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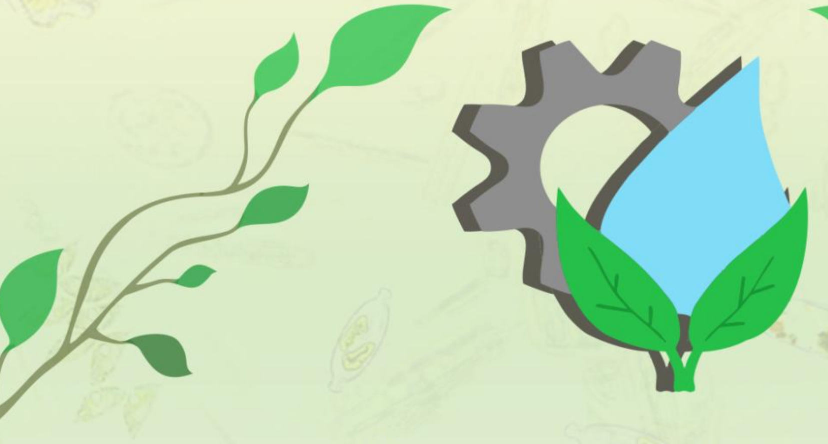

INTERNATIONAL CONFERENCE ON PLANT & ALGAE BASED BIOINDUSTRY

Institut Teknologi Bandung
17th - 18th November 2021

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'The use of plant and algae for bioindustrial purposes'

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Preface

As-salamu alaykum, distinguished guests, ladies and gentlemen.

On behalf of all members of Plant Science and Biotechnology Research Group and myself, I am very pleased to welcome you all to our International Conference on Plant and Algae-based Bioindustry, hosted by Plant Science and Biotechnology Research Group, School of Life Sciences and Technology, Institut Teknologi Bandung.

Unfortunately, this will be another virtual webinar, as we are all still experience an unprecedented situation due to the global Covid19 pandemic, but activities must go on. Like most people nowadays, I work most of the time from home. Let us hope that we will soon be able to physically meet again.

Let me highlight a few topics on our agenda. This proceeding will give us with an opportunity to exchange knowledge and technology with four keynote speakers and ten invited speakers from Indonesia, as well as Japan, the Netherlands, Malaysia, USA, and Canada, all of whom are recognized for their current expertise. The subjects covered span from fundamental knowledge of plant and algae biology to contemporary biotechnology approaches and their application to the development of biobased products beneficial for food, feedstock, pharmaceutical, and other industries. Additionally, a parallel session will have speakers discussing a variety of biobased technology. It gives me a great pleasure to share with you all the discoveries that may have an impact on the mid- to long-term achievement of the SDGs.

I would like to express my sincere appreciation to all of you who generously helped us make this event come together to become a success. Many thanks to the Dean of SITH, the Rector of ITB, and, of course, the organizer of today's webinar. Additionally, a heartfelt thank you to all presenters and participants who comes from ITB, Osaka University, Imperial College London, University of Gadjah Mada, IPB University, Andalas University, University of Sriwijaya, UIN Alauddin Makassar and soon for joining us today.

Allow me to start this event by extending a hearty Bismillah and wishing everyone good health and success. I hope we can discuss and challenge one another's perspectives to enrich our understanding of these subjects.

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

Welcoming Remarks

Dean of School of Life Sciences and Technology (SITH)

The honorable,
Group leader of Plant Science and Biotechology Research Group, SITH,
Dr. Rizkita Rahmi Esyanti,

Keynote speakers
Prof. Dr. J. Theo M. Elzenga from University of Groningen
Prof. Dr. Katsumi Suzuki from Shizuoka University
Prof. Dr. Eiichiro Fukusaki from Osaka University
Prof Dr. Ir. Siti Rozaimah from Universiti Kebangsaan Malaysia

Invited Speakers
Prof. Dr. Endang Semiarti from University of Gadjah Mada
Prof. Dr. Sri Nanan Widiyanto from Institut Teknologi Bandung
Assoc Prof. Dr. Roohaida from Universiti Kebangsaan Malaysia
Hadhimulya Asmara, Ph.D from University of Calgary Canada
Dr. M Arif Budiman from Orion Genomic, USA
Dr. Iriawati, Dr. Rizkita R. Esyanti, Dr. Taufikurahman, Dr. Apt. Elfahmi and
Neil Priharto, Ph.D from Institut Teknologi Bandung

Distinguished Guests and Participants,
My dear friends and colleagues,

Assalamu'alaikum warahmatullahi wa barakatuh,

It is my great pleasure, and on behalf of the School of Life Sciences and Technology, to welcome all of you in Proceeding of International Conference on Plant and Algae-based Bioindustry, 2021, published by Plant Science and Biotechnology Research Group, School of Life Sciences and Technology, Institut Teknologi Bandung.

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.

This proceeding will specifically discuss intensively one of the recent developments on plant and algae biotechnology research for for food, feedstock, pharmaceutical, and other industries. This scientific meeting will explore some of the potentials of plant and algae 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 read further from the writers in the proceeding. I truly hope that the exchanges of ideas will give a contribution to the advancement of new and effective ways to increase food production, tackle environmental issues, and also increase health development.

Distinguished guests and participants,

The topic that is raised in this proceeding 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 expertise 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 proceeding 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.

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

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'The use of plant and algae for bioindustrial purposes'

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Environmental Effects on Crop Production and Protected Horticulture

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ABSTRACT

Plants are unable to move, they are directly affected by the environment in which they grow. Crops are primarily grown in favorable places. However, crop production may be difficult to cultivate in the same way as before under the conditions of recent global warming and extreme weather. Crop production and stable food supply are very important as SDGs, no poverty, zero hunger, life on land, etc. In this presentation, I will explain the environmental effects on crop production and measures using agricultural materials and protected horticulture.

(1) Environmental stress and crops

When the crops are grown under optimal environmental conditions such as temperature, solar radiation and soil nutrients, they can achieve maximum yield without stress. However, the crops grow with various stresses during their growth. There is biotic stress such as pests or fungi as well as abiotic stress. Here, I deal with environmental stress as abiotic stress.

Plant growth is inhibited by temperatures outside of the optimal temperature range. The first visible sign appears in pollen that is the male organ. Rice cultivation has often damage in cold summer in Japan. In 1994, the yield was decreased due to cold weather damage. No major cold damage has occurred since then. In cold weather, when the temperature drops during the pollen development period, pollen development is inhibited, pollen becomes sterile, and fertilization is not possible, resulting in a severe reduction in yield. Furthermore, when the temperature drops during the flowering period or the period when the seed develops, the development of the rice paddy is inhibited.

Pollen development is affected even at high temperatures. In snap bean, when the temperature was high 10 days before flowering, the tapetum cells in the anther became abnormal, pollen development was inhibited, and pollen became sterile. High temperatures during the flowering period also reduce the pollen germination ratio. When the temperature rises during pod development, the growth of pod is inhibited. High temperatures cause damage to rice in the same way that cold damage does.

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One of the major factors affecting crop production is the nutrient or conditions in the soil, drought or excess wet damage, etc. The salt damage caused by the accumulation of salt in the soil also causes great stress on crop production. In addition, various nutrient deficiencies and nutrient excesses are also problems. There are also various factors that affect crop production, such as strong sunlight, lack of sunlight, and dryness.

(2) Environmental stress control

1) Breeding

As a countermeasure against environmental stress, it is important to produce tolerant crops by breeding. For example, heat-tolerant snap beans have been developed. Heat-tolerant snap beans have higher pollen fertility even at higher temperatures than sensitive cultivars. However, it is not easy to produce superior cultivars that are tolerant to various environmental stresses, and it takes long time to breed them.

2) Use of agricultural materials

Another method is to use agricultural materials and greenhouses that are usually used in horticultural crops. Protected horticulture is the term used to describe this type of horticulture. Protected horticulture enables stable crop production in an artificially controlled environment.

In Japan, the majority of tomato fruits are grown in greenhouses. Spring to summer in Japan is a good season for tomatoes to grow, but they are cultivated in a simple house that avoids rain. Avoiding the rain reduces the incidence of disease and improves fruit quality. In addition, by covering with a net, damage from pests can be prevented. Strong sunlight causes cracking of tomato fruits, but it can be prevented by shading.

A simple method is to cultivate the crops with a net. This is known as ‘betagake’, a technology that originated in Okinawa, Japan. Okinawa Prefecture is located in the subtropical region, and since it is warm even in winter, insects do not die in the cold, resulting in a high pest density. By covering net, it becomes a countermeasure against a pest, a strong solar radiation, a heavy rain, and a soil erosion. Changing the material provides a heat insulating technique, which is widely applied as row cover in Japan. It is often used in combination with mulch cultivation and drip irrigation using drip water tube.

3) Protected Horticulture

The production of protected horticulture protects not only crops but also workers in greenhouse. The advanced system is called a plant factory in Japan, and the inside is completely isolated from the outside, with air conditioning regulating temperature

and humidity for crops. Protected horticulture also uses various facilities to create a good environment for crops.

Heating is used against low temperature. By raising the temperature, it is possible to produce and supply vegetables such as tomatoes and cucumbers that can only be cultivated outside from spring to summer. However, High temperature measures are more difficult than low temperature. Please refer to the references about control high temperatures (Suzuki, 2020).

As a measure against excessive humidity, ventilation using window and fan, or dehumidification is performed with a heat pump. Fine mist is used to raise the humidity as a measure against drying.

As a measure against strong solar radiation, shading is performed using various materials. Recently, new materials that block heat lay have also been developed. Various hydroponic cultivation has been introduced as a counter-measure to soil. NFT cultivation is common for leaf vegetables, and medium cultivation is common for fruit vegetables. Hydroponic soil cultivation that makes the best use of soil cultivation is also carried out. In addition, the CO_2 enrichment can also control photosynthesis

The temperature and humidity suitable for crop production are created in a plant factory, which is completely shielded from the outside. CO_2 leves are also controlled about 1000 ppm. Although the amount of solar radiation can be artificially limited from financial problem, it is necessary to reduce costs for sale. The most advanced is the production of mushrooms, which are made by companies. In addition, sprout and leafy vegetable lettuce are cultivated.

It is possible to produce crops that are not affected by outside environment, but the major problem is cost. Therefore, research is being conducted on the development of low-cost about protected horticulture technology. Research about crops is also needed. Investigating how the environment affects crops and developing methods to effectively prevent them are important for protected horticultural production. The effects of low and high temperatures on crops was referred above, that information is very useful, for example, when choosing a heater.

As an example of our recent research, I introduce research about intumescence that occurs in tomato leaves. Intumescence is a long-known physiological disorder that occurs in bad weather. It may occur frequently when growing tomato seedlings using LEDs in a closed seedling system, recently. The cause is lack of UV. We studied about this disorder and suggested to control methods (Suzuki et al., 2020).

In order to find countermeasures, it is necessary to clarify the cause and what is happening. The use of agricultural materials and protected horticulture are effective measures for reducing environmental stress, but in order to reduce costs, both crop and material research are necessary.

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Intensifying Plant-based Treatment Technology in Recovering Secondary Resources from Wastewater

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ABSTRACT

Waste is no longer treated to comply stringent environmental regulations and directly disposed to the ecosystem. Waste nowadays can generate wealth and in the era of circular economy, sustainable waste management is crucial and demanded. The world is also experiencing exhausting natural resources forcing researchers to adventure on green technologies and seek for renewable resources as not leave detrimental impacts on the ecosystem. Wastewater, as waste in the form of liquid, either from industry or domestic, contains valuable elements to be recovered as secondary resources. Effluents from aquaculture, agriculture and food processing industries are rich with soluble nutrients and nutrient-rich solids in which these sources can be extracted and converted into value-added products such as feedstock, soil conditioner and fertilizers. To harvest nutrients in the form of soluble ions and solids, there are many available treatment methods including adsorption/extraction and coagulation-flocculation that can adsorb or extract soluble nutrients and capture nutrient-rich solids, respectively. The two approaches can be accomplished through biological and chemical means. If the chemical method were to be implemented, the resultant extracted nutrients and coagulated/flocculated solids will become toxic although originally the effluents from aquaculture, agriculture and food processing industries are considered non-toxic. Thus, treatment using natural resources such as plants is proposed since it will be more environmentally friendly and simultaneously open opportunities for resource recovery. Phytoremediation, a well-known technology utilizing plants and their associated microbes to degrade, transform, extract, accumulate and detoxify pollutants, can be used to extract soluble nutrients for their growth and eventually, the treating plants can be converted into other secondary resources such as feedstock, biofertilizers, bioenergy (biodiesel, bioethanol, biogas, biohydrogen), biosorbent, biochar and bio-oil, depending on type of plants used. Whilst coagulation-flocculation process via plant-based coagulants/flocculants to harvest nutrient-rich solids from non-toxic effluents can widen the potential of recovered sludge to be used as soil-conditioners or biofertilisers. These two plant-based treatment technologies have attracted increasing interests of researchers to further explore and look for suitable plants to be used as phytoremediator and biocoagulants/bioflocculants. Although phytotechnology is well established and applied in developed and temperate countries such as U.S., Europe, Japan and China, it is still scarcely applied in

developing countries which are mostly tropical countries, such as Indonesia and Malaysia, and rich of green plants and have no limitation on plant growth due to weather throughout the year. Research on phytoremediation in the Southeast Asia region is still new and at developing stage. On the other hand, biocoagulants/bioflocullants based on plants are still at research stage with no commercialized products at the market yet, mostly at lab-scale application and are actively researched in Asia countries such as Thailand, Malaysia, Indonesia, and India. More challenges and opportunities of these two plant-based treatment technologies are expected ahead before they can be commercialized and applied in real field application especially in tropical countries. Thus, research activities to search for suitable plants as phytoremediator and biocoagulants/bioflocculants need to be promoted and intensified for sustainable ecosystem.

Keywords: Phytotechnology, biocoagulant/bioflocculant, aquaculture, agriculture, primary resources, waste recovery, waste minimization

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A Plant Physiologist's View on Sustainable Agriculture in A Changing World

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ABSTRACT

Since the start of modern agriculture most of the innovations have been towards a higher dependence of plant development on human care and control. The ultimate example is the massive use of greenhouses where control is almost absolute: temperature, light intensity, quality and duration, nutrient availability and relative humidity are all maintained at preset values. Using hydroculture or inert substrate allows an unprecedented precision in the availability of nutrients. Any external influence of climate and variation in soil conditions are excluded from the culture system. The threat of pathogens is countered by chemical means and maintaining almost clinical protocols. In a way we treat our crops as incubator babies, unable to take care of themselves.

Breeding effort aimed at maximizing yield of crop plants have been very successful, resulting in highly productive and nutrient efficient varieties. But inadvertently, and sometimes unnoticed, valuable traits have been lost in the breeding process. Fairly late, increasing resistance, i.e. pathogens, insect herbivory and abiotic stresses, has been set as new breeding targets.

In order to fully use the potential a bio-based economy has to offer we need to develop crop varieties and culture practices that are robust, can handle unfavorable conditions for days, be self-reliant and less dependent on crop protection chemicals.

In my presentation I will give examples of useful plants traits that we have lost in the history of plant domestication and the potential to get them back in our crops and how these will be essential for sustainable agriculture under the global changes we have to anticipate.

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Food Security: From Research to Plant Based Food and Agriculture Industry

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ABSTRACT

Metabolomics is an omics science based on total metabolites, and it is expected to provide information that contributes to further understanding of genome functions through integrated analysis with upstream omics information (transcriptome and proteome). The metabolome, on the other hand, can be thought of as a precise phenotype more closely related to genomic information. Therefore, metabolomics can be operated independently of the upstream genomic information. The research targets of metabolomics are not limited to animals, plants, and microorganisms, but also cover a wide range of food materials, including processed foods.

Now, I would like to mention the application of metabolomics in food production research.

Indonesia is a country with rich bio-resources and produces huge amount of food. However, food loss also occurs frequently. There are many possible causes of food loss in Indonesia, such as "plant and seafood diseases", "suboptimal postharvest management", and "lack of quality". Metabolomics can provide useful information to solve the above problems. For example, metabolomic information can be obtained on the causes of disease, guidelines for optimizing postharvest management, and quality improvement.

In this section, we will briefly discuss metabolomics. We have already mentioned that metabolomics is an omics science based on comprehensive metabolite analysis. It requires steps such as "preparation of biological samples", "sampling including derivatization", "analysis", "organization of metabolite data matrix", "multivariate analysis", "knowledge creation", and "feedback". This requires techniques in biology, analytical chemistry, and informatics. Metabolic fingerprinting" is the most frequently used tactic in food metabolomics research. It is a technique that uses the metabolome as an explanatory variable to quantitatively explain the downstream macro phenotypes. In other words, it can be called chemometrics, where the metabolomic information is the explanatory variable and the quantitative macro

phenotypic information of the sample is the response variable. In this presentation, we will frequently use this metabolic fingerprinting technique.

Now, I will refer to phenotypes (response variables) in food metabolomics. Food has three functions, of which the second function is the most difficult to describe scientifically. In food metabolomics, Metabolic fingerprinting using Flavor and Grade of food as response variables enables us to analyze the complex correlation between many components in food and many secondary functions of food. The author has been conducting metabolomics research on various foods. In this talk, I will introduce some examples of research on tropical fruits, especially those produced in Indonesia. Specifically, the application of metabolomics research on ripening and post-harvest management of mangosteen, banana and pineapple will be presented.

(Our publications related)

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SHELL DNA Testing to Support the Sustainability of Indonesian Palm Oil Industry Especially Smallholders Oil Palm Sector

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ABSTRACT

Future demand for edible oil will keep increasing and can be estimated from growing population projections and per capita consumption. By 2050, the demand will probably be around 250 Mt, nearly twice today's total. Most of the additional oil may be from oil palm, Elaeis guineensis, the world's most important oil crop, so far accounting for over 35% of all traded vegetable oil, which has the lowest production cost among major oils. In Asia, palm oils have been consumed daily by nearly three billion people, however in Indonesia, aside for dietary components, palm oil has been used for a variety of vital non-food applications including biofuel raw material. In the future, biofuel demand might greatly exceed that for edible use, and the interchangeability of the major oils, for edible and biofuel uses, means that this demand will drive oil palm expansion, whether or not palm oil is eventually used for biodiesel.

The challenge for palm oil as it has been frequently associated with deforestation and significant greenhouse gas emissions, requires Indonesia and Malaysia, leading palm oil producers, to actively promote sustainable palm oil production and transition to net-zero emissions. Current traditional agricultural practices will not be able to supply palm oil demand and genomic research comes in to play, allowing discovery of important traits which will lead toward more intensive use of already planted lands, which should reduce pressures to expand the land area devoted for palm oil. An example of such trait isolated through collaborative effort of Malaysian Palm Oil Board and Orion genomics is *SHELL* gene controlling the shell thickness surrounding the oil palm kernel. The monogenic shell thickness is inversely correlated to mesocarp thickness, where the crude palm oil is extracted. The development of *SHELL* DNA testing and its use assures high purity Tenera be planted, improving sustainability by increasing yield on existing planted lands.

In the case of Indonesian oil palm, 42% of its plantation areas are smallholders which has been associated with low yield and low productivity. Recently, Jelsma et al, 2019, indicated the prevalence of non-Tenera contaminants among smallholders (> 50%) in Riau province, the largest smallholder plantation, confirming the existing low yield problem. Currently, the government of Indonesia undertakes a massive smallholder replanting effort for the next 25 year plantation cycle. The proposed use

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of SHELL DNA testing should complement the government replanting program and the economic model demonstrates that *SHELL* gene testing should confer substantial annual economic gains to the oil palm industry especially the smallholder oil palm sector, GNI and government tax.

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A Dynamic T-type Calcium Channels-calmodulin Complex on Signaling Cascade for Learning and Memory

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ABSTRACT

Calmodulin (CaM) is an important signaling molecule that regulates high voltage-activated calcium channels and second messenger cascades that activate CaM kinases, a second messenger enzyme, and CREB, a gene transcription factor. Calcium channels of the Cav3 family (T-type) mediate low threshold calcium influx but were not believed to interact with CaM. We reported a constitutive association between CaM and the Cav3.1 channel at rest that is lost through activity- and Cav3 calcium-dependent CaM dissociation, followed by activation of αCaMKII and CREB (Asmara, PMID 28800734). Recent work has examined how regulation of Cav3 channels, CaM, and αCaMKII could contribute to disorders of synaptic plasticity in the Fragile X Syndrome (FXS) model of Autism Spectrum Disorder. FXS derives from a loss of FMRP, a key regulator of protein translation that includes Cav3 channels, and CaM. We found that FMRP coimmunoprecipitates (coIPs) with Cav3.1 and forms an association close enough to support Foerster Resonance Energy Transfer (FRET) expressed in tsA-201 cells (Zhan, PMID 32488011). These findings are important in revealing a previously unrecognized association between FMRP, Cav3 calcium channels, and CaM that have a direct influence on the learning and memory mechanism. These findings will be important to address the root of the deficit learning and memory capability in Fragile X syndrome patients.

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Transcriptomic Changes of Banana Plantlets Exposed to Drought Stress

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ABSTRACT

Bananas (*Musa* spp.) are famously known as a commercial crop of the tropical and subtropicalcountries. Drought stress is considered the main environmental factor affecting plant growth and productivity of banana worldwide. A big loss of production yield of banana has been reported every year due to the drought condition, especially bananas with AAA genome. The objective of this project is to gain new insights into the molecular mechanism of banana plantlets to respond drought stresses. Drought refers to the condition of water deficit that occurswhen water levels dropped below a certain threshold. In this study, in vitro shoot cultures of banana (*Musa acuminata*, Colla) cv Pisang Barangan Merah (AAA genome) were exposed topolyethylene glycol (PEG) to induce water deficit stress condition. The PEG was added in culture medium in three concentration levels i.e. 2.5% (BP2), 7.5% (BP7), 10% (BP10). Banana plantlets grown on culture medium without PEG addition were used as the control treatment (BK). A transcriptome dataset was generated from four cDNA libraries of the BK, BP2, BP7 and BP10 samples, which were sequenced using Illumina MiSeqTM 2000 platform.Transcriptome assembly, annotation, differential gene expression (DEG) analysis, gene ontology enrichment, and gene networking analysis were accomplished. Statistical analysis identified 1744 genes as differentially expressed (DEGs) under the PEG treatment, and a totalof 1046 genes of them were mapped to the reference genomes. The DEGs were distributed in 25 functional clusters. Transcriptomic analysis revealed that the top 100 genes represented quite a large variety in function which were highly affected by the water deficit condition. Those affected genes are related to the process of photosynthesis, response to stress, cellular respiration, morphogenesis and organ development, and secondary metabolite biosynthesis. Further transcriptomic analyses are required to discover the effects of drought stress to major bioprocesses in more details. Further transcriptomic analyses are still ongoing to discover the effects of drought stress to major bioprocesses in detail.

Key words: Drought stress; *Musa acuminata*, Colla; transcriptome.

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Biotechnology Approach to Improve the Quality and Quantity of Orchids for the Agricultural Industry in Indonesia

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ABSTRACT

Agroindustry in Indonesia will continue to be developed. One of the plants that have the potential to be developed is orchids. Orchids are important ornamental plants, because of the beauty and durability of their flowers that do not wilt easily, some even contain phytochemicals for medicines, cosmetics, and perfumes. So that many are exclusively collected, or, traded to be used as the main plant in decoration at various events such as wedding ceremonies, state meetings and tourism vehicles. Therefore, the application of modern biotechnology is very appropriate to be used for the orchid agroindustry, especially the application of plant tissue culture techniques for mass propagation and the use of transgenic technology and genome editing system for producing Orchid GMOs with unique character(s) to improve the quality and manufacture of better traits. Academicians as biotechnology researchers will carry out the research innovations in biotechnology research at the laboratory in the University to produce prototypes with unique phenotypes, which will have to be managed by the industry for mass propagation so that it can be passed on to consumers and the public, which must be supported by the government for regulations both at the national and international. For the sustainability of agroindustry, a good and sustainable synergy is needed among academicians, businesspeople, the community, and the government (A-B-C-G).

Keywords: Agroindustry, Orchids, Micropropagation, GMO, A-B-C-G

1. Introduction

Indonesia is an archipelagic country that stretches along the equator, has tropical rain forests that are very beneficial as a natural habitat for tropical plants, one of which is orchids. Almost on every island in Indonesia found endemic species of orchids which became the icon of the area. Schuiteman [1,2] estimates that there are 5,000 of the 30,000 world orchids in Indonesia. Some of them are in great demand by the public and industry to be commercialized, including from the Phalaenopsis genus, namely Moth orchid (*Phalaenopsis amabilis*) which was appointed as the

national flower, Tie orchid (*Phalaenopsis gigantea*), *Dendrobium spectabile*, *D. lineale*, *D. lasianthera* which contain phytochemicals for antiaging, and antiviral, Tiger orchids (*Grammatophyllum scriptum*) from Papua (Fig. 1). Many people directly take these natural orchids from the forest to be traded, so that many natural orchid populations are declining, and they are threatened to be extinct from their natural habitats. There is also the threat of natural disasters and deforestation for plantations or shelter, or other purposes that cause the loss of these natural orchids as important germplasm [1,2]. Mass propagation of plants is needed for *ex situ* conservation of these orchids, which are then partially returned to their natural habitats for in situ conservation.

Figure 1. Indonesian Orchid Species. A. Moth Orchid-*Phalaenopsis amabilis* (L.) Blume; B. *Dendrobium spectabile* (Blume) Miq.; C. *Dendrobium lineale* Rolfe; D. Tiger Orchid (*Grammatophyllum speciosum* Blume); E. *Vanda tricolor* Lindl. var. suavis Forma Merapi. Bars: 1 cm

2. Plant Tissue Culture for Orchid Micropropagation

Tissue culture technique or in vitro culture technique is a technique for growing plant cells/tissues vegetatively on a nutrient medium aseptically under controlled conditions (physical and chemical) in a bottle to produce clones of cells/plants [3]. The basic principle is that every plant cell has autonomic properties and cell totipotency (Schleiden & Schwan), so that when plant cell is grown in a suitable medium and environment, it will grow and develop, regenerating into a complete and perfect new plant. The use of artificial mediums such as New Phalaenopsis, Knudson C and Vacin and Went proved successful for *in vitro* germination and micropropagation of *Dendrobium* spp, *Coelogyne* spp. *Phalaenopsis* spp, *Vanda* spp, *Cattleya* spp and *Cymbidium* spp orchids (Fig.2). The addition of phytohormones and growth regulators auxins and cytokinin to the growth media can grow protocorm like bodies (PLB) from the organ fragments of orchid seeds planted in the artificial medium, which then form somatic embryos and eventually grow into new plants identical to the parental plant [3,4,5].

This is very profitable, so it can be used for mass production of plants/plant clones for the purpose of preparing uniform agricultural plant seeds, as well as for industry [6]. The ability of plant cells to be grown in culture bottles under aseptic conditions also facilitates the development of genetic engineering by insertion of foreign genes to study the function of certain genes in plant growth & development [7, 8]. This genetic engineering technique continues to be developed to improve the

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quality of plants with new, more profitable, or unique properties that will increase the beauty or usefulness of the plant.

Figure 2. Orchid micropropagation results by using tissue culture. A. *Dendrobium stratiotes* Rchb.f. ; B. *Coelogyne pandurate* Lindl.

3. Genetic engineering for Orchid Quality and Quantity Improvement

Plant genetic engineering is modifying the nature of plants by inserting genes from other individuals or from the plant itself which is conditioned with the addition of certain promoters through genetic transformation either directly or indirectly by target/recipient plant cells so that new plants with different phenotypes are formed according to the characteristics of the plant inserted foreign gene [9,10,11,12]. The process of changing the constitution of the plant genome by insertion of foreign genes is called genetic transformation, and plants that have undergone the insertion of foreign genes in their genomes and have been expressed to form proteins that can appear in their phenotype are called transgenic plants.

In orchids, it has been reported the use of genetic engineering to change the characters of flower, drought resistance, the growth of somatic embryos, and production of multiple shoots with various foreign genes inserted into the protocorm (developing orchid embryo), leaves and roots.

Currently, the development of genetic engineering is advancing with the possibility of using a genome editing system since 2005. In general, the Genome Editing approach uses sequence-specific nucleases (SSNs) consisting of DNA-binding domains to provide sequence specificity associated with nuclease domains to generate DNA strand breaks (DSB) on SSNs. In this biotechnology application, it has been reported that there are 4 genome editing tools, namely: 1) zinc finger nucleases (ZFNs) (2005); 2) transcription activator-like effector nucleases (TALENs) (2011); 3) clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 (CRISPR/Cas9) (2013), and 4) CRISPR/CRISPR from Prevotella and Francisella 1 (CRISPR/Cpf1) (2016). However, the CRISPR/Cas9 genome editing system is considered the most appropriate to be used until now.

CRISPR/Cas9 adopts 3 important stages of genome editing in *Streptococcus pyrogenes* bacteria, namely 1) Adaptation: the process of forming a spacer sequence due to the

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entry of foreign genetic material into the bacterial body; 2) crRNA biogenesis: the formation of a precursor crRNA (pre-crRNA) which will be processed to form a mature crRNA characterized by the presence of repeating parts and spacers 3) Interference: a complex is formed between crRNA and Cas protein and recognizes a specific motif called with PAM (Protospacer Adjacent Motif) so that it starts the editing by cutting the recognized part [Fig.3].

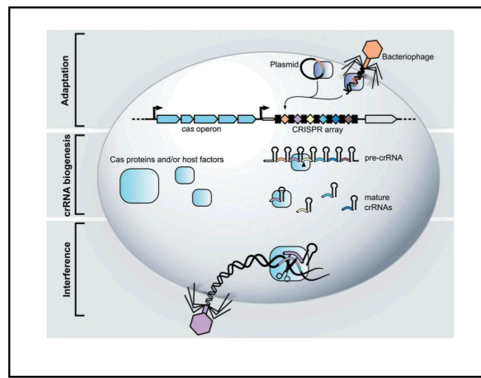
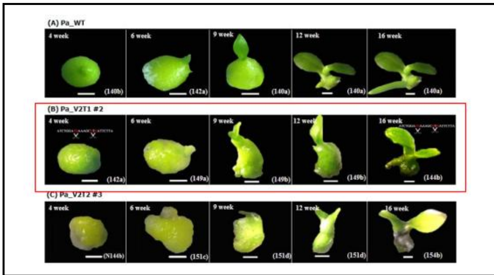


Figure. 3. Stages of CRISPR/Cas9 genome editing system in *Streptococcus pyrogenes* bacteria [13].



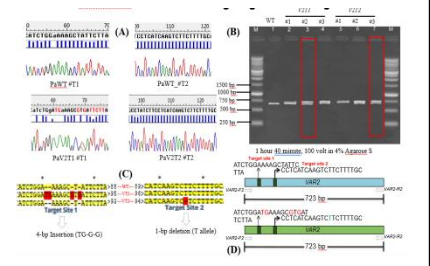


Figure. 4. Phenotypic changes of the CRISPR/Cas9 genome-edited *Phalaenopsis amabilis* protocorm.

A. Changes in leaf green color to pale green; B. The results of sequence analysis on the *VARIEGATA2* gene showed mutations in the target sequence, insertion in Target site 1 and deletion in Target site 2 [12].

Our team has succeeded in editing the genome of *P. amabilis* with the *VARIEGATA2* gene, the gene responsible for the thylakoid formation during chloroplasts development as a target site to obtain mutant orchid plants with a new phenotype (Fig. 4). By obtaining the V2T1 and V2T2 mutant plants, it is known that the CRISPR/Cas9 method can be applied to obtain orchids with a new phenotype [12].

The main advantage of this genome editing technology is that transgenes originally used to produce genetic changes can be easily removed from the genome by genetic

segregation, and gene-edited varieties show no phenotypic differences from those produced by conventional breeding methods.

4. Conclusion and Perspectives

The Biotechnology Approach for the Orchid Industry can be done by: 1) Tissue Culture/In vitro Culture Techniques; 2) Genetic engineering by inserting functional genes: embryonic genes, shoot-forming genes can increase the production of orchid seedlings, and flowering genes can accelerate flowering time; 3) The right genome editing system CRISPR/Cas9 method can be used to produce mutant orchid plants with the desired traits/characters.

To realize a good orchid industry and preserve Indonesian orchids in a sustainable manner, the perspective that can be given is a synergistic collaboration between the Academic-Business-Community-Government and the Mass Media (A-B-C-G-M). So that orchids as superior horticultural commodities will provide a maximum commercial contribution, but still exist in their natural habitat as endemic germplasm.

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Systems Biology Approach in Plant Product Development

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ABSTRACT

The sustainable use of plant genetic resources in plant product development is necessary to improve the economy while meeting the basic food needs in the current situation of diminishing agricultural lands and decreasing natural sources of water. Systems biology approach may provide a comprehensive and quantitative understanding of the processes in plants by utilising the huge amount of biological data generated through multiple omics technologies such as genomics, transcriptomics, proteomics, metabolomics, metagenomics and phenomics. This interdisciplinary research is possible due to the rapid developments in next generation sequencing technology and omics platforms. The integration of these biological data utilises advanced computer science concepts with the goal of computational modeling and simulation. The omics platforms have played an important role in understanding the molecular mechanism of various aspects of plant physiology such growth, senescence and response to biotic and abiotic stresses in addition to agriculturally important traits in numerous crops. Furthermore, systems biology can enhance our understanding of molecular regulator networks for these processes. It has facilitated the identification of some key genes and proteins involved in networks or pathways related to specific plant physiology processes and plant product biosynthesis. Advances in these fields have led to production of targeted molecules through genetic manipulation of key candidate genes and might make it possible to design smart crops with superior agronomic traits.

Keywords: Systems biology, transcriptomics, metabolomics, bioinformatics, plant products.

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Engineering of Biosynthetic Pathway of An Antimalarial Artemisinin from *Artemisia annua* to Enhance Its Production Level

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ABSTRACT

Artemisinin is a drug currently used to treat malaria caused by *Plasmodium falciparum*. Artemisinin is a secondary metabolite produced in *Artemisia annua* L., which is naturally produced in low concentration, therefore directly influence the cost of malaria therapy using artemisinin become expensive. One of the methods to increase artemisinin production is by engineering of the biosynthetic pathway which is focus on the key enzymes. We have engineered the key genes which are responsible in the artemisinin biosynthesis in *Artemisa annua*, *Saccaharomyces cereviceae* and *Eschericia coli*. Transform amorphadiene synthase (ads) and p19 gene as an antisilencing into *Artemisia annua* has been successfully done and could enhance the artemisinin content on the transformed leaves with ads-p19 up to 2.57 folds compared to the untransformed leaves, while for p19, co-transformed and ads were up to 2.25, 1.29, and 1.14 folds respectively. One of the main precursors in artemisinin biosynthesis is farnesyl pyrophosphate (FPP) which was catalyzed by farnesyl pyrophosphate synthase (FPS) enzyme. S. cerevisiae naturally produce FPS enzyme, so to increase FPP production, Recombinant plasmids which are pBEVY-GU_ads and pBEVY_GLfps were constructed. Recombinant plasmid pBEVY-GL_fps and pBEVY-GU_ads were constructed by the homologous recombination method. fps and ads gene were amplified using PCR with a couple of primers that are designed in order to provide the homolog recombination of fps and ads gene with expression plasmid of pBEVY-GL_and pBEVY-GU respectively. The template used were pUC_fps and pUC_ads containing yeast synthetically optimized fps and ads genes. Transformants were grown in selective media Synthetic Defined (SD) without leusine for transformants contain plasmid pBEVY-GL_fps and media

without uracil for transformants contain plasmid pBEVY-GU_ads. Confirmation of colonies contains plasmid recombinant was done by PCR colony with primers to amplify fps and ads. DNA from yeast was isolated from positive colony then transformed to *Eschericia coli*. Plasmid from *E. coli* was isolated for restriction analysis and sequencing. Based on PCR, restriction and sequencing analysis, it could be concluded that fps and ads genes were successfully constructed and transformed into *S. cerevisiae*. Transformation of 5 key genes in the biosynthesis of artemisinin in *E. coli* has been also successfully performed. It could alter the metabolite contents of recombinant E. coli harboring 5 key enzymes. It is assumed that the metabolite profile was due to effect of engineering of artemisinin biosynthetic pathway. Further analysis of the metabolites is still on progress

Keywords: Artemisinin, artemisian annua, ads, fps, biosynthetic pathway

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Exploring the Potential of Microalgae Bioindustry in Indonesia, Emphasizing synergism Between Universities, Research Institutes and Privates Sectors

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ABSTRACT

Currently, the capacity of domestic industry to produce microalgae is still negligible, while the need for products from microalgae in the market for various purposes such as for health, as supplements of protein and vitamins, animal feed, biodiesel, bioplastics and various other innovative products is increasing. Moreover, there are predictions which indicate that in the near future the world will face a food and energy crisis due to climate change and also the impact of a prolonged pandemic.

Constraints in the development of the microalgae industry in the country, among others, because people still think that the microalgae industry requires expensive costs for materials, nutritional media, equipments such as bioreactors and raceway ponds and the whole processes in general. In addition, the results of research in universities related to microalgae are generally still carried out on a laboratory scale and have not been implemented in the community and business to answer the challenges of the needs of microalgae product.

Several studies have shown that microalgae can be grown on media in the form of organic waste, such as bioslurry from cow dung which has been treated through a biogas reactor in the form of anaerobically digested dairy manure wastewater (ADDMW) and also from fishery waste which still contains nitrogen and other organic nutrients that can be used for microalgae cultivation. Some of the results of the research we conducted with ADDMW and fishery waste showed quite encouraging results for the production of microalgae biomass. In addition, microalgae cultivation using organic waste also has the potential to reduce the burden of organic pollution in the environment, especially in river ecosystems.

In addition, microalgae cultivation to produce various products can be carried out using a closed system such as using a Tubular Vertical Reactor (VTR) which can be modified from a 19 L capacity mineral water container, or an open system using raceway ponds with a pond equipped with a paddle wheel to create a continuous flow of water. Both systems have their own advantages and disadvantages, but in

general both systems can be used on a small scale to an industrial scale. We have tried these two systems mainly to use microalgae biomass as a supplement for fish and lobster feed in aquaculture, with the results showing that the addition of microalgae powder to fish feed can increase fish weight and color ligtness of ornamental fishes.

There is a need for a more serious collaboration between academics and researchers with the private sector to up-scaling research that is currently being carried out mostly on a laboratory scale, and to develop this microalgae industry from small scale (home industry) to medium and large scale. In addition, institutions that provide seeds of various quality microalgae strains are certainly an important requirement, and this role can be carried out by universities and research institutes in the country. Therefore synergy between researchers, entrepreneurs and government institutions is needed to develop national scale business to produce microalgae biomass which has great prospects in the market.

Keywords: Microalgae bioindustry, aquaculture, anaerobically digested diary manure wastewater

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Drop-ins Chemical and Bio-hydrocarbon Production from Microalgae via Thermochemical Conversion

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ABSTRACT

Two different microalgae species, marine microalga Nannochloropsis gaditana and freshwater microalga Scenedesmus almeriensis were used in two thermochemical process. Dried microalgae were first subjected to continuous fast pyrolysis at 380 and 480 °C in mechanically stirred bed reactor with fractional condensers. The produced heavy phase pyrolysis oils were then used as feed for catalytic hydrotreatment process at 400 °C and 15 MPa of H_2 pressure for 1 hour under the presence of NiMo catalyst. This study shows that fast pyrolysis combined with catalytic hydrotreatment of the feedstock would produce hydrotreated oils which contains higher HHV and low oxygen content than the original feedstock. The hydrotreated oils also contains a high number of, suggesting the potential use as drop-in chemical for existing petrochemical refinery. The utilization of microalgae-derived hydrotreated oils, however, was hindered by the higher amount of nitrogen compared to petroleum feed, thus a further deep catalytic hydrodenitrification was needed to mitigate the problem.

Keywords: microalgae, fast pyrolysis, catalytic hydrotreatment, hydrotreated oils, drop-in chemicals

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Improvement of Plant Defence Against Pathogenic Fungal: Case Study on Drought and Chitosan Elicitation to Enhance Chili Plant Resistance to *Phytophthora capsici* and *Colletotrichum scovillei*

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ABSTRACT

The chili pepper (*Capsicum* spp., family Solanaceae) is an economically important crop that dominates the trade of hot spices worldwide. However, the production of chili peppers has been significantly constrained due to the plant's susceptibility to a variety of pathogens. The range of pathogens afflicting pepper is extensive, including fungi (*Phytophthora capsici, Rhizoctonia solani, Verticillium dahliae, Colletotrichum scovillei, C. truncatum, Leveillula taurica,* and *Fusarium* spp.), bacteria (e.g., *Xanthomonas* spp.), viruses (e.g., Tospoviruses, Potyviruses, Tobamoviruses, Cucumoviruses, Geminivirus), and nematodes (Meloidogyne spp.). The disease can result in a production losses of up to 100%, depending on the plant's age and resistance level, the pathogen's virulence level, and the environmental conditions.

Numerous studies in the laboratory and in the field have been conducted to improve disease resistance in chili pepper plants, including physiological and molecular investigations. There are various methods to improve disease resistance in plant, among others is elicitation. Elicitation is a process to enhance synthesis of secondary metabolites by the plants to ensure their survival in adverse conditions. One of the major secondary metabolites produced by chili pepper is capsaicin, a substance that contributes to the fruit's pungency and also plays an important role in plant's defense against microbial infections and insect pests.

Our study examined the effect of drought and foliar application of chitosan on growth and capsaicin content in chilli pepper plants, as capsaicin also serves as disease resistance indicator. Growth parameters were quantified physiologically, whereas defense mechanisms were evaluated using visual indications of infection, capsaicin concentration, and expression analyses of defense genes.

The observed growth parameters include plant height, leaf number, and relative chlorophyll content suggest that chitosan-treated plants grew more rapidly than control plants, while drought-treated plants grew more slowly. The yield was quantified in terms of flowers and fruits, and the results showed that it followed a

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similar pattern to plant growth and development. Application of chitosan and drought stress to chili pepper plants increased the concentration of capsaicin, which may be responsible to reduce disease incidence and severity indices in chili plant.

Several defense-related genes were assessed, including 9-lipoxygenase (CaLOX), Ca^{2+}-bound calmodulin 1 (CaCaM1), receptor-like cytoplasmic protein kinase (CaPIK), Ptointeracting1 (CaPTI1), resistance gene analogue 2 (CaRGA2), and Transcription factor WRKY17 and 53. The results suggested that CaLOX, CaPTI1 and CaRGA2 genes were involved in defense mechanism against *Phytophthora capsici* when plant treated with chitosan, with increased expression during infection. Drought and chitosan treatment also enhance the expression of transcription factor WRKY17. Chitosan application was proposed to not only alleviate plants growth under drought stress, but also to enhance their resistance to drought stress, as well as disease incidence and severity indices. Similarly, this result was also observed when chili pepper plant was challenged by *C. scovillei*

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Polyploidization for Plants Improvement

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ABSTRACT

Polyploidization refers to the multiplication of a complete chromosome set of a certain species to produce a new species. Polyploid organisms possess three or more complete sets of chromosomes (triploid, tetraploid, hexaploid etc.), and this phenomenon is more common in plants compare to the other organism. Polyploidization is a widespread phenomenon, especially in flowering plants that exhibit genome duplication during their life time. These multiple sets of chromosomes occur in one nucleus and can be inherited to progenies. Wide range of plants, including many of crops, combines more than two sets of chromosomes originating from the same (autopolyploids) or related species (allopolyploids) (Pele *et al.,* 2018). Allopolyploid plants can be found in wheat and canola, meanwhile banana and potato are autoploypoids.

Plant polyploidization is considered as an important way for plant improvement. Polyploidy is usually accompanied with structural, developmental, physiological, and biochemical changes in plants. Therefore, polyploidization give a new opportunities for breeders to select optimal plants for a range of purposes, including medicinal and ornamental functions and enhanced resistance. Moreover, polyploid induction can increase breeding opportunities, promote the development of seedless triploid cultivars, enhance ornamental characteristics and environmental tolerances, increase biomass as well as restore fertility in wide hybrids. In citrus, tetraploid plants has been used as one of parental to be crossed with diploid plants to produce triploid seedless fruit plants. In *Catharanthus roseus* tetraploid plant synthesize higher concentration of indole alkaloid and vincristine than diploid plants (Xing *et al.*, 2011; Begum, 2011). Polyploidization also succeed to restore fertility in the hybrids *Rhododendron* (Contreras et al., 2007), and *Rudbeckia* sp. (Oates et al., 2012).

Various plant species are natural polyploids, including some vegetatively-propagated flower and fruit species, as well as agricultural crops. Chromosome doubling has potentially beneficial for plants, nevertheless natural polyploidy exist only in some genera in angiosperms. Polyploidization of some economically-important plants, therefore, was artificially induced over several decades. *In vitro* regeneration systems provide a powerful tool for manipulating ploidy to facilitate breeding and development of new crops (Touchell *et al.,* 2020). *In*

vitro polyploid manipulation is dependent upon disrupting polar migration of chromosomes during cell division, as a consequence the chromosomes do not separate resulting in polyploid cells. Several antimitotic substances, such as colchicine, oryzalin and trifluralin, have been widely used for successful *in vitro* polyploid induction. Colchicine still remains the most efficient and preferred antimitotic agents for polyploid induction in some plant species, ranging from in herbaceous to woody species.

Success in the *in vitro* polyploidization is also depend on explant type as well as genotype. Shoots, buds or shoot tips, callus, somatic or zygotic embryos, nodal segments and tuber segments have been used for polyploid induction in some species. Sometimes the suitability of several explant types needs to be further investigated. The ability to regenerate an entire plant from a single or only a few cells can improve the development of homogenous polyploid plants. Shoot organogenesis or somatic embryogenesis systems are preferred to be used for *in vitro* polyploidization in several plant species to reduces the possibility of chimeras (Touchell *et al.*, 2020). Following treatment with the antimitotic agent, the resulting cell population must be verified for polyploid production as well as mixoploidy. Various verification methods such as chromosome counting, flow cytometry and evaluation of anatomical/morphological characters have been used to confirm the ploidy level of treated tissues. Flow cytometry (Figure 1) still being the most commonly used method for determining ploidy level in plant cells due to its efficiency and accuracy.

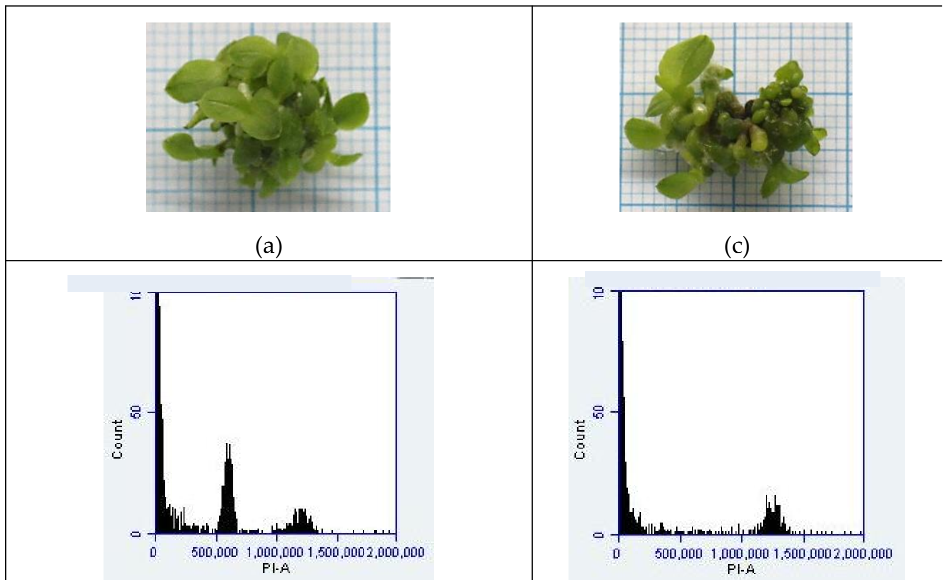

Figure 1. (a-b) Habitus and flow cytometry profile of diploid regenerants. (c-d) Habitus and flow cytometry profile of tetraploid regenerants in *Phalaenopsis violacea*.

In reponse to polyploidization, alterations in morphology have been widely reported Increasing of stomatal size and chloroplast number in guard cell (Figure 2),

decreasing in stomatal density, darker green colour of the leaves as well as larger flowers have also been reported in some plants following polyploidization. Some other traits, such as leaf size and thickness, internode length, inflorescence size and number, fruit and seed size have been reported to be affected by chromosome doubling. In general, polyploid plants usually have a thicker and larger leaves, larger flowers as well as larger fruit and larger seeds. The shoots of polyploids may also be shorter and thicker resulting in a dwarfed appearance.

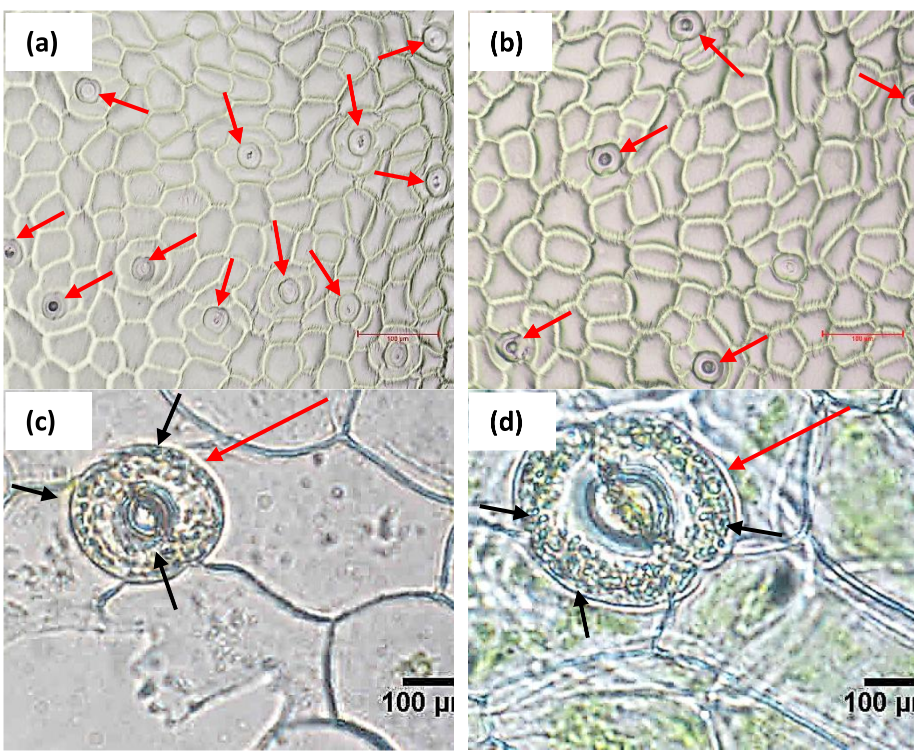

Figure. 2 Stomata (red arrow) of (a) diploid and (b) tetraploid leaves, with visible chloroplasts (black arrow) in guard cells of (c) diploid dan (d) tetraploid leaves. (LM, bar = 100 μm)

Polyploidization can also change some physiological characteristics, including drought stress tolerance, disease resistance and various characteristics related to postharvest functioning. Some polyploid species performs better heat and water stress tolerance in comparison to their diploid relative. These altered characteristics provide some advantages for many agricultural/horticultural crops. Polyploidisation is a powerful strategy for the development or improvement of plant species. Genome duplication in polyploid plants increase the capacity to provide novel sources of genetic variation, resulting in the potential adaptation to new environments (Ramsey, 2011).

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Production of Mycelium-Based Composite from Napier Grass (*Pennisetum purpureum*) Resulted from Phytoremediation of Chromium through Solid State Fermentation

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Abstract

Napier grass (*Pennisetum purpureum*) is a favorable biomass in phytoremediation due to its high growth rate and tolerance at chromium exposure and can be used as a solid-state fermentation (SSF) substrate to produce mycelium-based composites. This study aims to evaluate the effect of Napier grass from phytoremediation (control, 20 ppm, 40 ppm, tannery wastewater) which had Cr(VI) content of 1.24-3.57 ppm and fungi inoculum percentage (10% and 25% (w/w)) on the SSF process using white-rot fungus. The results showed that the growth rate and the final colonization of *Ganoderma lucidum*, *Pleurotus ostreatus*, and *Lentinula edodes* varied in the range of 1.298 ± 0.095 mm/day, 1.472 ± 0.095 mm/day, 0.468 ± 0.095 mm/day, and 2-5, 3-4, 2-4 respectively. The results showed that the variation of substrate did not affect the mycelium growth of *G. lucidum*, *P. ostreatus*, and *L. edodes* significantly ($p>0.05$). Meanwhile, inoculum quantity influences the growth of *G. lucidum* and *L. edodes* significantly ($p<0.05$), while it did not affect the growth of *P. ostreatus* ($p>0.05$). The results also showed that the final pH values from the SSF substrate of *G. lucidum*, *P. ostreatus*, and *L. edodes* decrease along with SSF duration. The decreasing pH value from the SSF substrate indicates the activity of fermentation.

Keywords: Mycelium-based composite, *Pennisetum purpureum,* phytoremediation, solid-state fermentation, white-rot fungi.

1. Introduction

Chromium waste has carcinogenic, mutagenic, and teratogenic characteristic that can endanger life if it disposed of into the environment without prior processing [1]. Therefore, this phenomenon required an alternative that can overcome this environmental pollution. One alternative that can be used is the phytoremediation process. Phytoremediation is a process to reduce the number of contaminants in the soil/water by utilizing the ability of plants to absorb heavy metals [2].

Napier grass (*Pennisetum purpureum*) is one type of grass that has good hyperaccumulator abilities. In addition, this plant has a large biomass, high growth rate, and high tolerance for pollutants [3]. Therefore, this plant can be utilized in the phytoremediation process of chromium. However, when the plant tissue died, the heavy metals contained would be reabsorbed into the planting medium. Phytoremediation plants can be used as substrates to produce bioproducts to prevent it.

One of the bioproducts that have been widely developed is a mycelium based biomaterial grown on solid substrates. In other words, the production of this biomaterial can be carried out through solid-state fermentation. Solid-state fermentation of lignocellulosic biomass can be carried out by filamentous fungi. Fungi are the most ideal organisms and have adapted to suitably being applied to the solid-state fermentation process, thus facilitating the fungal colonization process through the utilization of nutrients on the substrate [4]. Solid state fermentation on lignocellulosic substrates can produce mycelium based biocomposites. Mycelium is a vegetative growth of filamentous fungi that can bind to organic matter through the formation of a network of hyphal microfilaments [5]. During the fungal growth process, the fungus will decompose the solid organic substrate and gradually cover the entire surface of the substrate with its mycelium to produce a lightweight composite [6, 7].

The fungi that can be used in the production process of this biocomposite are white-rot fungi or fungi belonging to the class Basidiomycota. This fungal species can degrade cellulose, hemicellulose, and lignin through enzymatic or non-enzymatic mechanisms [8]. The matrix from the substrate will be penetrated by hyphae which continue to grow making the substrate matrix denser. Over time, substrates that have been degraded by fungi will be replaced with fungal mycelium which binds all the substrates and produces a biocomposite product [9]. Some examples of white-rot fungi that can be used for biomaterial production are *Ganoderma lucidum, Pleurotus ostreatus,* and *Lentinula edodes.* Therefore, this study aims to review the effectiveness of solid-state fermentation by *G. lucidum, P. ostreatus,* and *L. edodes* on Napier grass (*P. purpureum*) substrates as a result of phytoremediation of Cr(VI) waste through aspects of mycelium growth rate, degree of colonization, and pH value and compare the performance of three fungi for biocomposite production.

2. Materials and Methods

2.1 Materials

Biocomposite substrate are consisted of Napier grass from phytoremediation treatments, rice husk, tapioca flour, calcium carbonate ($CaCO_3$), distilled water, and mushroom grain spawn of lingzhi (*Ganoderma lucidum*), white oyster (*Pleurotus ostreatus*), and shiitake (*Lentinula edodes*). Napier grass resulted from phytoremediation of tannery wastewater with various treatment such as control,

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synthetic waste of $K_2Cr_2O_7$ with concentration of 20 ppm, 40 ppm, and tannery wastewater from waste water treatment plant (WWTP) of small-scale industry in Garut. Cr(VI) content of Napier grass are shown in Table 1. Mushroom spawn collected from CV Asa Agro Corporation, meanwhile rice husk, tapioca flour, and $CaCO_3$ were obtained from e-commerce.

Table 1. Cr(VI) content of Napier grass from phytoremediation

Treatments	Cr(VI) (ppm)		
	Roots	Stems	Leaves
Control	1.91	1.36	2.07
20 ppm Cr(VI)	2.41	1.24	2.29
40 ppm Cr(VI)	3.57	1.34	2.30
Tannery wastewater	1.55	1.28	1.51

2.2 Methods

2.2.1 Biocomposite Substrate Preparation

Production of biocomposite used in 4 variations of phytoremediation Napier grass (control, tannery waste, 20 ppm, and 40 ppm) mixed with rice husk as a substrate with ratio 60:40. Napier grass have gone through a drying process for two weeks at room temperature and size reduction to a size of < 2 mm [10]. Apart from that, tapioca flour, $CaCO_3$, and distilled water were added to the substrate mixture.

The substrate mixture used in this study was adjusted to achieve the optimum C/N ratio for the growth of *G. lucidum*, *P. ostreatus*, and *L. edodes* fungus, namely a C/N ratio of 70, 60, and 50 respectively [11-14]. Production of new research biocomposites is carried out in plastic containers + molds. The plastic container used is heat-resistant plastic and the mold container used is a square container with a size of 6x6x3 cm. The composition of the biocomposite substrate used can be seen in Table 2.

Table 2. Composition of biocomposite substrate

Composition	Mass (g)		
	G. lucidum	*P. ostreatus*	*L.edodes*
Napier grass resulted from phytoremediation	14.0	15.8	18.0
Rice husk	9.33	10.53	12.0
Tapioca flour	16.58	13.59	9.32
$CaCO_3$	0.23	0.26	0.30
Distilled water	26.10	26.12	25.75

The mixture of this substrate was put in plastic and sterilized with an autoclave at a temperature of 121˚C, a pressure of 1 atm, and for approximately 15-60 minutes [15].

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2.2.2 Inoculation of Mushroom Grain Spawn

Mushroom spawn was inoculated on a mixed substrate in plastic with variations of 10% (w/w) and 25% (w/w) and carried under sterile conditions with the help of a spirit burner, alcohol, tissue, and a spatula (Table 3). The inoculated substrate was then closed using a baglog ring and closed with cotton.

Table 3. Variation of mushroom grain spawn addition on substrate

Amount of grain spawn (%)	Mass (g)		
	G. lucidum	*P. ostreatus*	*L.edodes*
10	6.62	6.63	6.54
25	16.55	16.58	16.34

2.2.3 Solid-state Fermentation

The solid phase fermentation (SSF) process was divided into two stages, SSF phase I (in a plastic bag) and SSF phase II (in a square container). SSF phase 1 was conducted with incubation of inoculated substrate mixture with three varieties of mushrooms in a heat-resistant plastic in a dark room with a temperature of 24.8-29.3 and relative humidity (RH) of 55-85%. SSF phase 1 in *G. lucidum*, *P. ostreatus*, and *L. edode*s treatment has a different fermentation period which are ten days, 12 days, and 15 days respectively. The SSF I biocomposite was then crushed, mixed, and reconstructed on a 6 cm x 6 cm x 3 cm container to begin the fermentation phase II (SSF II). Samples were stored at room temperature for 15 days in the dark. Measurement of room temperature and RH was carried out every three days during the fermentation process. After the SSF phase II was completed, the samples were dried using an oven at 80°C for 24 hours [16].

2.2.4 Analysis of Fermentation Process

The fermentation process was analyzed with fungal mycelium growth, colonization stage, and change of pH in substrate. Fungal mycelium growth on biocomposite samples at the SSF II stage was carried out with measurement of the mycelium length in horizontal and vertical directions every three days during the fermentation. Furthermore, the fungal mycelium growth (mm/day) was calculated based on the average mycelium growth in two directions against the number of days [10, 17]. Mycelium colonization rate was determined visually and grouped according to 5 levels of the scale, namely (1) no mycelium; (2) rarely seen mycelium; (3) there is mycelium, but no aggregation to the substrate; (4) there is mycelium and some aggregation to the substrate; (5) there is a lot of mycelium and aggregation to the substrate in a large extent [18].

3. Results and Discussion

3.1 Mycelium Growth

The effectiveness of fermentation process can be analyze from mycelium growth aspect. In this study, a comparison of the mycelium growth from three types of fungi will be reviewed and can be seen in Figure 1 and Figure 2.

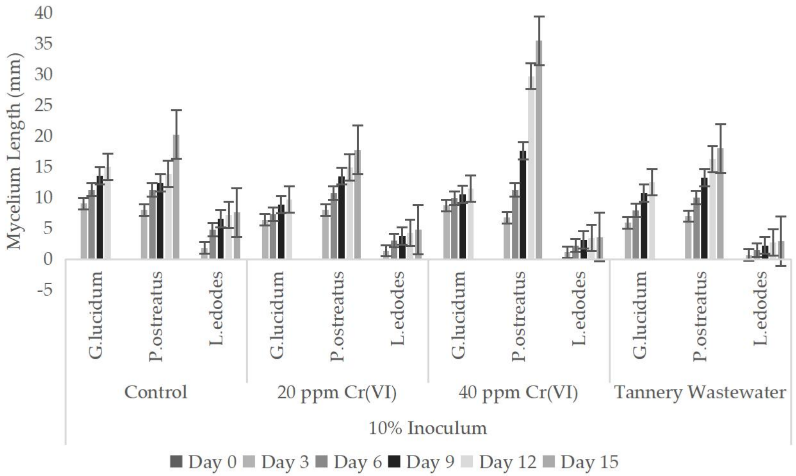

Figure 1. White-rot fungus mycelium growth with 10% inoculum and variations of Napier grass from phytoremediation

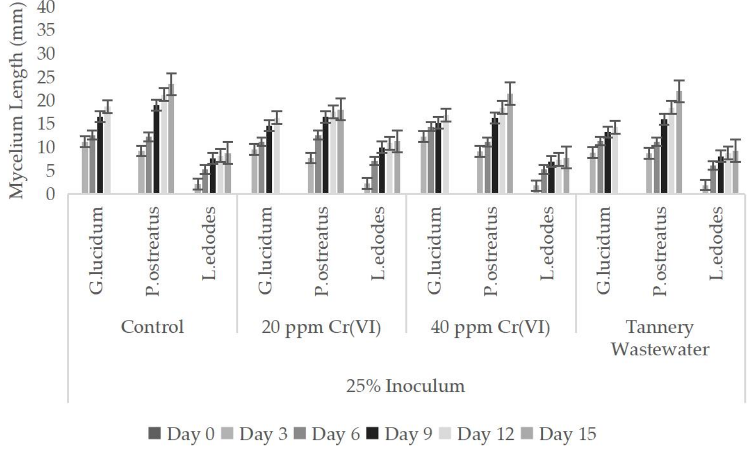

Figure 2. White-rot fungus mycelium growth with 25% inoculum and variations of Napier grass from phytoremediation

The highest mycelium length increase of *G. lucidum* was found at the beginning of the fermentation (Day 3) (Figure 1 and 2). The deceleration of mycelium growth over time can be caused by several factors such as (1) depletion of simple nutrients

[19]; (2) decreasing xylanase activity after early fungal growth [20]; (3) accumulation of inhibitory metabolites [21]. Mycelium length of *G. lucidum* varied from 5.91–18.73 mm (Figure 1 and 2.). On the 3rd day of fermentation, Napier grass from phytoremediation affects the *G. lucidum* mycelium growth significantly ($p = 0.034$). It can be caused by Napier grass content changes in the phytoremediation treatment. Napier grass treated with 20-60 ppm of Cr(VI) showed a trend of increasing nitrogen and decreasing sulfur content [22] Thus, changes in components of Napier grass from phytoremediation can affect the nutrient composition of the substrate.

On the 3rd day of fermentation, the lowest *G. lucidum* mycelium length was shown in the Napier grass from tannery wastewater treatment (Figure 1 and 2). It can be caused by the presence of other heavy metals in tannery wastewater compared to other treatments that only use $K_2Cr_2O_7$ solution. *G. lucidum* has different tolerance levels at other heavy metals [23, 24]. Therefore, the low value of mycelium growth in the tannery wastewater treated grasses could be due to differences in the tolerance level of *G. lucidum* in the presence of other heavy metals. On later fermentation duration (6, 9, 12), Napier grass from phytoremediation does not affect the *G. lucidum* growth significantly ($p > 0.05$). It could be due to the heavy metal content of Napier grass from phytoremediation is still in the range of *G. lucidum* tolerance level. According to Gupta et al. [23], *G. lucidum* can tolerate Cr(VI) up to a concentration of 1,000 ppm. Therefore, the growth of mycelium did not show a significant difference in the difference between the grass substrate resulting from phytoremediation of waste with Cr content.

Meanwhile, variations of inoculum quantity affect the *G. lucidum* growth significantly ($p < 0.05$). The *G. lucidum* growth at 25% inoculum was greater than *G. lucidum* growth at 10% inoculum, which varied from 5.91-14.98 mm and 8.89-18.73 mm respectively (Figure 1 and 2). Inoculum size is an important factor for mycelium growth rate because it can determine the substrate degradation rate and shorten the lag phase of fungal growth [25]. The increase in inoculum size can increase the mycelium growth rate, which is also in accordance with the other studies [11, 26].

Based on Figure 1 and 2, it can be seen that the mycelium growth of the fungus *P. ostreatus* in all treatments showed a significant growth for 15 days of the fermentation process. The length of the mycelium obtained was in the range of 6.75–35.50 mm. Based on statistical analysis, it was stated that there was no significant effect of Napier grass from phytoremediation on the growth of fungal mycelium ($p>0.05$). This is suitable with the research conducted by Aguilar et al. [27] who reviewed the mycelium growth of the fungus *P. ostreatus* at 5 variations of Cr concentration, which is 20-100 ppm. Based on this study, it was stated that the mycelium growth of the fungus *P. ostreatus* was not inhibited at all concentration variations and did not experience significant differences [27]. The same study was also conducted by [28], which stated that the mycelium growth of the fungus *P. ostreatus* could grow well up to 150 ppm Cr concentration.

In this study, the largest variation of Cr concentration used was at a Cr concentration of 40 ppm and it can be seen in Figure 1 and 2 also that the largest mycelium length was obtained in Napier grass with a Cr concentration of 40 ppm. This could be because generally, low concentrations of heavy metals can stimulate fungal growth [28]. Fungi have two mechanisms to adapt to an environment contaminated with heavy metals, which is extracellularly and intracellularly [29]. In the extracellular mechanism, some organic molecules that are not included in the cell wall matrix will be excreted by fungal cells for the chelation process with metals, whereas in the intracellular mechanism, transport proteins that can play a role in heavy metal tolerance can prevent toxic heavy metals from reaching the cytosol [29]. According to research Yang et al. [28], heavy metals that are absorbed into fungal biomass will be converted to insoluble metal components extracellularly. For Cr (chromium), this metal is found in an insoluble form as a transformed product with the rest of the metal accumulating in the intracellular compartment [28]. In addition, *P. ostreatus* produces oxalic acid which plays an important role in the chelating process with heavy metals so that it can reduce the toxicity of heavy metals [28].

Based on statistical analysis, it was stated that there was no significant effect of inoculum percentage on the growth of fungal mycelium ($p>0.05$). In general, increasing the percentage of mushroom inoculum used will accelerate mycelium growth and shorten colonization time [30]. However, in this study, the addition of mushroom inoculum by 10% and 25% did not provide a significant difference. This is suitable with the statement in the research of Oei & Nieuwenhuijzen [31] which states that the fungal inoculum used does not need to exceed 10% of the substrate weight because it does not show a significant increase in biological efficiency and only causes economic losses. In addition, the addition of inoculum to exceed the limit will reduce laccase production which can cause decreased metabolic activity [32].

Based on Figure 1 and 2, the increase in *L. edodes* mycelium length in each variation of phytoremediation napier grass and variations in the addition of fungi on day 0 to day 9 indicate the occurrence of high metabolic activity carried out by fungi to break down substrate components. Due to SSF 1 process in the first 15 days, the metabolic activity in *L.edodes* uses dissolved or simple carbon sources to form hyphae [33]. Inoculated mycelium vigor affects the ability of fungi to break down lignocellulosic substrates. During the SSF 2 process, simple carbon sources on the substrate are limited, so *L. edodes* mycelium will produce various enzymes that are capable of converting lignocellulose, such as hydrolases and oxidases [34, 35]. The oxidase enzymes released during the mycelium phase are cellulase, laccase, and manganese peroxidase (MnP) [36].

The growth rate of *L.edodes* mycelium in this study has a range of 0.19-0.76 mm/day and average of 0.468 ± 0.095 mm/day. The difference in the rate of mycelium growth with variations in the addition of fungi, namely 10% and 25% (w/w), also differed for fungi to adapt to the substrate which was proportional to the growth of fungi in

the substrate. At the addition of 10% fungus, the highest and lowest mycelium growth rates occurred in napier grass as a result of phytoremediation of control and waste treatments. Napier grass as a result of phytoremediation of waste treatment can contain various types of inorganic compounds such as heavy metals (Zn, Cu, Cd, Cr, Fe, and Co) which can be a source of micronutrients for mycelium but can suppress the growth of fungal mycelium if the concentration of these compounds exceeds the required levels. needed. These heavy metals can suppress the production of MnP enzymes and inhibit the growth of mycelium [37].

The mycelium of the *L. edodes* fungus did not grow optimally on the substrate due to several things, namely the type of cultivation substrate used in the form of elephant grass is an unfavorable substrate for the fungus, the age of the fungus that has entered the adult phase, there are heavy metal compounds of chromium in the form of Cr(III) and high Cr(VI) on the substrate, the environmental conditions for mycelium growth were not ideal, and the substrate conditions were contaminated by other microorganisms (mold) during the SSF process. At the time the SSF 2 process took place, the age of *L. edodes* mycelium was 16-30 days, while the growth of *L. edodes* mycelium occurred in the inoculation phase, namely during a period of 25 days [38] where the highest mycelium growth rate was between the period of the mycelium inoculation phase on the substrate with development of fruiting bodies which ranged from day 18 to day 23 [39]. This makes the fungal mycelium only active in the early SSF 2 (0-9 days) and matures towards the formation of fruit bodies in 9-15 days, where there is an increase in the production of laccase enzymes, lignin peroxidase, and MnP to break down lignin on the substrate. The mature condition, namely the formation of the fruiting body, is characterized by a visual change, namely the change in the color of the fungal mycelium from white to brown on the substrate (browning) [38]. The browning process was driven by contamination with antagonistic fungi such as Trichoderma sp. during the SSF process takes place as a defense mechanism of *L. edodes* mycelium. Trichoderma sp. release antifungal compounds, produce polysaccharide enzymes and induce laccase formation through the release of secondary metabolites to compete for nutrients with L. edodes [40].

Growth rates of *G. lucidum*, *P. ostreatus*, and *L. edodes* on variations of Napier grass from phytoremediation and inoculum size are shown in Table 4. Based on One-Way ANOVA statistical analysis, it was stated that the variety of fungal species affected the value of mycelium growth rate ($p < 0,05$). This is in accordance with other study which states that fungal species can affect the mycelium growth rate [10]. The mycelium growth rates of *G. lucidum*, *P. ostreatus*, and *L. edodes* varied in the range of 0.805-1561 mm/day, 0.68-2.60 mm/day, and 0.194-0.756 mm/day, respectively.

Table 4. The average growth rates of *G. lucidum*, *P. ostreatus*, and *L. edodes* on Napier grass from phytoremediation of chromium with inoculum size 10–25%

Fungi	Mycelium Growth Rate (mm/day)
G. lucidum	1.298 ± 0.095
P. ostreatus	1.472 ± 0.095
L. edodes	0.468 ± 0.095

The highest growth rates were shown in *P. ostreatus*, *G. lucidum*, and *L. edodes*, respectively. However, the growth rate of *L. edodes* showed a more significant difference compared to *G. lucidum* and *P. ostreatus*. It can be caused by *G. lucidum* and *P. ostreatus* tolerance to Cr(VI) content of Napier grass from phytoremediation [23, 28], while exposure of heavy metals to *L. edodes* can decrease the production of manganese peroxidase [37]. The lower growth rate of L. edodes can be caused by several factors: (1) Napier grass is considered as an unfavorable substrate for *L. edodes*; (2) the age of *L. edodes* that has entered the adult phase; (3) unideal condition due to Cr(III) and Cr(VI) content of Napier grass; (4) mold contamination during SSF process [41]. However, these fungus still showed a good tolerance to grow in substrates containing heavy metals which is indicated by mycelium growth during fermentation process.

3.2 Mycelium Colonization

The degree of colonization is one aspect which can indicate the ability of fungal growth in a substrate. The degree of colonization from each type of fungus will certainly have differences depending on the ability of the fungal mycelium to grow on a substrate [16]. In this study, a comparison of the final degree of colonization from three types of fungi will be reviewed and can be seen in Figure 3 and Figure 4.

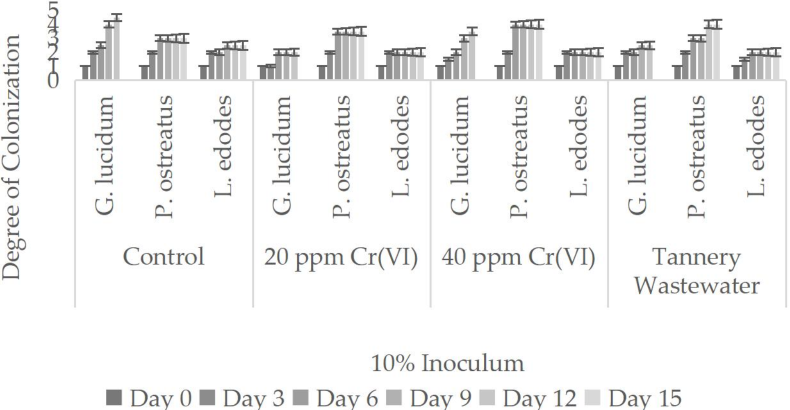

Figure 3. White-rot fungus degree of colonization with 10% inoculum and variations of Napier grass from phytoremediation

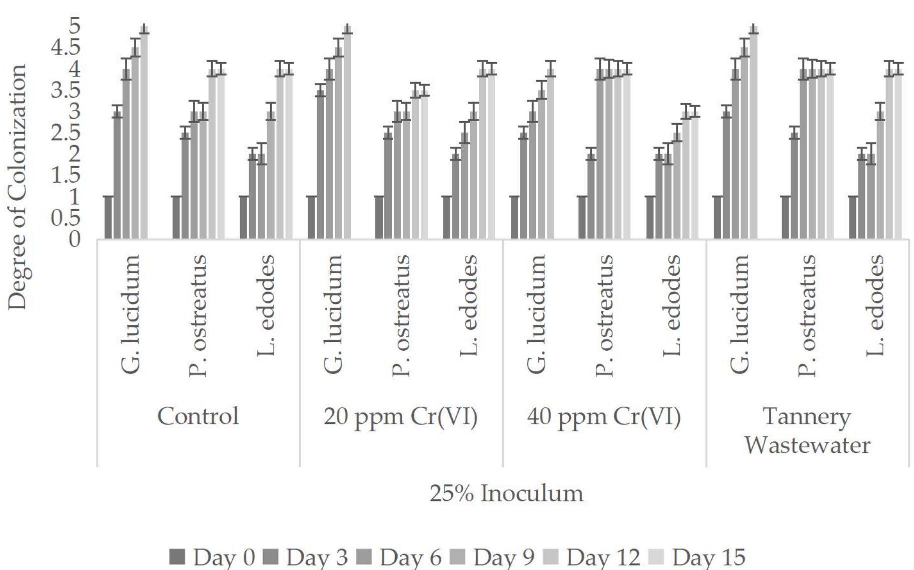

Figure 4. White-rot fungus degree of colonization with 25% inoculum and variations of Napier grass from phytoremediation

In general, variations of Napier grass from phytoremediation do not affect the colonization rate of *G. lucidum* significantly ($p > 0.05$). It could be due to the heavy metal content of Napier grass from phytoremediation is still in the range of *G. lucidum* tolerance level According to Gupta et al. {23], *G. lucidum* can tolerate Cr(VI) up to a concentration of 1.000 ppm. Therefore, *G. lucidum* colonization did not show a significant difference in the Napier grass from phytoremediation treatments. *G. lucidum* tolerance level indicates the potential of using lignocellulosic biomass from phytoremediation as a solid-state fermentation substrate.

Variations of inoculum size affect the *G. lucidum* colonization significantly ($p < 0.05$). In the addition of 10% inoculum, there were samples that reached colonization level 5 at the end of the fermentation time. Meanwhile, with the addition of 25% inoculum, there were samples that reached colonization level 5 at the end of the fermentation time (Figure 3 and 4). The colonization rate of *G. lucidum* mycelium was better at the higher inoculum size. This could be caused by an increase in the substrate degradation rate and a shorter lag phase in the addition of higher mushroom seeds [25].

Based on Figure 3 and 4, it can be seen that the degree of colonization by *P. ostreatus* in all treatment showed a significant increase for 15 days of the fermentation process. The degree of colonization obtained in this study ranged from 1-4. Based on statistical analysis, it was stated that there was no significant effect of Napier grass from phytoremediation and inoculum percentage on the degree of

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colonization (p>0.05). This is suitable with the explanation that has been described in mycelium length section, which is the *P. ostreatus* that can still grow well up to a concentration of 150 ppm Cr(VI) and the presence of Cr in low concentrations can stimulate the growth of fungal mycelium [28]. In addition, the mushroom inoculum of 10% and 25% also did not provide a significant difference because the fungal inoculum used more than 10% by weight of the substrate did not show a significant increase in biological efficiency and only caused economic losses [31].

Based on Figure 3 and 4, it can also be seen that generally, there was a drastic increase in the degree of colonization from day 3 to day 6 of the fermentation process. This could be due by the fact on the mycelium growth curve of the fungus *P. ostreatus*, it was stated that the lag phase occurred for 5 days before entering the exponential phase for 10 days after that and the 15th day had begun to enter the stationary phase [42].

Based on Figure 3 and 4, the percentage of fungi of 25% (w/w) resulted in the colonization rate of *L.edodes* mycelium compared to the percentage of fungi of 10% (w/w) that indicating the presence of Cr(VI) resistance capacity on the substrate for the mycelium of the fungus *L.edodes* [37]. However, this increase has not reached the level of colonization scale 5 that caused by the type of substrate used, the age of the fungal mycelium, the process of transferring the substrate from SSF 1 (plastic) to SSF 2 (mold), and the occurrence of contamination by microorganisms during the SSF 2 process. In general, mycelium can increase the activity of cellulase and hemicellulase as lignolytic enzymes. However, in certain strains, hemicellulose harms fungal growth, while lignin is converted into nutrients for mycelium growth [43]. Cr(VI) compound is more toxic than Cr(III) because when the mycelium absorbs Cr(VI) on the 40 ppm treatment napier grass substrate, Cr(VI) is reduced to Cr(V) which can react with H_2O_2 to form hydroxyl and can damage DNA and oxidizes every subcellular structure and organelle in the mycelium [44].

The fungus age affects the level of fungal colonization of *L.edodes* since, during the 2nd solid-phase fermentation process, the age of *L.edodes* mycelium has reached 16 days and the simple carbon source on the substrate is limited, so the fungus will increase enzymatic activity to break down lignocellulose such as hydrolases and oxidases such as cellulase, laccase, and manganese peroxidase [34, 35]. The re-construct process at the start of SSF 2 aims to stimulate and strengthen the mycelium structure which will encourage the stiffness and strength of the final biocomposite to mix *Trichoderma* sp spores. with *L.edodes* mycelium that separated again, so the mycelium grew more slowly and did not colonize the substrate quickly due to competition for nutrients with antagonistic fungi. The emergence of *Trichoderma* sp during the SSF process inhibits the growth of *L. edodes* mycelium by spreading green spores to colonize the substrate, releasing antifungal compounds to the *L. edodes* mycelium, producing polysaccharides, and encouraging the formation of laccase by *L.edodes* to enter the browning phase [45]. Proper sterilization

operating conditions can reduce the possibility of contamination by other fungi such as *Trichoderma* sp.

Based on Figure 3 and 4, it can be seen that the final degree of colonization from *Ganoderma lucidum, Pleurotus ostreatus*, and *Lentinula edodes* fungi, respectively, is in the range of 2-5; 3-4; and 2-4. In addition, the average value of the degree of colonization obtained from the fungi *G. lucidum, P. ostreatus,* and *L. edodes*, respectively, was 3.94; 3.75; and 2.94. This shows that the highest colonization rate was *G. lucidum*, followed by *P. ostreatus* and *L. edodes*. Based on One-Way ANOVA statistical analysis, it was stated that the variety of fungal species affected the value of degree of colonization. This is similar with the research conducted by Appels et al. [16] which states that fungal species affect the level of colonization because each fungus has different growth abilities on a substrate. In this study, the highest colonization rate was *G. lucidum*, which reached the final colonization level of 5, followed by *P. ostreatus* and *L. edodes*. This could be due to the ability of each fungus to tolerate Cr(VI) metal contained in its growth substrate.

The fungus *G. lucidum* has a high tolerance for metal Cr(VI) up to a concentration of 1,000 ppm [23], the fungus *P. ostreatus* can only tolerate metal Cr(VI) up to a concentration of 150 ppm [28], while the *L. edodes* fungus exposed to heavy metals can decreased the production of manganese peroxidase [37] where the manganese peroxidase enzyme played an important role in the fungal colonization process [46]. However, in general, these three fungi still have a good ability to grow in substrates containing heavy metals which are characterized by the value of a degree of colonization more than 1, which there's still a little mycelium observed [18].

3.3 pH Values Profile of Substrate

The pH values tested in this study were the initial and the final pH value of the biocomposite after the fermentation process ended. The pH values at the beginning and end of the fermentation process were measured to review the chemical changes of organic compounds before and after the growth of fungal mycelium [47]. In this study, a comparison of the initial and the final pH from three types of fungi will be reviewed and can be seen in Figure 5 and Figure 6.

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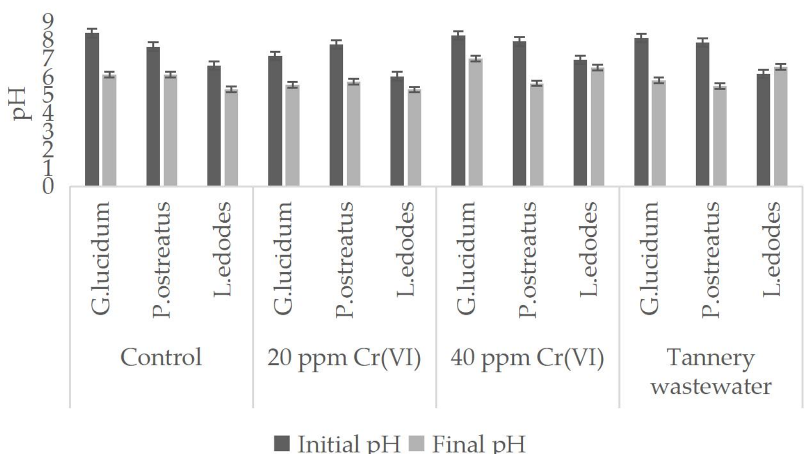

Figure 5. Change of pH in substrate with 10% inoculum and variations of Napier grass from phytoremediation and fungus species

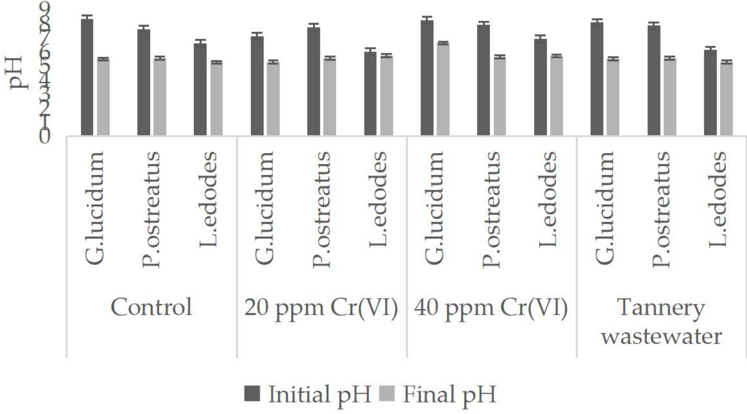

Figure 6. Change of pH in substrate with 25% inoculum and variations of Napier grass from phytoremediation and fungus species

Based on Figure 5 and 6, it can be seen that there was a decrease in the pH value from the beginning of the fermentation to the end of the 15th day of fermentation for each treatment of *G.lucidum*, *P.ostreatus*, *L.edodes* except on the napier grass from tannery wastewater treatment substrate at 10% (w/w) inoculum of *L.edodes*. This is similar with the statement in the research of [47], which the pH value of the substrate is expected to decrease due to the formed fungal mycelium. The variety of initial pH of each species may caused by the difference in the amount of napier grass and rice husk substrate eventhough the ratio of each treatment is 60:40. However, the initial pH did not effect the trend of pH as time goes on. The decreasing in pH value of each fungus species in the fermentation process can be

caused by fungi that consume nutrients from the substrate and produce organic acids that are released into the substrate [48]. Nevertheless, the result of statistical analysis with ANOVA showed that the variation of napier grass resulted from phytoremediation, type of fungal species, and the amount of inoculum doesn't affect the decrease of pH value ($p>0.05$).

The pH value of the *G. lucidum* fermentation substrate in this study was in the range of 5.03-8.39 (Figure 5 and 6). According to [49], the optimal pH for Ganoderma spp. is in the range of 5-9. Therefore, the *G. lucidum* growth in this study is still ongoing at optimal pH conditions. The initial and final substrate pH values were in the range of 7.14-8.39 and 5.03-7.01 respectively (Figure 5 and 6). The decreased pH value at the end of the fermentation process may indicate the fermentation activity. It can be caused by the production of organic acids that can cause a decrease in the pH value [50]. However, an extreme decrease in pH can lead to unfavorable conditions for *G. lucidum* solid-state fermentation. Therefore, it is necessary to control the pH of the substrate to ensure the stability of the pH value. In this study, the addition of $CaCO_3$ can help stabilize the pH of the substrate [51]. $CaCO_3$ acts as a substrate buffer by binding the organic acids formed during fermentation activities, thus keeping the pH value not too low [52].

The initial pH of the fungal growth substrate in this study was in the pH range of 7.65–8.47 and the mycelium of the fungus *P. ostreatus* was still able to grow well. This can be happened because the *Pleurotus* fungus can grow at a relatively high initial pH value (around 8) when compared to other contaminant fungi [47]. Based on research conducted by Gorai & Sharma [53], the optimum pH range for the growth of the fungus *Pleurotus ostreatus* is in the pH range of 6.5–7.5. In addition, the fungus *P. ostreatus* which produces oxalic acid as a result of the chelating process with heavy metals to reduce heavy metal toxicity [28] can also reduce the pH value of the substrate. In addition, it is stated that the substrate breakdown process by fungi will reduce the pH value of the substrate to around pH 5 [47]. The final pH value obtained in this study was in the pH range of 5.38–6.12. All final pH values in each treatment variation in *P.ostreatus* were around pH 5, but for control Napier grass treatment with 10% mushroom inoculum had a final pH value of 6.12. This could be due by the fact that the growth of *P. ostreatus* mycelium was not complete and did not cover the entire substrate, so there was a possibility that the fermentation process did not accomplish completely when compared to other samples. Based on the parameters of mycelium length, degree of colonization, and pH value, *Pleurotus ostreatus* showed an optimum fermentation activity in all treatment, so that this fungus could be utilized in Napier grass plants from phytoremediation to produce biocomposites.

The initial pH value of the fermentation substrate on *L.edodes* was in range of 6.03-6.94. During the fermentation process, the initial pH value did not affect the growth of the mycelium because the fungus was able to regulate the pH of the substrate independently to achieve the optimum pH for its growth [54]. However, change in

pH is influenced by metabolic activity in the fungus. At 10% (w/w) inoculum, the final pH of the substrate of Napier grass from tannery wastewater treatment increase due to enzymatic reactions causing pH fluctuations during the fermentation process [54]. Decreasing pH value indicates the occurrence of enzymatic activities such as MnP and laccase to break down lignin compounds and cellulase enzymes to break down cellulose compounds, the formation of organic acids, and the result of mycelium digesting ammonium salts in the substrate [55].

4. Conclusions

Different varieties of white-rot fungi, *G. lucidum, P. ostreatus*, and *L. edodes*, showed good tolerance to grow on substrates containing heavy metals. The growth rate and the final colonization of *G. lucidum, P. ostreatus*, and *L. edodes* varied in the range of 0.805-1.561 mm/day, 0.68-2.60 mm/day, 0.194-0.756 mm/day, and 2-5, 3-4, 2-4 respectively. The highest growth rate was shown in *P. ostreatus, G. lucidum*, and *L. edodes*, respectively. While the highest colonization was shown in *G. lucidum, P. ostreatus,* and *L. edodes* respectively. The results also showed that the final pH values from the SSF substrate of *G. lucidum, P. ostreatus*, and *L. edodes* varied in the range of 5.30-7.01, 5.5-6.12, 5.28-6.56 respectively. However, the fungal growth response in this study was still not optimal. Therefore, further research can be carried out regarding the contamination prevention and oxygen supply to support the optimal growth of these fungi.

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The Potential and Mechanism of Globe Fimbry (*Fimbristylis globulosa* (Retz.) Kunth) in Phytoremediation of Acid Mine Drainage in Wetland PIT 07 Banko Barat

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Abstract

Coal mining is an activity that has an impact on the environment by producing acid mine drainage (AMD). Acid mine drainage has a low pH and high metal solubility. One of the efforts to control AMD biologically is by utilizing globe fimbry (*F. globulosa*). Globe fimbry is a hyperaccumulator plant that can absorb metals. Therefore, it is necessary to monitor the success of phytoremediation of AMD by considering the accumulation of heavy metals, such as iron (Fe) and Manganese (Mn), absorbed in globe fimbry (*F. globulosa*) in Wetland PIT 07 Banko Barat managed by PT. Bukit Asam. Metal concentrations accumulated in the roots and leaves of *F. globulosa* were analyzed using Atomic Absorption Spectroscopy (AAS). The ability of heavy metal accumulation in plants can be determined by calculating the bio-concentration factor (BCF) and translocation factor (TF). The BCF values of Fe and Mn in the roots were 7.32 and 1.07, respectively. These numbers shows that the ability of roots to accumulate Fe and Mn is very high because the BCF values are > 1. Meanwhile, the TF values for Fe and Mn were 0.4 and 3.4, respectively. *F. globulosa*in absorbing Mn uses a phytoextraction mechanism which is characterized by a TF value of > 1. Meanwhile, in absorbing Fe, *F. globulosa* uses a phytostabilization mechanism which is indicated by a TF value of < 1.

Keywords: Acid Mine Drainage, Phytoremediation, *Fimbristylisglobulosa* (Retz.) Kunth, BCF, TF.

1. Introduction

Acid mine drainage (AMD) is an environmental pollutant waste as a result of mining activities. This waste is created due to the oxidation process of pyrite mineral material (FeS2) and other sulfide mineral materials which are exposed to the ground surface in the process of extracting mining minerals. Acid mine drainage can cause a decrease in water quality both in surface flow and groundwater patterns [1]. Acid mine drainage is a source of environmental contamination because, in addition to having a low pH, it also contains harmful

heavy metals, such as Fe, Al, Mn, Cu, Zn, Cd, Pb, and As. Furthermore, it usually also contains high sulfate [2].

The high content of heavy metals and sulfates in acid mine drainage may cause environmental damage so that special handling is highly needed. One of the technologies for treating acid mine drainage is phytoremediation. Phytoremediation is a technology to restore certain contaminant substances in soil, sediment, dirt or mud, groundwater, surface water, and wastewater by utilizing plants [3]. The mechanism of phytoremediation includes several processes, such as phytoextraction, rhizofiltration, phytodegradation, phytostabilization, phytovolatilization, and phytotransformation [4].

Globe fimbry (*Fimbristylis globulosa* (Retz.) Kunth) (Indonesian: *mendong*) which belongs to the Cyperaceae family is one of the plants that have the potential for phytoremediation of AMD. Globe fimbry plants are hyperaccumulators that have the ability to phytoextract heavy metals, grow fast, have high resistance, and are not staple [4]. [5] reported that globe fimbry (*F. globulosa*) is able to reduce mercury levels in traditional gold mine wastewater. The concentration of mercury in gold mine wastewater before being treated with globe fimbry plants (*F. globulosa*) was 4.019 mg/L. After being treated with globe fimbry plants, the mercury concentration in wastewater decreased to 0.170 – 0.340 mg/L. The highest absorption of mercury (96%) was found in the treatment using globe fimbry plants (*F. globulosa*), by 2 kg.

The ability of heavy metal accumulation in globe fimbry plants can be found out by calculating the bio-concentration factor (BCF) and translocation factor (TF). [6] revealed that BCF in leaves and roots can be calculated to determine how much metal concentration in leaves and roots comes from the environment. In addition to BCF, TF can be also calculated. It is the ratio between metal concentrations in leaves and plant roots. The TF value is calculated to determine metal accumulation from roots to leaves. The TF value can be used to identify whether the plants conduct phytoextraction (TF > 1) or phytostabilization (TF < 1).

The purpose of this study was to determine the TF and BCF values of *F. globulosa* and to find out the phytoremediation mechanism that occurs in *F. globulosa* in Wetland PIT 07 Banko Barat managed by PT. Bukit Asam.

2. Materials and Methods

2.1. Materials

The materials needed in this study were distilled acid mine drainage, and roots & stems of *F. Globulosa* taken from PIT 07 Banko Barat managed by PT. Bukit Asam, Tanjung Enim, South Sumatra.

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2.2. Methods

2.2.1 Sampling Method

Root and leaf samples of globe fimbry (*F. globulosa*) and sediments were taken from Wetland PIT 07 Banko Barat managed by PT. Bukit Asam, Tanjung Enim, South Sumatra. Root samples were roots that entered the sediment, while leaf samples were green leaves [20]. Root and leaf samples were analyzed in the Physiology and Development Laboratory, Department of Biology, Faculty of Mathematics and Natural Sciences, Sriwijaya University.

2.2.2 Measurement of Fe and Mn

Measurement of Fe and Mn levels was carried out by analyzing the metals content obtained from roots and leaves of *F. globulosa* using the Atomic Absorption Spectroscopy (AAS) method and sample analysis was conducted at the Palembang Industrial Research and Standardization Center (Baristand). This measurement was done by adding concentrated HNO_3 and then allowed to stand for 24 hours. After that, it was heated on a hotplate for 5 to 10 minutes at a temperature of 105 – 200°C. Furthermore, it was added with distilled water until the volume reached 50 mL, and then it was precipitated [20]. The sample was then filtered with filter paper for the solution. The solutions were than analysed by using AAS tools with regards to the SNI 6989.4 of 2009 about the method to measure iron (Fe) while to measure manganese (Mn) according to SNI 06-6989.5 of 2004.

2.2.3. Bio-Concentration Factor (BCF)

BCF calculation was conducted to find out how much heavy metal concentration in roots comes from the environment. According to [7] the value of BCF can be calculated using the following formula.

$$BCF = \frac{Heavy\ Metal\ Concentration\ in\ Roots}{Heavy\ Metal\ Concentration\ in\ Sediment}$$

According to [8], a plant in accumulating heavy metals can be divided into 3 category, namely BCF > 1 = accumulator, BCF < 1 = excluder, and BCF = 1 = indicator.

2.2.4. Translocation Factor (TF)

TF calculation was conducted to determine the transfer of accumulated heavy metals from roots to shoots [19]. The values from BCF and TF calculations can be

used to determine the status of plants as phytoextraction (TF > 1) or phytostabilization (TF < 1). The value of TF can be calculated using the following formula.

$$TF = \frac{Heavy\ Metal\ Concentration\ in\ Leaves}{Heavy\ Metal\ Concentration\ in\ Roots}$$

3. Results and Discussion

The levels of heavy metals accumulated in the roots and leaves of *F. globulosa* were used as an effort to monitor the success of phytoremediation of acid mine drainage in Wetland PIT 07 Banko Barat. The levels of heavy metals in the roots and leaves of *F. globulosa* in Wetland PIT 07 Banko Barat are presented in Table 1.

Table 1. The levels of Fe and Mn in roots and leaves of *F. globulosa* taken from Wetland PIT 07 Banko Barat

Parts of the Plant	Fe (mg/l)	Mn (Mg/l)
Roots	2866.77 ± 155.87	29.07 ± 4.50
Leaves	1199.17 ± 593.33	100.21 ± 29.16

Note: ± = standard deviation

The ability of *F. globulosa* to accumulate Fe and Mn can be calculated using the values of BCF (bio-concentration factor) and TF (translocation factor). BCF calculation was carried out to find out how much heavy metal in roots comes from the environment. Meanwhile, TF calculation was conducted to determine the transfer of accumulated heavy metals from roots to shoots. The values of BCF and TF in *F. globulosa* are presented in Table 2.

Table 2. BCF and TF values of Fe and Mn in *F. globulosa* taken from Wetland PIT 07 Banko Barat

Metals	BCF		TF
	Roots	Leaves	
Fe	7.32	3.06	0.4
Mn	1.01	3.50	3.4

Note: BCF < 1 = Excluder; BCF = 1 = Indicator; BCF > 1 = Accumulator/hyperaccumulator. TF < 1 = Phytostabilization; TF > 1 = Phytoextraction.

The BCF calculation indicates the value of > 1, meaning that *F. globulosa* is an accumulator or hyperaccumulator plant capable of accumulating Fe and Mn metals. According to [9], certain plants have the ability to absorb and accumulate metal contaminants through the root system and store them in various plant compartments. [8] plants considered for phytoremediation consist of three main groups based on their physiological mechanisms, namely excluders (as used in phytostabilization), accumulators (for phytoextraction), and indicators. Excluders are plants that limit the uptake and accumulation of contaminants, while accumulators translocate contaminants from roots to shoots.

Fe metal was more accumulated in the roots than in the leaves, as indicated by the value of translocation factor (TF) of < 1. Meanwhile, Mn metal had a value of translocation factor (TF) of > 1. The TF value of < 1 in plants indicates that *F. globulosa* slightly translocates Fe in the shoots, in which the accumulation of Fe is only limited in the roots. [10] translocation factor (TF) generally indicates the movement of metals from soil (sediment) and water to roots and shoot. TF provides an indication of whether the types of plants used are accumulators/hyperaccumulators, excluders, or indicators.

Table 2 shows that *F. globulosa* in accumulating Mn, as seen from the TF value greater than 1, has phytoextraction potential. In other words, *F. globulosa* can absorb Mn contained in the sediment and translocate it to the leaves. Meanwhile, in accumulating Fe, as seen from the low value of TF, *F. globulosa* has the potential for phytostabilization, in which these plants transform pollutants into non-toxic compounds. [11] reported that measurement for Mn in cattail plants shows a BCF value of > 1 and a TF value of > 1, while the measurement for Mn in *F. globulosa* indicates a BFC value of > 1 and a TF value of > 1. It means that the phytoextraction mechanism works in those plants.

Therefore the TF value in the accumulation of Mn is > 1, it means that *F. globulosa* performs a phytoextraction mechanism in accumulating Mn. According to [11], phytoextraction is the absorption of pollutants by plants from water or soil. After that, the pollutants are accumulated or stored in leaves and stems [4]. These plants are called hyperaccumulators. After the accumulation of pollutant had reach to the maximum capacity, the plants must be destroyed with an incinerator. The phytoextraction mechanism begins with the roots absorbing heavy metals and accumulating them to other parts, such as stems and leaves [12]. [13], stated that plants with a phytoextraction mechanism will collect metals in their tissues, especially in the canopy, and have high metal concentrations in plant tissues even exceeding the soil concentration.

F. globulosa has 2 phytoremediation mechanisms: phytoextraction and phytostabilization. The results of the calculation of the TF value indicate that, for Mn, *F. globulosa* functions as phytoextraction because the TF value is > 1. [16] phytoaccumulation or phytoextraction is the absorption of heavy metals (Ag, Cd, Co, Cr, Cu, Hg, Mn, Ni, Pb, or Zn) in the soil by plant roots, accumulating these

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compounds to other plant parts, such as roots, stems, or leaves. [16] added that phytoextraction is the absorption of polluting compounds from the soil or water by plant roots. Furthermore, it is also the translocation and accumulation of the polluting compounds in the upper part of the plant, one of which is the plant canopy [14].

The mechanism used by *F. globulosa* for removing Fe is called a phytostabilization mechanism, which is indicated by a TF value of < 1. Fe metal is absorbed more in the roots than in the leaves, as shown by a TF value of < 1. [15] phytostabilization is a phenomenon of the production of certain chemical compounds to immobilize contaminants in the rhizosphere area. [16] added that phytostabilization is a process carried out by plants to transform pollutants in the soil into non-toxic compounds without absorbing these pollutants first into the plant body. The results of the transformation of these pollutants remain in the soil. Plants are able to stabilize pollutants in the soil, thus pollutants harmless [13].

Phytostabilizing plants are able to reduce the mobility of metals so that they do not spread to other places [17]. This is in line with the results of a study conducted by [14] that plants can immobilize heavy metals in the soil through absorption by roots, deposition, complexation, or decrease in metal valence in the rhizosphere by secreting special redox enzymes. Plants can convert harmful metals into relatively less toxic forms, thereby reducing the possibility of plant damage from metal stress.

Based on the TF value concerning the Fe metal, *F. globulosa* performs a phytostabilization mechanism because the TF value is < 1. Phytoextraction and phytostabilization are mechanisms in phytoremediation. [18] the way phytostabilization works is to use the ability of roots to change environmental conditions. Plants will block the movement of metals that are absorbed and accumulated by the roots and then deposited in the rhizosphere. A study conducted by [11] reported that the values of the accumulation of Fe by cattail plants are > 1 for the BCF value and < 1 for the TF value, meaning that the phytostabilization work significantly.

4. Conclusions

The BCF values for Fe in the roots and leaves of *F. globulosa* were 7.32 and 3.06, respectively. Meanwhile, the BCF values for Mn in the roots and leaves of *F. globulosa* were 1.01 and 3.50, respectively. The BCF value of > 1 indicates that *F. globulosa* is an accumulator or hyperaccumulator plant. *F. globulosa* in absorbing Mn uses a phytoextraction mechanism, which is indicated by a TF value of > 1. Meanwhile, for absorbing Fe, *F. globulosa* uses a phytostabilization mechanism, which is indicated by a TF value of < 1.

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Public Perception to Orchids and Entrepreneurial Behavior of Orchid Farmers and Sellers

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Abstract

Orchids are well known as unique and high price ornamental plants, not only Indonesia but also worldwide. Indonesia has approximately 5.000 original species, therefore orchids market could potentially be flourished in the country. The increase in orchids market is very much depend on public perception to this flower as well as entrepreneurial behavior of orchid farmers and sellers. In order to understand it, we conducted a survey to some orchid business players and distributed questionnaire to orchid hobbyists and non-hobbyists. Results showed that the most liked orchids attributes are their shape (83,4%), color (82,4%), and pattern (72%). Hybrid orchids (59,2%) are more desirable than wild species (40,8%). The most desirable orchids are *Dendrobium* (65%), *Phalaenopsis* (55%), and *Cattleya* (22,20%), while the most expensive are *Cattleya, Vanda, Grammatophyllum,* and *Dendrobium.* Factors affecting orchids price are its scarcity (55%), beauty (25%), and consumer demand (15%). The sellers obtained orchids seeds by doing their own nursery (55%), from out-of-town collector (40%), nursery center (35%), another personal nurse (30%), import (25%) and local forests (5%). Public seem still perceived orchids as an expensive and not easy to grow, therefore sellers should be more proactive in familiarizing public to various lower-price orchids available and convincing public how easy it is to grow orchids.

Keywords: Orchid, Market, Public Perception, Entrepreneurial Behavior, Attributes, Hybrid, Wild Species

1. Introduction

Agricultural sector is the second highest contributor to Indonesia's gross domestic product (GDP) in 2020 after industry. It contributes Rp 2.115,1 T or 13,7% to GDP. There are 7 subsectors in agricultural sector, such as crops, horticulture, farm, livestock, agricultural and hunting services, forestry and logging, and fisheries [1]. Ornamental plant is one of important horticulture commodities in Indonesia. Its demand continues to increase along with the increase of people's welfare and income [2] and population growth [3]. Increasing demand opens up business opportunities thus causes increasing activities of this sector in several region [3].

Commodities of ornamental plant that contribute a lot in horticulture production are orchid and chrysanthemum [4]. Orchids have high aesthetical value because of its unique shape, size, pattern, and color of flower and long freshness endurance as cut flower, therefore it is named as "Queen of Flower". It is often chosen as favorite flower for decoration [5]. Indonesia has approximately 5.000 orchid species. 986 species distributed in the forests of Java Island, 971 species in Sumatera, 113 species in Maluku, and the rest are distributed in Sulawesi, Irian Jaya, Southeast Nusa, and Kalimantan [6].

Orchid production in 2020 reached 11,68 million stalks, decreased by 37,22% (6,93 million stalks) from 2019 production. Orchid harvested area reached 95,38 hectares, decreased by 0,44% (80,63 hectares) from 2019 harvested area. Provinces with the highest orchid production are East Java, West Java, and Banten. East Java contributed about 36,38% to the national production with 4,25 million stalks of production and 19,96 hectares of harvested area. West Java contributed about 34,91% with 4,08 million stalks of production and 47,88 hectares of harvested area. Banten contributed about 11,62% with 1,36 million stalks of production and 8,99 hectares of harvested area [4].

Orchids have botanical as well as economical values [7]. Orchids take advantage of a large market share, both nationally and internationally. The diversity of orchids color, shape, size, structure, and textures provide excellent market prospects [8]. Since last decade, orchids business activities have been growing rapidly in several regions in Indonesia and could play a significant contribution to the country's economic growth [9]. Orchid agribusiness development has a positive impact on the income source of orchid farmers and sellers, the provision of employment and foreign exchange earnings through exports [5]. The increase in orchids market is very much depend on public perception to this flower as well as entrepreneurial behavior of orchid farmers and sellers. The aim of this study is to understand public perception to this flower as well as entrepreneurial behavior of orchid farmers and sellers.

2. Methods

This study was conducted online on 26 of September 2021 to 08 of October 2021 for orchid hobbyist and non-hobbyist, while for orchid sellers conducted on 26 of September 2021 to 14 of October 2021. Methods used in this study is distributed questionnaire via google form for hobbyist and non-hobbyist, while sellers were given two choices, interview via zoom meeting for those who please and distributed questionnaire for those who don't.

There are 63 respondents from non-hobbyist including 56 women and 7 men, 48 respondents from hobbyist including 38 women and 10 men, and 20 respondents from sellers including 14 men and 6 women (4 of them conducted interview and 16 others filled the questionnaire).

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3. Results and Discussion

There are some indicators including public perception and entrepreneurial behavior of orchids sellers and farmers in this study, such as the most liked orchid attributes, perception to wild species and hybrid orchid, the most desirable orchid genus, orchid price, factors affecting orchid price, and seller's obtaining orchid seeds.

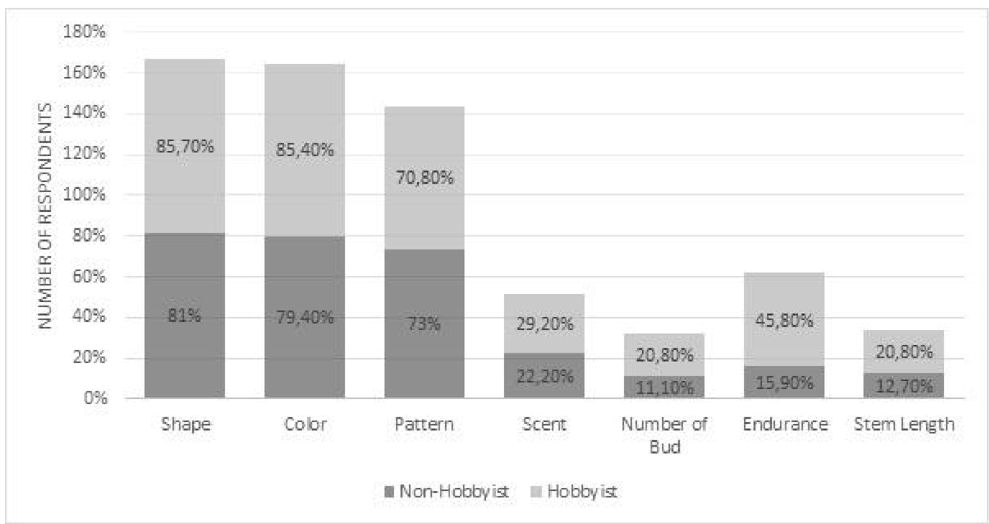

Figure 1. The Most Liked Orchid Attributes

Based on figure 1, there are 81% respondents from non-hobbyist like shape of orchid, 79,40% respondents like color of orchid, 73% respondents like pattern of orchid, 22,20% respondents like scent of orchid, 15,90% respondents like endurance of orchid, 12,70% respondents like stem length of orchid, and 11,10% respondents like number of buds. While from hobbyist, there are 85,70% respondents like shape of orchid, 85,40% respondents like color of orchid, 70,80% respondents like pattern of orchid, 45,80% respondents like endurance of orchid, 29,20% respondents like scent of orchid, 20,80% respondents like number of buds and 20,80% respondents like stem length of orchid. Based on the discussion above, the most liked orchids attributes are their shape (83,4%), color (82,4%), and pattern (72%). Consumer preference to flower as well as consumer preference to fashion. It means that consumer preference to flower rapidly change to be better like flower beauty, color, size, shape, arrangement, and endurance [10].

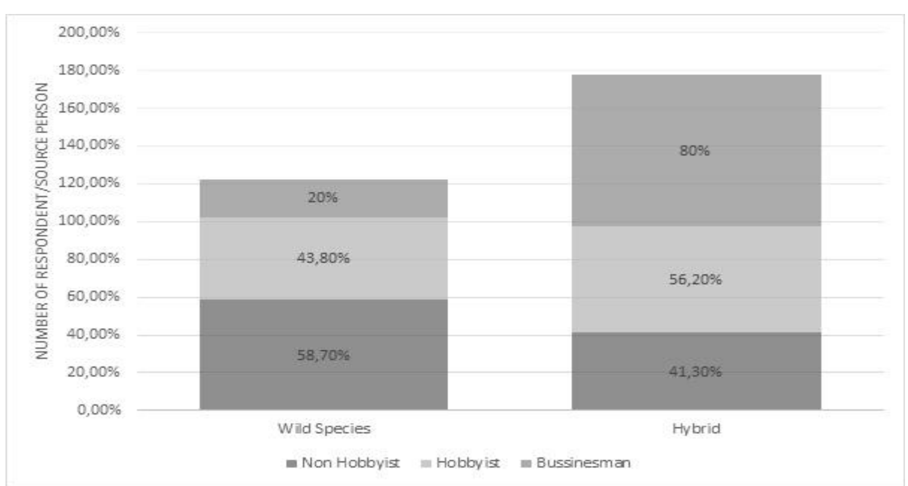

Figure 2. Perception to Wild Species and Hybrid Orchid

Based on figure 2, there are 58,70% respondents from non-hobbyist preferred wild species and another 41,30% respondents preferred hybrid orchid. While from hobbyist, there are 56,20% respondents preferred hybrid orchid and another 43,80% respondents preferred wild species. As well as hobbyist, there are 80% sellers stated that hybrid orchid is more desirable, and another 20% stated that wild species is more desirable. Based on the discussion above, hybrid orchid is more desirable than wild species, with percentage of 59,2% and 40,8% respectively.

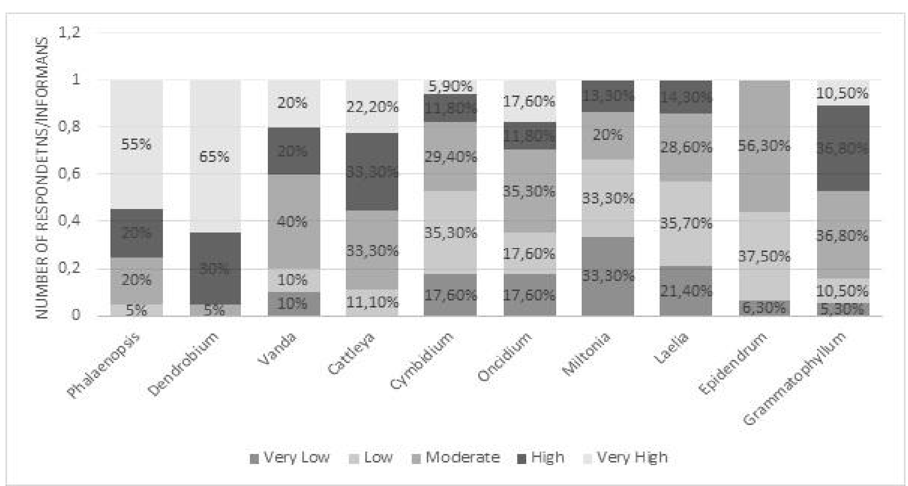

Figure 3. The Most Desirable Orchid

According to sellers, consumers interest in various types of orchids are diverse. Consumer interest are measured in a range of 1-5, with 1 is very low, 2 is low, 3 is moderate, 4 is high, and 5 is very high. 55% sellers stated that consumer interest in *Phalaenopsis* was very high, 20% sellers said it was high, 20% sellers said it was

moderate, and 5% seller said it was low. As for consumer interest in *Dendrobium*, 65% sellers said it was very high, 30% sellers said it was high, and 5% seller said it was moderate. As for consumer interest in Vanda orchids, 40% sellers said it was moderate, 20% sellers said it was high, 20% sellers said it was very high, 10% sellers said it was low, and 10% sellers said it was very low. As for consumer interest in *Cattleya*, 33,30% sellers said it was high, 33,30% sellers said it was moderate, 22,20% sellers said it was very high, and 11,10% sellers said it was low. As for consumer interest in *Cymbidium*, 35,30% sellers said it was low, 29,40% sellers said it was moderate, 17,60% sellers said it was very low, 11,80% sellers said it was high, and 5,90% seller said it was very high. As for consumer interest in *Oncidium*, 35,30% sellers said it was moderate, 17,60% sellers said it was very high, 17,60% sellers said it was low, 17,60% sellers said it was very low, and 11,80% sellers said it was high. As for consumer interest in *Miltonia*, 33,30% sellers said it was very low, 33,30% sellers said it was low, 20% sellers said it was moderate, and 13,30% sellers said it was high. As for consumer interest in *Laelia*, 35,70% sellers said it was low, 28,60% sellers said it was moderate, 21,40% sellers said it was very low, and 14,30% sellers said it was high. As for consumer interest in *Epidendrum*, 56,30% sellers said it was moderate, 37,50% sellers said it was low, and 6,30% seller said it was very low. As for consumer interest in *Grammatophyllum*, 36,80% sellers said it was moderate, 36,80% sellers said it was high, 10,50% sellers said it was very high, 10,50% sellers said it was low, and 5,30% seller said it was very low. Based on the discussion above, the most desirable orchid genus are *Dendrobium* (65%), *Phalaenopsis* (55%), and *Cattleya* (22,20%). Here are some species examples of those orchid genus:

Table 1. Species Examples of Orchid Genus

Genus	Example
Phalaenopsis	*Phalaenopsis amabilis* https://en.wikipedia.org/wiki/Phalaenopsis_amabilis

Dendrobium	*Dendrobium anosmum* https://commons.wikimedia.org/wiki/File:Dendrobium_anosmum_Lindl.,_
Vanda	*Vanda ampullaceal* https://en.wikipedia.org/wiki/Vanda#/media/File:Ascocentrum_ampullaceum.jpg
Cattleya	*Cattleya labiate* https://en.wikipedia.org/wiki/Cattleya#/media/File:Cattleya_labiata_Orchi_1013.jpg

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Cymbidium	*Cymbidium* Clarisse Austin Best Pink https://en.wikipedia.org/wiki/Cymbidium#/media/File:Cymbidium_Clarisse_Best_Pink.jpg
Oncidium	*Oncidium excavatum* https://id.wikipedia.org/wiki/Oncidium#/media/Berkas:Oncidium_excavatum_RTBG.jpg
Miltonia	*Miltonia candida* https://en.wikipedia.org/wiki/Miltonia#/media/File:Miltonia_candida.jpg

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Laelia	*Laelia albida* https://en.wikipedia.org/wiki/Laelia#/media/File:Laelia_albida_Orchi_005.jpg
Epidendrum	*Epidendrum ibaguense* https://en.wikipedia.org/wiki/Epidendrum_ibaguense#/media/File:A_and_B_Larsen_orchids_-_Epidendrum_ibaguense_DSCN5011.JPG
Grammatophyllum	*Grammatophyllum speciosum* http://www.plantsoftheworldonline.org/taxon/urn:lsid:ipni.org:names:636310-1

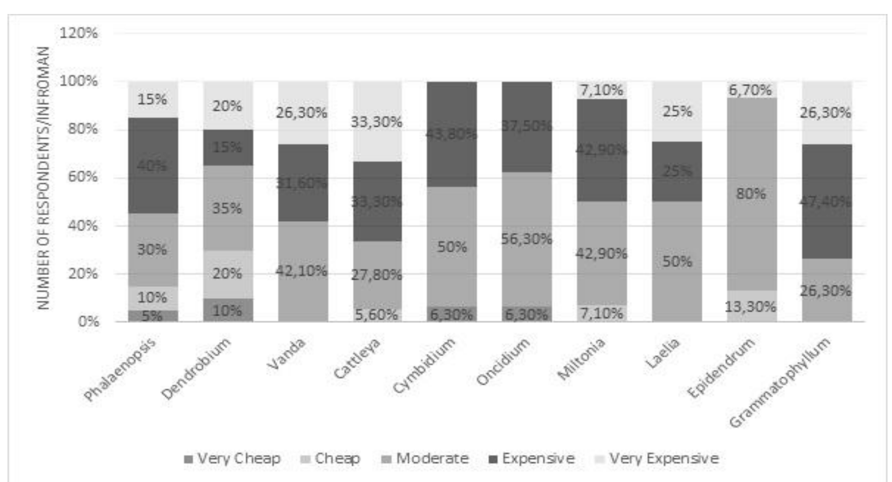

Figure 4. Orchid Price

Orchid price is measured in a range of 1-5, 1 is very cheap, 2 is cheap, 3 is moderate, 4 is expensive, and 5 is very expensive. Very cheap is Rp 25.000,00 to Rp 70.000,00, cheap is Rp 71.000,00 to Rp 90.000,00, moderate is Rp 91.000,00 to Rp 250.000,00, expensive is Rp 251.000, 00 to Rp 900.000,00, and very expensive is Rp 901.000,00 to Rp 10.000.000,00. Based on figure 4, 40% sellers stated that the price of *Phalaenopsis* was expensive, 30% sellers said it was moderate, 15% sellers said it was very expensive, 10% sellers said it was cheap and 5% seller said it was very cheap. As for the price of *Dendrobium*, 35% sellers said it was moderate, 20% sellers said it was very expensive, 20% sellers said it was cheap, 15% sellers said it was expensive, and 10% sellers said it was very cheap. As for the price of the *Vanda*, 41,20% sellers said it was moderate, 31,60% sellers said it was expensive, and 26,30% sellers said it was very expensive. As for the price of *Cattleya*, 33,30% sellers said it was very expensive, 33,30% sellers said it was expensive, 27,80% sellers said it was moderate, and 5,60% seller said it was cheap. As for the price of *Cymbidium*, 50% sellers said it was moderate, 43,80% sellers said it was expensive, and 6,30% seller said it was very cheap. As for the price of *Oncidium*, 56,30% sellers said it was moderate, 37,50% sellers said it was expensive, and 6,30% seller said it was very cheap. As for the price of *Miltonia*, 42,90% sellers said it was moderate, 42,90% sellers said it was expensive, 7,10% seller said it was very expensive, and 7,10% seller said it was cheap. As for the price of the *Laelia*, 50% sellers said it was moderate, 25% sellers said it was expensive, and 25% sellers said it was very expensive. As for the price of *Epidendrum*, 80% sellers said it was moderate, 13,30% sellers said it was cheap, and 6,70% seller said it was very expensive. As for the price of *Grammatophyllum*, 47,40% sellers said it was expensive, 26,30% sellers said it was very expensive, and 26,30% sellers said it was moderate. Based on the discussion above, it is concluded that the types of orchids that have the highest prices are *Cattleya, Vanda, Grammatophyllum*, and *Dendrobium.*

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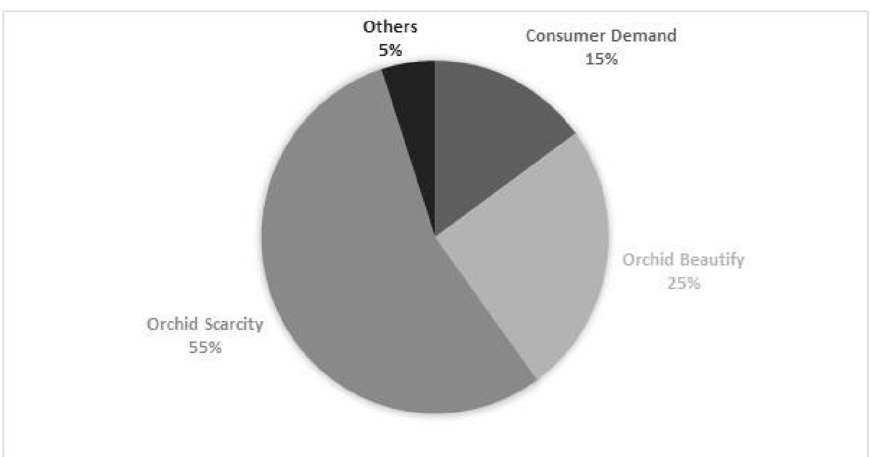

Figure 5. Factors Affecting Orchid Price

In term of factors affecting orchid price, sellers have different opinions. Based on figure 5, 55% sellers stated that orchid price was influenced by orchid scarcity, 25% sellers stated orchid beauty, 15% sellers stated consumer demand, and 5% seller stated another thing, that hybrid orchids were influenced by import regulations and the availability of seeds, while species orchids were affected by orchid scarcity.

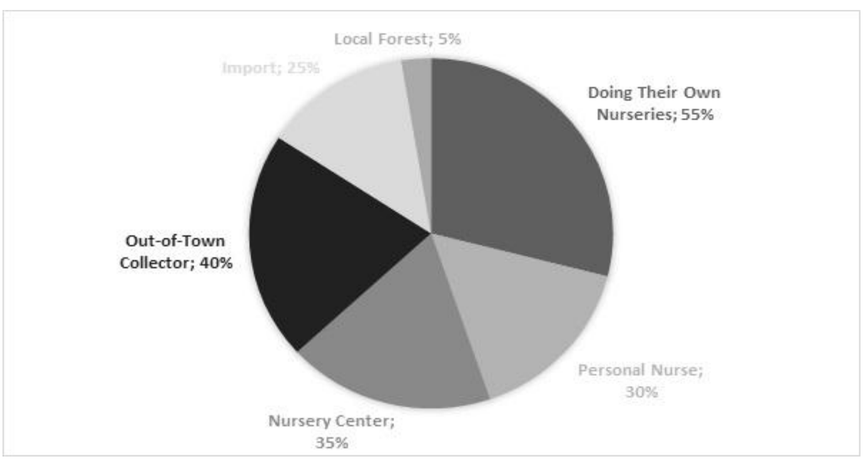

Figure 6. Seller's Obtaining Orchid Seed

Before marketing orchids to consumers, sellers have their own way of obtaining the seeds. Based on figure 6, 55% sellers doing their own nurseries with different methods, including in vitro with tissue culture techniques, keiki, splitting, and hybridization. There are two methods in orchid propagation, generative and vegetative. Generative method is propagation through seeds that are preceded by pollination flower. Some vegetative methods are splitting, keiki, and cuttings [11].

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Splitting is breaking the shoots of sympodial orchid such as *Dendrobium* and *Cattleya*. Orchid with 3-5 branches is better [12]. Some orchids are able to spontaneously produce keiki, i.e., offshoots from the nodes along the pseudobulbs or floral stems. In the natural environment, the implication by offshoots is an important strategy for the survival and expanding populations of some orchid asexually without pollinators and seed production [13]. Hybridization is method producing new plant by crossing two or more plants which have different genetic. This method is used to combine some good characteristic in one plant, to expand genetic plant variability through genes recombination and to get vigor hybrid [14]. Tissue culture is the most efficient technique to germinate orchid seed by providing nutrition aseptically [15]. Tissue culture can produce a huge amount orchid seeds in a short time [16]. 40% sellers obtained from out-of-town collectors and 35% sellers from nursery centers, such as Bandung, Magelang, Gunung Sindur, and Malang. 30% sellers from personal nurse, 25% sellers imported seeds from abroad such as Singapore and Thailand, and 5% seller obtained from the forest.

4. Conclusions

Sellers should be more proactive in familiarizing public to various lower-price orchids available and convincing public how easy it is to grow orchids in their houses.

Acknowledgments

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Production of Banana Seedling (*Musa acuminata*) using Shoot Culture with TIS RITA® Bioreactor

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Abstract

Cavendish banana (*Musa acuminata*) is one of the most common fruit commodities that potential for supporting food sustainability in Indonesia. Optimization of banana seedling production process can be done by using tissue culture technology with significantly produce higher number of regenerant and faster seed maturation. The Bioreactor Temporary Immersion System (TIS) is a system that combines the advantages of using a liquid medium, but avoids continuous immersion so as to provide aeration and agitation of nutrients evenly. In this research, observations were made to measure growth and economic analysis by calculating the survival rate of Cavendish banana shoot culture in a liquid system and TIS-RITA® bioreactor with immersion time variations of 5 and 20 minutes using half-strength Murashige & Skoog liquid medium and supplemented with 0.5 ppm gibberellic acid. The growth of shoot culture was determined by calculating the growth rate of biomass. Survival rate is determined through plant's ex-vitro growth observation. Economic analysis is calculated using net present value. The average growth rate of Cavendish banana shoot culture at 5 minutes and 20 minutes immersion time variation was 0.05 cm/day and 0.053 cm/day, respectively. In Thin-layer Culture, the survival rate obtained was 66.7%, while in the TIS-RITA® bioreactor it was 100%. The net present value of the application of TIS-RITA® bioreactor technology is Rp.13,796,000.00, while the thin-layer culture has a net present value of Rp.11,990,670.00. The payback period required for TIS-RITA® is 2 years and 5 months

Keywords: Cavendish Banana, Survival Rate, TIS-RITA® Bioreactor, Tissue Culture,

1. Introduction

Banana is a very common fruit commodity and has the potential to support food sustainability because of its nutritional value, which high in carbohydrates, calories, and vitamins. Banana plants are one of the most abundant primary fruit crops in Indonesia with total production of 6,448,018 tons according to the Badan Pusat Statistika Republik Indonesia in 2016. Cavendish banana (*Musa acuminata*) are type of fresh fruit that can be consumed directly, which widely known for its attractive appearance and distinctive taste. This banana is easily found in markets and have

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enormous economic potential. However, conventional banana cultivation takes significantly longer time for tree maturation [1]. Thus, a more optimized and economical approach of seedling production is required.

Banana seedlings production can be done through tissue culture technology. Tissue culture is a technology used to maintain and grow plant tissue in sterile conditions with a calculated medium composition to ensure the absorption of nutrients can take place ideally and efficiently. Tissue culture is widely used to produce clones of plants on a large scale. Through tissue culture, environmental factors can be controlled to maintain explant growing conditions for optimal seedlings production [2]. Tissue culture has many advantages over conventional cultivation, including seedling production can be done in controlled environmental conditions regardless of climate change and soil quality, cultures that will be free from microbes, significantly larger number of cultivation, and faster seed maturation [3]

In cavendish banana tissue culture, shoot tips (around 3-4 mm in size) is usually used as an explant and cultured in Murashige dam Skoog (MS) medium. Multiplication is also conducted in MS medium, added with 30 g/L sucrose and 8 g/L agar and supplemented with phytohormones to support the growth and multiplication of banana explants. For further cultivation, explants were subcultured and then can be transferred to a bioreactor [4].

The Temporary Immersion System is a semi-automated or fully automated periodic cultivation system, based on a cycle of temporary immersion of plant tissue culture into a liquid medium followed by drying and exposing the plant tissue to the air. Usually, the immersion time is shorter compared to the exposure period in air. Appropriate immersion and exposure time will create optimal conditions for explant to develop and still provide adequate nutrition with minimal contact with liquid [5]. The variation of temporary immersion time is based on the approaching value of previous research immersion period [6].

In order to meet the respiration needs of plant tissues, forced diffusion of oxygen is made through pump air aeration in the bioreactor [7]. Increased oxygen transfer has a major effect on better gas exchange, reduces oxygen limitation, and minimizes the chance of physiological disturbances. However, the TIS system did not result in any agitation, if there was little agitation, then it was due to the plant tissue being displaced due to hydrodynamic forces during the immersion period. Under these conditions, the cultivation of plant tissue through minimal shear stress can maintain the culture and also improve the morphology and physiology of the plant organs. TIS are usually consists of transparent glass or transparent plastic containers. Thus, light from outside can be used to illuminate the cultured plants in the container [5].

One of the most frequently used TIS bioreactors is the TIS RITA ® (Recipient for Automated Temporary Immersion System). TIS RITA ® was developed for intensive in vitro culture. The system consists of a single autoclavable polypropylene container (500 mL) with 2 compartments, separated by a tray

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attached with a support mesh and a plastic tube attached to the center. The container is closed by a wide screw lid. The compartment above the container is the culture room, and at the bottom is the storage medium. The advantage of using TIS RITA is that this bioreactor is very simple and can be used properly, it also does not take up much space for the equipment. This bioreactor is supported by proper humidity with full separation of plant tissue and liquid medium. All internal elements in this bioreactor are connected to each other and can be manipulated as a single unit that handles biomass development. Disadvantages of this reactor are the inability to renew the nutrient medium and few options for ventilation and CO_2 entry [5].

The aims of this study were to compare the yield of biomass, determine the relative growth rate and the efficiency of sucrose consumption of banana (Musa acuminata) seedling in TIS RITA and full immersion systems, and to evaluate the economic survivability rate of banana shoots cultured seedlings (Musa acuminata) in temporary immersion systems (TIS RITA) compared to thin layer culture.

2. Materials and methods

2.1 Materials

2.1.1 Cavendish Banana Explants

In vitro cavendish banana, cultured in solid medium, were used as an explant. These explants were approximately 1 month in culture and having 3-4 leaves.

2.1.2 Chemical Material

The chemicals used in this study were divided into chemicals for cultivation, extraction, and testing. Chemicals for cultivation consisted of Murashige & Skoog (MS) medium, sucrose, Growth Regulators (ZPT) in the form of Gibberellic Acid (GA), agar, fungicides, and HCl and NaOH to adjust the pH of the medium.

2.1.3 Liquid Medium

Liquid medium is used for the cultivation process in bioreactors and liquid systems, in the form of liquid MS medium. The liquid medium consisted of half-strength Murashige & Skoog (MS), sucrose 20 g/L, and 0.5 ppm Gibberellic Acid (GA).

2.2 Methods

2.2.1 Medium Preparation

The acidity condition in each medium was set at pH 5.8. The medium was sterilized by autoclaving at 121℃ for 15 minutes..

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2.2.2 Cavendish Banana Shoot Subculture

The shoot culture method used has been optimized in the laboratory at SITH for 4 years. The shoot culture used had passed the explant preparation stage and shoot initiation stage for 4 weeks beforehand, so it was ready for subculture. In the subculture process, the explant medium used was a solid growth medium. The subculture process was carried out in Laminar Air Flow under sterile conditions. The banana culture was then incubated at room temperature and lighting for 24 hours. Subcultures were performed every two weeks with three subcultures and the blackened tissue was removed before subculture. After three subcultures, the newly formed shoots were ready to be acclimatized in liquid acclimatization medium.

2.2.3 Cavendish Banana Shoot Acclimatization

Liquid medium was used for the acclimatization process. The acclimatization process was carried out using a 100 mL Erlemyer under sterile conditions. Banana cultures were incubated at room temperature with lighting conditions for 24 hours. Incubation was carried out for 1 week and when finished, the banana shoot culture was ready to be cultured in a liquid system and TIS RITA bioreactor.

2.2.4 Shoot Culture in Liquid System

The acclimatized Cavendish banana shoot culture was cultivated in a liquid or thin-layer culture (TLC) system in the form of 100 mL Erlenmeyer with a medium volume of 25 mL. The medium used is a liquid medium. The weight of the explants before inserted into TIS system was weighed (Sartorius ED822) as the initial weight (g). Meanwhile, the explant height was measured by millimeter block and recorded as initial height (cm). The culture was incubated on a shaker with a rotational speed of 60 rpm. This system is operated for 14 days at room temperature with a lighting period of 24 hours. The data observation were done 3 times.

2.2.5 Shoot Culture in TIS RITA Bioreactor

The acclimatized Cavendish banana shoot culture was cultivated in the TIS RITA bioreactor with a medium volume of 200 mL. The medium used is a liquid medium. The weight of the explants before inserted into TIS system was weighed as the initial weight (g). Meanwhile, the explant height was measured by millimeter block and recorded as initial height (cm). This system is operated for 14 days at room temperature with a lighting period of 24 hours. The data observation were done 2 times..

2.2.6 Analysis of Average Growth Rate and Cultivation Medium

Plant height and weight at the end of the TIS RITA liquid system and bioreactor treatment were calculated as final height (cm) and final weight (g). The average

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growth rate consists of the growth rate of biomass and growth rate of plant height. The average growth rate was calculated as the difference between the initial conditions and the final conditions, then divided by the cultivation time.

2.2.7 Analysis of Sucrose Content and Conductivity of Medium

Sucrose levels and conductivity in the cultivation medium at the beginning and end of each treatment were measured using a refractometer (Milwaukee Refractometer MA871) and a conductivity meter (Eutech Instruments Con – 100). The mass of sucrose contained in the test solution is known by entering the measurement results into the sucrose standard curve.

Aquadest is used as a standard zero on refractometers. The culture medium that has gone through the sterilization process, added cefotaxime, and is free of contamination is taken a few drops to be tested for the sucrose content of the initial medium. The final medium sucrose level was obtained by testing a few drops of culture medium after 14 days of culture in a bioreactor. The difference in medium sucrose content was calculated as the level of sucrose consumption of banana seedling.

2.2.8 Seedling Survivability Test

To test and calculate the survival rate of the banana seed explants produced, the explants were washed with running water to remove the remaining medium, and immersed in fungicide. After immersion in fungicide and rinsing with water, the explants were immersed back into growmore. Then the explants were planted in acclimatized soil that had been sterilized beforehand, and covered with plastic. After 14 days, the percentage of the number of seed explants that survived was calculated. The results of the survival test of these seeds are then used as the basis for processing economic analysis calculations. The economic analysis carried out is the calculation of the Net Present Value and Payback Period.

3. Results and discussion

Before cultivating banana seedlings using the TIS RITA bioreactor, banana explants were first subcultured. Explants from solid subculture were then acclimatized for 7 days in liquid medium before being cultivated in a bioreactor. The results of subculture and acclimatization can be seen in Figure 1.

(a) (b)

Figure 1. Banana Shoot Culture within (a) solid medium, (b) acclimatization process

During the subculture process, both in solid and liquid medium, fresh green explants have higher and faster leaf growth. The use of an appropriate medium and a supportive growth environment is a factor in the success of growth. The use of MS medium proved to be effective for the growth of banana explants. MS medium had higher ammonium, nitrate, and calcium content compared to other mediums. The high concentration of ammonium in the MS medium can probably affect *in vitro* shoot formation by increasing the synthesis of cytokinins. The nitrate and calcium contained also support shoot growth [8].

The addition of the gibberellic acid (GA) was used as a key regulator of growth promoting banana explants, which was mixed into the MS growth medium. GA hormone is able to stimulate tissue growth and elongation, such as for shoot apical and young internodes elongation, as well as for leaf expansion [9]. These caused the growth of banana explants to become faster and taller.

3.1 The Effect of Immersion Time on the Growth of *Musa acuminata* Banana Seedling

Evaluation of the effect of immersion on growth was carried out by comparing explants with temporary immersion in the TIS RITA bioreactor and control explants with continuous immersion. In the temporary immersion, the immersion time was varied, to immerse the explants in the medium for 5 minutes and 20 minutes in an interval of 4 hours. The analysis was carried out by comparing the growth of explant biomass in terms of wet weight, and the average growth rate. The increase in biomass and the average growth rate are shown in Figure 2 and Figure 3.

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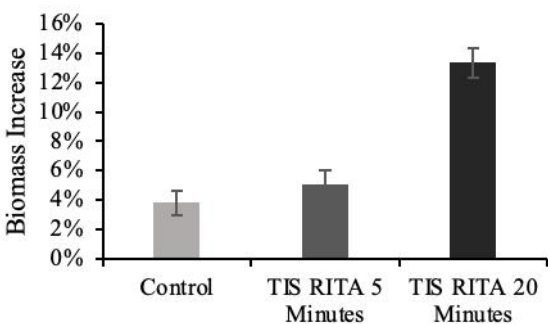

Figure 2. Increase of explant biomass in each of immersion variation treatment

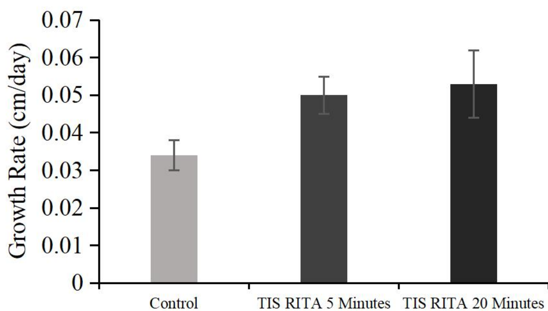

Figure 3. The average growth rate for each immersion variation

After 14 days of cultivation, all explants experienced growth accompanied by an increase in biomass, where explants with temporary immersion for 20 minutes had the largest increase in biomass and average growth rate, namely an increase in biomass of 13% with a growth rate of 0.053 cm/day. Then followed by temporary immersion for 5 minutes which has an increase in biomass of 5% with a growth rate of 0.05 cm/day. Then followed by the lowest increase in biomass in a continuous immersion culture system of 4% and a growth rate of 0.034 cm/day. The lowest growth was in the continuous immersion culture system, indicating hyperhidricity in the explants.

Visually, the quality of the seed explants looks different. The results of the temporary immersion system cultivation showed greener and fresher plants as shown in Figure 4a. On the other hand, explants in the control system using continuous immersion showed yellow-brown and withered shoots as shown in Figure 4b, which indicated browning in seedling explants in that system.

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(a) (b)

Figure 4. Cavendish Plant Visual a) Cultivation Result of TIS RITA; b) Cultivation Result of Continued Immersion

Hyperhydricity or what can be referred to as vitrification, is a change in plant shape morphologically, anatomically, and physiologically caused by excessive water absorption which results in swelling of plant tissues. This phenomenon is correlated with excess water availability, excess microelements, or hormonal imbalance in tissue culture media [10]. Too much water in plants will cause structural abnormalities in shoots and leaves, and inhibit apoplast water transfer so that it can inhibit the transfer of water and nutrients to plants [9].

Immersing in medium for too long can also result in excessive water accumulation in the leaves resulting in a lack of oxygen in the cells, which triggers a hypoxic condition of the plant. Under conditions of hypoxia and osmotic stress, plants will produce free radicals in the form of reactive oxygen species (ROS). This chemical species of ROS is a by-product of oxygen metabolism, as a form of self-protection against environmental stresses with highly reactive properties [11]. However, if ROS accumulates too much in plants, it will become toxic. ROS species can cause damage to the DNA structure of plants because they are able to oxidize cell components and change the functions of plant organelles. Chloroplasts and mictochondria became one of the cell organelles that were damaged from ROS toxicity, which resulted in the fading of green to brown color in explants and disruption of the photosynthetic process [12].

Browning phenomenon in plants is a form of protection mechanism for non-enzymatic pathways carried out by plants to decompose ROS species in tissues, namely through enzymatic and non-enzymatic pathways. The mechanism of protection of the enzymatic pathway is carried out by breaking free radicals in ROS to be converted into water. Meanwhile, the protection mechanism of the non-enzymatic pathway is carried out by interrupting the ROS free radical chain reaction. Phenolic compounds are one of the compounds produced by plants to carry out non-enzymatic protection mechanisms. Phenolic compounds are produced through the activity of the enzyme phenylalanine ammonia lyase (PAL) which is able to convert phenylalanine into trans-cinnamic acid [13]. After going

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through several processes, this trans-siamic acid turns into several types of phenolic acids. Plant cells that are damaged by membranes or wounds, their vacuoles will split and release phenolic compounds. This phenolic compound is then oxidized to produce a blackish brown color in plants. This is what causes the browning phenomenon in plants [14]. The phenolic released during the browning phenomenon will diffuse into the medium and be absorbed by the explants. The browning phenomenon that takes place on an ongoing basis can result in environmental stress conditions for explants, where this stress will inhibit the growth of explants, and can even lead to death if it lasts for a long time.

The use of the TIS RITA bioreactor system has proven to be effective as a culture system that combines aeration with the positive effects of liquid medium culture. Negative effects in the form of inefficiency of liquid medium culture that can cause hyperhydricity can also be avoided using the TIS RITA bioreactor system. The aeration connected to the TIS RITA serves as a medium for immersion, provides air circulation, and at the same time homogenizes the liquid medium. This is what causes during immersion in the TIS RITA system, the supply of water, nutrients, and air can be maintained to ideally maintain the cycle of primary components in banana seedlings [15].

There are several factors that determine the efficiency of seedling cultivation in the TIS RITA bioreactor, including immersion time, medium volume, tank volume, and oxygenation. Immersion time that is able to ensure optimal nutrient absorption is achieved at 20 minutes every 4 hours. The volume of the liquid medium of 200 ml is the ideal volume because it does not induce hyperhydricity [16]. The volume of the TIS RITA tank used during the study of 5 L also encourages the multiplication of biomass, because it has provided sufficient space for explants to absorb nutrients evenly [17]. Oxygenation through pneumatic agitation carried out on the TIS RITA bioreactor is effective in preventing hypoxia (lack of oxygen levels in cells and tissues), and reducing the relative humidity of the container to stimulate transpiration in plants, making it more adaptive when grown ex-vitro [15].

3.2 Evaluation of Nutritional Bioconversion Efficiency in Banana (Musa acuminata) with Variation of Immersion

Plants need macro and micro nutrients for cell division and material synthesis. The analysis of the medium was carried out to observe changes in the composition of the medium which were directly proportional to the absorption of nutrients and to see its effect on plant growth. Parameters reviewed in the analysis of the medium were conductivity, pH and sucrose content. The change in the conductivity value is shown in Figure 5, while the change in pH is shown in Figure 6.

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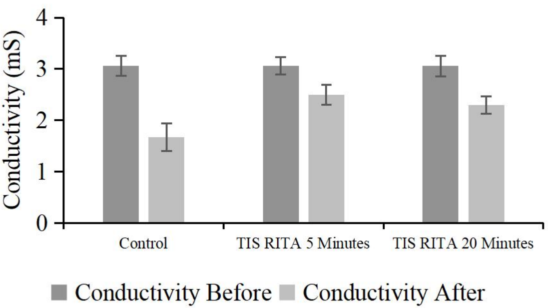

Figure 5. Conductivity changes of the medium in each immersion variation

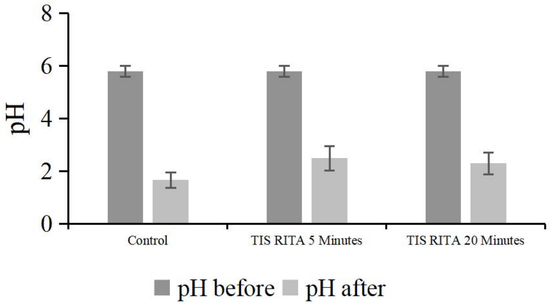

Figure 6. pH level changes in the medium of each immersion variation

Based on Figure 5, the conductivity of the medium decreased for each treatment. The decrease in conductivity indicates that the ions contained in the medium are reduced and this reduction is due to the absorption of ions into the plant. The decrease in the conductivity of the medium occurred in all treatments with variations in immersion time. The maximum absorption of minerals occurs in the control system, this probably due to the medium immersion was conducted for 14 days, continously. As stated by Persson in 2012 [18], the decrease in conductivity indicates a decrease in the amount of minerals in the medium due to nutrient absorption by plants.

The decrease in pH was also observed in all treatments as shown in Figure 6. The decrease in pH in the medium, especially in the control system medium, could be caused by the production of metabolites resulting from metabolism, non-enzymatic protection of phenols in plants stimulated by environmental stresses growing explants [13]. The decrease in pH in the medium was also caused by the respiration carried out by the explants. CO_2 gas compounds produced by plants during respiration will dissolve and interact with water to form H_2CO_3, which in equilibrium the plants will form HCO_3^- and H^+. Henceforth, HCO_3^- will release H^+

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and CO_3^-. The release of H^+ ions in equilibrium conditions in the medium resulted in a decrease in the pH degree to become more acidic [19].

Explants also require a carbon source as one of the important macronutrients in cell division and material synthesis. One of the macronutrients needed is the element carbon. In this study, the carbon source of the explants was sucrose dissolved in a liquid medium. Sugar consumption by banana explants for 14 days is shown in Figure 7.

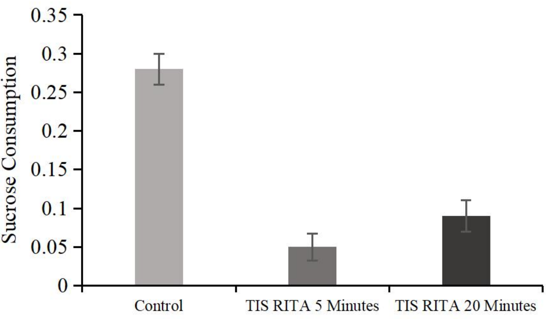

Figure 7. Sucrose Consumption of each immersion variation

The control system with continuous immersion showed the highest consumption of sucrose with a consumption rate of 28%. Followed by a temporary immersion system TIS RITA for 20 minutes with a consumption rate fraction of 9%. Meanwhile, the fraction with the lowest consumption rate of 5% was found in the TIS RITA immersion system for 5 minutes. This indicates the maximum consumption of sucrose in the control system due to continuous immersion of the growing medium. However, the very high level of control sucrose consumption was not accompanied by good biomass growth. This shows that the banana seed metabolism that takes place in the control system does not take place primary, but secondary metabolism caused by environmental stress in the form of prolonged immersion of the medium. The high and efficient level of sucrose consumption was shown by immersing TIS RITA for 20 minutes accompanied by optimal growth of biomass.

3.3 Economic Analysis Based on Survivability Rate

To calculate the economic feasibility analysis of banana seed production using the TIS RITA bioreactor, data on the survival rate of seedlings were used which were compared with controls using Thin-layer Culture, after ex-vitro cultivation for 2 weeks. The survival rates of the two systems can be seen in Table 1 below.

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Table 1. Survivability rate in culture system

System	Survivability rate
Thin-layer Culture	66,7%
TIS RITA immersion 20 minutes	100%

The survival rate of using a temporary immersion system for cultivation of banana seedlings in the TIS RITA bioreactor was higher than the control. The TIS RITA bioreactor system which is able to effectively prevent the hyperhidricity in banana seedlings, supports the survival of banana seedlings when grown in an *ex-vitro* environment. Banana seeds released from TIS RITA are stronger and fresher, so they can adapt to new environments quickly and optimally. In contrast to banana seedlings obtained from controls that showed symptoms of hyperhydricity, when removed for *ex-vitro* cultivation, their physical condition was more wilted and fragile for planting. As a result, 33% of banana seedlings died after being planted within 3-4 days due to not being able to adapt optimally to their new environment.

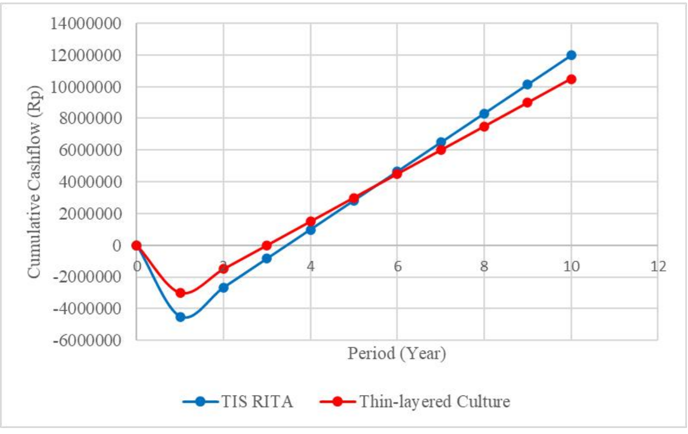

Figure 8. Cumulative Cash Flow on the use of RITA and Control TIS bioreactors

Based on the results of calculations and economic analysis (Figure 8), it indicates that the seedling cultivation system carried out using either the TIS RITA bioreactor or the control has a positive economic value, although with different time periods. The application of the TIS RITA bioreactor technology was able to produce positive economic values better than the control. The NPV value compared to the application of TIS RITA bioreactor technology was able to reach Rp. 13,796,000.00, while the control had an NPV value of Rp. 11,990,670.00.

In terms of payback period, the use of TIS RITA technology has a longer payback period compared to Thin-layer culture. TIS RITA takes 2 years and 5 months

compared to Thin-layer culture which only takes 2 years. This is because a slightly higher initial capital is required as a form of initial investment to use TIS RITA technology. However, the long-term production of banana seedlings using TIS RITA is much more economical and sustainable.

4. Conclusions

From the research to produce Cavendish banana (*Musa acuminata*) seedlings that have been carried out, it is concluded that the biomass gain of banana seedling culture in the temporary system (TIS RITA) has a higher biomass recovery rate of 13% compared to banana seedling culture in the thin layer culture immersion system. which is only 4%. The average growth rate of banana seedling culture was higher in the temporary immersion system, while TIS RITA was 0.053 cm/day, compared to the growth rate in the full immersion system, which was 0.034 cm/day. The level of sucrose consumption for biomass conversion of banana seed culture (*Musa acuminata*) higher in the thin layer culture system, which is 28%, but the level of sucrose consumption for conversion to biomass is more efficient and optimal in the TIS RITA system by 9%. The results of the economic evaluation of the survivability rate of banana seed culture (*Musa acuminata*) showed that the TIS RITA temporary immersion system was more productive and economical with an NPV of Rp. 13,796,000.00 than the thin layer culture immersion system with an NPV of Rp. 11,990,670.00.

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Specific Growth Rate of Microalgae in Mixed Culture of *Nannochloropsis* sp. and *Chlorella vulgaris* in comparison to their Monocultures, Using Aquaculture Wastewater as Growth Medium

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Abstract

In nature, microalgae grow in water body with various species interacting in it. In laboratory-scale however, for practical reasons, cultivation is usually conducted in monoculture system. Mixed culture cultivation of microalgae in laboratory scale can only be conducted in limited scale due to difficulties to mimic complexity of various factors in the ecosystem. In this study we observed specific growth rate (SGR) of mixed culture microalgae which consisted of *Nannoschloropsis* sp. and *Chlorella vulgaris*. Growth media used was aquaculture wastewater which contain residues of fish feed and fish metabolites. The experiment was conducted in Vertical Tubular Reactor with a working capacity of 20 L. The results showed that in mixed culture of microalgae *C. vulgaris* and *Nannochloropsis* sp. a higher SGR (0.309 day-1) was obtained, which was higher than the SGR of monoculture microalgae *Nannochloropsis* sp. (0.224 day-1), but lower than the SGR of monoculture *C. vulgaris* (0.505 day-1). Furthermore, in mixed culture, microalgae has longer exponential phase than in both monocultures, and produce 3 to 7 times higher biomass productivity. It can be concluded that mixed culture microalgae which contain *Nannochloropsis* sp. and *C. vulgaris* is more recommended to be applied than monoculture system to produce more microalgal biomass. Moreover, the use of aquaculture wastewater showed good result, and hence can be used as a more economic alternative than an expensive commercial growth media.

Keywords: Aquaculture wastewater, *Chlorella vulgaris*, mixed culture, *Nannochloropsis* sp., specific growth rate.

1. Introduction

Currently, microalgae have attracted worldwide attention as one of the biological agents that have the potential produce biomass and various metabolites that are useful for meeting human needs because of their photosynthetic efficiency and high metabolites concentration [1]. Current studies are starting to focus on the growth of

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microalgae by culturing several types of microalgae which are commonly called mixed cultures [2]. The mixed culture is carried out to facilitate the culturing 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 usually occurs in microalgae monocultures [3]. Research conducted by Ugetti et al. [4] and Bohutsky et al. [2] concluded that mixed culture on microalgae still obtained an increase in biomass. *Chlorella vulgaris* and *Nannochloropsis* sp. are species of microalgae capable of producing high amounts of lipids and proteins [5]. Availability of nutrients, carbon sources, and other factors also need to be considered to produce biomass and other microalgae-based bioproducts at relatively low cost and are environmentally friendly [2]. Through this research, we observed specific growth rate (SGR) of mixed culture microalgae which consisted of *Nannochloropsis* sp. and *C. vulgaris* using aquaculture wastewater as medium which contain residues of fish feed and fish metabolites. Therefore, a production system that involves both aspects can be designed to produce bioproducts at a certain scale.

2. Materials and Methods

2.1 Materials

The bioreactor used in this study for the treatment of single and mixed cultures is a vertical tank reactor, a tubular photobioreactor type made from acrylic with a working volume of 20 L. The bioreactor scheme used is presented in Figure 1. Light source of the bioreactor comes from LED lamp with a light intensity of 25 mol m^{-2} s^{-1}. There is a sparger at the bottom of the bioreactor which functions to break up air particles with borosilicate base material. This research utilizes analytical balance, pH meter, UV-VIS spectrophotometer, centrifuge, autoclave and digital microscope which are facilities of the School of Life Sciences and Technology (SITH), Institut Teknologi Bandung. *C. vulgaris* and *Nannochloropsis* sp. inoculum was obtained from Balai Besar Perikanan Budidaya Air Payau Jepara, Central Java. Aquaculture wastewater was obtained from Kebun Pendidikan SITH ITB Haurngombong, Jatinangor.

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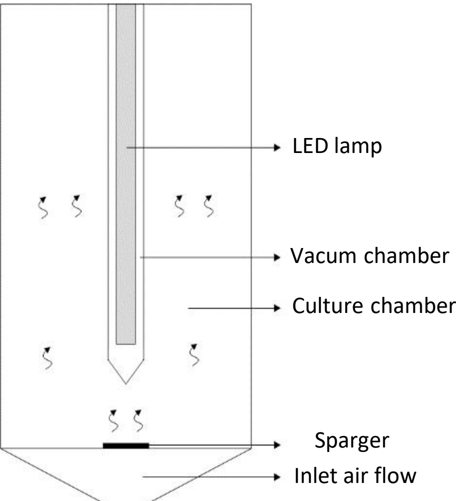


Figure 1. Tubular photobioreactor for microalgae cultivations

2.2 Methods

2.2.1 Cultivation Medium Preparation

The inoculum cultivation medium used was Walne synthetic medium which was used as much as 1 mL for each addition of 1 liter of cultivation water. And the growth medium used in this study was Aquaculture Wastewater medium. Aquaculture wastewater medium is filtered first using a filter cloth to separate the solid and liquid fractions. The liquid waste fraction was then put into a glass bottle and then sterilized using an autoclave at a temperature of 121°C and a pressure of 1.5 bar for 15 minutes. Sterile aquaculture wastewater is ready to be used as a cultivation medium. The characteristic of aquaculture wastewater in common is shown in Table 1.

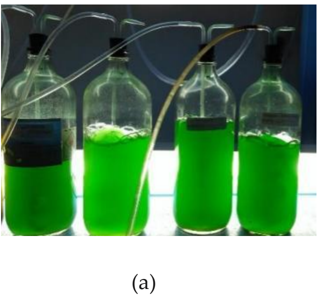
Table 1. Charachteristic of Aquaculture Wastewater [6]

Parameter	Concentration
pH	6,68±0,03
Temperature (°C)	27,8±0,12
TSS (mg/L)	0,7±0,15
EC (mg/L)	400±45,39
Nitrate (mg/L)	8,5±0,06
Ammonia (mg/L)	0,08±0,01
Phososphate (mg/L)	2,5±0,08
DO (mg/L)	4,17±0,08
COD (mg/L)	108,7±6,66
BOD (mg/L)	46,8±0,88

2.2.2 Cultivation of Microalgae

Cultivation of each microalgae inoculum was carried out in the glass bottle with a working volume of 800 mL using a Walne synthetic medium. Cultivation was carried out with a ratio of inoculum volume: medium solution = 1 : 4 (v/v). Cultivation was carried out for 1 week with room temperature conditions (23-28°C) and photoperiodism L:D = 2 : 1.

Microalgae cultivation was then continued on a tubular photobioreactor with a total working volume of 20 L. The variations given were single culture of each species and mixed culture. Mixed cultures were prepared with a volume ratio of inoculum: medium = 1: 4 with a ratio of the volume of culture *Nannochloropsis* sp.: *C. vulgaris* = 1: 1. The cultivation medium used in this research is aquaculture wastewater. Microalgae cultivation was carried out for 1 week. All cultivation was carried out at an aeration rate of 3 L/min, room temperature (25-28°C), and photoperiodism L:D = 2:1.



(a)

(b)

Figure 2. Microalgae cultivation in (a) glass bottles (b) Tubular Photobioreactor

2.2.3 Determining the Specific Growth Rate

A sampling of each treatment was carried out by isolating the culture in 10 mL plastic bottles. Sampling was carried out once a day. Measurements of pH and salinity samples were carried out simply using a pH meter and the determination of the number of cells was carried out using a hemacytometer. The SGR is then determined based on the gradient of the exponential phase of microalgae growth using eq. (1).

$$\ln C_t = \ln C_t + \mu\, t \qquad (1)$$

Where C are cell density in time t, μ are the SGR (day^{-1}), dan t are time sampling.

To calculate the maximum volumetric biomass yield and productivity, which represents the produced biomass per day and the reactor volume unit eq. (2) and eq (3) has been used respectively.

$$Y = \frac{X_t - X_0}{t - t_0} \qquad (2)$$

$$P = \frac{Y}{t} \qquad (3)$$

Where Y are volumetric biomass yield, X are biomass in mg, and P are volumetric biomass productivity.

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3. Result and Discussions

In this study, microalgae growth was observed by counting the number of microalgae cells every 24 hours using a hemacytometer and a binocular microscope. In addition to directly counting the number of cells, microalgae growth can also be observed by measuring culture's Optical Density (OD) value [7]. OD is a value that indicates the high and low population of microorganisms, such as bacteria or microalgae, which is seen as culture turbidity [8]. Although it tends to be more accessible, the measurement of culture OD is considered less accurate in representing the number of cells because the OD value depends on the culture temperature which can affect the color of the culture, especially when it is in the death phase [9]. Microalgae growth curves for each treatment variation are shown in Figure 3.

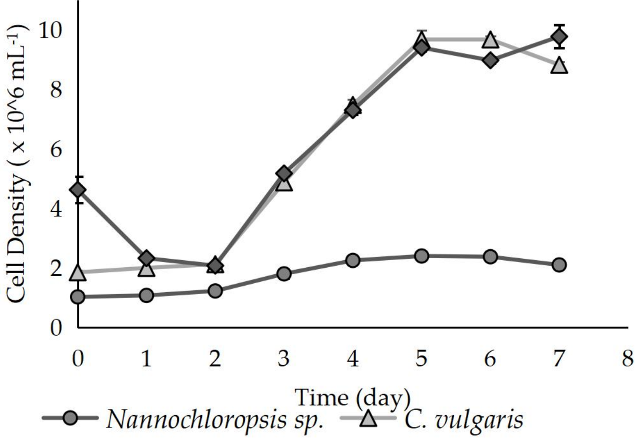

Figure 3. Growth Curve of Each Variation Treatment

In the monoculture treatment variation, both species of microalgae began to enter the exponential phase at t = 2 days and entered the death phase at t = 6 days. However, both of them show different growth performances. *C. vulgaris* monoculture cells showed much better growth, which was indicated by the high SGR reaching 2 times when compared to *Nannochloropsis* sp monoculture. Various studies related to monoculture cultivation of *C. vulgaris* or *Nannochloropsis* sp. are shown in Table 2.

Table 2. Growth Parameter of Each Species

Spesies	Cultivation Medium	μ (day $^{-1}$)	Y (gL^{-1})	P ($gL^{-1}day^{-1}$)	Reference
C. vulgaris	Aquaculture waste water	0,505±0,005^{a}	1,89±0,053$^{\alpha}$	0,315±0,008*	This Research
	Urban Wastewater	0,15±0,12	1,05±0,12	0,13±0,005	[5]
	Basal Medium	0,24	0,49	0,254	[10]
	Walne Medium	0,452	-	-	[11]
Nannochloropsis sp.	Aquaculture waste water	0,224±0,019^{b}	0,745±0,035$^{\beta}$	0,124±0,006**	This Research
	Urban Wastewater	0,2±0,04	1,29±0,2	0,17±0,009	[5]
	Basal Medium	0,2	0,66	0,194	[10]
	Walne Medium	0,2616	2,652		[12]
Mixed Culture	Aquaculture waste water	0,300±0,005^{c}	0,955±0,060$^{\gamma}$	0,955±0,06***	This Research
	Urban Wastewater	0,19±0,05	1,42±0,15	0,19±0,012	[5]

Research conducted by Fallahi et al.[5] and Metsoviti et al. [10] showed that *Nannochloropsis* sp. tends to have the same or higher SGR and biomass gain than C vulgaris. Meanwhile, in this study, *Nannochloropsis* sp showed a lower value than *C. vulgaris*. The growth rate is known to be one indicator of the success of a species adaptation to environmental conditions [13]. So the difference in values indicates that in this study *C. vulgaris* grew under optimal conditions and could adapt better than *Nannochloropsis* sp when cultivated in aquaculture watstewater medium. This could be influenced by the structure of *C. vulgaris* which has a thicker and tougher cell wall which makes *C. vulgaris* have higher resistance when cultivated in a waste medium [14].

In addition, in terms of its optimum conditions, *C. vulgaris* can grow in a wider range of pH and salinity, namely pH 5-9 and salinity 0-35 ppt. While *Nannochloropsis* sp. is known to be susceptible to changes in salinity [15] In this study, although monoculture of *Nannochloropsis* sp. was in the optimal pH range, the salinity level of the culture was far below the optimal salinity of *Nannochloropsis* sp. which is 30 ppt or equivalent to 30,000 ppm [15]. Therefore it can be said that in this study *Nannochloropsis* sp did not grow in optimal culture conditions, so it could

inhibit the growth of *Nannochloropsis* sp. Changes in pH and salinity of cultures in each treatment variation are shown in Figure 4.

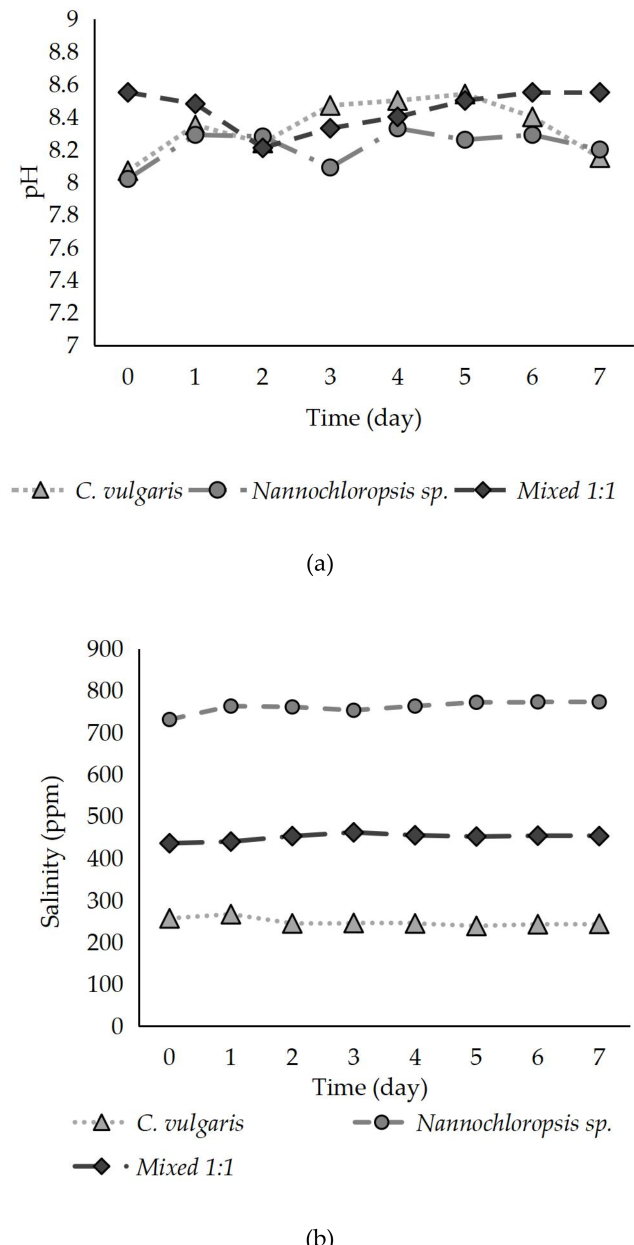

Figure 4. Culture condition of each treatment pH (a) and salinity (b)

The mixed culture treatment in this study had the same growth curve trend as the monoculture growth curve of *C. vulgaris*. but has a slower growth rate and biomass recovery. When compared with monoculture *Nannochloropsis* sp. treatment, mixed

culture had a higher SGR and biomass gain. This shows that the mixed culture treatment has better cultural stability compared to monoculture so that it can overcome the adaptation of *Nannochloropsis* sp monoculture in fish culture liquid waste media that is not good [16]. This statement is supported by the growth curve attached in Figure 4.1. The mixed culture began to enter the exponential phase at t = 2 days and continued for up to 7 days of observation. The mixed culture treatment showed an exponentially longer phase range than the monoculture treatment. This is in line with the research conducted by Militao et al. [16] and Akronrat et al. [17] which showed that the mixed culture treatment of

Pseudopediastrum boryanum, *Scendesmus obliquus* and *Nannochloropsis* sp-*Tetraselmis* sp. those cultivated in the synthetic medium had better growth because they did not experience a lag phase. In addition, the mixed culture treatment in this study also showed significantly higher biomass productivity ($p<0.05$) compared to the monoculture.

When compared with research conducted by Fallahi et al. [5] who performed mixed culture of *C. vulgaris*, *Nannochloropsis* sp., and *Scenedesmus*, the mixed culture treatment in this study showed higher growth parameter values. This could be due to the dominance of different species. In a study conducted by Fallahi et al. [5] the dominant species is *Nannochloropsis* sp. while in this study the dominant species was *C. vulgaris* which had a very high SGR compared to the SGR of *Nannochloropsis* sp. in the study of Fallahi et al. [5]. So that the dominance of different species can produce different growth parameter values. The graph of the interaction between *C. vulgaris* and *Nannochloropsis* in this study is shown in Figure 5.

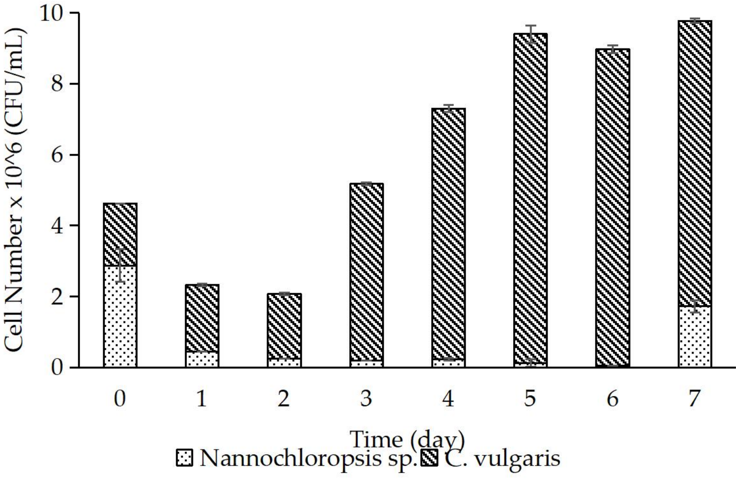

Figure 5. Cell Number of Each Species in Mixed Culture

At the beginning of cultivation, *Nannochloropsis* sp cells made up almost 80% of the culture. However, from day 1 that *C. vulgaris* continued to dominate until day 7 of

observation. This could be due to the high SGR of *C. vulgaris* which doubled when cultivated in monoculture, causing competition for nutrients for the growth of the two species. The high SGR of *C. vulgaris* supported by its larger cell size compared to the cell size of *Nannochloropsis* sp. [18,19] can cause shelf shading events, where microalgae cells that are in the top layer to cover the cells below from exposure to light needed for growth [20].

In addition, differences in growth performance of *C. vulgaris* and *Nannochloropsis* sp. in the mixed culture and monoculture showed that in the mixed culture treatment there were interactions between species that could inhibit each other's growth. Phatarpekar et al. [21] in their research conducted a mixed culture between I. galbana and C. calcitrans and got similar results. the decreased growth of C. calcitrans was cultivated when mixed cultures expected that there was a possibility of I. galbana suppressing the growth of C. calcitrans. However, the growth characteristics of C. calcitrans in mixed cultures did not change when C. calcitrans became the predominant species. This statement is supported by the statement of Huang et al. [22] and Uchida et al. [23] who added that in mixed culture cultivation, one species can secrete certain compounds that can inhibit the growth of other species. Uchida et al .[23] added that this interaction is on what species dominates and its cell density during cultivation. Nevertheless, research related to competition and growth inhibition between species in mixed cultures is still needed for further research.

4. Conclusion

Based on this research. mixed culture microalgae which contain *Nannochloropsis* sp. and *C. vulgaris* is more recommended to be applied than monoculture system to produce more microalgal biomass. Moreover, the use of aquaculture wastewater showed good result, and hence can be used as a more economic alternative than an expensive commercial growth media.

Acknowledgments

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Kinetic modeling of cultured *Spirogyra* sp. growth and lipid synthesis for lipid production under various concentrations of sodium chloride

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Abstract

Spirogyra sp. is known to contain 11-21% dry weight (DW) lipid and lipid content in algae can be enhanced by growing the algae under different salt concentrations. Therefore, the objective of this study was to determine the effect of various NaCl concentrations on growth and lipid content of *Spirogyra* sp. and to determine the optimum NaCl concentration. *Spirogyra* sp. was grown in modified Bold Basal Medium (BBM) with an addition of NaCl at various concentrations i.e. 0.025; 0.05; and 0.075 g L^{-1} for 8 days. The kinetic of the growth and lipid production of *Spirogyra* sp. were determined using logistic and Luedeking-Piret model. The results showed that the highest biomass growth was obtained under 0.05 g L^{-1} NaCl with maximum biomass (X_{max}) i.e. 344.93 mg L^{-1} and maximum specific growth rate (μ_{max}) i.e. 0.153 day^{-1}. This study indicated that the addition of NaCl could enhance the lipid content in *Spirogyra* sp. with the highest lipid yield of 90.71 mg g^{-1} was obtained under 0.075 g L^{-1} NaCl. The highest lipid productivity of 8.79 mg $g^{-1}day^{-1}$ was obtained under 0.075 g L^{-1} NaCl. This result showed that lipid productivity increase up to 52% compared to the control.

Keywords: *Spirogyra* sp., lipid, growth, NaCl, kinetic modeling

1. Introduction

Spirogyra sp. is a filamentous green macroalgae from the order Zygnematales which is often found in freshwater habitats. The utilization of *Spirogyra* sp. in Indonesia is still limited as fish bait and sold at a low price. *Spirogyra* sp. is known to contain 11-21% dry weight (DW) lipid content and Polyunsaturated Fatty Acid (PUFA) content of these macroalgae can reach 37.35% consisting of omega-3 and omega-6 [1, 2].

Lipid is a group of biomolecules that are insoluble in water and soluble in nonpolar solvents. Lipid is essential compounds for the human body that have biological functions as structural components of cell membranes, storing energy, and signaling [3]. One of the most widely used lipid is PUFA. One type of PUFA that is important for the human body and widely used is omega-3. A common source of omega-3 is from fish oil, but there has been a decrease in fish oil production caused by marine

pollution, this causes fish oil to contain harmful substances such as dioxins, mercury, and PCBs [4] . In addition, fish oil is not suitable for vegetarians and has a strong odor [5] . One of the alternatives to this problem is to utilize lipid from algae. *Spirogyra* sp. is one of the freshwater macroalgae that has the potential to be a source of lipid. Macroalgae is a promising alternative source of lipid because of its high photosynthetic efficiency in producing biomass, has a fast productivity rate and growth when compared to other lipid-producing plants, and its cultivation does not require large and fertile land so that it will not compete with terrestrial plants. In addition, macroalgae have the ability to reduce CO_2 in the atmosphere [6].

There are several factors that can enhance lipid synthesis in algae, i.e. temperature, light, nitrogen, phosphorus, salinity, and carbon. Salinity is the level of saltiness or amount of salt dissolved in water. Generally, NaCl is chosen to increase salinity in algae cultivation medium because it is inexpensive and easy to obtain [7] . According to Shetty et al. (2019), most green algae accumulate lipid under salinity stress [8] . For example, in freshwater macroalgae *Ulva intestinalis*, the addition of 0.05 g L^{-1} NaCl caused lipid content to increase from 18% to 36.15% [9] . Research conducted by Lawton et al. (2015) also showed an increase of lipid content in *Oedogonium* sp. under a certain concentration of NaCl [10]. Under conditions of high salinity, algae are stimulated to increase lipid production as an osmoprotectant from given stress [11]. Therefore, the objective of this study was to determine the effect of various NaCl concentrations on growth and lipid content of *Spirogyra* sp. and to determine the optimum NaCl concentration for the highest lipid productivity. The kinetic of the growth and lipid production of *Spirogyra* sp. were also determined using logistic and Luedeking-Piret model. Logistic model was used because this model is commonly used to study cell growth, doesn’t depend on substrate consumption, and is very suitable for autotrophic algae culture and Luedeking-Piret model was used because this model is commonly used to model product formation.

2. Materials and Methods

2.1 Materials

Spirogyra sp. were obtained from a fishing pond in Majalaya, West Java. All chemicals used in this study were obtained from CV Putra Mebelindo Solusi and CV Titian Scientific provided by SITH Warehouse, Bandung Institute of Technology.

2.2 Methods

2.2.1 Bold’s Basal Medium (BBM) Preparation

The BBM was used as a medium to *Spirogyra* sp. with concentration of 2.5% and the following composition : $NaNO_3$ 0.074 mM; $CaCL_2.2H_2O$ 0.004 mM; $MgSO_4.7H_2O$ 0.008 mM; K_2HPO_4 0.011 mM; KH_2PO_4 0.032 mM; NaCl 0.011 mM; EDTA 0.428 mM;

KOH 1.383 mM; $FeSO_4.7H_2O$ 0.004 mM; H_2SO_4 0.004 mM; H_3BO_3 0.463 mM; $ZnSO_4.7H_2O$ 0.008 μM; $MnCl_2.4H_2O$ 0.182 μM; MoO_3 0.123 μM; $CuSO_4.5H_2O$ 0.157 μM; dan $Co(NO_3)_2.6H_2O$ 0.042 μM. The medium was dissolved using distilled water.

2.2.2 *Spirogyra sp.* Cultivation

Spirogyra sp. was cultivated in plastic containers (14 x 8.5 x 7.5 cm) with the total volume of the container is 1 L. Each container was filled with 500 mL of medium. A total of 3 grams of *Spirogyra* sp. inoculum was placed in each cultivation container. *Spirogyra* sp. was cultivated at room temperature (25 ± 3℃) with a light intensity of 27,88 ± 5,88 mol photons m^{-2} s^{-1} (light: dark period is 12:12 hours). The aeration rate was 0.2-0.4 L $minute^{-1}$. The pH of the medium was measured using litmus paper every 2 days and the measurement results showed the pH value of 7 on the universal pH indicator. The variation of NaCl concentration used was 6.25 x 10^{-4} (control); 0.025; 0.05; 0.075 g L^{-1}. Each treatment was carried out in triplicate. Cultivation was carried out for 8 days. Measurement of growth parameters (fresh and dry weight) was carried out every 2 days.

2.2.3 Fresh and Dry Weight Measurement

Biomass of *Spirogyra* sp. were separated from the medium using a flour sieve, 60 mesh. The filtered biomass was then weighed and the result was the fresh weight of biomass. The wet biomass was then placed on filter paper and dried using a fan for 24 hours at 25 ± 3℃. The sample was then placed in the oven for 15 minutes at 105℃. The dried biomass was weighed and the results were the dry weight of biomass.

2.2.4 Lipid Extraction and Analysis

Lipid extraction was carried out using the Bligh & Dyer method [12] . The dried biomass was ground with mortar and pestle to make the powdered biomass. The powdered biomass was placed into a falcon tube. A total of 4 mL of methanol and 2 mL of chloroform were pipetted into a falcon tube containing the powdered biomass. The mixture was then vortexed for 2 minutes. A total of 2 mL of chloroform was added and then vortexed again for 2 minutes. A total of 3.6 mL of distilled water was added and vortexed again for 2 minutes. The mixture was then centrifuged for 10 minutes at 2000 rpm. The methanol phase at the top of the mixture was pipetted out of the falcon tube and discarded. The chloroform phase was then filtered using Whatman paper no.1. The filtrate from the first extraction was stored and the remaining residue was taken for the second extraction. The second extraction was carried out with 4 mL of 10% (v/v) methanol in chloroform and vortexed for 2 minutes. The mixture was then centrifuged for 10 minutes at 2000 rpm. After centrifugation, the mixture was filtered using filter paper and the filtrate from the second extraction was mixed into the first extraction filtrate. The mixture was then placed in an aluminum cup and heated using a water bath at 70℃ until the solvent evaporated. The extract was dried in an oven at 105℃ for 15

minutes. Extraction results were analyzed by gravimetric method to obtain lipid weight. Lipid were weighed on an analytical scale and lipid weights were determined.

2.2.5 Kinetic Modeling of *Spirogyra* sp. Growth

The growth of *Spirogyra* sp. was modeled with a logistic equation. To estimate the X_{max} and μ_{max} parameters, data fitting was done by Curve Fitting application in MATLAB where the x-axis is the time (day) and the y-axis is the dry weight of the biomass (mg L^{-1}) using Eq. (2). The results are parameter estimation value along with the value of R^2.

$$\frac{dX}{dt} = \mu_m X\left(1 - \frac{X}{X_m}\right) \quad (1)$$

$$X = \frac{X_0 e^{\mu_m t}}{1 - \frac{X_0}{X_m}\left(1 - e^{\mu_m t}\right)} \quad (2)$$

Where $\frac{dX}{dt}$ is the biomass growth rate (mg day^{-1}), X is biomass concentration (mg L^{-1}), X_m is maximum biomass concentration (mg L^{-1}), μ_m is maximum specific growth rate (day^{-1}), t is time (day), and X_0 is initial biomass concentration or when t=0 (mg L^{-1}).

The maximum specific growth rate from the experimental results was also calculated using Eq. (3)

$$\mu_m = \frac{\ln(X_i) - \ln(X_0)}{t_i - t_0} \quad (3)$$

2.2.6 Kinetic Modeling of Lipid Production

Lipid production was modeled using the Luedeking-Piret model. To estimate the α and β parameters, data fitting was done by Curve Fitting application in MATLAB where the x-axis is the time (day) and the y-axis is the lipid content in biomass (mg L^{-1}) using Eq. (5). The results are parameter estimation value along with the value of R^2.

$$\frac{dP}{dt} = \propto \frac{dX}{dt} + \beta X \quad (4)$$

$$P = Po - \propto X_0 \left\{ \frac{e^{\mu_m t}}{\left[1 - \left(\frac{X_0}{X_m}\right)\left(1 - e^{\mu_m t}\right)\right]} \right\} + \beta \frac{X_m}{\mu_m} ln\left\{1 - \frac{X_0}{X_m}\left(1 - e^{\mu_m t}\right)\right\} \quad (5)$$

Where P is lipid concentration (mg L^{-1}), X is biomass concentration (mg L^{-1}), t is time (day), α is growth correlation coefficient (mg mg^{-1}), β and is non-growth correlation coefficient (mg mg^{-1} day^{-1}) [13]. The relationship between mode of product

formation and growth was classified as follows: Class 1, when $\alpha \neq 0$ and $\beta = 0$, the product formation is related to biomass growth; Class 2, when $\alpha = 0$ and $\beta \neq 0$, the product formation is unrelated to biomass growth; Class 3, when $\alpha \neq 0$ and $\beta \neq 0$, product formation is partially associated with biomass growth [14].

2.2.7 Estimation of Lipid Productivity

Lipid productivity was determined using the estimated lipid yield obtained from the logistic and Luedeking-Piret model. Productivity was calculated on the 8th day of cultivation with Eq. (6).

$$Productivity\left(\frac{mg}{g}.day\right) = \frac{Lipid\ yield\ (\frac{mg}{g})}{Time\ (day)} \quad (6)$$

2.2.8 Data Analysis

The average value and standard deviation of the biomass growth and lipid yield data were calculated. The data were then plotted on a graph to obtain growth curves and product formation curves. The dry weight of biomass and lipid yield data were also analyzed using statistical analysis. The first analysis was the Normality Test to determine whether the data is normally distributed or not. The Normality Test of the data was carried out using the Kolmogorov-Smirnov method. If the results showed that the data is normally distributed, then the data can be used for parametric statistical tests. The next statistical analysis was One Way-Analysis of Variance (ANOVA). One way-ANOVA is used to determine whether there are any statistically significant differences between the means of two or more. If the value of $p>\alpha$ ($\alpha=0.05$) then there is no significant difference between the variations, but if the value of $p\leq\alpha$ then there is a significant difference between the variations. If the ANOVA test showed that there is a significant difference between the variations ($p\leq 0.05$), further test was carried out to compare one variation with other variation. The further test carried out was the Duncan's Multiple Range Test (DMRT). All data calculations and statistical analysis were carried out using the Microsoft Excel 2019.

3. Results and Discussion

3.1 The Effect of Various NaCl Concentration on the Growth of *Spirogyra* sp.

The results showed that the growth of *Spirogyra* sp. lasted for 6 days of cultivation on BBM 2.5% (control). *Spirogyra* sp. undergo an exponential growth phase until 6th day of cultivation and entered a stationary phase from 6th to 8th day of cultivation (Figure 1). The highest biomass accumulation occurred on 6th day of cultivation with a dry weight of biomass i.e. 335,82 mg L^{-1}. A similar growth trend was reported by Sirirustananun (2016) where *Spirogyra* sp. was cultivated for 3 weeks and the results

showed that the highest biomass weight and growth rate occurred in the first week of cultivation and continued to decrease in the second and third weeks of cultivation [15]. A different growth trend was reported by Sulfahri et al. (2017) who cultivated *Spirogyra hyalina* and *Spirogyra peipingensis* for 20 days. *Spirogyra hyalina* continued to grow until 10th day of cultivation and then entered a stationary phase, while *Spirogyra peipingensis* could grow up to 15th day of cultivation [16].

There are several factors that can cause differences in the growth of *Spirogyra* sp. ie. differences in *Spirogyra* species, cultivation medium, physical conditions of cultivation, and the amount of inoculum. Sulfahri et al. (2017) used *Spirogyra hyalina* and *Spirogyra peipingensis* inoculum of 1 g L^{-1}, while this study used an inoculum of 6 g/L. The inoculum used in this study was denser than the study by Sulfahri et al. (2017). According to Sirirustananun (2016), biomass accumulation and growth rate of *Spirogyra* sp. decreased as the amount of inoculum increased. A high amount of inoculum can reduce photosynthesis rate which leads to a decrease in biomass productivity [15]. Apart from that, the medium used is also different. Sulfahri et al. (2017) used Sulfahri-01 medium consisting of KNO_3 40 mg L^{-1}, P_2O_5 30 mg L^{-1}, K_2O 30 mg L^{-1}, $MgSO_4$ 2 mg L^{-1}, $Ca(NO_3)_2.4H_2O$ 2 mg L^{-1}, and micronutrients. In this study, the medium used is BBM 2.5%, which has a different composition from the Sulfahri-01 medium. These different mediums can affect the biomass growth of *Spirogyra* sp [16].

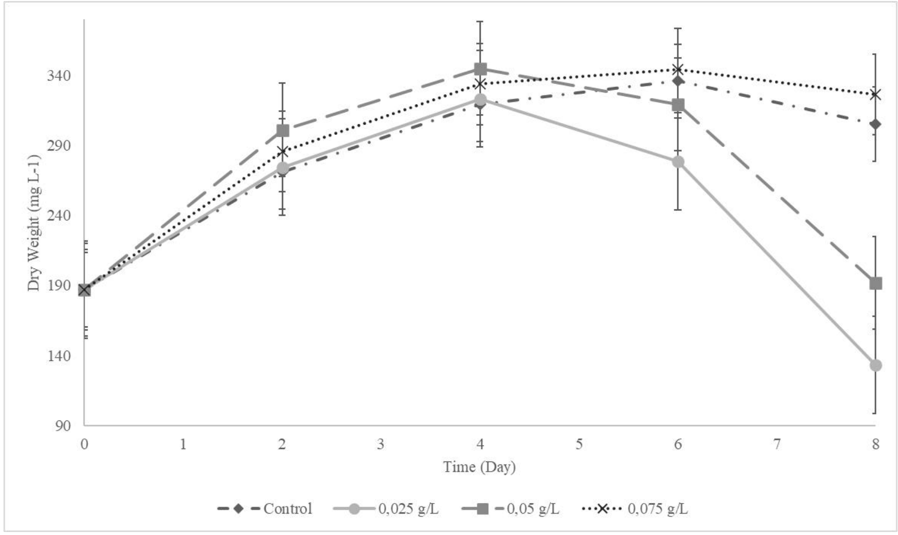

Figure 1. *Spirogyra* sp growth curve at various concentrations of NaCl

The result showed that there are differences on growth trend when *Spirogyra* sp. cultivated at different NaCl concentrations (Figure 1). With the addition of 0.025 and 0.05 g L^{-1} NaCl, the highest biomass accumulation occurred on 4th day of cultivation with biomass dry weight reaching 323.27 mg L^{-1} and 344.93 mg L^{-1}, respectively. With the addition of 0.075 g L^{-1} NaCl, the highest biomass

accumulation occurred on 6th day of cultivation with biomass dry weight of 344.4 mg L^{-1}. This showed that *Spirogyra* sp. can tolerate NaCl concentration up to 0.075 g L^{-1}. The results were in accordance with the statement that moderate salt stress can increase the growth of algal biomass and an optimal concentration of NaCl to be added to the culture depends on the algal species [17].

In this study, it was found that the highest biomass accumulation was achieved under the addition of 0.05 g L^{-1} on 4th day of cultivation. This result is similar to the study by Gopal & Ruma (2021) which showed that the highest biomass accumulation (2, 19 g L^{-1}) of *Spirogyra punctulata* was achieved under 0.05 g L^{-1} NaCl with a dry weight 1.4 times higher than control. The maximum tolerance level of NaCl concentration for *Spirogyra punctulata* was determined as 0.05 g L^{-1}. The negative trend in growth was observed when the NaCl concentrations were further increased to 0.1 g L^{-1} [9]. Another result reported by Kumar et al. (2016) showed that the highest *Spirogyra* sp. biomass accumulation was achieved under 0.006 g L^{-1} NaCl and the growth of *Spirogyra* sp. was observed to decrease with the increase in NaCl concentration [18]. The difference in optimal NaCl concentration could be due to the different *Spirogyra* species used. The optimal concentration of NaCl for algae growth depends on the species and variety of algae [17].

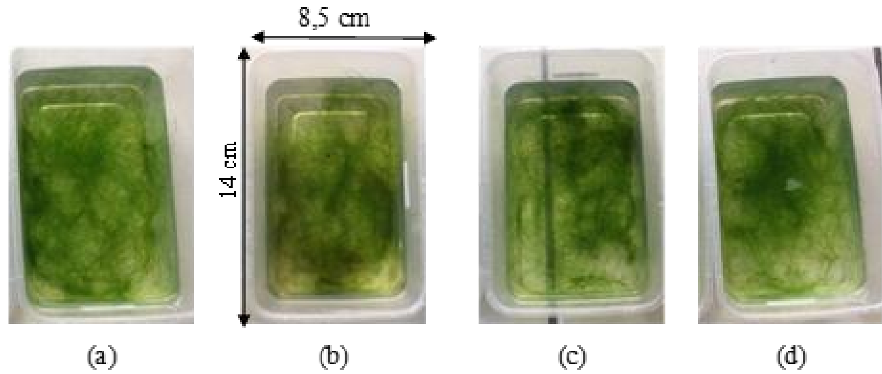

Figure 2. *Spirogyra* sp. on the 4th day of cultivation at various concentrations of NaCl (a) control, (b) 0,025 g L^{-1}, (c) 0,05 g L^{-1}, (d) 0,075 g L^{-1}

One way-ANOVA test showed that the variation of NaCl concentration of 0.025; 0.05; and 0.075 g L^{-1} did not significantly affect the growth of *Spirogyra* sp. ($p>\alpha$,α=0.05). Therefore, to determine the effect of the NaCl addition to growth of *Spirogyra* sp further, it is necessary to use variations of NaCl concentration with significantly different ranges.

3.2 Parameter Estimation of *Spirogyra* sp. Growth

The growth model of *Spirogyra* sp. was determined to estimate the growth parameters i.e. the maximum specific growth rate (μ_{max}) and maximum biomass concentration (X_{max}). The kinetic model used in this study was the logistic model,

logistic model is commonly used to study cell growth because it is independent of substrate consumption and is highly suitable for autotrophic culture of algae. However, this model can only represent cell growth from the exponential phase to the stationary phase, not until the death phase [19].

The results of curve fitting have a correlation coefficient (R^2) of more than 0.9 which indicates the model used has a good fit with the experimental data (Table 1). Although the values of R^2 were more than 0.9, there were differences in model and experimental data (Figure 3). This could be due to the logistic model can only represent *Spirogyra* growth until the stationary phase, so that curve fitting was only carried out until that phase, while the experimental data showed the growth until death phase.

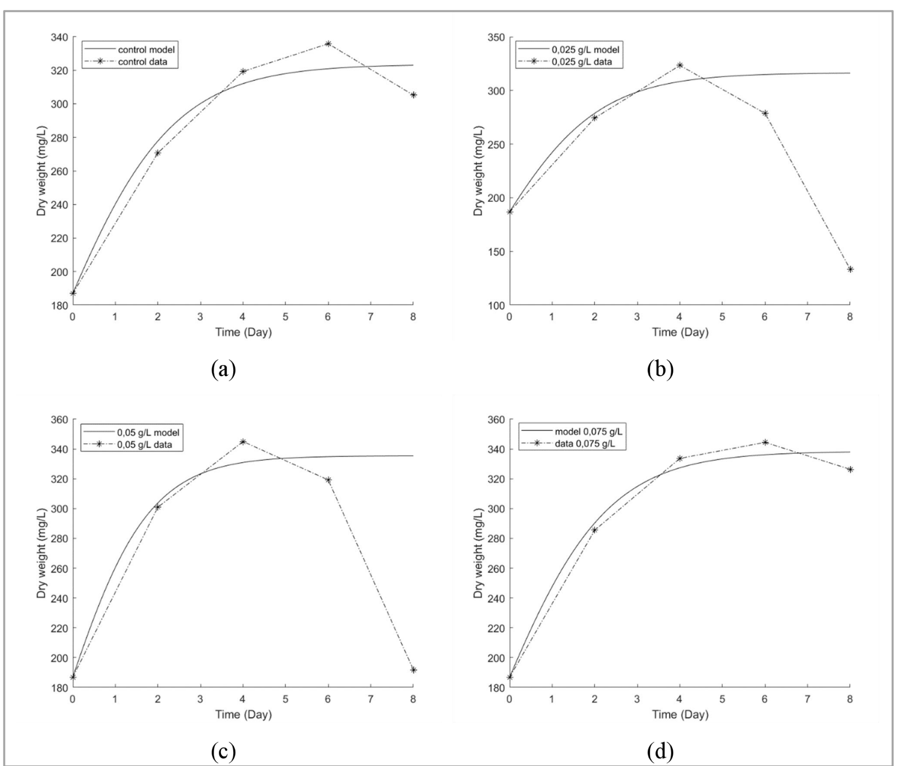

Figure 3. The growth model of *Spirogyra* sp. at various concentrations of NaCl (a) control, (b) 0,025 g L^{-1}, (c) 0,05 g L^{-1}, (d) 0,075 g L^{-1}

Table 1. Parameter estimation of *Spirogyra* sp growth at various concentrations of NaCl

Parameter	Control	0.025 g L^{-1}	0.05 g L^{-1}	0.075 g L^{-1}
	Experimental data			
X_{max} (mg L^{-1})	335,82	323,27	**344,93**	344,4
μ_{max} ($hari^{-1}$)	0,098	0,137	**0,153**	0,102
	Model			
X_{max} (mg L^{-1})	323,6	316,4	335,4	**338,4**
μ_{max} ($hari^{-1}$)	0,746	0,817	**1,017**	0,794
R^2	0,95	0,96	0,98	0,98

The X_{max} value obtained from model showed an increasing trend along the increase in NaCl concentration with the highest value of X_{max} obtained under 0,075 g L^{-1} NaCl. On the other hand, the highest X_{max} from experimental data was obtained under 0.05 g L^{-1} NaCl, but the X_{max} value of 0.075 g L^{-1} NaCl was not significantly different from the X_{max} value of 0.05 g L^{-1} NaCl (Table 1). X_{max} obtained from the model also have a lower value than the experimental data. This difference can be caused because the value of %R^2 is not completely 100%. This % R^2 value explains the proportion of experimental data that can be represented by the model [20] . In the NaCl variation of control, 0.025, 0.05, and 0.075 g L^{-1}, the model could explain 95%; 96%; 98%; and 98% of the experimental data. Although the R^2 value for all variations were close to 100% which indicated a fairly good fit between the model and the experimental data, there were still an error of 5%; 4%; 2%; and 2% in the control; 0.025; 0.05; and 0.075 g L^{-1} NaCl, respectively.

Based on the parameter estimation using the logistic model, the value of μ_{max} in all variations of NaCl concentration have a higher value than control with the highest μ_{max} was obtained under 0.05 g L^{-1} NaCl. The value of μ_{max} tend to increase until the addition of 0.05 g L^{-1} NaCl and decrease when the NaCl concentration was increased up to 0.075 g L^{-1} (Table 1). If based on the experimental data, the value of μ_{max} in all variations of NaCl concentration also have a higher value than the control and the highest value of μ_{max} was obtained under 0.05 g L^{-1} NaCl. The μ_{max} value of the experimental data were also observed to decrease when the NaCl concentration was further increased to 0.075 g L^{-1}. This showed that there were similarities in the μ_{max} trend between the model and experimental data.

The highest μ_{max} from model was obtained under 0.05 g L^{-1} NaCl i.e. 1.017 day^{-1}, followed by 0.025 g L^{-1} NaCl i.e. 0.817 day-1, 0.075 g L^{-1} NaCl i.e. 0.794 day^{-1}, and

control with the lowest μ_{max} i.e. 0.746 day^{-1}. The μ_{max} value was influenced by the period of the exponential phase of *Spirogyra* sp. A shorter exponential phase will result in a higher value of μ_{max}. At variations of 0.025 g L^{-1} and 0.05 g L^{-1} NaCl, the exponential phase only lasted for 4 days, while the exponential phase lasted for 6 days in the control and 0.075 g L^{-1} NaCl (Figure 1).

Similar results were reported by Lawton et al. (2015), the growth rate of freshwater macroalgae *Oedogonium* sp. increased under the addition of 1 g L^{-1} NaCl, but the growth rate decreased when the NaCl concentrations were further increased to 2 and 3 g L^{-1} [10]. A similar trend was also reported by Zhang et al. (2018), the growth rate of freshwater microalgae *Chlorella sorokiniana* increased when NaCl is added until certain concentration but the excessive concentrations of NaCl can inhibit their growth and reduce the growth rate of biomass. At variation of 10 g L^{-1} NaCl, *Chlorella sorokiniana* was observed to have the highest growth rate compared to other NaCl concentrations and control. When the NaCl concentration was increased to 20 and 30 g L^{-1}, the biomass growth rate subsequently decreased [21].

The addition of NaCl until a certain concentration can increase the growth rate of algae, but an excessive concentrations of NaCl can inhibit their growth [17]. Sodium ions (Na^+) could facilitate photosynthesis in algae through inorganic nutrient uptake, intracellular pH regulation, and alkalotolerance so algae are able to optimize their growth under certain NaCl concentration. However, excessive concentrations of NaCl in eukaryotic algae can cause water loss from cells and ionic imbalances that can disrupt cell metabolism and lead to decrease in growth and even cell death [17] [22] . The excessive concentrations of NaCl can also cause the degradation of chlorophyll in algae and lead to a decrease in the rate of photosynthesis and growth [23]. This is in accordance with the study by Kumar et al. (2016) which showed that the chlorophyll content of *Spirogyra* sp. continued to decrease with increasing NaCl concentration from 0.006 to 0.03 g L^{-1} [18]

In this study, the highest concentration of NaCl given to the culture was 0.075 g L^{-1} and it was observed that *Spirogyra* sp. can still tolerate NaCl concentration up to 0.075 g L^{-1}. Although the value of μ_{max} was observed to decrease when NaCl concentration was increased up to 0.075 g L^{-1}, the value of μ_{max} obtained was still higher than control (Table 1). Therefore, it is necessary to review the addition of NaCl higher than 0.075 g L^{-1} in the culture of *Spirogyra* sp. to see the inhibition of biomass growth due to excessive concentrations of NaCl

3.3 The Effect of Various NaCl Concentration on the Lipid Yield of *Spirogyra* sp.

The lipid yield of *Spirogyra* sp. was observed in BBM 2.5% and medium with various NaCl concentrations (0.025, 0.05, and 0.075 g L^{-1}). The results showed that all variations of NaCl concentration could enhance lipid yield in *Spirogyra* (Figure 4).

One-way ANOVA test showed that the addition of NaCl had a significant effect on lipid yield (p<α, =0.05).

The highest lipid yield was obtained under 0.075 g L^{-1} NaCl on 8th day of cultivation i.e. 90.71 mg g^{-1}. This result was 1.4 times higher than the highest lipid yield in control (63.83 mg g^{-1}). Variations in NaCl concentration of 0.025 and 0.05 g L^{-1} also showed higher lipid yield than control i.e. 73.53 mg g^{-1} and 87.22 mg g^{-1}, respectively. The lipid yield of *Spirogyra* sp. was observed to increase with the increase in NaCl concentration up to 0.075 g L^{-1}.

An increase in the lipid yield of *Spirogyra* when the NaCl concentration increased was also reported by Gopal & Ruma (2021). *Spirogyra punctulata* cultivated at various concentrations of NaCl had a higher lipid accumulation than the control. The highest lipid accumulation achieved in the *Spirogyra punctulata* culture was under 0.05 g L^{-1} NaCl with the highest lipid yield of 32.56% on 8th day of cultivation, which was 2 times better than control (16.3%). However, lipid accumulation was observed to decrease with increasing concentrations of NaCl higher than 0.05 g L^{-1} in *Spirogyra punctulata* cultures [9] . Lipid yield in freshwater microalgae *Chlamydomonas mexicana* and *Scenedesmus obliquus* were also increased up to two times compared to control under 1.5 g L^{-1} NaCl, but lipid yield decreased when the NaCl concentrations were increased to 3 and 6 g L^{-1} [22]. The enhancement of lipid yield under the addition of NaCl to a certain concentration were also observed in freshwater macroalgae and microalgae *Oedogonium* sp., *Ulva intestinalis*, *Chlorella sorokiniana* [9-10, 21]. This showed that the addition of NaCl at a certain concentration could enhance lipid yield, but lipid yield could decrease due to excessive concentrations of NaCl. In this study, the highest concentration of NaCl given to the culture was 0.075 g L^{-1} so that no decrease in lipid yield was observed due to excessive concentrations of NaCl. Therefore, it needs to be reviewed further by using a variation of NaCl concentration higher than 0.075 g L^{-1}.

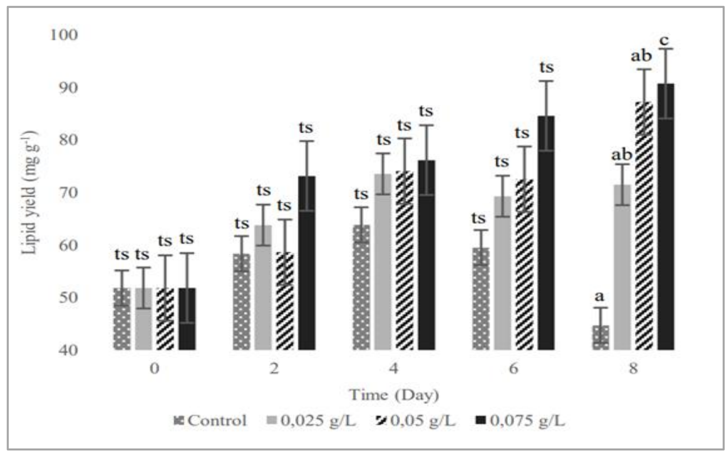

Figure 4. Lipid yield of *Spirogyra* sp. at various concentrations of NaCl

Note: The mean value of ± STD with the notation "ts" showed a non-significant difference (p>0.05); different letter notation showed a significant difference (p≤0.05).

According to Shetty et al. (2019), most green algae accumulate lipid under salinity stress [8]. Under conditions of high salinity, algae are stimulated to increase their lipid production, especially triacylglycerols, to protect them from salt stress [22]. Medium with the addition of NaCl might affect intracellular osmolarity due to hypertonic medium condition over the algae cells. It can cause the release of water from algae cells into the environment. Under these conditions, algae produce lipid in large quantities to prevent the escape of water from cells [24]. The rapid increase of lipids could enhance membrane rigidity which help algae to tolerate osmotic pressure caused by high salinity [25]. Furthermore, acetyl CoA carboxylase enzyme (ACCase) plays a major role in fatty acid biosynthesis. It has also been suggested that in fatty acid biosynthesis, the activity of ACCase may increase under the addition of NaCl which leads to the increase of synthesis Acetyl-CoA to malonyl-CoA [26]

3.4 Parameter Estimation of Lipid Production in *Spirogyra* sp.

Lipid production model of *Spirogyra* sp. was determined to estimate the α and β parameters. The model used was the Luedeking-Piret model. The Luedeking-Pirett model is commonly used to model product formation [27]. The model has fairly good fit with the experimental data because the correlation coefficient (R^2) were more than 0.8 (Table 2).

Table 2. Parameter estimation of lipid production in *Spirogyra* sp at various concentrations of NaCl

Parameter	Control	0.025 g L^{-1}	0.05 g L^{-1}	0.075 g L^{-1}
α (mg mg^{-1})	0.118	0.156	0.105	0.126
β (mg mg^{-1} day^{-1})	-4.61×10^{-3}	-7.1×10^{-3}	-2.01×10^{-3}	-2.03×10^{-3}
R^2	0.84	0.83	0.83	0.98

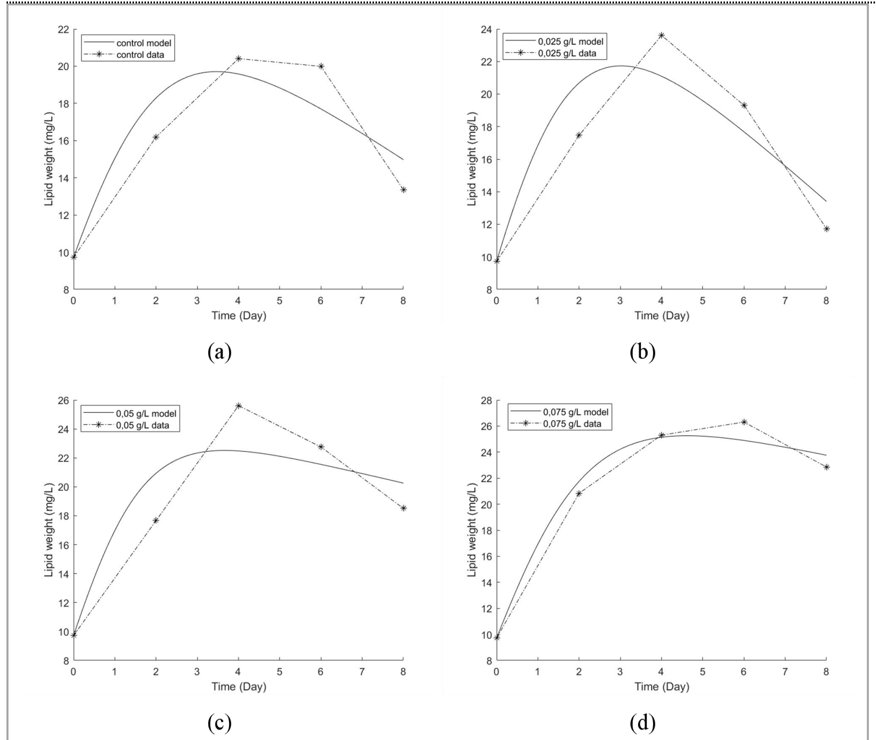

Figure 5. The lipid production model of *Spirogyra* sp. at various concentrations of NaCl (a) control, (b) 0.025 g L^{-1}, (c) 0.05 g L^{-1}, (d) 0.075 g L^{-1}

The parameter estimation from the model showed that the values of α and β for control and all variations of NaCl concentrations were not 0 (Table 2). α is growth correlation coefficient (mg mg^{-1}), β and is non-growth correlation coefficient (mg mg^{-1} day^{-1}) [13]. The values of α and β obtained fluctuate and didn't show a trend, but it was observed that all values of α were much higher than β in all variations. The values of $\alpha \neq 0$ and $\beta \neq 0$ showed that the lipid production in *Spirogyra* sp. is classified as class III, where lipid formation is partially associated with biomass growth. Class III involves a more complex process where the product is involved in many pathways, and it is indirectly related to the primary metabolism pathway [28]. Under conditions of high salinity, algae will increase the synthesis of neutral lipid, or triacylglycerols and certain glycerols, that will help the algae tolerate high salinity and protect them from stress [11, 23].

Several studies have also reported that the formation of lipid in algae is classified as class III metabolism. A study by Tevatia et al. (2012) showed the formation of lipid in the microalgae *Chlamydomonas reinhardtii* classified as class III and the modeling of lipid production in *Isochrysis galbana* reported by He et al. (2016) also showed lipid classified as class III [28-29]

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3.5 Estimation of Lipid Productivity

The estimation of lipid productivity was carried out at the end of cultivation on the 8th day. The highest estimation of lipid productivity was achieved under 0.075 g L^{-1} NaCl i.e. 8.79 mg g^{-1} day^{-1}, followed by 0.05 g L^{-1} NaCl i.e. 7.52 mg g^{-1} day^{-1}, control i.e. 5.79 mg g^{-1} day^{-1}, and 0.025 g L^{-1} NaCl with the lowest productivity of 5.3 mg g^{-1} day^{-1} (Figure 6). Lipid productivity at 0.075 g L^{-1} NaCl showed an increase up to 52% compared to the control. Under NaCl concentration of 0.075 g L^{-1}, the highest lipid yield was obtained in culture (Figure 4) and biomass accumulation did not decrease significantly on 8th day of cultivation (Figure 1). This can cause the highest lipid productivity obtained under the addition of 0.075 g L^{-1} NaCl.

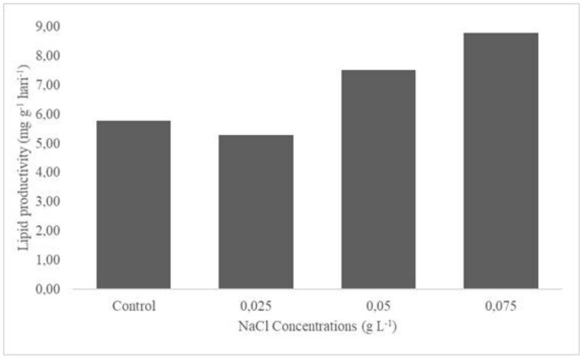

Figure 6. Estimation of *Spirogyra* sp. lipid productivity at various concentrations of NaCl

In this study, it was observed that the lipid productivity at 0.05 and 0.075 g L^{-1} NaCl had a higher value than the control, but the lipid productivity at NaCl 0.025 g L^{-1} had a lower value than the control. This could be due to the fact that during the 8th day of cultivation, the accumulation of biomass at 0.025 g L^{-1} NaCl had decreased significantly (Figure 1) so that the lipid productivity also decreased.

Lipid productivity was observed to increase with increasing NaCl concentration (Figure 6). Similar results were also reported by Zhang et al. (2018), where the lipid productivity of freshwater microalgae *Chlorella sorokiniana* was observed to increase with the addition of NaCl up to a certain concentration. The highest lipid productivity was achieved under 20 g L^{-1} NaCl with productivity 2 times higher than control. [21] . In a study conducted by Gopal & Ruma (2021), the optimal concentration of NaCl for growth and lipid production of *Spirogyra* punctulata culture was 0.05 g L^{-1} [9] . The optimal NaCl concentration is not much different from the results obtained from this study, where the addition of 0.075 g L^{-1} NaCl can achieve the best lipid productivity. The slight difference may be due to the different species of *Spirogyra* used. The optimal concentration of NaCl for growth and lipid production depends on the species and variety of algae [17].

4. Conclusions

The addition of NaCl could increase the biomass growth of *Spirogyra* sp. as indicated by the experimental results where the highest biomass growth was obtained under 0.05 g L^{-1} NaCl with maximum biomass (X_{max}) i.e. 344.93 mg L^{-1} and maximum specific growth rate (μ_{max}) i.e. 0.153 day^{-1}. The values of μ_{max} and X_{max} were observed to increase until the addition of 0.05 g L^{-1} NaCl and decrease when the NaCl concentration was further increased to 0.075 g L^{-1}. Based on parameter estimation from logistic model, the same trend was obtained for μ_{max} with the highest μ_{max} (1.017 day^{-1}) was obtained under 0.05 g L^{-1}. On the other hand, the X_{max} value obtained from the model showed an increasing trend along the increase in NaCl concentration with the highest value of X_{max} (338.4 mg L^{-1}) was obtained under 0.075 g L^{-1}. The addition of NaCl could enhance the lipid content in *Spirogyra* sp., lipid yield was observed to increase with increasing NaCl concentration. The highest lipid yield of 90.71 mg g^{-1} was obtained under 0.075 g L^{-1} NaCl. Parameter estimation from Luedeking-Piret model showed the value of α and $\beta \neq 0$, this result indicated that lipid production in *Spirogyra* sp. was partially associated with growth. The highest lipid productivity of 8.79 mg $g^{-1}day^{-1}$ was obtained under 0.075 g L^{-1} NaCl. This result showed that lipid productivity increases up to 52% compared to the control.

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Heterotrophic Growth of *Galdieria sulphuraria* in Wastewater for Food Ingredients Development : A Systematic Review

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Abstract

The rise in food-borne diseases makes consumers more health-conscious and demand natural food ingredients. The potential source of natural ingredients is microalgae, a microorganism with a wide range of functional components. Microalgae cultivation in heterotrophic conditions somehow increases biomass production and accelerates the cultivation period, which is valuable in upscale production. *Galdieria sulphuraria* is one of numerous heterotrophic microalgae species that exhibit considerable metabolic versatility and flexibility. Those unique abilities provide a new perspective in the development of food ingredients. This study aims to compare the growth of *G. sulphuraria* in utilizing various types of waste, by-products, or side-stream. A systematic review was carried out by reviewing works of literature and collecting secondary data from relevant papers. The outline presented in this review compares biochemical profile,biomass production and the suggestion on using wastewater from various sources like agricultural and food production. This review indicates that *G.sulphuraria* can grow heterotrophically and potential to be used as agent foralternative food ingredients production

Keywords: *Galdieria sulphuraria*, heterotrophic growth, natural food ingredient, systematic review, waste

1. Introduction

Microalgae are microscopic unicellular organisms capable of converting solar energy into chemical energy through photosynthesis. Microalgae have various benefits for humans, both in the industrial and commercial fields. One of the uses of microalgae in the industrial sector is the food industry, as microalgae is a rich source of carbohydrates, proteins, enzymes, fiber, fatty acids, vitamins, and antioxidants (Sathya *et al.* 2021; Bleakly and Hayes 2017; Kang *et al.* 2012).

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There have been many food products that involve microalgae as raw materials, and there has been an increase in microalgae-based food products on the market since 2015-2019, globally. Therefore, it is indispensable to conduct further research to find out other roles of microalgae in the food industry.

Microalgae is remarkably adaptable organism that may thrive in a variety of environments. Microalgae can be grown in three different ways: autotrophic, heterotrophic, and mixotrophic. Autotrophic microalgae develop by using energy from photosynthesis, whereas heterotrophic microalgae can grow in the dark using organic carbon. In production, mixotrophic microalgae can use both provided organic carbons and light energy. Because light penetration is inversely related to cell concentration, autotrophic growth leads to low biomass yields **(**Wan *et al.* 2016; Moraes *et al.* 2008**)**. Additionally, heterotrophic growth has none of these limitations and can provide significantly better growth rates and biomass yields. Today, the heterotrophic growth of microalgae with sugar as the energy and carbon source rather than sunlight and carbon dioxide is a commercialized approach. Due to fast heterotrophic growth, a tremendous amount of biomass can be obtained during a short time. Heterotrophic growth has been analyzed for several species of microalgae such as *Spirulina sp*. [27], *Scenedesmus sp*. [28], *Chlorella sp*. [29]. Some of these studies discovered enhancement of the growth rate up to 100 %. However, some species of microalgae are obligate autotrophs. Low light conditions has inhibited the synthesis of several biochemical components such as photosynthetic pigments.

The extremophilic red alga *Galdieria sulphuraria* is capable of chemoheterotrophic metabolism. *G. sulphuraria* belongs to the cyanidales, a group of polyextremophilic microalgae that thrive at temperatures as high as 56 ^{o}C. *G. sulphuraria* digests and metabolizes at least 27 sugars and 50 carbon sources as nutrients [2]. As a consequance, *G. sulphuraria* may thrive on any substrate, including wastes. *G. sulphuraria* for instance accumulates around 40% proteins when grown in presence of digestate and glucose [18]. *G. sulphuraria* not only has organic substrate absorption mechanisms but it also retains the photosynthetic apparatus [16]. The identical thing explained by [9] that photopigment production is suppressed in most heterotrophic microalgae, but *G. sulphuraria* can produce photopigments both when growing autotrophically in the light and heterotrophically in the dark. The antioxidant activity of biomass produced by a selected strain of *G. sulphuraria* as well as the proximate composition (protein, carbohydrates, fiber, and lipids) and micronutrient content (carotenoids, phycobiliproteins, chlorophylls, and vitamins) was determined by [5]. According to [7], *G. sulphuraria* macro and micronutrient profiles can be used to design food preparations and extract of some high-value phytochemicals. As a result, to manufacture the important chemical, heterotrophic culture of *G. sulphuraria* to enhance biomass may be used. Biomass development might be a beneficial technique in the food sector.

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Organic carbon sources, such as sugars or organic acids, are required to grow heterotrophic microalgae culture, which represents the main cost in the medium formulation [4]. Some wastewater, food by-products, or side-stream can be used as sole carbon and energy sources, resulting in significant cost savings. Industrial processing of plant-derived raw materials generates enormous amounts of by-products. On the one hand, these by-products pose a serious disposal problem because they emerge seasonally and are susceptible to microbial decay. They are, on the other hand, a rich source of valuable compounds, particularly secondary plant metabolites and cell wall materials, which can be recovered [25]. Two benefits can be obtained by utilizing waste, including waste treatment and biomass recovery. Furthermore, the biomass obtained can be used to create high-value materials. In this review, we want to answer a simple research question about factors affecting the effectiveness of using waste in the heterotrophic cultivation of *G.sulphuraria* and the challenges as well as opportunities in the food industry.

2. Methods

2.1 Formulation of the research question

The formulation of PICO was used for systematic reviews [23]. Each letter of PICO has a meaning as follow, the P for "population" is the subject that given treatment; I for "intervention" is the independent variable or the treatment; C for "comparison" as a control or comparison; and O for "outcome" which is the dependent variable of a relevant measure as the influence of the intervention given. In this study, each PICO approach was described as P: *Galdieria sulphuraria*, I: wastes, C: organic substrate, and O: biomass productivity and biochemical content of microalgae.

2.2 Literature Research

Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) criteria were used to conduct the literature search [24, 22]. Science Direct, Google Scholar, Pubmed, and Springer were used to conduct this step. "*Galdieria sulphuraria*," AND "heterotrophic," AND "waste," OR "wastewater," OR "by-product", AND "food functional" OR "ingredient" were some of the basic keywords utilized.

2.3 Study selection

The selection was done based on the inclusion and exclusion criteria. Inclusion criteria is a list of potential or any related papers that can be used to this systematic review [17]. In contrast, exclusion criteria mostly are unrelated, duplicated, unavailable full texts, or abstract-only papers. The inclusion criteria used in this study were full-text articles that reported biomass production and biochemical content of *G.sulphuraria*, articles that used wastes as media cultivation, published in 2017-2021, and published in reputable international peer-reviewed journals. The exclusion criteria used were grey literature (government reports, theses, and dissertations that have not been published), review articles, Life-cycle Assessment, and incomplete articles (abstract only).

3. Result and Discussion

According to the PRISMA flow chart (Figure 1), the initial searches resulted in 2857 articles. There were 1141 eliminated because it is duplicates articles and its remain 1716 articles. After reviewing the title and the abstract, 90 articles were selected. In total, 6 out of the 90 reports were eligible to answer the research questions in this systematic review based on relevancy.

3.1 Cultivation media affect the nutritional profile

Tabel 1 represents the characteristics of wastes used by selected articles. A study on six types of wastes indicated that the nutrient composition of the culture media had a substantial effect on the macro and micronutrient profiles of *G. sulphuraria*.

Table 1. The characteristics of wastes used

Author	Waste	Parameters	Value
Massa *et al.* 2019	spent Cherry-Brine Liquid (sCBL)	pH	4.2
		Total Suspended Solid (g/L)	0.64
		Dry matter (g/L)	88.4
		NH_4^-N (mg/L)	1.15
		NO_3^-N (mg/L)	11
		NO_2^-N (mg/L)	0.3
		N total (mg/L)	150
		P total (mg/L)	30.1
		TOC (g/L)	23.8
		SO_4^{2-} (mg/L)	162
		BOD (mg/L O2)	1200
		COD (mg/L O2)	65000
		Total sugar (%)	8.5
		Reducing sugar (%)	6.1
Russo *et al.* 2021	Second Cheese Whey (SCW)	pH	5.9
		Ash (g/L)	5.5
		Dry weight (g/L)	58.4
		Volatile solids (g/L)	53.3
		NH_4^-N (mg/L)	25
		NO_3^-N (mg/L)	80

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		N total (mg/L)	0.59
		Free amino nitrogen (mg/L)	231.14
		P total (mg/L)	96.1
		Reducing sugar (g/L)	43.4
		Protein content (g/L)	3.1
Scherhag and Ackerman 2020	Sugar-fond	Sucrose (g/L)	31.12
		Glucose (g/L)	39.02
		Fructose (g/L)	32.88
Scherhag and Ackerman 2020	Wastewater from the filling process	Sucrose (g/L)	35.22
		Glucose (g/L)	42.67
		Fructose (g/L)	36.33
Scherhag and Ackerman 2020	Apple-cleaning bath	Sucrose (g/L)	7.33
		Glucose (g/L)	5.5
		Fructose (g/L)	10.33
Sloth *et al.* 2017	Restaurant waste	Dry matter (%)	18.1
		starch (%)	33.5
		protein (%)	14.8
		fat (%)	12.9
		free sugars (%)	8.5
Sloth *et al.* 2017	Bakery waste	dry matter (%)	93.4
		strach (%)	61.6
		protein (%)	8.3
Zimmerman *et al.* 2020	Whey permeate	pH	4.65
		protein (%)	ND
		Ash (%)	0.47
		Total sugar (g/L)	20.3
		Lactose (g/L)	10.7
		Nitrate (g/L)	1.5

Pleissner *et al.* 2021	Hydrolyzed Straw	Ash (%)	3.7
		Lignin (%)	11.7
		Hemicellulose (%)	36.4
		protein (%)	4.9
		cellulose (%)	26.5
		fat (%)	ND
		starch (%)	ND
		free sugars (%)	1.2
		Glucose (g/L)	4.5
		ammonium (mg/L)	1.9
		Free amino nitrogen (mg/L)	19.7
		Phosphate (mg/L)	46
Pleissner *et al.* 2021	Hydrolyzed Digestate	Ash (%)	11.9
		Lignin (%)	ND
		Hemicellulose (%)	6.6
		protein (%)	47.7
		cellulose (%)	26.7
		fat (%)	ND
		starch (%)	ND
		free sugars (%)	ND
		Glucose (g/L)	0.5
		ammonium (mg/L)	23.8
		Free amino nitrogen (mg/L)	290.7
		Phosphate (mg/L)	7.1

The role of lactose in the metabolism of *G. sulphuraria* has been described by [15] as the author using whey permeate containing 10.7 g/L lactose (Table. 1). The presence of astaxanthin diester in the *G. sulphuraria* extracts derived from cultures containing 15-40% whey permeate. The biomass did not present the typical bluish color of phycocyanin-producers butthe color observed was yellowish. This report corresponds with [13], which discovered that heterotrophic growth on lactose produced bleaching in *G. sulphuraria* strain 107.79. [13] also found that a typical red algal chloroplast, as seen in autotrophic cells, was missing. Astaxanthin is one of the carotenoids that are minor pigments found in *G. Sulphuraria* [21]. Astaxanthin in the

form of di- and mono- ester have great interest due to their natural origin and their bioactive properties preventing certain eye diseases for humans, such as age-related macular degeneration and cataract.

In contrast, results were described by [12], which showed that *G. sulphuraria* produced phycocyanin on waste of restaurant and bakery hydrolysates can reach 5.5 mg/L and 4.4 mg/L, respectively, with the addition of 0.5 g/L NH_4^+ in the media. Ammonium is present only in limited concentrations in restaurant and bakery hydrolysates, which indicates that the microalgae still need inorganic supplementation in the use of waste. The addition of ammonium to the hydrolysates increased the specific phycocyanin content. Phycocyanin is a protein from the phycobiliprotein (or phycobilin) group, the structure made up of protein and non-protein components known as phycocyanobilins. The production of phycocyanin occurs concurrently with the synthesis of proteins, which is influenced by the predominant ammonium content [6,30,31] This statement is consistent with the results of [15], which revealed that the composition of whey permeate (Table 1.) used contains a slight amount of nitrogen sources. Nitrogen sources as a limiting factor of the biochemical components of heterotrophic *G.sulphuraria*, especially protein [18]. The protein content of *G. sulphuraria,* which was obtained from several different studies, is varied. Low protein content may indicate nitrogen limitation and continuous utilization of intracellularly stored protein.

As reported by [17], the proportion of glucose, protein, and lipids in microalgal biomass was influenced by the growth media composition. In spent Cherry-Brine Liquid (sCBL), organic nitrogen was present in trace amounts (Table 1), forcing the microalga to assimilate organic nitrogen, which probably shifts the metabolism of microalgae into carbohydrate and lipid synthesis. However, the sCBL biomass showed high carbohydrate content and polyphenols (5.3 vs. 1.6 mg/g). The biomass of *G.sulphuraria* had a high carbohydrate content in SCW-containing growth media as discovered by [9]. Reducing sugar might be enhanced the cellular glycogen content in heterotrophic cultures of *G. sulphuraria* as explained by Sakurai *et al.* (2016). However, the glycogen content of *G. sulphuraria* grown heterotrophically with waste has never been investigated. Most microalgae have glycogen as a food reserve. Glycogen is preferred to starch in nutritional applications due to its cold water solubility and enzyme accessibility. A unique feature of red microalgae is storing highly branched glycogen, such as amylopectin, which is used in products like sports drinks. *G~~aldieria~~ sulphuraria* can produce up to 50% glycogen (Mamoous *et al.* 2020, Schmidt *et al.* 2005, Graziani *et al.* 2013). This could be investigated further in terms of media formulation research to obtain the maximum amount of glycogen.

Microalgae can be metabolically very flexible, and their total lipid concentration and fatty acid composition can change significantly depending on medium composition. A study conducted by [8] discovered significant differences between *G~~.~~sulphuraria* biomass growth on SCW media and standard media (control). The control showed a greater concentration of oleic acid (up to 78% of total fatty acids, TFA) respect to the SCW samples (30–60% of TFA). The biomass grown on media containing 1.5% SCW showed an attractive fatty acid profile (g/100g), which consisted of 40.1 SFA, 35.43

monounsaturated fatty acids (MUFA), and higher polyunsaturated fatty acids (PUFA). Linoleic acid (C18:2) and linolenic acid (C18:3) are the predominant PUFA in the biomass of *G. Sulphuraria* [9], which have received considerable attention for their nutritional functions due to the functions of regulating blood lipids, clearing blood clots, regulating immune functions, etc. (He *et al.* 2020).

3.2 Biomass production

The waste contains nutrients that can be utilized by *G.sulphuraria* to produce biomass and the synthesis of biochemical components, as described in the previous chapter.

Table 2. *Galdieria sulphuraria* growth on a variety of wastes

Author	Year	Media	Maximum Biomass	μmax/day
Russo *et al.*	2021	second cheese whey (SCW) + 1% reducing sugar (RS)	3.87 g/L	0.19
Scherhag and Ackerman	2020	apple-cleaning bath	15 g/L	1.21
Sloth *et al.*	2017	restaurant waste hydrolysates + 5 g/L of glucose and 0.5 g/L NH_4^+	4.5 g/L	1.22
Pleissner *et al.*	2021	digestate and hydrolize straw	5 x 10^8/mL	1.20

The presence of free amino nitrogen (FAN) in SCW depleted after 12 days of cultivation (data not shown) yielded final biomass of 3.87 g/L (Table 2), indicating that when the presence of carbon sources in the medium is low, nitrogen dominates *G. sulphuraria* metabolism [17]. The combination of SCW and 1% RS resulted in a slow growth rate (0.191/day), presumably because the carbon source in SCW is lactose, and lactose absorption is slower than glucose uptake for *G. sulphuraria*, as described by [26]. Microalgae cultivation using dairy product waste offers opportunities to produce valuable biomass for various industrial applications. Rapid algal growth can be efficiently maintained in this waste despite the abundance of D-Lactose cannot be metabolized by any microalgal species [26].

However, *G. sulphuraria* growth rate on other wastes has a constant value. The waste materials used by [9, 12, 18] all contain glucose, although in varying concentrations. According to Khan *et al.* (2016), glucose produces significantly higher growth and respiration rates because glucose has more energy per mole than other substrates. According to the biomass gain, this result is likewise not

comparable to those obtained when glucose is used as the sole carbon source in the media. According to [3, 8, 11], using glucose as the sole carbon source yields biomass of 10-100 g/L.

3.3 Carbohydrates Consumption of *Galdieria sulphuraria*

Galdieria sulphuraria has a great metabolic flexibility, and has grown using not only glucose but also other components of the wastes. Carbohydrates consumption is often expressed as biomass substrate yield per gram of substrate consumed. A study by [10] shows that 0.64 grams of *G. sulphuraria* biomass are formed for every one gram of waste that comes from the apple-cleaning bath. The composition of the waste (Table 1.) consists of sucrose, glucose, and fructose. The specific substrate consumption rate achieved in this study was 2.41±0.14 gSub/gDW per day,- measured at pH 2 and 42◦C. Similar result by [9], shows that 0.61 grams of *G. sulphuraria* are formed for every one gram of waste from restaurant hydrolysates. Unlike the previous chapter, this chapter discusses the effect of environmental factors (pH and temperature) on consumption rate.

Galdieria sulphuraria is known to grow on sucrose, and it is thought that these species create extracellular hydrolases enzyme to breakdown di- or poly-saccharides. When glucose and fructose are in low concentration and sucrose is not entirely digested, substrate availability depends on pH value. Therefore, pH value is something that must be consideredwithin the treatment of wastewater containing sucrose. Environment with pH 3 or higher is not optimal condition for *G. sulphuraria*, because *G. sulphuraria* is demonstrated to be an acidophilic species. Several extracellular enzymes from acidophilic microbes are known to be functional at much lower pH than that inside the cells [27]. However, high cultivation temperatures increased the rate of sucrose cleavage. As mentioned by [10], at 42◦C, the specific growth rate was 216 % higher than at 30 ◦C and about 250 % higher than at 50 ◦C.

Consumption rate has a linear correlation with biomass production related with time profiles of growth of *Galdieria sulphuraria*. In the industrial context, the shorter the growth period, the more profitable it is. In this study, we recommend using waste in the form of hydrolyzate, which means that there are more simple components than complex components, so that microalgae do not have to prolonged the lag phase. Suppose waste in the form of hydrolyzate cannot be obtained. In that case, it is preferable to monitor the growth pH and temperature, because the enzymes that work to hydrolyze the complex components can only be active at certain temperatures and pH.

3.4 Scale-up Opportunities and Challenges

Microalgae cultivation and biorefinery platforms have attracted considerable attention for their ability to synthesize large quantities of value-added compounds. In addition to significant challenges to overcome, including biomass cultivation, compound recovery, downstream processing energy consumption, and scaling-up methods,there are different technical difficulties when it comes to introducing

microalgal-based ingredients into the food industry. The most concerning issue about the use of waste is we must adhere to FDA regulations.

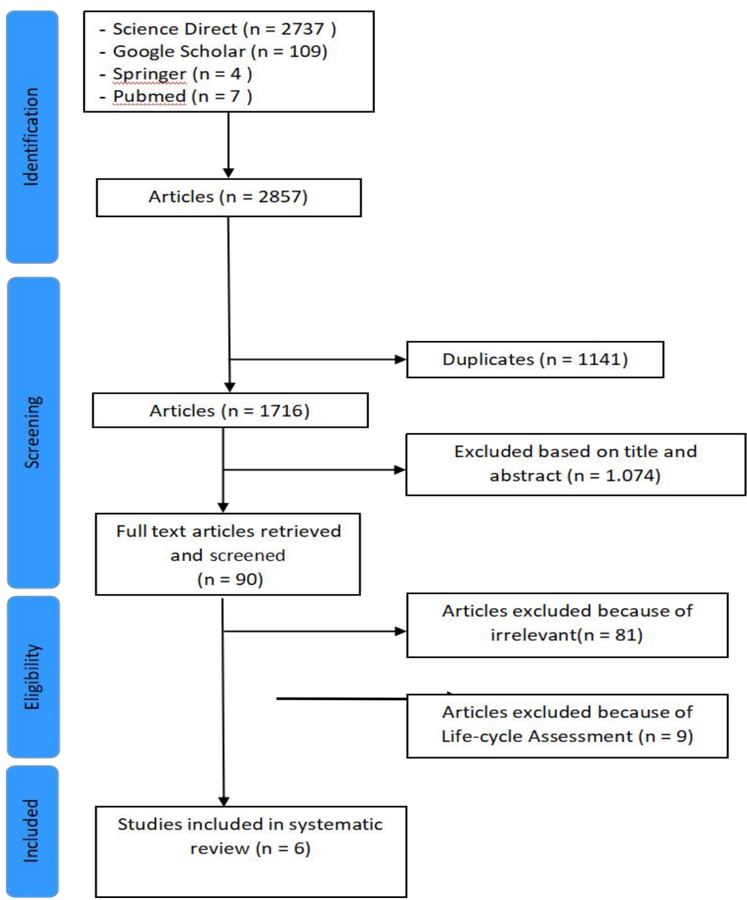

Figure 1. The preferred reporting items for systematic reviews and meta-analysis (PRISMA) flow diagram of the literature search process

4. Conclusion(s)

Heterotrophic microalgae cultivation using food waste represents an environmentally friendly method of producing valuable compounds. With some modifications to the production process, heterotrophic growth of *G. sulphuraria* in waste or by-product media may be feasible. However, waste cannot completely substitute organic carbon and inorganic compounds for the heterotrophic growth of *G.sulphuraria.* Therefore, we need a comprehensive review to build a formulation model to design suitable waste-based media for producing a specific compound. With additional research on the use of waste in food production, this systematic review has the potential to be expanded into a meta-analysis.

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Kinetic modelling of cultured *Spirogyra* sp. growth and lipid synthesis for lipid production under different concentrations of nitrogen

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Abstract

Spirogyra sp. is a freshwater macroalgae that can be used as fatty acids source because its lipid contained relatively high fatty acids content. The objective of this study was to determine the effect of nitrogen concentration to biomass growth and lipid accumulation on *Spirogyra* sp. with kinetic modelling approach. *Spirogyra* sp. was grown in modified Bold Basal Medium for 8 days, with concentrations of nitrogen as much as 6.25 (control); 3.125; 1.25; and 0.625 mg/L; respectively. Biomass accumulation on *Spirogyra* sp. that grown in sufficient nitrogen was higher than that grown on nitrogen deficiency, with the highest biomass was 335.8 mg/L at control. From logistic growth modelling, the lower the nitrogen, the higher the maximum specific growth rate, and the lower the maximum biomass. Lipid yield on *Spirogyra* sp. that was grown in nitrogen sufficiency was lower than that grown in nitrogen deficiency, with the highest lipid yield was 84.4 mg/g at 0.625 mg/L. From Luedeking-Piret lipid production modelling, the lower the nitrogen, the higher the lipid formation coefficient, the lower the non-growth correlation coefficient. The obtained model parameters were used to estimate lipid productivity, and the highest lipid productivity obtained in this study was 16.92 $mg.g^{-1}day^{-1}$ at 0.625 mg/L.

Keywords: growth, lipid, modelling, nitrogen deficiency, *Spirogyra* sp.

1. Introduction

Lipids are group of natural compounds that have high solubility on nonpolar solvent. On living organisms, lipids have many functions such as energy storage, membrane structure, enzyme cofactor, electron carrier, and light absorbing pigment [1]. Most of the lipids on living organisms are used as energy storage, that stored in the form of fatty acids [2] . Fatty acids have many benefits to human health [3] . There are some fatty acids that can't be synthesized by humans, and those fatty acids are called essential fatty acids, such as omega-3 fatty acids. The source of omega-3 fatty acids that have been consumed by humans are obtained from fish [4]. However, fish as omega-3 fatty acids source started to be seen as an unsustainable resource because the fish that needed for human use have exceeded the ocean capacity, and because there is a chance of some dangerous chemicals in the fish oil because the sea ecosystem that can't be controlled [5]. To replace fish that viewed as an unsustainable resource, an alternative is needed, and one alternative that can be used is macroalgae.

Macroalgae have several characteristics such as high growth rate, high productivity, can process CO_2 waste, and didn't use arable land to cultivate [6] . These characteristics made macroalgae have potential to be used as lipid that contains omega-3 fatty acid. Freshwater macroalgae also not used as a food, so it didn't interfere with food security. One of the freshwater macroalgae species that can be found in Indonesian freshwaters is *Spirogyra* sp. This macroalgae contained relatively high lipid content, reaching 14.8-21% [7] , while other species of macroalgae only contained 1-4% (w/w dry weight) [8]. Omega-3 fatty acids content in *Spirogyra* sp. could reached 40% [9].

Lipid content in algae could be enhanced by giving it some stresses during cultivation. One of the stress that can increase lipid content in algae is nitrogen deficiency. Nitrogen deficiency could affect lipid biosynthesis pathway in algae, so that the biomass growth and lipid production in algae was also affected [10] . Carbon on algae was converted to lipids and carbohydrates when algae reached the growth phase [11] . Algae that grown on nitrogen deficiency condition degraded compounds that contains nitrogen (such as chlorophyll and protein), and used the nitrogen for cellular maintenance [12]. There were already some studies that proved that nitrogen deficiency condition enhanced lipid production in some algae species, such as *Chlorella vulgaris*, *Neochloris oleoabundans*, *Botryococcus braunii*, dan *Ulva pertusa* [13-16].

Nitrogen deficient treatment could enhance lipid production, but could also decrease biomass growth, so it is necessary to optimize the best lipid productivity [17]. Modelling was done to simplify the optimization of lipid production process, and to reduce the costs [18]. Therefore, in this study, *Spirogyra* sp. growth was modelled with logistic equation, and lipid production was modelled with Luedeking-Piret equation, so that the lipid productivity in *Spirogyra* sp. could be estimated and the optimum results could be obtained.

2. Materials and Methods

2.1 Materials

2.1.1 *Spirogyra* sp. Culture

Spirogyra sp. culture was obtained from fishing ponds in Majalaya, West Java.

2.2 Methods

2.2.1 Medium Preparation

Medium that used to cultivate *Spirogyra* sp. was modified Bold Basal Medium (BBM). The components of the medium were presented in Table 1.

Table 1. Bold basal medium composition with 2,5% concentration

Component	Concentration (mg/L)
$NaNO_3$	6,25
$CaCL_2.2H_2O$	0,625
$MgSO_4.7H_2O$	1,875
K_2HPO_4	1,875
KH_2PO_4	4,375
NaCl	0,625
Alkaline EDTA *solution*	
EDTA (Titriplex III)	12,5
KOH	7,75
Acidified iron solution	
$FeSO_4.7H_2O$	1,245
H_2SO_4	1,245
H_3BO_3	2,855
Trace metal solution	
$ZnSO_4.7H_2O$	2,205
$MnCl_2.4H_2O$	0,36
MoO_3	0,1775
$CuSO_4.5H_2O$	0,3925
$Co(NO_3)_2.6H_2O$	0,1225

All the components of the medium were mixed and added with aquadest until the volume reached 500 mL. $NaNO_3$ concentrations was varied with the concentrations used were 6.25 (control); 3.125; 1.25; and 0.625 mg/L [19].

2.2.2 Cultivation

Cultivation of *Spirogyra* sp. was initiated by inoculating 3 grams of fresh algal biomass on 1 L plastic container with a size of 14 x 8.5 x 7.5 cm, with 0.5 L BBM medium in it (Figure 1). *Spirogyra* sp. was cultured on room temperature, which was 25±3°C, with a light intensity of 30,38±3,38 μmol photon $m^{-2}s^{-1}$, photoperiods light:dark 12:12 hours light-dark, and with an aeration of 0,3±0,1 L/min. The pH

level was measured periodically with pH indicator paper, and showed a pH of 7. In control condition, *Spirogyra* sp. was cultured on BBM medium with 2.5% power, with $NaNO_3$ concentration was 6.25 mg/L. In nitrogen deficiency conditions, $NaNO_3$ concentrations was modified, with the concentrations used were 3.125; 1.25; and 0.625 mg/L. *Spirogyra* sp. was grown for 8 days, with 3 replications.

Figure 1. *Spirogyra* sp. cultivation in the control medium on the 4th day

2.2.3 Sampling

Sampling was done every 2 days in 8 days of cultivation. The biomass was filtered with a mesh 60 sieve, and weighed with analytical balance to get the fresh weight. *Spirogyra* sp. was dried by spreading the fresh biomass on filter paper and placing it under running fan for 24 hours on room temperature (25±3°C), and after that it was dried in an oven at 105°C for 15 minutes [20]. The dried sample was weighed to get the dry weight. Experimental maximum specific growth rate was calculated using Eq. 1.

$$\mu_{max} = \frac{\ln(X_t) - \ln(X_0)}{t_t - t_0} \quad (1)$$

Where X is biomass concentration (mg/L), t is time (day), μ_m is maximum specific growth rate (/day).

2.2.4 Lipid Extraction

Lipid extraction was carried out using Bligh & Dyer (1959) method [21]. Dry algal biomass was put in a centrifuge tube. After that, 4 mL of methanol and 2 mL of chloroform was poured into the falcon tube, and mixed with a vortex for 2 minutes. The 2 mL of chloroform was added to the mixture, and mixed with a vortex for 2 minutes. The 3.6 mL of aquadest was added to the mixture and mixed with a vortex for 2 minutes. The mixture was centrifuged at 2000 rpm for 10 minutes. The top part of the solution was removed by pipette. The bottom part of the solution was filtered with Whatman Paper No.1, so the solids and the supernatant were separated. The solids that were filtered was put in another falcon tube for a second extraction. The second extraction was carried out by pouring 4 mL of 10% (v/v) methanol in chloroform into the filtered solids, and mixed with a vortex for 2 minutes. The mixture was centrifuged, filtered with filter paper, and the chloroform phase of the mixture was taken out. The chloroform phase from the second extraction was added to the first extract. The mixture was evaporated on a heater at 70°C to get the lipid

extract. To completely evaporate the solvent, the lipid extract was dried in an oven at 105°C for 15 minutes.

2.2.5 Kinetic Modelling of Biomass Growth

Biomass growth was modelled with logistic equation shown in Eq. 2. Growth parameters, such maximum specific growth rate (μ_m) and maximum biomass concentration (X_m), was estimated by fitting the biomass growth over time data against the growth model that was obtained with Eq. 2. Curve Fitting function on MATLAB was used to fit the data, whereas time as x axis and biomass growth as y axis.

$$X = \frac{X_0 e^{\mu_m t}}{1 - \frac{X_0}{X_m}(1 - e^{\mu_m t})} \qquad (2)$$

Where X is biomass concentration (mg/L), t is time (day), μ_m is maximum specific growth rate (/day), X_m is maximum biomass concentration (mg/L), and X_0 is initial biomass concentration (mg/L).

2.2.6 Kinetic Modelling of Lipid Production

Lipid production was modelled with Luedeking-Piret equation shown in Eq. 3. Growth parameters, such maximum specific growth rate (μ_m) and maximum biomass concentration (X_m), was obtained from biomass growth model. Lipid production parameters, such as lipid formation coefficient (α) and non-growth correlation coefficient (β) was estimated by fitting the lipid concentration over time data against the lipid production model that was obtained with Eq. 3. Curve Fitting function on MATLAB was used to fit the data, whereas time as x axis and lipid concentration as y axis.

$$P = P_o - \alpha X_0 + \propto \left(\frac{e^{\mu_m t}}{\left(1 - \frac{X_0}{X_m}\right)(1 - e^{\mu_m t})} \right) + \beta \frac{X_m}{\mu_m} ln\left(1 - \frac{X_0}{X_m}(1 - e^{\mu_m t})\right) \qquad (3)$$

Where P is lipid concentration (mg/L), t is time (day), α is lipid formation coefficient (mg/mg), β is non-growth correlation coefficient (mg/mg-day), and P_0 is initial lipid concentration (mg/L).

2.2.7 Analysis Mehods

Biomass and lipid weight data that obtained every 2 days in 8 days was averaged using Equation (4), and the standard deviation from the data obtained was calculated using Equation (5).

$$\bar{x} = \frac{\sum_{i=1}^{n} xi}{n} \qquad (4)$$

$$s = \sqrt{\frac{1}{N-1}\sum_{i=1}^{n}(x_i - \bar{x})^2} \quad (5)$$

The obtained data were tested using Kolmogorov-Smirnov test to see the normality of the data. If the data obtained were evenly distributed, the data were tested using ANOVA test to determine whether there were differences between treatments.

3. Results and Discussion

3.1 Effect of Nitrogen Concentrations to Growth

Spirogyra sp. growth on nitrogen sufficiency (6.25 mg/L $NaNO_3$) showed differences with that grown on $NaNO_3$ concentrations of 3.125; 1.25; and 0.625 mg/L (Figure 2). On sufficient nitrogen condition, biomass accumulated until the 6th day with a weight of 335.82 mg/L, and after that it was stationary. On deficient nitrogen condition, biomass accumulated until the 2nd day, entered the stationary phase until the 4th day, and after that it decreased. Biomass weight at day 4 in $NaNO_3$ concentrations of 3.125; 1.25; and 0.625 mg/L, respectively, was 317.67; 286.73; 276.87 mg/L. Based on the ANOVA statistic test, biomass growth data that obtained in this study were insignificantly different on day 0, 2, and 4, but significant on day 6 and 8.

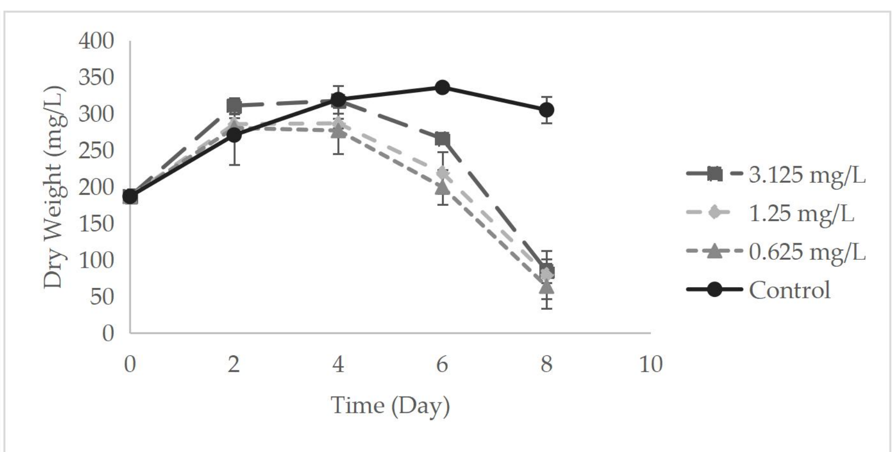

Figure 2. *Spirogyra* sp. growth in medium with $NaNO_3$ concentrations of 6.25; 3.125; 1.25; and 0.625 mg/L

In this study, results indicated that the lower the nitrogen concentration, the lower the biomass that accumulated. This could happen because nitrogen deficiency condition caused algae couldn't have enough nitrogen to synthesize biomass, thus causing less biomass to be formed. Nitrogen itself was one of the elements that needed in large quantities for algal growth, because it was used in many compounds such as protein and chlorophyll, so the lack of nitrogen will cause less biomass to be formed. The decrease in biomass could also occur because nitrogen deficiency condition caused algae to degrade chlorophyll to obtain nitrogen, thus

causing the decreased in photosynthetic activity, and the less biomass was formed [12] . The decrease in biomass growth on algae that grown on nitrogen deficiency condition also occurred in several algal species, such as *Chlorella vulgaris, Botryococcus braunii,* and *Ulva pertusa* [13, 15, 16].

The color difference of *Spirogyra* sp. that grown on nitrogen sufficient condition and nitrogen deficiency condition could be seen, where in the nitrogen sufficient condition, biomass was still green, but in nitrogen deficient condition, biomass started to turn brown (Figure 3). This indicates that in nitrogen deficiency condition, *Spirogyra* sp. reached the death phase faster, so the decrease in biomass accumulation happened.

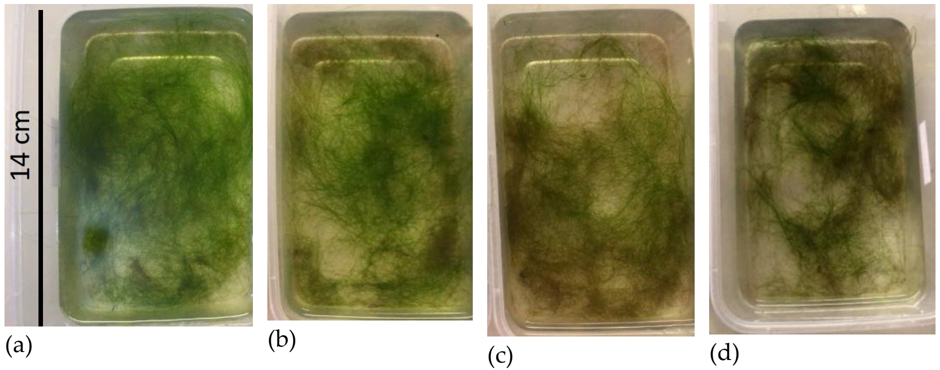

Figure 3. *Spirogyra* sp. culture at day 6 with $NaNO_3$ concentrations (a) 6.25, (b) 3.125, (c) 1.25, and (d) 0.625 mg/L

In nitrogen sufficient condition, *Spirogyra* sp. could continue to accumulate biomass until day 6, while in nitrogen deficiency condition, biomass could only accumulate until day 2. This could be due to the difference in nitrogen concentration used. Nitrogen deficiency condition caused photosynthetic activity decreases due to the use of chlorophyll as nitrogen source for protein synthesis, causing cell division to stop [22]. In nitrogen sufficient condition, there was more nitrogen available, chlorophyll was not used as nitrogen source, photosynthetic activity was maintained, so the algae could form biomass for longer than the algae that grown on nitrogen deficiency condition. This also happened on *Ulva prolifera*, where those that grown in nitrogen deficiency condition had lower chlorophyll content and growth rate than those that grown on nitrogen sufficient condition [23].

3.2 Biomass Growth Modelling Analysis

In this study, *Spirogyra* sp. growth data was modelled to determine growth kinetic parameters of *Spirogyra* sp. in medium with various nitrogen concentrations. Biomass growth modelling was carried out using logistic equation, with Curve Fitting function on MATLAB (Figure 4).

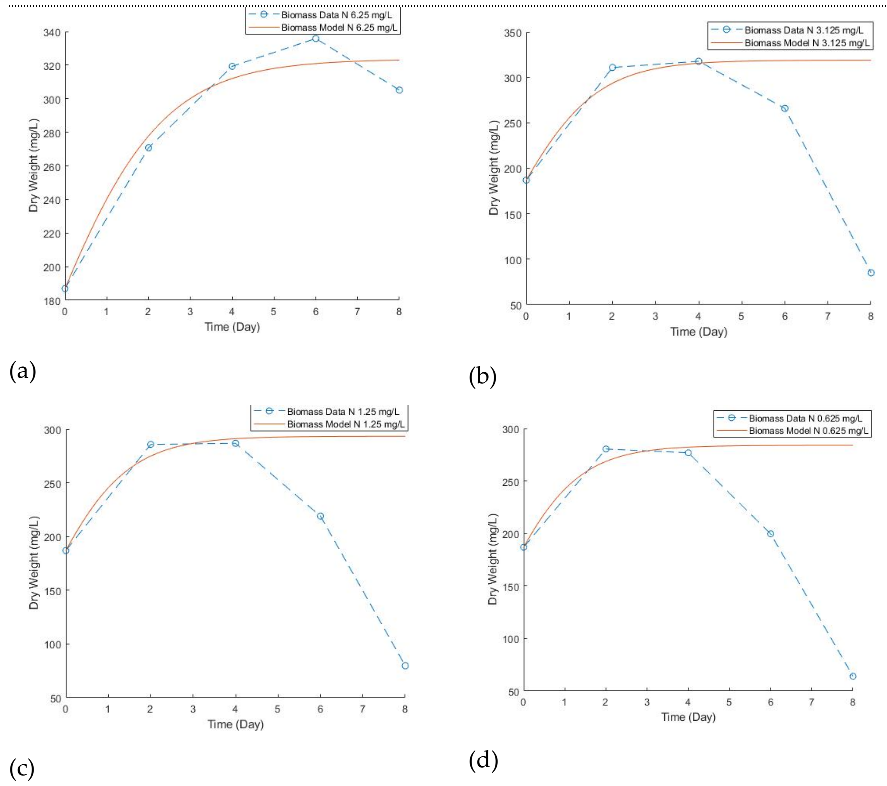

Figure 4 Biomass growth model of *Spirogyra* sp. with $NaNO_3$ concentrations of (a) 6.25, (b) 3.125, (c) 1.25, and (d) 0.625 mg/L

Table 2. Experimental and model growth parameter

Experimental Parameter				
Parameter	**Control**	**3.125 mg/L**	**1.25 mg/L**	**0.625 mg/L**
μ_{max}	0.098	0.254	0.212	0.203
X_{max}	335.8	317.7	286.7	280.5
Modelling Parameter				
Parameter	**Control**	**3.125 mg/L**	**1.25 mg/L**	**0.625 mg/L**
μ_{max}	0.7456	1.050	1.069	1.103
X_{max}	323.6	318.9	293.3	284.0
R^2	0.95	0.97	0.98	0.97

Logistic equation is an equation that could be used to model algal growth. Logistic equation is a sigmoidal function, so it could model the lag, exponential, and stationary phase of algal growth [24]. Logistic equation could not model the decrease in biomass, so in this study, the logistic model was carried out just until the stationary phase, for the control treatment until the 8th day, and for the nitrogen deficiency treatment until the 4th day. With the curve fitting function on MATLAB, the R^2 value obtained when comparing the model with the experimental data was above 0.9, so it can be concluded that logistic model could be used to model *Spirogyra* sp. growth in various nitrogen concentration.

The experimental growth parameters obtained were different from the modelling parameters (Table 2). The difference between μ_{max} value from experiment and from model was the μ_{max} value from model was higher than from experiment, and the order of the largest to the smallest value of μ_{max} was also different. The similarity between the experimental and the modelling parameters were on both parameters, the μ_{max} value in the nitrogen deficiency condition was bigger than in the nitrogen sufficient condition. The difference in value could occur because μ_{max} value from the experimental parameters were calculated with exponential equation, while the modelling parameters were calculated with logistic equation. In experimental parameters, the μ_{max} value was directly proportional with the X_{max} value because the bigger the difference between initial biomass weight with the X_{max} value, the bigger the μ_{max} value obtained. In nitrogen sufficient condition, the μ_{max} value from the experimental parameter was smaller than in nitrogen deficiency condition because in nitrogen sufficient condition, the growth lasted until the 6th day, while in nitrogen deficiency was only until 2nd day. For the X_{max} parameter, the obtained value showed little difference between experimental and modelling parameters. The difference between experimental and modelling parameters indicated that there were differences between the experimental data and resulting model.

Maximum specific growth rate and maximum biomass concentration of *Spirogyra* sp. was determined by experiment and by modelling. According to the experimental and modelling growth parameter values, it was found that the value of μ_{max} and X_{max} of each variation were different, so it could be determined that *Spirogyra* sp. biomass biosynthesis was influenced by nitrogen concentration (Table 2). Experimentally, order of μ_{max} values from largest to smallest, respectively, was obtained at $NaNO_3$ concentrations of 3.125 mg/L, 1.25 mg/L, 0.625 mg/L, and the last in the control treatment, and for the X_{max} value, the order from highest to lowest, respectively, was obtained in the control treatment, 3.125 mg/L, 1.25 mg/L, and the last in 0.625 mg/L. By modelling, order of μ_{max} values from largest to smallest, respectively, was obtained at $NaNO_3$ concentrations of 0.625 mg/L, 1.25 mg/L, 3.125 mg/L, and the last in the control treatment, and for the X_{max} value, the order from largest to smallest, respectively, was obtained in the control treatment, 3.125 mg/L, 1.25 mg/L, and the last in 0.625 mg/L. The obtained parameters indicated that *Spirogyra* sp. has higher growth rate when cultured on nitrogen deficiency condition, but the biomass maximum concentration was smaller than that grown on nitrogen sufficient condition. There was a study that shows some algae species have higher nitrogen uptake when grown on nitrogen deficient medium [25]. This could happen because one of the strategies that algae used to adapt to nitrogen deficiency

situation is luxury consumption of nitrogen, which will allow the algae to accumulate nitrogen for future usage [26]. Higher nitrogen uptake on nitrogen deficiency medium will make algae have higher growth rate on the start of the cultivation, but because the nitrogen supply was low, algae will reach death phase faster, while on nitrogen sufficient medium, the growth rate was lower but more stable, making the maximum biomass concentration higher.

Based on the obtained parameters, the trend was the higher the nitrogen concentration, the lower the μ_{max} value, the higher the X_{max} value. This also happened on algae *Isochrysis galbana*, when cultivated on medium with various nitrogen concentration, the higher the nitrogen concentration, the lower the μ_{max} value, the higher the X_{max} value [27]. But, on algae *Ulva lactuca*, it shows that the higher the nitrogen concentration, the higher the μ_{max} and X_{max} value [28]. In this study, the μ_{max} values on nitrogen deficiency condition were higher because the increase in biomass on nitrogen sufficient condition lasted until day 6th, while on nitrogen deficient condition, only lasted until day 2nd, and stationary untul day 4th. This difference caused the value of μ_{max} on nitrogen deficient condition was higher than on nitrogen sufficient condition. The X_{max} value that was obtained from the modelling was in accordance with the results of the experiment, where the higher the nitrogen concentration, the higher the maximum biomass concentration. However, because the logistic model couldn't model the decrease in biomass, the model obtained in this study could only be used until the end of the stationary phase.

3.3 Effect of Nitrogen Concentrations to Lipid Production

Lipid yield on *Spirogyra* sp. that cultivated on several nitrogen concentrations, which is 6.25 mg/L (control); 3.125; 1.25; and 0.625 mg/L, showed differences (Figure 5). It can be seen that there were differences between *Spirogyra* sp. that cultivated on nitrogen sufficiency and those on nitrogen deficiency. On nitrogen sufficiency, lipid yield increased until the 4th day, reaching 63.83 mg/g, and after that it decreased. On nitrogen deficiency, the lipid yield was higher than on nitrogen sufficiency, with the lipid on the 8th day for $NaNO_3$ concentrations of 3.125; 1.25; and 0.625 mg/L, respectively, were 69.35; 72.13; and 84.40 mg/g. The order of lipid yield from highest to lowest, respectively, was at $NaNO_3$ concentration 0.625 mg/L, 1.25 mg/L, 3.125 mg/L, and the last in control treatment. Based on the ANOVA statistic test, lipid yield data that obtained in this study were insignificantly different on day 0, 2, and 4, but significant on day 6 and 8.

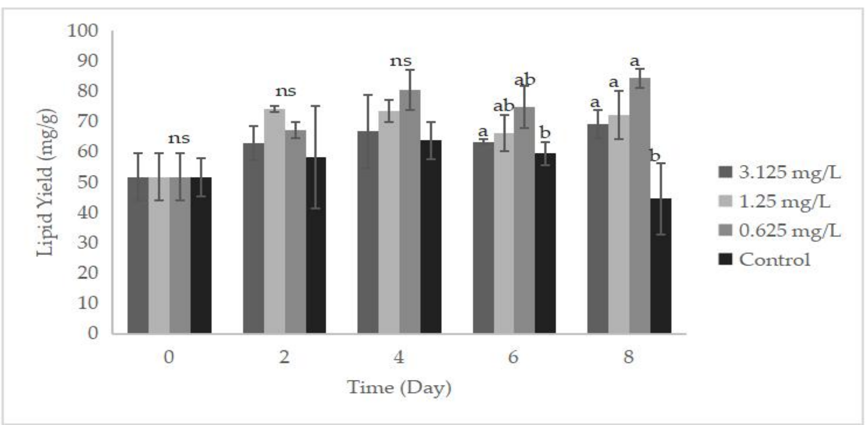

Figure 5. Lipid yield profile on *Spirogyra* sp. with $NaNO_3$ concentrations of (a) 6.25, (b) 3.125, (c) 1.25, and (d) 0.625 mg/L

[a,b,c,ns] Mean ± standard deviation with different notation on the same column show significant difference with $p < 0.05$, while ns notation show not significant

In this study, it was found that lipid yield on nitrogen deficient condition was higher than on nitrogen sufficient condition, with a trend that the lower the nitrogen concentration, the higher the lipid yield. This could happen because nitrogen deficiency condition caused algae to degrade macromolecules that contain nitrogen, to accumulate carbon reserves compounds, such as carbohydrates and lipid [29]. Other than that, nitrogen deficient condition also caused radicals on algae overproduced, and due the lack of antioxidant production, cellular oxidative stress level will increase and triggered lipid accumulation [12]. Increase in lipid yield by algae that grown on nitrogen deficient condition also occurs in several algae species, such as *Chlorella vulgaris*, *Botryococcus braunii*¸ and *Ulva rigida* [13, 30, 31].

In this study, it was found that on the 8th day, lipid yield in the control treatment decreased, while in nitrogen deficiency, the lipid yield increased. This also happened on algae *B. braunii*, where those that grown on nitrogen sufficient condition produced constant lipid yield, while those that grown in nitrogen deficiency produced increased lipid [15]. The decrease in lipid yield could occurred because the biomass produced decrease, so the lipid produced was also reduced, whereas, in *Spirogyra* sp. that grown on nitrogen deficient condition, lipid yield increased because nitrogen deficiency triggered algae to accumulate lipid, so that even though the biomass produced decreased, the lipid yield still increased. This phenomenon also occurred in other algae species, such as *Chlorella vulgaris* and *Neochloris oleoabundans* [14].

3.4 Lipid Production Modelling Analysis

In this study, *Spirogyra* sp. lipid production data was modelled to determine lipid production kinetic parameters of *Spirogyra* sp. in medium with various nitrogen concentrations. Lipid production modelling was carried out using Luedeking-Piret

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equation, with Curve Fitting function on MATLAB (Figure 6).

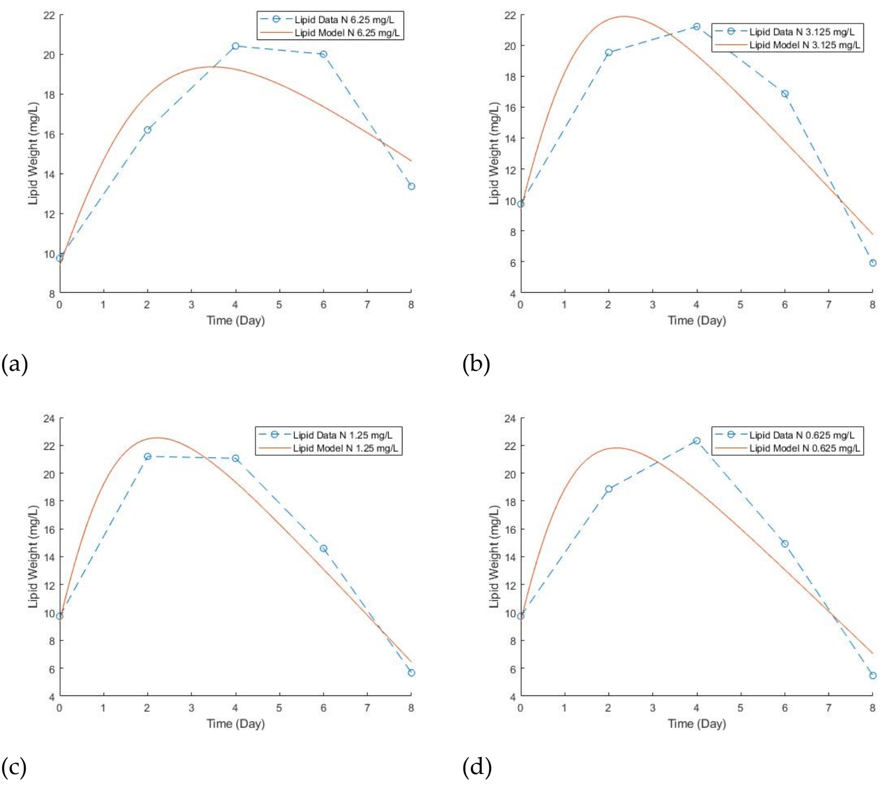

Figure 6 Lipid production model of *Spirogyra* sp. with $NaNO_3$ concentrations of (a) 6.25, (b) 3.125, (c) 1.25, and (d) 0.625 mg/L

Table 3. Lipid production model parameter

Parameter	**Control**	**3,125 mg/L**	**1,25 mg/L**	**0,625 mg/L**
α	0.1183	0.16	0.2114	0.2136
β	-0.0046	-0.0095	-0.0114	-0.0106
R^2	0.84	0.88	0.96	0.85

Luedeking-Piret equation was an equation that could be used to model lipid production on algae. Luedeking-Piret equation used μ_{max} and X_{max} value from growth parameters to model lipid production and produced kinetic parameters, such as α (lipid formation coefficient) and β (non-growth coefficient). With the curve fitting function on MATLAB, the R^2 value obtained when comparing the model with the experimental data was above 0.8, so it could be concluded that Luedeking-Piret model could be used to model lipid production on *Spirogyra* sp. that grown on various nitrogen concentration, but still not be able to display a

model that was exactly the same with the experiment data. This could be overcome by taking more sample points, so the curve fitting process could be done more accurately.

From the modelling results, it was found that α and β value from each variation were different, so it can be determined that lipid biosynthesis on *Spirogyra* sp. was affected by nitrogen concentration. The order of the α value from the highest to the lowest, respectively, was obtained on $NaNO_3$ concentration of 0.625 mg/L; 1.25 mg/L; 3.125 mg/L; and the last on the control treatment, whereas the order of the β value from the highest to the lowest, respectively, was obtained on the control treatment; on $NaNO_3$ concentration of 3.125 mg/L; 0.625 mg/L; and the last on 1.25 mg/L (Table 3). The parameters indicated that *Spirogyra* sp. had higher α value and lower β value if grown on nitrogen deficient condition. The α and β values were not zero, thus indicating that lipid formation on *Spirogyra* sp. was partially related to biomass growth. This could happen because *Spirogyra* sp. could grow well with low lipid accumulation on nitrogen sufficient condition, while on nitrogen deficient condition, biomass growth was inhibited but with increased lipid production [27]

Based on the parameters obtained, the higher the nitrogen concentration, the lower the α value, and the higher β value. This also happened on *Chlorella salina* and *Nannochlorophis oculate*, where α value was higher when grown on nitrogen deficient condition [24]. The α value from Luedeking-Piret equation was affected by the increased lipid production, the higher the increase in lipid, the higher the α value. The negative β value occurred because the lipid weight obtained in this study decreased due to the decrease in biomass.

3.5 Lipid Productivity Estimation

Growth and lipid production kinetic parameters for *Spirogyra* sp. that obtained were used to determine which nitrogen concentration yields the highest lipid productivity. The order of lipid productivity from highest to lowest, respectively, was obtained at $NaNO_3$ concentration of 0.625 mg/L; 1.25 mg/L; 3.125 mg/L; and the last on control treatment (Figure 7).

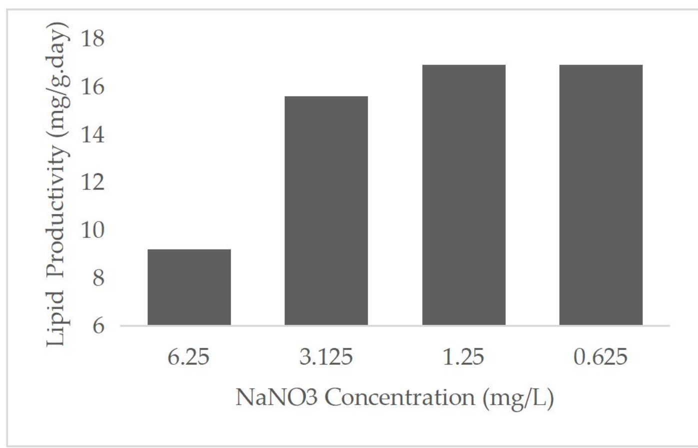

Figure 7. *Spirogyra* sp. lipid productivity estimation

Lipid productivity of *Spirogyra* sp. that grown on medium with various nitrogen concentration (Figure 7) indicated that lipid productivity on nitrogen sufficient condition was lower than on nitrogen deficient condition. This shows that on *Spirogyra* sp. that grown on nitrogen deficient condition, the increase in lipid production exceeded the decrease in biomass production, so the lipid productivity still increased [14]. In this study, the highest lipid productivity was achieved on $NaNO_3$ concentration of 0.625 mg/L, with the lipid productivity obtained was 16.92 mg $g^{-1}day^{-1}$. This value indicates that at this concentration, although the biomass formed was lower, the lipid yield obtained was higher than other nitrogen concentrations, thus causing the lipid productivity on this concentration was higher than the others.

4. Conclusions

Nitrogen deficient condition affected *Spirogyra* sp. growth, where the higher the nitrogen condition, the more biomass was formed. Based on the growth experimental parameters that obtained, the higher the nitrogen concentration, the higher the X_{max} value, while for the μ_{max} value, the order from highest to lowest, respectively, was obtained on $NaNO_3$ concentration of 3.125 mg/L; 1.25 mg/L; 0.625 mg/L; and the last on control treatment. Based on the modelling results, the kinetic parameters that obtained from logistic equation was, the higher the nitrogen concentration, the lower the μ_{max} value, the higher the X_{max} value. Nitrogen deficient condition also affected lipid production on *Spirogyra* sp., where the lower the nitrogen concentration, the more lipid was produced. Based on the modelling results, the kinetic parameters that obtained from Luedeking-Piret equation was, the lower the nitrogen concentration, the higher the α value, the lower the β value. The obtained modeling parameters were used to estimate lipid productivity, and the highest lipid productivity obtained in this study was 16.92 mg g^{-1} day^{-1} at 0.625 mg/L nitrogen.

Acknowledgments

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Spirogyra sp. growth and lipid synthesis kinetic model supplemented with sodium bicarbonate

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Abstract

Macroalgae *Spirogyra* sp. has promising potential as an alternative resource of lipid. This study is aimed to increase lipid production in *Spirogyra* sp. by varying the C:N ratio through the addition of sodium bicarbonate. Sodium bicarbonate was added from the beginning of cultivation period to see how it affected algal biomass growth. *Spirogyra* sp. was grown in Bold Basal Medium with sodium bicarbonate added at 2:1, 4:1 and 6:1 C:N ratios. Cultivation was carried out for 8 days, and the data on biomass and lipid yields were collected every two-days. A Logistic model was used to estimate the biomass growth parameter values, whereas Luedeking-piret product formation model was used to predict lipid production. The result showed that as the C:N ratio in the medium increases, the culture's maximum specific growth rate increases but the biomass yield decreased. The highest biomass yield was achieved with a 2:1 C:N ratio i.e. 404.3 $mg.L^{-1}$ on the 8th day. The treatment was also showed increase lipid accumulation in all variations, with the highest lipid yields at a 2:1 C:N ratio i.e. 79.51 $mg.g^{-1}$ on the 2nd day. Maximum lipid productivity value was reached by adding sodium bicarbonate at 4:1 C:N ratio with 6.99 $mg.g^{-1}day^{-1}$.

Keywords: Lipid, Logistic model, Luedeking-piret, Sodium bicarbonate, *Spirogyra* sp.

1. Introduction

Spirogyra sp. is one of the green macroalgae that grows well in Indonesian waters. The presence of spiral-shaped chloroplast organelles characterizes these freshwater macroalgae [1]. *Spirogyra* sp. has a high enough lipid content of 11-21% [2], which is rich in polyunsaturated fatty acid (PUFA). According to study results, *Spirogyra* sp. contains up to 37,35% PUFA with the composition of omega-3 fatty acids such as α-linolenic (ALA), dihomo-γlinolenic (DGLA), and eicosapentanoic (EPA), and omega-6 fatty acids such as linoleic (LA) [3].

PUFA is an important nutrient needed to support brain growth and development. Omega-3, one type of PUFA, is critical for human biological systems and the development of the brain's nervous system at the end of the pregnancy period. Until recently, fish oil was the primary source of PUFA due to the high concentration of these fatty acids in fish. Overfishing happens as a result of rising market demand for fish oil, which can lead to the extinction of fish populations. If this continues

without any action, it is estimated that the world's fish stocks will run out in the next 40 years [4]. Excessive hunting also causes environmental pollution due to human activities that involve the use of hazardous chemicals in the fishing process. Therefore, a renewable and sustainable alternative resource is required to produce PUFA-rich lipids.

The omega-3 fatty acids in fish oil originally come from algae. Algae is the primary producer of PUFAs and is consumed by fish [5]. According to recent studies, algae have a promising potential to be a source of lipid due to the presence of PUFA in them [6]. Algae has high photosynthetic efficiency because of its simple photosynthetic apparatus, allowing it to produce high amounts of biomass in a relatively short length of time compared to other terrestrial organism biomass [7]. Furthermore, the lipids produced by algae do not have an unpleasant taste or odor as compared to fish oil. The use of algae as an alternative raw material to replace fish can overcome the problem of fish extinction while also enabling the renewable and sustainable production of PUFA-rich lipids. So, *Spirogyra* sp. has potential as an alternative raw material for lipid production.

Lipid accumulation in algal cells is influenced by many factors, one of which is the availability of carbon in the culture medium. Carbon is the main compound involved in the formation of biomass in photosynthetic processes. In optimal culture conditions, carbon becomes a limiting factor nutrient, so the addition of a carbon source to the medium can increase the yield of algal biomass [8]. Besides affecting biomass, carbon source addition can also influence lipid accumulation. The addition of a carbon source will raise the C:N ratio in the medium, which can trigger the conversion of carbon from sugars to lipids, resulting in an increased lipid accumulation in algal cells [9,10].

In algal cultivation with an open pond system, carbon is generally acquired via carbon dioxide (CO_2) from the atmosphere or aeration. Due to the low solubility of CO_2 at atmospheric pressure (1.45 g/L at 25℃), algal growth becomes relatively slower due to the lack of dissolved carbon sources in the medium. To increase the dissolved carbon concentration in the medium, it is possible to add carbon sources from chemical compounds, such as sodium bicarbonate ($NaHCO_3$). When compared to CO_2, $NaHCO_3$ has a higher solubility at atmospheric pressure (96 g/L at 20 °C) [11]. Based on earlier research [12-14], the addition of $NaHCO_3$ to the optimum concentration can improve the yield of biomass and lipid in the green algae culture of *Chlorella* sp. and *Scenedesmus* sp.. Therefore, this study is aimed to increase the lipid production in the culture of *Spirogyr*a sp. by varying the C:N ratio through the addition of $NaHCO_3$ to the medium and to determine the effect of the treatment on the kinetics of biomass growth and lipid accumulation.

2. Materials and methods

2.1 Materials

The macroalgae *Spirogyra* sp. was collected from the Majalaya Fishing Pond, West Java, Indonesia. The algae were gradually cleaned with tap water before use. The culture media used was modified Bold Basal Medium (BBM) with the composition

of $NaNO_3$ 6.25, $CaCL_2.2H_2O$ 0.625, $MgSO_4.7H_2O$ 1.875, K_2HPO_4 1.875, KH_2PO_4 4.375, NaCl 0.625, EDTA 1.250, $FeSO_4.7H_2O$ 0.125, H_2SO_4 0.125, H_3BO_3 0.286, $ZnSO_4.7H_2O$ 0.221, $MnCl_2.4H_2O$ 0.036, MoO_3 0.018, $CuSO_4.5H_2O$ 0.039 and $Co(NO_3)_2.6H_2O$ 0.012 mg L^{-1}. The media was diluted in distilled water. The chemical compounds were purchased from the local distributor, i.e. CV Putra Mebelindo and CV Titan Scientific.

The source of inorganic carbon was obtained from the addition of $NaHCO_3$ with variation in the C:N ratio of 2:1 (0.16 g L^{-1}), 4:1 (0.32 g L^{-1}) dan 6:1 (0.48 g L^{-1}). The materials used for the lipid extraction process are technical methanol, chloroform, and distilled water. All materials for medium and lipid extraction were provided by the School of Life Science and Technology Warehouse of ITB. The instruments used were aerators, oven, fume hood, hotplate, and centrifuge, which are facilities provided by the School of Life Science and Technology Laboratory of ITB.

2.2 Methods

2.2.1 Cultivation of *Spirogyra* sp.

Three grams wet weight of inoculum was grown in 500 ml of BBM medium in a 1L volume container with dimensions of 14×8.5×7.5 cm. The concentration of inorganic carbon source $NaHCO_3$ was varied in the medium, with C:N ratio of 2:1, 4:1, and 6:1. In the control treatment, *Spirogyra* sp. was grown only in modified BBM without $NaHCO_3$ addition. The culture was cultivated for 8 days at room temperature (25±3℃) with the light intensity of 30.37±3.37 μmol foton.m^{-2}.s^{-1} using Phillips Cool White TL-D 36 W lamp (light:dark period 12:12 hours). Air was flowed continuously with an aeration rate of 0.2-0.4 L min^{-1}. The pH of the medium was maintained at a scale of 7 which was measured periodically with universal pH meter. The cultivations were completed in triplicate.

2.2.2 Determination of biomass dry weight

The samples of culture were taken on the 2nd, 4th, 6th, and 8th days. The fresh algal biomass was filtered out of the medium using a 60 mesh sieve and the wet weight was measured. Next, it was dried for 24 hours by spreading the biomass on filter paper and placing it under a fan airflow exposure at room temperature (25±3℃) [15], and then dried for another 15 minutes in an oven at 105℃. The dried samples were weighed and used for further analysis.

2.2.3 Determination of lipid yield

The Bligh and Dyer method was used for lipid extraction with a procedure according to Sündermann et al. (2016). The dried algal biomass sample were pulverized by mortar and pestle. Four ml methanol and two ml chloroform were added to the sample in a falcon tube. The sample was homogenized using vortex for 2 minutes. Then, 2 ml chloroform was added to the sample in the tube and vortexed for 2 minutes. Next, 3.6 ml distilled water was added to the mixture in the tube and vortexed again for 2 minutes. The mixture was then centrifugated at 2000 rpm for 10 minutes. As a result of the centrifugation process, three layers were formed: the methanol phase, residual biomass, and chloroform phase. The top layer,

which was clear-colored methanol, was taken by a pipette. The remaining two-layer were then filtered out into a new tube using Whatman paper no.1 to separate the residual biomass from the the chloroform phase, which contains lipid.

Then a second extraction was carried out for the residual biomass using 4 ml 10% (v/v) methanol in chloroform as solvent. The mixture was vortexed for 2 minutes, then centrifugated. After the centrifugation process, the mixture was filtered through filter paper and the chloroform phase was added to the first extract. The extract was then placed in an aluminum foil cup and heated on a hotplate at 70℃ until the chloroform evaporated. After the evaporation process, the cup containing lipid was dried in the oven at 105℃ or 15 minutes [16]. The lipid extract was then weighed to determine the lipid yield.

2.2.4 Determination of maximum specific growth rate

The actual maximum specific growth rate was calculated by Eq. (1).

$$\mu_{max} = \frac{\ln X_t - \ln X_0}{t - t_0} \tag{1}$$

where μ_{max} is the maximum specific growth rate (day^{-1}), t is cultivation time (day), t_0 is the beginning of cultivation time (day), X_t is the biomass concentration at t ($mg\ L^{-1}$), and X_0 is the biomass concentration at t=0 ($mg\ L^{-1}$).

2.2.5 Modeling of biomass growth kinetics

The logistic equation in Eq. (2) was used to model the biomass growth kinetics. In this model, the cell growth rate is proportional to the cell concentration at a given time. Under sufficient nutrient availability, the growth rate increases exponentially until the nutrient sources are depleted, at which point cell growth enters a stationary phase.

$$\frac{dX}{dt} = \mu_{max} X \left(1 - \frac{X}{X_{max}}\right) \tag{2}$$

Eq. (3) is derived from Eq. (2) to obtain a model that describes the lag phase, the initial exponential phase's growth rate, and the stationary phase's cell concentration.

$$X = \frac{X_0 e^{\mu_{max} t}}{1 - \frac{X_0}{X_{max}}\left(1 - e^{\mu_{max} t}\right)} \tag{3}$$

where $\frac{dX}{dt}$ is biomass growth rate ($mg\ L^{-1}\ day^{-1}$), t is cultivation time (day), X is biomass concentration ($mg\ L^{-1}$), X_{max} is the maximum biomass concentration ($mg\ L^{-1}$), X_0 is the biomass concentration at t=0 ($mg\ L^{-1}$), and μ_{max} is the maximum specific growth rate (day^{-1}).

X_{max} and μ_{max} parameters in the logistic equation and also the goodness of fit (R^2) were estimated by fitting the logistic equation with the data of the dried weight biomass measured from the experiment ($mg\ L^{-1}$) against time (day) for each treatment variation. The data fitting process was carried out by curve fitting in MATLAB software, where the x-axis is the cultivation time and the y-axis is the biomass dry weight.

2.2.6 Modeling of lipid production

Lipid production in the *Spirogyra* sp. cultures was modeled by the Luedeking-Piret equation. Luedeking-Piret is an equation that is commonly used to model the formation of a product. The Luedeking-Piret model is described in Eq. (4).

$$\frac{dP}{dt} = \alpha \frac{dX}{dt} + \beta X \tag{4}$$

Eq. (4) was then integrated with the logistic model in Eq. (3) so that Eq. (5) was obtained.

$$P = Po - \alpha X_0 \left\{ \frac{e^{\mu_{max} t}}{\left[1 - \left(\frac{X_0}{X_{max}}\right)(1 - e^{\mu_{max} t})\right]} \right\} + \beta \frac{X_{max}}{\mu_{max}} ln \left\{1 - \frac{X_0}{X_{max}}(1 - e^{\mu_{max} t})\right\} \tag{5}$$

where P is product concentration (mg L^{-1}), t is cultivation time (day), X is biomass concentration (mg L^{-1}), X_{max} is the maximum biomass concentration (mg L^{-1}), X_0 is the biomass concentration at t=0 (mg L^{-1}), μ_{max} is the maximum specific growth rate (day^{-1}), α is growth-associated constant (mg mg^{-1}) and β is nongrowth-associated constant (mg mg^{-1} day^{-1}).

Data fitting was carried out using the model equation and the lipid yield data (mg L^{-1}) which was measured in the experiment, against the cultivation time (day) for each treatment variation. The parameter values of X_{max} and μ_{max} were obtained from the modeling of biomass growth kinetics, which was then used in Eq (4) to estimate α and β parameters.

2.2.7 Determination of lipid productivity

Lipid productivity was estimated using the equation from the modeling result to determine biomass and lipid yield at the end of the cultivation period. Eq. (6) was used to calculate the lipid productivity.

$$P\ (\text{mg}\ g^{-1} day^{-1}) = \frac{[lipid]}{t} \tag{6}$$

where [lipid] is lipid concentration at the end of cultivation period (mg g^{-1}) and t is cultivation time (day).

2.2.8 Statistical analysis

The experiment was carried out by triplicate in each variation. The data results were expressed by mean ± standard deviation. The statistical tests used are the Kolmogorov-Smirnov normality test, the one-way ANOVA (Analysis of Variance) test, and Duncan's multiple range test, respectively. Statistical analysis was performed using Microsoft Excel 2018 software.

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3. Result and discussion

3.1 Effect of sodium bicarbonate supplementation on biomass growth

According to the experimental result of the *Spirogyra* sp. culture in the modified BBM, it was found that the macroalgae in the control grew exponentially until the 4th day, then entered the stationary phase until the 8th day (Figure 1). The biomass of *Spirogyra* sp. cultured with a C:N ratio of 2:1 remained in the exponential phase until the 8th day. At a 4:1 ratio, biomass grew exponentially until the 2nd day, entered a stationary phase until the 6th day, then declined on the 8th day, but at a 6:1 ratio, biomass grew exponentially until the 2nd day and then declined on the 4th day.

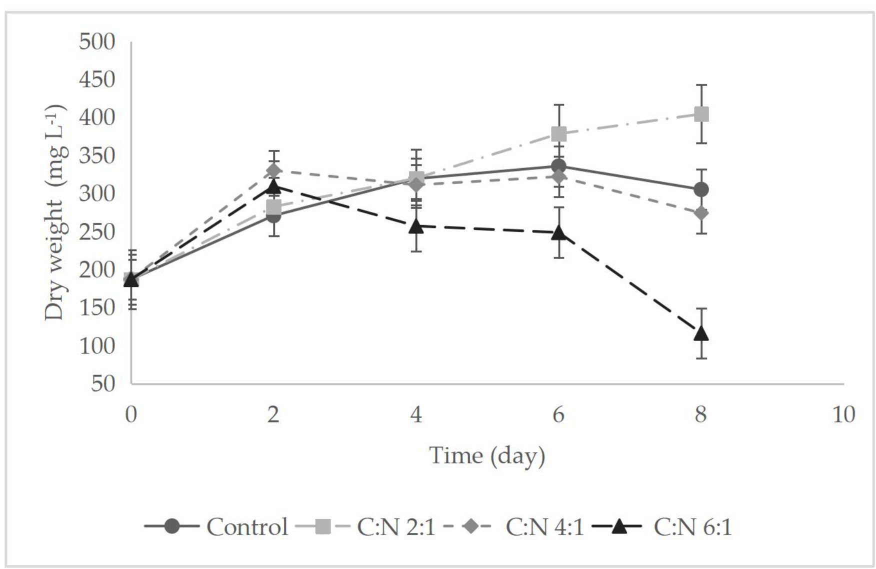

Figure 1. The growth curve of a *Spirogyra* sp. cultures in control medium and with the addition of $NaHCO_3$ at a C:N ratio of 2:1, 4:1, and 6:1

Despite having a lower growth rate than other treatments, the addition of $NaHCO_3$ with a ratio of 2:1 resulted in a better biomass growth, which could be seen from the highest value of maximum biomass yield. The maximum biomass yield obtained was 404.3 mg L^{-1} on the 8th day. The maximum biomass yield was greater than the control (335.8 mg L^{-1}) and treatments with a C:N ratio of 4:1 (330 mg L^{-1}) and 6:1 (309.3 mg L^{-1}). The statistical test one-way ANOVA showed that the value of $p<\alpha$ (α=0,05) indicates that adding $NaHCO_3$ to the culture of *Spirogyra* sp. resulted in a significant difference in biomass growth between treatments, that was indicated by the best results at C:N ratio of 2:1.

The results obtained are in line with other reports who conducted on different species of macro and microalgae. It was reported that adding $NaHCO_3$ to the medium increased biomass yield considerably because bicarbonate supplementation increased cell division and algal metabolism [14]. A similar result was reported by Zhou et al. (2016) in the macroalgae cultures of *Gracialariopsis*

lemaneiformis, *Gracilaria vermicular-phylla*, and *Gracilaria chouae.* From the study, it was found that the addition of $NaHCO_3$ to the optimum concentration could improve biomass yield [17].

In the research of Pancha et al. (2015), it was discovered that the optimum concentration of $NaHCO_3$ for producing the highest biomass yield in *Scenedesmus* sp. is 1.2 g L^{-1} [14]. In contrast to the optimum concentration of $NaHCO_3$ for Gracilariales culture in the study of Zhou et al. (2016), that is 0.25-0.33 g L^{-1}. These two optimum concentrations differ from the findings of this study, which revealed that the best concentration to produce the maximum biomass yield of *Spirogyra* sp. cultures is a C:N ratio of 2:1 or a $NaHCO_3$ concentration of 0.16 g/L. The optimum $NaHCO_3$ concentration or C:N ratio for biomass growth can be different for each species [18].

Another cause of decreased biomass yield is the high concentration of $NaHCO_3$ in the medium, which can cause osmotic pressure from the salt and stress due to toxic Na^+ ions from the dissociation of $NaHCO_3$ in water. As a result of this stress, the chlorophyll content in algae decreases, so the rate of photosynthesis also decreases [18]. This can be observed from the change in the color of the culture at the ratios of C:N 4:1 and 6:1, which turned yellow on the 8th day (Figure 2). The color change at the 6:1 ratio was seen to be more significant due to the higher $NaHCO_3$ concentration.

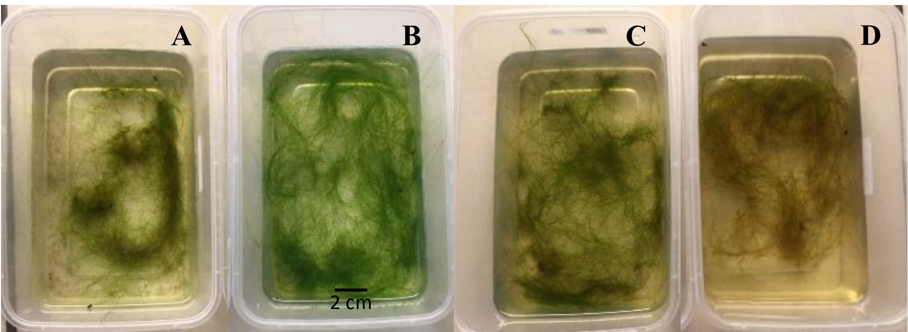

Figure 2. *Spirogyra* sp. cultures on the 8th day in (A) control medium and with the addition of $NaHCO_3$ at a C:N ratio of (B) 2:1, (C) 4:1, and (D) 6:1

Based on the experimental results on the cultivation of *Spirogyra* sp. with the addition of $NaHCO_3$ to the medium, it was found that the treatment with a C:N ratio of 2:1 gave the best biomass yield of *Spirogyra* sp. compared to the control and other treatments. The addition of $NaHCO_3$ with this ratio can increase the yield of biomass without causing negative effects of the high C:N ratio and toxic Na^+ ions on culture. This can be observed from the culture color, which still looks fresh green on the 8th day of cultivation compared to other treatments (Figure 2).

3.2 Growth kinetics modeling of *Spirogyra* sp. cultures at various C:N ratios

The logistic equation was used to model the growth of *Spirogyra* sp. cultures from the exponential to stationary growth phase. The modeling results have a good

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match with the experimental data because the data fitting results have a correlation coefficient value (R^2) that is close to 1 (Figure 3). A good R^2 value was obtained in the treatment of control, a C:N ratio of 2:1 and 4:1, i.e., 0.95, 0.99, and 0.95, respectively. The treatment of the C:N ratio of 6:1 gave the smallest R^2 value, 0.7. This was due to the reason that the biomass weight decreased faster in the 6:1 treatment than in the other treatments, which began on the 4th day. The logistic equation can accurately model biomass growth from the exponential to the stationary phase, but it cannot correctly model growth until the death phase.

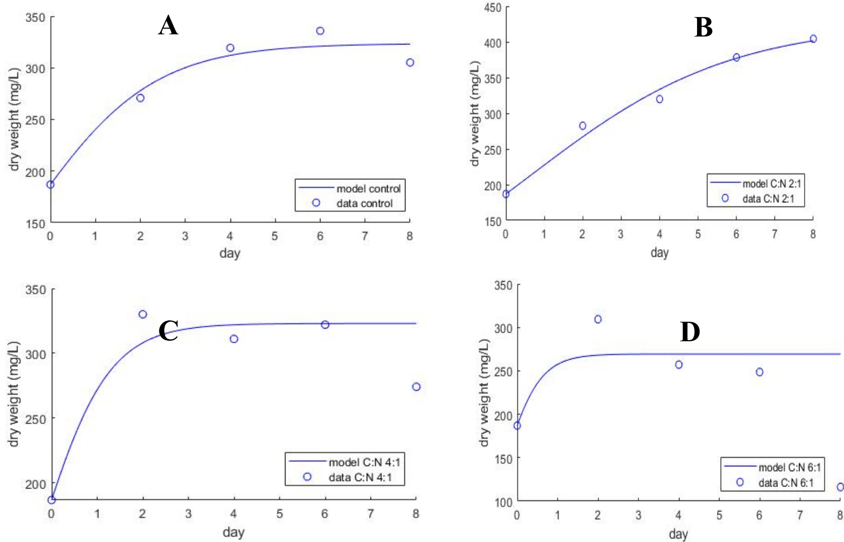

Figure 3. The logistic growth model *of Spirogyra sp.* cultures in (A) control medium and with the addition of $NaHCO_3$ at a C:N ratio of (B) 2:1, (C) 4:1, and (D) 6:1

The maximum specific growth rate (μ_{max}) for control and the variations of the ratios 2:1, 4:1, and 6:1 were 0.746 day^{-1}, 0.383 day^{-1}, 1.356 day^{-1}, and 2.257 day^{-1}, respectively, based on the estimation results of growth kinetics parameters (Table 1). It was discovered that the more $NaHCO_3$ was added, the more μ_{max} value increased. The obtained trend is consistent with the findings of Lohman et al. (2015), who reported that the addition of $NaHCO_3$ to the culture medium of *Chlorella vulgaris* at low concentrations at the beginning of cultivation could increase the value of the μ_{max} parameter. The same result is stated from the research that was done by Zhou et al. (2016), which found that adding $NaHCO_3$ to a Gracilariales macroalgae culture increased the specific growth rate of biomass. This is because the treatment can cause an increase in the concentration of chlorophyll pigment at the beginning of the cultivation time, resulting in a higher rate of photosynthesis in the culture [20]. However, when $NaHCO_3$ was added at a C:N ratio of 2:1, the value of (μ_{max}) was lower than the control. This is due to the time difference in the exponential phase. The control's exponential phase occurred from day 0 to day 4 of the cultivation period, whereas the treatment with a 2:1 ratio's exponential phase continued until day 8. The quicker the growth rate reaches the stationary phase, the higher the μ_{max} value is obtained.

Table 1. Estimation of logistic growth model parameters of *Spirogyra* sp. cultures in control medium and with the addition of $NaHCO_3$ at a C:N ratio of 2:1, 4:1, and 6:1

Parameter	Control	C:N 2:1	C:N 4:1	C:N 6:1
	Measurement			
μ_{max} (day^{-1})	0.134	0.096	0.284	0.252
X_{max} (mg L^{-1})	335.8	404.3	330.1	309.3
	Modeling			
μ_{max} (day^{-1})	0.746	0.383	1.356	2.257
X_{max} (mg L^{-1})	323.6	425.5	323.0	269.3
R^2	0.95	0.99	0.95	0.7

Although the addition of $NaHCO_3$ increases the value of the μ_{max} parameter, the opposite trend was observed in the trend of maximum biomass concentration value (X_{max}). According to the modeling results, the higher the addition of $NaHCO_3$ to the medium, the lower the X_{max} value obtained. The highest X_{max} value was obtained at a 2:1 C:N ratio, i.e., 425.5 mg L^{-1}, followed by control of 323.6 mg L^{-1}, a 4:1 ratio of 323 mg L^{-1}, and a 6:1 ratio with the lowest value of 269.3 mg L^{-1}. The decrease in the value of the X_{max} parameter was caused by the inhibition of high concentrations of carbon sources, as well as the stress of the toxic ion Na^+ from the dissociation of $NaHCO_3$ in water, which resulted in lower levels of chlorophyll in culture, reducing biomass yield [18]. These findings are also consistent with the findings of Yang et al. (2011), who found that a high C:N ratio can reduce biomass yield.

Despite differences in values, the growth model parameters μ_{max} and X_{max} from modeling and experimental results show a similar trend (Table 1). The experimental X_{max} value increased with a C:N ratio of 2:1 and decreased with a higher ratio. However, there is a different trend in the value of μ_{max} at the final point, i.e. C:N ratio of 6:1. According to modeling results, the value of μ_{max} at a ratio of 6:1 is higher than at a ratio of 4:1, but actually the measurement showed that the value of μ_{max} at a ratio of 6:1 is less than 4:1. Growth modeling at 6:1 ratio has the lowest R^2 value of all treatments (0.7). It might indicate that the model was less suitable for this condition.

3.3 Effect of sodium bicarbonate supplementation on lipid production

The lipid yield of *Spirogyra* sp. cultures in control increased until the 4th day, then decreased until the 8th day. In cultures with the addition of $NaHCO_3$, the lipid yield in algal cells tended to increase more rapidly, starting from the 2nd day to the 8th day. Each treatment resulted in a different increase in lipid yields (Figure 4). At a ratio of 2:1, the lipid yield in algal cells increased on the 2nd day and then decreased until the 8th day. At a ratio of 4:1, the lipid yield in cells decreased after the 2nd day to the 6th day, then increased on the 8th day, whereas at the 6:1 ratio, the lipid yield tended to be constant since the 2nd day.

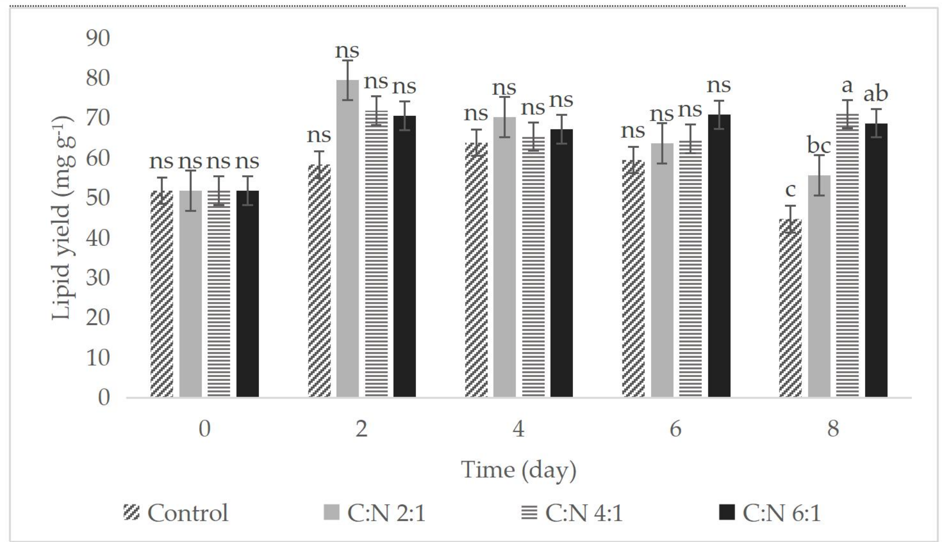

Figure 4. The lipid yield curves of a *Spirogyra* sp. cultures in control medium and with the addition of $NaHCO_3$ at a C:N ratio of 2:1, 4:1, and 6:1

i indicates the statistical difference between treatments on the same day, α=0,05
ns = nonsignificant

The addition of $NaHCO_3$ in all C:N ratio variations can increase the lipid yield of *Spirogyra* sp. biomass. This can be seen from the lipid yield of the biomass on the 2nd day. With a lipid yield control of 58.34 mg g^{-1}, the addition of $NaHCO_3$ with a C:N ratio of 2:1, 4:1, and 6:1 resulted in the lipid yield in dry biomass of 79.51 mg g^{-1}, 71.89 mg g^{-1}, and 70 mg g^{-1}, respectively. The statistical test one-way ANOVA showed that the value of $p<\alpha$ (α=0,05) indicates that adding $NaHCO_3$ to the culture of *Spirogyra* sp. resulted in a significant difference in lipid accumulation between treatments at the end of cultivation. These study results are consistent with the findings of Mokashi et al. (2017), who reported that the addition of $NaHCO_3$ to a freshwater algal culture of *Chlorella vulgaris* increased the lipid yield of the algal biomass with the addition of $NaHCO_3$ to 1 g L^{-1}. The optimum concentration of $NaHCO_3$ obtained by Mokashi et al. (2017) differs from the concentration obtained by this study, which is 0.16 g/L or with a C:N ratio of 2:1. This could be due to differences in the ability of each algae species to withstand stress conditions caused by the addition of $NaHCO_3$.

The increase in lipid content in algal cells due to the addition of $NaHCO_3$ was caused by bicarbonate, which was able to induce carbon storage metabolic activity and trigger lipid accumulation. These compounds can also increase the accumulation of triacylglycerol (TAG) in algal cells [12]. Pancha et al. (2015) and Mondal et al. (2017) also stated that adding inorganic carbon supply from $NaHCO_3$ to nitrogen-deficient algal cultures can trigger de novo lipogenesis, a complex process that converts excess carbohydrates into fatty acids that can be used to synthesize triglycerides or other lipid molecules [14,21]. The same result was

confirmed by Nayak et al. (2018), who reported that bicarbonate is the primary key substrate in the de novo pathway of fatty acid biosynthesis.

The C:N ratio in the culture medium has an important role in the lipid synthesis process in algal cells. The macronutrient nitrogen consumed by algae was used more for synthesis into biomass in culture conditions without additional carbon source treatment. As a result of the high intracellular conversion to protein, lipid synthesis is reduced at high nitrate utilization. However, in culture conditions with the addition of a carbon source, increasing the C:N ratio can modify cellular metabolic pathways in algal cells by limiting protein synthesis and using excess energy for the biosynthesis of carbon storage materials, such as lipids for cell maintenance. Hence, increasing the C:N ratio to the optimum ratio can increase lipid synthesis in algal cells [5].

In this study, the highest lipid yield was obtained in cultures with the addition of $NaHCO_3$ at C:N ratio of 2:1 on the 2nd, i.e. 79.51 mg g^{-1} dry weight, or equivalent to 7.95%. In the treatment with a larger C:N ratio (4:1 and 6:1), it was found that the lipid yield was lower than the treatment with a 2:1 ratio on the 2nd day. This can be caused by the inhibition of stress that occurs during growth, resulting in an inefficient synthesis of lipids in cells [18]. Nevertheless, there was a tendency for the lipid yield to decrease from the 2nd day to the 8th day in the treatment with a C:N ratio of 2:1. This could be because the C:N ratio is not too high, resulting in high nitrogen utilization in the cells the following day. As a result, cells preferentially use energy for the synthesis of biomass or protein over lipids. This is also supported by the trend of biomass yield, which continues to rise until the 8th day.

On the 8th day of cultivation, the lipid yield was higher at C:N ratios of 4:1 and 6:1 than at 2:1. Increasing the C:N ratio can induce the conversion of carbon from sugars to lipids, leading to an increase in lipid accumulation in cells [9,10]. Furthermore, in water, the carbon source $NaHCO_3$ dissociates into bicarbonate ions (HCO_3^-) and toxic ions Na^+. Toxic Na+ ions stress algal cells, causing them to accumulate more lipids as a form of cell defense. Stress conditions cause metabolic fluxes to be channeled into lipid biosynthetic pathways because these compounds can help cells survive [18].

3.4 Lipid production modeling of *Spirogyra* sp. cultures at various C:N ratios

The Luedeking-Piret model is well-fitted to the experimental data, as indicated by correlation coefficient value (R^2) is close to unity (Figure 5 and Table 2). The control treatment, the addition od $NaHCO_3$ with a C:N ratio of 2:1, 4:1, and 6:1 had R2 values of 0.84, 0.78, 0.99, and 0.86, respectively. From this result, it seemed that not all condition was suited to the Luedeking-Piret model.

The parameter estimation results show that the values of α and β are not zero. The values of α and β which are not equal to 0 are classified in the production of class III metabolites [22]. This class indicates that lipid synthesis in *Spirogyra* sp. culture is partially associated with biomass growth. It was also discovered in all treatments that the value of a>β, indicating that lipid production in *Spirogyra* sp. culture is associated with biomass growth or that lipids are growth-associated products [23].

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A negative value was obtained, indicating a decrease in the trend of lipid yield in culture. The parameter estimation results are in line with the research of Baskar and Selvakumari (2018), who also modeled lipid accumulation for freshwater green algae, *Scenedesmus* sp., with the results of a>β values [24].

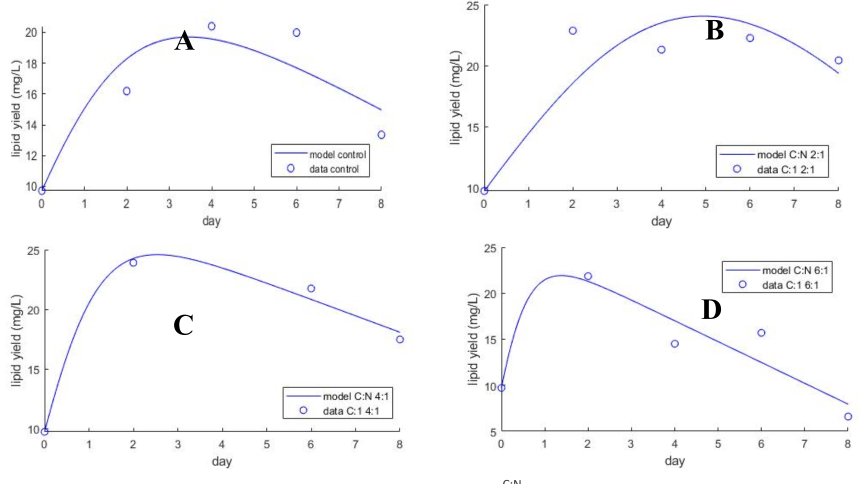

Figure 5. The Luedeking-piret lipid production model of *Spirogyra* sp. cultures in (A) control medium and with the addition of $NaHCO_3$ at a C:N ratio of (B) 2:1, (C) 4:1, and (D) 6:1

Table 2. Estimation of Ludeking-piret model parameters of *Spirogyra* sp. cultures in control medium and with the addition of $NaHCO_3$ at a C:N ratio of 2:1, 4:1, and 6:1

Parameter	Control	C:N 2:1	C:N 4:1	C:N 6:1
α (mg mg^{-1})	0.118	0.170	0.139	0.194
β (mg mg^{-1} day^{-1})	-0.005	-0.011	-0.004	-0.008
R^2	0.84	0.78	0.98	0.86

Because lipids is a primary metabolite, it is required for both metabolic and cell survival processes. Lipid can influence cell growth and development in algal cells because they are building blocks or cell-forming elements that also play a role in the cell signaling process [25]. Lipid accumulation in algal cells is also a cell defense mechanism for survival under stress conditions [5]. Hence, the formation of lipids in algal cells is associated with cell growth.

3.5 Lipid productivity estimation at various C:N ratios

The modeling result of biomass growth kinetics with the logistic model and lipid formation with the Luedeking-piret model was then used to estimate lipid productivity in *Spirogyra* sp. with the addition of $NaHCO_3$ carbon sources at various C:N ratios. Productivity was calculated on the 8th day of cultivation. The estimation results show that the highest lipid productivity of 6.99 mg g^{-1} day^{-1} was obtained with the addition of $NaHCO_3$ at a C:N ratio of 4:1, followed by a 2:1 ratio of 6.05 mg

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g^{-1} day^{-1}, control of 5.80 mg g^{-1} day^{-1}, and a ratio of 6:1 with the lowest productivity of 3.68 mg g^{-1} day^{-1} (Figure 6).

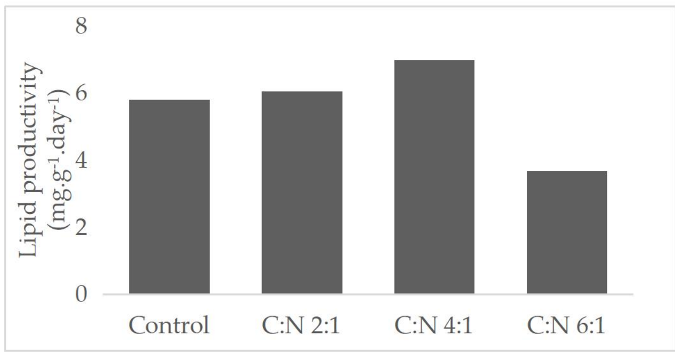

Figure 6. Lipid productivity estimation of *Spirogyra* sp. cultures in control medium and with the addition of NaHCO3 at a C:N ratio of 2:1, 4:1, and 6:1

The treatment with the addition of $NaHCO_3$ at a C:N ratio of 4:1 has higher lipid productivity than the control and other treatments. Although the biomass production in this treatment was less than the C:N ratio 2:1 result, higher lipid productivity was obtained based on modeling of lipid production. Stress caused by the addition of $NaHCO_3$ causes algal cells to accumulate more lipids. At a larger ratio of 6:1, stress from the addition of $NaHCO_3$ resulted in a very significant decrease in biomass yield, resulting in lower lipid productivity. This suggests that adding $NaHCO_3$ at a C:N ratio of 4:1 can provide the best lipid productivity in *Spirogyra* sp. macroalgae cultures, yielding 6.99 mg g^{-1} day^{-1}.

4. Conclusion

The addition of an inorganic carbon source $NaHCO_3$ to the culture medium of *Spirogyra* sp. can increase the maximum specific growth rate of biomass (μ_{max}) with the highest value of μ_{max} obtained at a C:N ratio of 4:1 i.e., 0.284 day^{-1}. However, the higher the C:N ratio in the medium, the maximum biomass concentration value (X_{max}) decreases, with the highest X_{max} value obtained at a 2:1 C:N ratio of 404.3 mg L^{-1}. The addition of inorganic carbon sources $NaHCO_3$ can also increase the lipid yield of *Spirogyra* sp. cultures in all variations of the C:N ratio, with the highest increase of 36% greater than the control at 2:1 C:N ratio on the 2nd day of cultivation. From the modeling result of biomass growth kinetics and lipid production, it can be estimated that the best lipid productivity was obtained from the addition of $NaHCO_3$ with a C:N ratio of 4:1 i.e., 6.99 mg g^{-1} day^{-1}.

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