with non-fat cotton. Filtration residue was washed with hexane and then filtered again to gain more filtrate for each sample. The color of the extracts appeared yellow to greenish which indicated the contents of carotenoid and chlorophyll pigments. To eliminate both pigments, bleaching process was done by adding 10% activated carbon into the extracts. After settling for 48 hours and obtaining a translucent colored of the extracts, activated carbon was separated from hexane extract by filtration using non-fat cotton.

The filtrate was then evaporated with a vacuum evaporator. This process was performed until approximately 98% solvent hexane evaporated. Total lipid/oil which has been separated from the solvent of hexane was transferred into small bottles and left bottles opened in room temperature to evaporate the remaining hexane.

2.4 Quantification of Algae oil

After all of hexane evaporated, the quantity of algae oil or lipid in the bottle was determined by measuring weight (weight of the bottle containing algal oil reduced by empty bottle). This measurement was done to all obtained oil.

2.5 Characterization of Algae oil

Characteristic of algae oil were analyzed based on the value of acid number, saponification and iodine value using standard methods for assessing quality of biodiesel, which issued by *Badan Standardisasi Nasional* (BSN) Indonesia [7].

3. Result and Discussion

3.1 Collection of Algae

Five species of macroalgae have been successfully collected from various fresh waters ecosystems in West Java areas. The collected species (Figure 1.) were identified as *Spirogyra* sp. obtained from ponds at the Bogor Botanical Gardens, Bogor; *Hydrodiction* sp. from rice fields in Dago Biru, Bandung; *Cladophora* sp. from upstream river in Bogor Botanical Gardens, Bogor; *Chara* sp. from the lake Situ Bagendit, Garut and *Nitela* sp. from the Lido lake in Sukabumi. Morphologically, *Spirogyra* sp. *Cladophora* sp., and *Hydrodiction* sp. are filamentous algae. C*hara* sp. and *Nitela* sp. are higher algae resemble vascular plants which possess stems and branches

3.2 Quantity of Algae oil

Results of oil quantification in the extracts (Figure 2.) indicate that the highest oil content was obtained from *Nitella* sp. as much as 0.61%. The other extracts show lower content than that of *Nitella* sp i.e., 0.56% in *Cladophora* sp., 0.54% in *Hydrodictyon* sp., 0.30% in *Chara* sp. and 0.15% in *Spirogyra* sp, respectively. Currently there is limited information about the oil content in the species of *Nitella* sp., *Cladophora* sp., *Hydrodiction* sp., and *Chara* sp. which oil profile comparison can be made. Therefore, our results can be used as preliminary consideration if one intends to use green algae as a source of biodiesel.

A previous study by Hossain *et al*., 2008 reported the potency of oil from *Spirogyra* sp. for biodiesel production [4]. In that study, *Spirogyra* sp. was reported to contain 7.3% oil w/w. Our results however, showed much lower oil content in *Spirogyra* sp. i.e. 0.15% w/w. This probably due to the large variability of chemicals (nutrient) composition in aquatic plant species, which is influenced by the composition of the water and other physical environments where they grow [8]. Hossain *et al*., used *Spirogyra* sp. that had been collected from certain freshwater ecosystem in Malaysia, while our study used *Spirogyra* sp. obtained from pond ecosystems at West Java, that possibly have different nutrient composition and different physical environments.

3.3 Characteristic of Algae oil

Most of the extracted oils from the collected algae showed dark color, except the extracted oil from *Spirogyra* sp. Which is yellow translucent (Figure 3.). Chemical analysis of the oils showed that acid number values ranging from 55 to 143 mg KOH/g as shown on Table 1. These values were higher than that of Indonesian Biodiesel Standard (SNI), i.e. 0.8 mg KOH/g. This result indicated that oil of these algae is too acidic and uneconomic to be converted into biodiesel. Acid number however could be affected by the process of extraction. Therefore, it is necessary to improve the extraction method that are more suitable to extract macroalgal biomass.

The algae oils have saponification values with the range of 400-500 mg KOH/g (Table 1). The values were quite high when compared with other crude oils, like coconut oil which has the value of saponification number only 246-260 mg KOH/g. The fatty acids of coconut oil usually show 8 to 10 carbon chains. Thus, the algae oil which showed a higher saponification value was assumed to have about 4 carbon chains. Meanwhile, potential oil which can be used as raw material for biodiesel typically has 12-20 carbon chains [9].

Iodine values of the extracted oils from five species of macroalgae were between 3-18 g I_2/100g (Table 1). The values meet the standard of Indonesia Biodiesel (SNI) with maximum value of 115-gram I_2/100g. However, the Iodine value in the tested oil was considered very low.

The assumption that the oils extracted from the collected macroalgae to have 4 carbon chain in their fatty acids, as was indicated by the high saponification value, may be caused by the unsuited method of extraction. Therefore, it is necessary to improve the extraction method which particularly suited to gain higher oil content and better oil characteristic in macroalgae.

Bastianoni, et.al. (2008), reported that improvement of oil extraction methodology in macroalgae could results in a good residue which are usable for biofuel production [10].

Thus, based on these chemical characteristics, it could be assumed that freshwater macroalgae, such *Spyrogyra* sp., *Hara* sp., *Hydrodiction* sp., *Cladophora* sp., and *Nitela* sp. are less potential to be used for biodiesel product. However, the oil/lipid could be used as material source for fine chemicals [11] and many applications in pharmaceuticals industry, food and feed supplement. Moreover, after extraction of the oil, the remaining biomass of macroalgae may be useful as source of other forms of product such as biofertilizer and bioethanol. As reported by Chun Sheng Goh and Keat Teong Lee (2010), certain species of algae have the ability to produce high level carbohydrate instead of lipid. This carbohydrate can be extracted to produce fermentable sugar to convert into bioethanol by involving some bacteria or fungi [12].

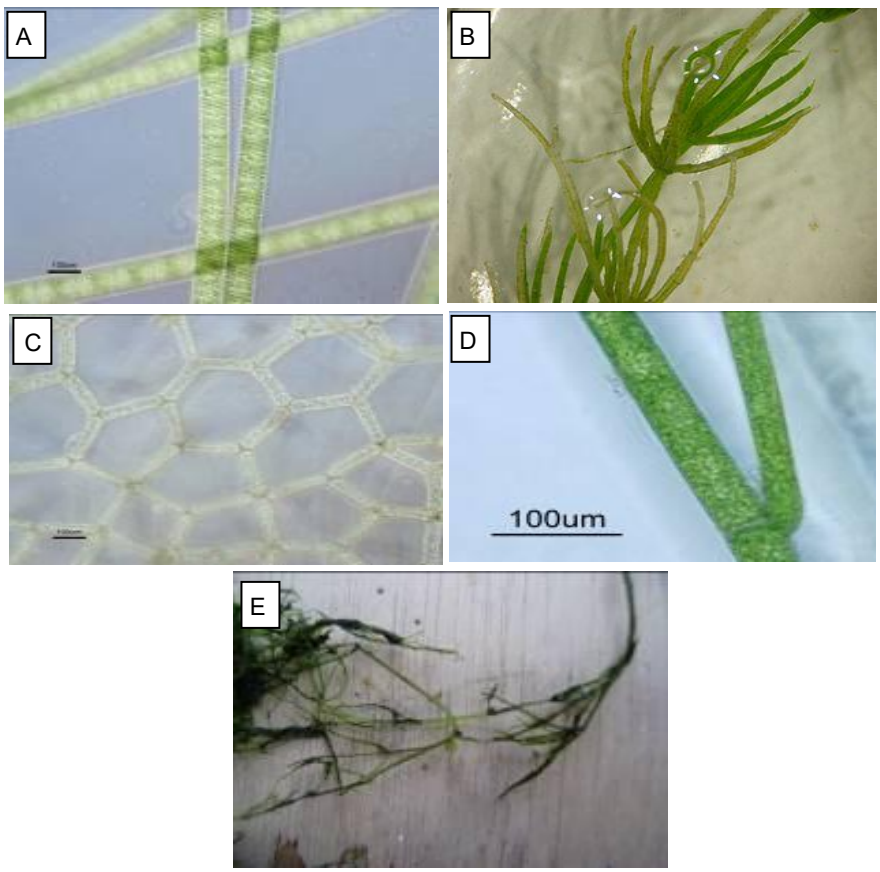

Figure. 1. Collected Macroalgae from different places of freshwater ecosystems, (A) a filament of *Spyrogyra* sp., (B). a branch of filament of *Chara* sp., (C) a single colony of *Hydrodiction* sp., (D) a branch of filament *Cladophora* sp, (E) a branch of filament of *Nitella* sp.

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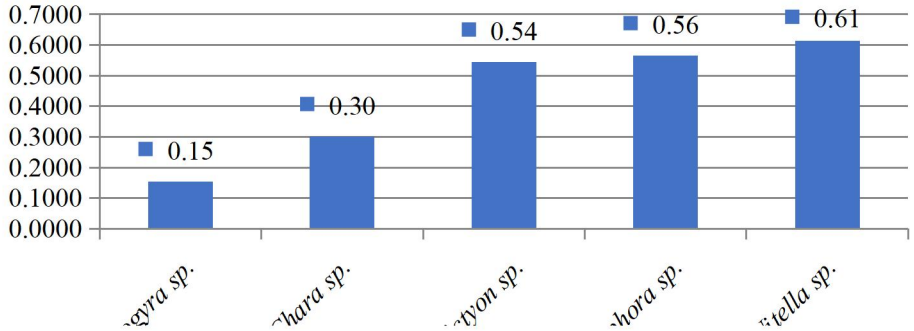

Figure 2. The percentage of oil in extracts of *Spirogyra* sp., *Chara* sp.. *Hydrodiction* sp., *Cladophora* sp., *Nitella* sp.

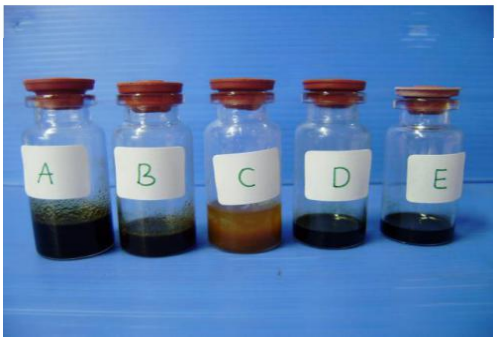

Figure. 3. The algae oil of (A) *Chara* sp., (B) *Cladophora* sp., (C) *Hydrodiction* sp., (D) *Nitella* sp., (E) *Spirogyra* sp.

Table 1. Acids number, safonification value, iodine value of the macroalgae oils

No	Sample	Acid Number (mg KOH/g sample)	Saponification Value (mg KOH /g sample)	Iodine Value (g I_2 / 100 g sample)
1	*Spirogyra* sp.	142.98	457.27	27.14
2	*Chara* sp.	88.35	400.29	16.27
3	*Hydrodiction* sp.	63.62	408.75	3.38
4	*Cladophora* sp.	58.80	435.77	15.98
5	*Nitella* sp.	55.88	482.52	17.33

4. Conclusion

Five different species of freshwater macroalgae were abundantly in areas of West Java i.e. Bandung, Bogor, Sukabumi and Garut. Based on morphological characteristics, the collected macroalgae were identified as Spyrogyra sp., *Hydrodiction* sp., *Cladophora* sp., *Hara* sp., and

Nitela sp. Content of oil in five species of freshwater macroalgae are considered low which are 0.61% in *Nitella* sp., 0.56% in *Cladophora* sp., 0.54% in *Hydrodiction* sp., 0.30% in *Chara* sp., and 0.15% in *Spyrogyra* sp. Chemical properties of each oil indicate that the macroalgae oils have short carbon chains resemble to butyric acid (C-4) which does not meet the standard requirement of national biodiesel. Thus, *Nitela* sp., *Cladophora* sp., *Hydrodiction* sp., Chara sp. and *Spirogyra* sp. are less potential to be used as raw material for biodiesel oil. However, the oils can be used as source of naturally fine chemicals and the remaining biomass after extraction of oil may be useful for producing bioethanol as well as for producing biofertilizer. Macroalgae extraction method need to be improved in order to gain higher oil content and better oil characteristics.

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