PROCEEDING

The 3rd Joint Symposium on Plant Sciences and Products

November 16th- 17th, 2022
ITB Ganesha Campus, Bandung

"Utilization of Biomass towards Strengthening Bioeconomy"

ITB Press

PROCEEDING OF

The 3rd Joint Symposium on Plant Sciences and Products

'Utilization of Biomass towards Strengthening Bioeconomy'

Volume 2, 2023

Institut Teknologi Bandung

16th-17th November 2022

Published by:

ITB PRESS

Preface

On behalf of all members of Plant Science and Biotechnology Research Group and myself, I am very pleased to welcome you all to our Proceeding of the 3rd Joint Symposium on Plant Science and Products, 2022, published by Plant Science and Biotechnology Research Group, School of Life Sciences and Technology, Institut Teknologi Bandung.

This proceeding of Conference highlighted a few topics on our agenda, but it provides only partial record of what was presented, since some papers are published elsewhere. During the two days of conference, the participants had an opportunity to exchange knowledge and technology speakers from Indonesia, as well as Japan, the Netherlands, Malaysia, and Canada, all of whom are recognized for their current expertise. The subjects covered span from fundamental knowledge of plant and algal biology to contemporary biotechnology approaches and their application to strengthening bioeconomy. Additionally, in a parallel session there were 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.

Sincere thanks to the following Keynote speakers and invited speakers in the International Conference on Plant and Algae-based Bioindustry

1) Prof. Dr. Muranaka Toshiya: Food Security: Gene editing to improve plant secondary metabolite pathway
2) Assoc. Prof. Dr. Roohaida Othman: Functional genomics for carrageenan producing macroalgae
3) Prof. Dr. Ismanizan Ismail: Multi omics in system biology: Data integrations for crop improvement
4) Dr. Nurulhima Md Isa: Improving environmental stress resilience in crops for high yield production
5) Dr. Rizkita Rachmi Esyanti: System engineering to enhance steviol glycoside production
6) Prof. Dr. Hamim, M.Si.: Pyhtomining: A biomass production for precious metal
7) Dr. Ir. Asep Harpenas: Technology to produce high quality seed for farmers welfare
8) Prof. Dr. J. Theo M. Elzenga: How to optimize the breakdown of cell wall material in order to increase the yield of valuable compound from biomass sources
9) Prof. Dr. Siti Rozaimah Sheikh Abdullah: Bioprocess engineering: Converting environmental problem into bioproducts
10) Prof. Dr. Nurul Taufiqu Rahman: Nanotechnology development and commercialization based on natural resources: Technology transfer from lab to the industry in Indonesia.
11) Prof. Dr. Sri Nanan B. Widiyanto: Application of smart agricultural technology and innovation in the development of an integrated and sustainable agricultural sector to strengthening bioeconomy in Indonesia
12) Dr. Ahmad Faizal: Strategies for research and technology development to produce high value bioproducts for strengthening a bioeconomy in Indonesia

I would like to express my sincere appreciation to all of you who generously helped us make the event successful. Many thanks to the Dean of SITH, the Rector of ITB, and, of course, the

organizer of the webinar. Additionally, a heartfelt thank you to all presenters and participants who comes from ITB, Osaka University, University of Gadjah Mada, IPB University, Andalas University, University of Sriwijaya, UIN Alauddin Makassar, and many more that are not mentioned here.

Allow me to thank those who served as the Steering, Scientific, and Organizing committee, as well to supporting teams in organizing the symposia and preparing this proceeding. I hope that the conference and this proceeding will become a valuable insight to all participants.

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

WELCOMING REMARKS FROM CHAIRMAN OF

The 3rd Joint symposium on Plant Sciences and Products

First of all, we thank the Almighty God for enabling us to be together in ITB campus in healthy and safe conditions after we have experiencing almost 3 years of covid-19 pandemic. Thank God that the pandemic is almost gone by now, although there are still some cases found in several countries, including Indonesia and Malaysia I suppose. Certainly, we should take lessons from our experienced from the exposure of pandemic, so we could prepare much better in case other pandemic will emerge again in the future.

Within two days, today and tomorrow, 16th and 17th of November 2022, we will be conducting the 3rd Joint Symposium on Plant Sciences and Products (JPSP) in ITB campus. This symposium is a collaboration between SITH-ITB with UKM Malaysia which we started the JSPSP before pandemic time in 2019 and continued the 2nd JSPSPS in 2020 (virtual). This year, the symposium will be conducted through both offline and online (hybrid). Since the beginning of this semester, ITB urged its lectures and students to conduct academic activities such as courses, practical class and seminars through hybrid system in order the academic atmosphere at the campus will be raised and felt again by its civitas academica of ITB.

The theme for this 3rd JSPSP is "**Utilization of Biomass towards Strengthening Bioeconomy**". Tropical plant products, apart from being used as food and wood, also have many benefits for the community, such as vitamins, medicines, herbs, furniture, handicrafts, etc. The touch of biotechnology through genetic engineering, biological engineering and nanotechnology, for example, can increase plant growth, plant yields, increase levels of plant secondary metabolites, micro and macroalgae, increase plant resistance to environmental stress conditions. Farming techniques with a "smart farming" system in a controlled environment can also increase crop products.

We would also like to express our gratitude to all of our Keynote and invited Speakers: Prof. Muranaka Toshiya from Osaka University, Prof. J. Theo M. Elzenga from Groningen University; Prof. Siti Rozaimah, Prof. Ismanizan Ismail, Assoc. Prof. Roohaida Othman, Dr. Nurulhikma from UKM; Prof. Hamim from IPB; Prof, Nurul Taufiqu Rochman from BRIN; Ir. Asep Harpenas from PT East West Seed Indonesia; Prof. Srinanan B. Widiyanto, Dr. Rizkita R. Esyanti, Dr. Ahmad Faizal from ITB for their acceptance to our request to share their knowledge and experiences on the issue.

We also thank all presenters from various universities and institutions for their participation in this event. Thank you also to all participants, including our students, who join this symposium.

Last but not the least, as the chairman of this conference I would like to express our gratitude to Dean of Faculty of Science and Technology UKM, Dean of SITH ITB and to all Scientific and organizing Committee members for their support to make this conference a successful event. We would like to apologize for any shortcoming that may arise in conducting this event.

We hope that all of us will learn a lot from all keynote speakers and presenters and from warm discussion at each session we will be able to get some inspirations.

Sincerely Yours,

Chairman

Dr. Taufikurahman

PROCEEDING OF

The 3rd Joint Symposium on Plant Sciences and Products

'Utilization of Biomass towards Strengthening Bioeconomy'

Supported by

3 Bio: Journal of Biological Science, Technology, and Management
Current Research on Biosciences and Biotechnology Journal (CRBB)
Journal of Integrated and Advanced Engineering (JIAE)

Kelompok Keilmuan Sains dan Bioteknologi Tumbuhan
Sekolah Ilmu dan Teknologi Hayati
Institut Teknologi Bandung

Gedung Labtek XI
Jalan Ganesa No. 10, Bandung, 40132, Jawa Barat
Telp. +62222511575; +62222500258
Email : icsbt@sith.itb.ac.id
: kksbtsith@gmail.com
Website : sbt.sith.itb.ac.id

Table of Contents

A.1

Multi Omics in Systems Biology:

Data Integration for Crop Improvement

Ismanizan Ismail

Department of Biological Sciences and Biotechnology, Faculty of Science and Technology,
Institute of Systems Biology (INBIOSIS),
Universiti Kebangsaan Malaysia, 43600 UKM Bangi, Selangor, Malaysia

Abstract

For the past few decades, omics platform and technology have significantly contributed for plant system and crop improvement. The advent of next-generation sequencing (NGS) and mass spectrometer (MS) has enabled the generation of the main four omics platform, namely genomics, transcriptomic, proteomics and metabolomics. Due to its powerful technology combined with high throughput techniques, this multi omics technology have played an important role for the plant scientist to understand plant growth and development, senescence, yield, and their responses towards biotic and abiotic stresses. So far, many important crops among others such as wheat, *Medicago truncatula,* and rice has benefited from the application of this technology for their improvement. The huge omics dataset not only involve assembly, storage, processes and analysis but may also offer the integration of data among the omics. Omics dataset integration potentially allow the understanding and explore the relationships between crop genomes and phenotypes under specific physiological and environmental conditions. Substantial amount of research has been carried to understand the role and integration of multi-omics technologies for crop breeding science. While there is huge gain from this multi-omics technology, challenges facing the integration of multi-omics with regard to the functional analysis of genes and their networks as well as the development of potential traits for crop improvement need to be addressed. The emerging and powerful panomics platform allows for the integration of complex omics to construct models that can be used to predict complex traits. In the context of system biology, the integration of multi-omics technology has paved a way for a holistic study and analysis towards explaining various biological processes and complexity in plants. Systems biology integration with multi-omics dataset will also enhance the understanding of molecular regulator networks for crop improvement.

A.2

Functional Genomics for Carrageenan Producing Macroalgae

Roohaida Othman

Department of Biological Sciences and Biotechnology, Faculty of Science and Technology,
Institute of Systems Biology (INBIOSIS),
Universiti Kebangsaan Malaysia, 43600 UKM Bangi, Selangor, Malaysia
E-mail address: roohaida@ukm.edu.my

Abstract

Functional genomics as the field of study to elucidate the function and regulation of genes has facilitated understanding various biological processes in an organism. Its contribution towards the field of systems biology has been clearly depicted in terrestrial plants in unravelling their biology, physiology, gene regulation and metabolism. Such studies have also been conducted on marine macroalgae due to their proven economic value to decipher the processes that are unique to these organisms. *Eucheuma denticulatum* and *Kappaphycus alvarezii* are economically important red algae which serve as important source of carrageenan. The phycocolloid, carrageenan is an important ingredient and receives high demands in the food, pharmaceutical and manufacturing industries. Transcriptomic studies on these species to study the biosynthesis of carrageenan have been undertaken which shed light into the regulation of gene expression in this process. Functional analysis of the enzymes involved in carrageenan biosynthesis for these algae may reveal the biochemical aspect of this event. Since carrageenan is obtained through extraction of the entire algae, the sustainable source of these algae must be maintained. These macroalgae are traditionally propagated from cuttings which is very labor intensive. Studies on tissue culture generation of these algae may alleviate this issue as well as ensuring a uniform source of algae. Transcriptome analysis of algal tissue culture may highlight regulation of carrageenan biosynthetic genes in these tissues. This talk will highlight the role of functional genomics in unravelling the physiology and characteristics of algae in developing algal improvement strategies.

Keywords: Carrageenan, functional genomics, macroalgae, tissue culture, transcriptomics

A.3

Improving Environmental Stress Resilience in Crops for High Yield Production

Nurulhikma Md Isa[1*], Nur Aminah Mohd Hazbir[1], Sitti' Aisyah Mohd Roszelin[1], Khairun Nisa Japlus[1], Su Datt Lam[2] and Sobri Hussein[3]

[1]Department of Biological Sciences and Biotechnology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600 UKM Bangi, Selangor, Malaysia.

[2]Department of Applied Physics, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600 UKM Bangi, Selangor, Malaysia.

[3]Division of Agrotechnology and Bioscience, Malaysia Nuclear Agency, 43000, Kajang, Selangor, Malaysia.

E-mail address: hikma@ukm.edu.my*

Abstract

The unpredictable changing of environmental conditions has given huge impact on agricultural productivity. Along with the recent Covid-19 Pandemic, food safety and security issue has become the main focus in many countries. Agricultural productivity and sustainability issue need to be addressed properly in order to increase and secure national self-sufficiency level. Various approaches have been applied to improve crop sustainability and yield production which including molecular breeding, genetic manipulation as well as agricultural management. Rice is a main crop for many Asian countries and has face severe impact due to the fluctuation of environmental stress. Crops are sensitive to extreme environmental conditions such as drought, salinity, high temperature, cold and hypoxia. However, crops do have a plasticity phenotypic that can make them adapt to the change's environment. Besides, many molecular breeding approach has also look into the wild relative plant for their abiotic resilience traits in the breeding program. For example, understanding the wild relative rice such as highland rice behavior and how they sustain and response to less water has been most important in order to target specific gene for breeding program. In terms of functional genomics study, many researchers have focus on gene regulation and co-ordination in response to multiple abiotic stress. Understanding the cell signaling and gene regulation can facilitate researchers to target specific pathway for crop improvement. One of the gene families that are recently have been highlighted is the Stress Associated Protein families which encodes for zinc-finger binding protein and have a potential role to be used in the genetic manipulation program for crop tolerance to multiple stress.

A.4

System Engineering to Enhance Steviol Glycosides Production

Rizkita Rachmi Esyanti

School of Life Sciences and Technology, Institut Teknologi Bandung, West Java, Indonesia

rizkita@sith.itb.ac.id

Abstract

Study in our lab showed that stevia plantlets showed the optimum growth in TIS RITA® bioreactor system with the thirty-minutes immersion every 6 hours (Melviana & Esyanti, 2016, Melviana et al., 2021). Treatment with far red light during the culture increased the level of expression (up-regulated) in all genes related to steviol glycosides synthesis i.e.: gen ent-KO (~1,16 fold), ent-KS1 (~1,27 fold), ent-KAH 13 (~1,28 fold), SrUGT85C2 (~1,25 fold), SrUGT74G1 (~1,77 fold) and SrUGT76G1 (~1,14 fold). Increased gene expression was also supported by a higher content of stevioside and rebaudioside-A metabolites. Stevioside metabolite in far-red light LED RITA® increased up to 37.15% compared with control bioreactor (no far-red exposure) whereas rebaudioside-A content increased up to 22.98% (Melviana et al. 2021.). Treatment with GA inhibitor Daminozide also showed that Transcription levels of SG-related genes, SrKA13H, SrUGT85C2, and SrUGT76G1 were significantly upregulated by 10 ppm Daminozide, showing 0.5, 4 and 8 folds of transcripts accumulation. However, more concentrated Daminozide decreased transcription levels of the genes. Moreover, the result was also consistent with SG yield data in this experiment, in which SG yield increased significantly with 10 ppm Daminozide while with 20 ppm it was equal to control (Saptari et al, 2019., Saptari et al, 2022). The beneficial of in vitro application of farred light and daminozide are practical and more effective rather than in the field which is affected by a fluctuating environment. The results of this study can give scientific information about how steviol glycoside production can be enhance through a direct modification on the metabolic pathway by shifting or blocking its interlinked pathway using physical and chemical treatments which is practical and inexpensive, this method will be a useful technique for scale up production of steviol glycoside from stevia.

Keywords: Stevia, farred light, steviol glycosides

A.5

Phytomining: A Biomass Production for Precious Metal

Hamim

Department of Biology, IPB University, Bogor, Indonesia

hamimhar@apps.ipb.ac.id

Abstract

Economic development has unwittingly caused environmental problem, including heavy metal pollution. Gold mining, for example, is a sector that contributes large amounts of heavy metal emissions in Southeast Asia, including Indonesia. Although heavy metals cause poisoning and damage to all living things, some types of plants can accumulate heavy metals beyond the threshold of other living things, which is known as hyper accumulator plants. Indonesia has big potential for hyper accumulator plants because Indonesia has the largest ultramafic land in the world, and therefore exploring this potential hyper accumulator is challenging. This plant has the potential to be used in phytoremediation and phytomining program. Gold phytomining, for example, can be applied on gold mine tailings, both in community as well as industrial mining areas, so that in addition to improve polluted environment, this program can produce precious metals for economic reason. Several experimental results using gold mine tailings showed that there was a variety of plants that grow well in the tailings area and able to absorb gold. *Amaranthus spinosus* and *Typha angustifolia* are among the prospective species to be used in phytomining due to capability to accumulate relatively high gold and high biomass production. The use of microbes such as Dark Septate Endophyte and chelating agent ammonium thiocyanate was able to increase the growth and uptake of gold metal in phytomining. In addition, the development of phytomining in the future will be very interesting if it is directed to produce nanoparticles with high economic value. The experimental results showed the success of plants such as *Brassica juncea,* alfalfa and other species in accumulating and producing gold nanoparticles, giving hope for this expectation. Meanwhile, Phytomining needs support from many parties because it is ecologically and economically beneficial.

Keywords: Phytoremediation, phytomining, gold mine tailings, gold nanoparticle, dark septate endophyte, thiocyanate

A.6

Technology to Produce High Quality Seeds for Farmers Welfare

Ir. Asep Harpenas

PT. East West Seed Indonesia

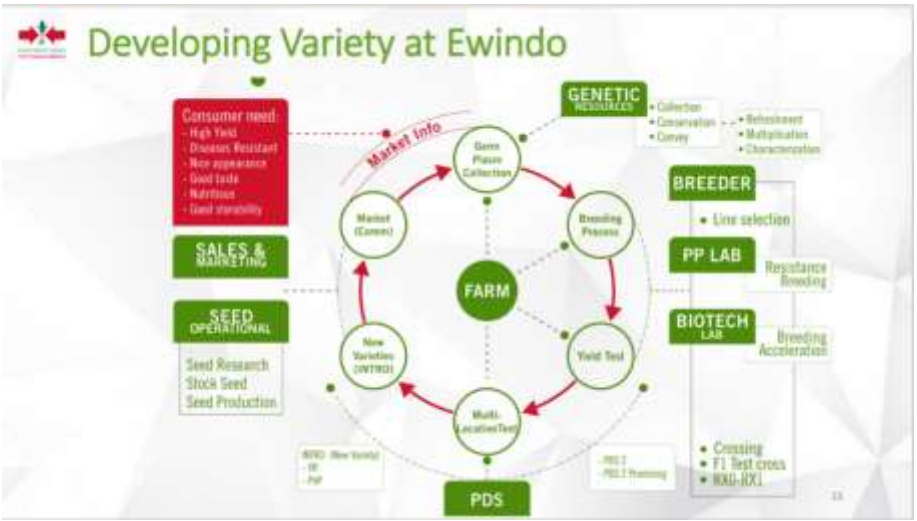
harpenas@panahmerah.id

Abstract

Why EWINDO exist?

We want to be a farmer's best friend. We need high quality vegetable seeds, customized to different customer segments; and we need excellent services for strong relationships with customer and, easily attainable products (multiple & varying distribution channels). EWINDO core values are Farmer's best friend, Happy employee and, Strive for excellence. Vision: We believe in high quality vegetable seeds and excellent services for farmer's prosperity.

Mission: EWINDO provides high quality vegetable seeds; helps farmers through excellent services; and promotes vegetables consumption



Keywords: Seed, farmers welfare, seed distribution

A.7

Bioprocess Engineering: Converting Environmental Problem into Bioproducts

Siti Rozaimah Sheikh Abdullah

Universiti Kebangsaan Malaysia, 43600 UKM Bangi, Selangor, Malaysia

E-mail address: rozaimah@ukm.edu.my

Abstract

Environmental pollution is caused by many factors and activities including day-to-day activity in human's life, industry, mining, agriculture, logging, plantation, transportation, and others. All these activities will consume water, energy and raw materials and simultaneously generate products and waste. Waste can be categorized as toxic and non-toxic, biodegradable, and biodegradable, inorganic and organic, industrial and municipal ones. Even if it is not considered toxic as it does not contain hazardous compounds and ions, for example nutrients from agriculture, aquaculture, and food processing industries, if released to the environment exceeding certain environmental limits, it still can harm the environment through eutrophication that directly impact aquatic life and water scarcity and security. Detrimental effects on the environment due to hazardous waste will obviously lead to negative impacts to human's life and its ecosystem. We continually astonished with Japan's citizens, companies and NGOs who are coming together in order to create environmental sustainability through 3Rs (reduce, reuse, recycle). The word "mottainai" (もったいない) which can be translated as "don't waste anything worthy" or "what a waste" has been inculcated to represent the island nation's environmental awareness. An increasing rate of recycling is undeniably the key in driving the transitional process from a linear "take-make-dispose" economy to a circular "make-use-return" economy (Kurniawan et al. 2021). Thus, over a decade, various countries have partially integrated the recovery concept into national laws, such as Japan's 2002 Basic Law for Establishing a Recycling-Based Society (Su et al., 2013). In a report written by Japan Ministry of the Environment (2014), about 385.98 million tonnes of industrial waste was produced in 2010. Out of that amount, only 14.25 million tonnes (3.7%) were sent for final disposal. How does Japan handle their industrial wastes as such that only 3.7% of total generated industrial waste was sent to the final disposal? Thus, we can challenge ourselves to take Japan's best practices as a benchmark for managing industrial hazardous waste sustainably. 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. So now, it is a challenge for us to convert environmental pollution into valuable resources even for toxic waste. Treatment using natural resources such biological processes including phytoremediation, mycoremediation and phycoremediation will be more environmentally friendly and simultaneously open opportunities for resource recovery. More challenges and opportunities of these green technologies are expected ahead before they can be commercialized and applied in real field application especially in tropical countries.

Keywords: mining, aquaculture, agriculture, primary resources, waste recovery, waste minimization

A.8

Nanotechnology Development and Commercialization Based on Natural Resources: Technology Transfer from Lab to The Industry in Indonesia

Nurul Taufiqu Rochman

Research Center for Advanced Material, National Research and Innovation Agency, Serpong, Indonesia

E-mail address: nurul@nano.or.id

Abstract

Nanotechnology will become the next industrial revolution. Indonesia, a country with abundant of natural resources and 4th largest in population, has to take advantage for development of nanotechnology. This required appropriate strategy regarding to Indonesia's potential and capability in advancing technology. This presentation overviews a potential application of nanotechnology to increase the added value of Indonesian natural resources. First, a brief introduction of nanotechnology and its current status are described. Potential applications of nanotechnology in Indonesia, particularly for increasing the added value of Indonesian natural resources are highlighted. The models of technology transfer (commercialization) at LIPI/BRIN are explained and its respective achievements over the past 5 years are also reported. The role of the start-up incubation program in "the valley of death of innovation" is described in detail. As a case study, the strategy of commercialization of nanotechnology and the experience of building nanotechnology-based startups with young technopreneurs in Indonesia are presented.

Keywords: Nanotechnology, Indonesia natural resources, added value, commercialization.

B.1

Application of Biosurfactant-Producing Rhizobacteria in Phytoremediation of Crude Oil Sludge

Siti Shilatul Najwa Sharuddin[1]*, Siti Rozaimah Sheikh Abdullah[1], Hassimi Abu Hasan[1,2], Ahmad Razi Othman[1], Nur 'Izzati Ismail[1] & Israa Abdulwahab Al-Baldawi[a]

[1] Department of Chemical and Process Engineering, Faculty of Engineering and Built Environment, Universiti Kebangsaan Malaysia, 43600 UKM Bangi, Selangor, Malaysia.

[2] Research Centre for Sustainable Process Technology (CESPRO), Faculty of Engineering and Built Environment, Universiti Kebangsaan Malaysia, 43600 UKM Bangi, Selangor, Malaysia.

[a]Department of Biochemical Engineering, Al-khwarizmi College of Engineering, University of Baghdad, Baghdad, Iraq

*Corresponding author/presenter: Email: sitishilatulnajwa@gmail.com

Abstract

Crude oil sludge is one of the key waste streams contain higher concentration of total petroleum hydrocarbon (TPH). Due to its hydrophobic characteristics and slow process of biodegradation, TPH can remain in the environment for a long time and its toxicity can cause negative impact on ecosystem for both animals and humans. The objective of this study was to examine the effectiveness of phytoremediation process bioaugmented with rhizobacteria-producing biosurfactant for the remediation of crude oil sludge containing organic contaminants. In this study, rhizobacteria isolated from rhizosphere soil of *Scirpus grossus* comprising *Bacillus* sp. strain SB1, *Bacillus* sp. strain SB3 and *Lysinibacillus* sp. strain SB6 were capable to simultaneously produce biosurfactant and biodegrade hydrocarbon. A set of tanks containing crude oil sludge with different variations was prepared to study the effect of inoculation of these three rhizobacteria species and biosurfactant on phytoremediation of crude oil sludge. The planted crude oil sludge contaminated soil with *S. grossus* was inoculated with these three rhizobacteria, extracted biosurfactant, culture supernatant, and sodium dodecyl sulfate (SDS) as synthetic surfactant was monitored for 90 days in a greenhouse. The findings proved that inoculation of rhizobacteria and biosurfactant extracted by the same rhizobacteria effectively increase the bioavailability of organic pollutants and thus enhanced their microbial degradation in soil. Plants has enhanced the rhizosphere environment for microorganisms and thus promoted the bioremediation of contaminants. Hence, the combination treatment of biosurfactant and

phytoremediation process becomes an effective method in reducing the soil pollution due to petroleum waste.

Keywords: Biosurfactant, *Scripus Grossus*, Crude oil sludge, Rhizobacteria

1. Introduction

Petroleum refinery is one of industrial plant which refined the crude oil for different type of useful products such as liquefied petroleum gas, gasoline, kerosene, diesel dan asphalt base [1,2] However, there are some unwanted leakages of petroleum oil and its products during the refining process which might act as a persistent water and soil pollutants. According to [3], among the waste produced, solid wastes which is crude oil sludge is becoming the centre of attention due to its toxicity that can contaminate the environment and damage industrial activity. The soil pollution caused by crude oil sludge generate huge changes in the chemical and physical properties of soil consequence in negative fallout for plant growth. The toxicity of waste generated from petroleum products reduce the presence of nutrients, water, and oxygen in soil resulting in decrease in the rate of seed germination and high effect on plant growth [4].

Several technologies all around the world have been proposed for treatment of oil contaminated sites but the most promising method to reduce the pollution is through bioremediation combining with microorganism technique [5,6]. As presented in many literatures review, the optimal rate of biodegradation of hydrocarbon in soil pollution by microorganism can be maintained by the continuing source of oxygen, nutrients, and water supply [7]. However, high hydrophobicity characteristic and low solubility of petroleum hydrocarbon compounds make them hardly available for microorganism and thus remediation of soil polluted with these compounds is limited [5]. In this regard, the use of biosurfactant producing plant growth rhizobacteria in combination with phytoremediation using native plant species is proposed. The use of biosurfactant in phytoremediation study is proven can enhance the contact surface of bacteria cell by increasing the bioavailability and solubility of pollutants in which at the same time promote the growth of bacteria and the rate of bioremediation [8].

According to [9], some microorganism such as bacteria isolated from plant rhizospheres can metabolize petroleum-based compounds and produce biosurfactants. Generally, biosurfactants are amphiphilic molecules that can be produced extracellularly by a wide range of microorganisms [10] including bacteria [5, 10], fungi [3] and yeast [1]. Most of related research explores the potential implication of biosurfactant isolated from oil contaminated area which the identified species shows better degradation of hydrocarbons in their native environment [3]. Therefore, this study was conducted with the focus is on the removal of toxic effect of total petroleum hydrocarbon (TPH) using most important physiological properties of rhizobacteria such as biosurfactant combination with plant that can be used to enhance the efficiency of phytoremediation of petroleum contaminated soil.

2. Materials and Methods

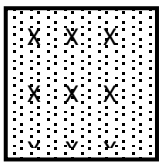
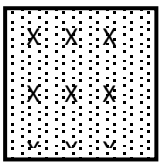
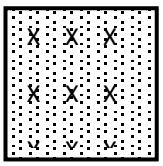
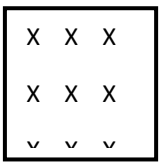
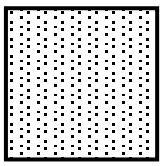
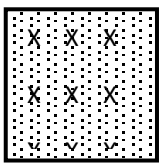
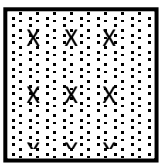
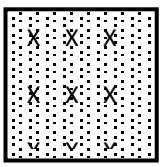
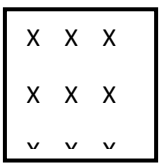
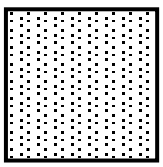
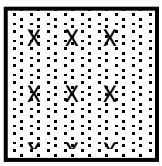
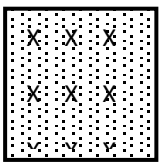
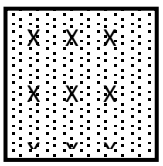
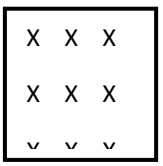
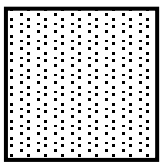
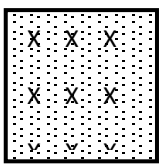
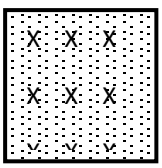
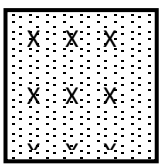
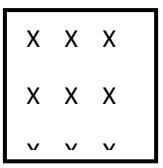
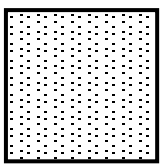
2.1 Crude oil sludge and plant preparation

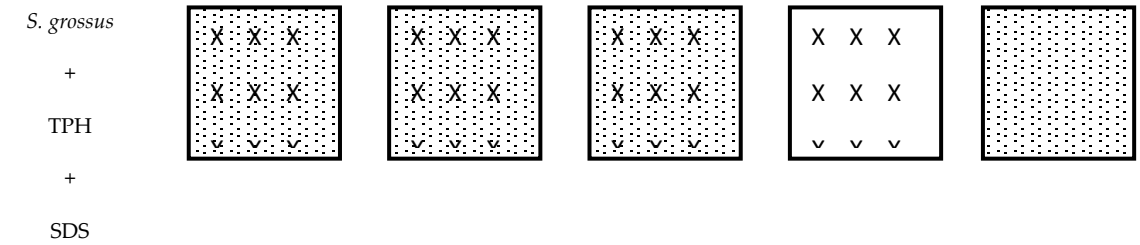
The real crude oil sludge used in this research study was collected from one of the crude oil terminals in Malaysia, stored in a lever lock closure drum and transported to greenhouse. Native plant species know as *S. grossus* was taken from its natural habitat (wetlands) Tasik Chini, Pahang. All plants were propagated for experimental use in a greenhouse located in Universiti Kebangsaan Malaysia, Selangor, Malaysia. During plant propagation, the plant growth was closely monitored by ensuring good conditions with sufficient water supply for plants and used in the next experimental run after two months of propagation.

2.2 Set-up of phytoremediation application

Table 1 depicts the schematic diagram of application study for *Scirpus grossus* with five treatments (different additives) consists of *S. grossus*+TPH, *S. grossus*+TPH+Rhizobacteria, *S. grossus*+TPH+Biosurfactant, *S. grossus*+TPH+Supernatant, *S. grossus*+TPH +SDS and two controls. The treatment study was conducted in the same type of polyethylene tank with a dimension of 60 cm × 40 cm × 30 in which about 30 kg of 100% concentration of real crude oil sludge was placed in each treatment tank. Each medium was planted with nine one-month old of *S. grossus* plants. As presented in Table 1, three replicates of treatment (R1, R2 and R3) of each additive type were set up. An unplanted tank was used as a contamination control (CC) and another tank without crude oil sludge was used as a plant control (PC). After the preparation of tank was completed, about nine healthy, two-month-old *S. grossus* plants were transferred into each tank containing crude oil sludge. All experimental plants were frequently watered every day throughout the exposure days.

Table 1: Schematic diagram for application process

Parameter	Treatment			Plant Control (PC)	Control Contaminant (CC)
	R1	R2	R3		
S. grossus + TPH					
S. grossus + TPH + Rhizobacteria					
S. grossus + TPH + Supernatan					
S. grossus + TPH + Biosurfactant					



2.3. Addition of the Rhizobacteria, Biosurfactant, Surfactant commercial (SDS) and culture supernatant for phytoremediation

This study examined the effects of adding the combination of three different rhizobacteria known as *Bacillus* sp. strain SB1, *Bacillus* sp. strain SB3 and *Lysinibacillus* sp. strain SB6 in which they were mixed to form a mixed culture with an equal ratio, surfactant commercial (SDS) and culture supernatant to the respective tank containing crude oil sludge planted with *S. grossus* accordingly for the growth analysis of *S. Grossus.* In this study, the best three bifunctional hydrocarbon degraders rhizobacteria *Bacillus* sp. strain SB1, *Bacillus* sp. strain SB3 and *Lysinibacillus* sp. strain SB6 were isolated from roots of *S. grossus* contaminated by hydrocarbons as conducted in the previous study [11].

2.4. Analysis of plant growth

The exposure period was conducted for 90 days. The sampling days for examining the concentration of TPH in crude oil sludge was conducted at day 0 and day 90. The physical appearance of plants such as turn yellowish colour for the leaves and some part of the stem, welting and death were also observed during initial (Day 0) and last day (Day 90) of exposure.

2.5. Analysis of TPH

The TPH concentration of the real crude oil sludge was extracted and examined through the modified USEPA 3550C method using ultrasonic solvent extraction [6]. The determination of the TPH concentration in crude oil sludge was calculated using the following equation:

$$TPH\ \text{Concentration} = \frac{\text{GC–FID Results}\left(\frac{\text{mg}}{\text{L}}\right)\text{x GC–vial volume (2 mL)}}{\text{Crude oil sludge (g)}} \quad \text{Eq.1}$$

The percentage of TPH degradation on Day 90 was determined by dividing the difference of the current TPH value with the initial TPH value, as in following equation:

$$\text{TPH Removal (\%)} = \frac{TPH_0 - TPH_{SD}}{TPH_0} X\ 100 \quad \text{Eq. 2}$$

with TPH_0 is the total petroleum hydrocarbon on sampling day 0, and TPH_{SD} is the total petroleum hydrocarbon on each sampling day.

2.6. Statistical analyses

Each set of data from experiments were performed in triplicate to compensate for experimental errors and were reported as the mean ± standard deviation (SD).

3. Results and Discussion

3.1. Growth performance of *S. grossus* and percentage removal TPH

As presented in Table 2, it can be concluded that the growth performance of *S. grossus* in plant control (PC) remained fresh with no physical change and the growth performance increased until 90 days of exposure. This result indicated that plant could survive without being affected by the presence of pollutants in which the leaves of plants remained green, and the stems were still fresh. However, the observation of day 90 (Table 2) exhibited that *S. grossus* had symptoms of death in all treatment tanks after 30 days of exposure. This condition was demonstrated by the physical changes (yellowing and withering leaves) of *S. grossus*. The leaves, stems and roots of *S. grossus* turned yellow and obviously dried after 30 days of exposure indicating that the plant cannot grow well and about to die.

Table 2: *S.grossus* growth performance and percentage removal of TPH

Parameter	Physical Observation		Percentage Removal of TPH (%)
	Day 0	Day 90	Day 90
Plant Control (PC)			0

S. grossus + TPH			73.6
S. grossus + TPH +Rhizobacteria			96.5
S. grossus + TPH + Supernatan			88.1
S. grossus + TPH + Biosurfactant			90.2
S. grossus + TPH + SDS			84.1

The presence of these symptoms in plant in all treatment tanks for the first 30 days exposure was due to the toxicity of crude oil sludge [11]. The higher concentration of TPH in crude oil sludge causes root damage and prevents the absorption of water and minerals needed by *S. grossus* [2, 6]. As indicated in Table 2, the lack of crucial plant nutrients, as highlighted by [12], results in a decline in plant biomass, ultimately leading to plant mortality by the end of the exposure study. This occurrence is observed mostly in the absence of any supplemental additives.

According to [7], the hydrophobic nature of oil-contaminated soil causes a reduction in oxygen concentration of root zone, and consequently a decrease in root elongation, and viability which reduced the biomass production and growth of the plants which is obviously showed by the condition of *S. grossus* in this study without the addition of any additives.

However, as shown in Table 2, there a few new saplings (new generation) of *S. grossus* in the presence of supernatant and SDS and more available new saplings were recorded in medium tanks containing rhizobacteria and biosurfactant which the new saplings grew healthily up to 90 days of research study. One of the effective ways to increase the bioavailability of petroleum hydrocarbon pollutants in soil is by using microbial interaction to enhance their desorption and solubilization, thereby facilitating microbial degradation [8]. In this combination treatment with phytoremediation, plants provide sufficient nutrients and habitation; while in return, the bacteria improve plant health and enhance the biodegradation of organic contaminants in the rhizosphere [12].

The results of percentage removal of TPH in application study as presented in Table 2 illustrate the lowest percentage removal of TPH after 90 days exposure was 73.7% that occurs in the medium having only *S. grossus*, followed by 84.6% by the presence of SDS, 88.1% when the surfactant was added, 90.3% with the addition of biosurfactant and the highest percentage TPH removal was 96.6% by rhizobacteria. In this study, three different rhizobacteria added in the medium were known as plant growth promoting rhizobacteria which play a significant role in the manipulation of the rhizosphere by increased production of plant growth promoting hormones, enzymes, siderophores, biosurfactants and 1-aminocyclopropane, 1-carboxylate (ACC) deaminase [8]. Moreover, symbiotic relationships between the additives and plants also facilitate the successful survival of plants under toxic environmentBiosurfactants produced by bacteria are more effective for TPH remediation because they solubilize, emulsify, and mobilize better than chemical surfactants (SDS).

These results agree with those of [13] who reported the removal of crude oil with rhamnolipid was one of the best options rather than with SDS because of the advantages of biodegradation and low environmental toxicity. In addition, as stated by [14,15] biodegradation in soil seems to be positively influenced by the presence of plants and plant–bacterial interactions either directly from rhizobacteria or from extracted product called biosurfactant. Each partner of the plant–

microbe system works in a variety of ways and better tolerance in contaminated soil results in a more significant role in phytoremediation [16].

4. Conclusions

In conclusion, the addition of various additives with *S. grossus* demonstrated that the use of a bacteria producing biosurfactant are excellent for the phytoremediation of crude oil contaminated soil. The addition of culture supernatant, rhizobacteria, SDS and biosurfactant shows increasing percentage removal of TPH due to increasing number of bacterial colonies at the root surface and stimulates microbes to decompose pollutants through rhizodegradation mechanism. The long, fibrous roots of *S. grossus* have a large surface area that aids in the multiplying of rhizosphere microbial communities and increases interactions with TPH, promoted especially by bacterial and biosurfactant additives. Therefore, the biosurfactant extracted from those three rhizobacteria has strong potential for the application of phytoremediation of TPH-contaminated soil.

Acknowledgements

The authors would like to thank the Malaysia Ministry of Higher Education, University Kebangsaan Malaysia (UKM) (GUP-2022-022) and Tasik Chini Research Centre (PPTC) for supporting this research project.

References

[1] Mecheri DL, Gan KS, Djenane, D. Biosurfactant production from newly isolated Rhodotorula sp.YBR and its great potential in enhanced removal of hydrocarbons from contaminated soils. *World Journal of Microbiology and Biotechnology*. 2021;*37*(1). https://doi.org/10.1007/s11274-020-02983-3

[2] Das, AJ, Kumar, R. Bioremediation of petroleum contaminated soil to combat toxicity on Withania somnifera through seed priming with biosurfactant producing plant growth promoting rhizobacteria. *Journal of Environmental Management*. 2016;*174*:79–86. https://doi.org/10.1016/j.jenvman.2016.01.031

[3] Othman AR, Ismail NS, Abdullah SRS, Hasan HA, Kurniawan SB, Sharuddin SSN, Ismail NI. Potential of indigenous biosurfactant-producing fungi from real crude oil sludge in total petroleum hydrocarbon degradation and its future research prospects. *Journal of Environmental Chemical Engineering*. 2021;*10*(3). https://doi.org/10.1016/j.jece.2022.107621

[4] Khan MAI, Biswas B, Smith E, Naidu R, Megharaj M. Toxicity assessment of fresh and weathered petroleum hydrocarbons in contaminated soil- a review. In *Chemosphere*. 2018;212:755–767. https://doi.org/10.1016/j.chemosphere.2018.08.094

[5] Sharuddin SSN, Abdullah SRS, Hasan HA, Othman AR, Ismai NI. Potential bifunctional rhizobacteria from crude oil sludge for hydrocarbon degradation and biosurfactant production. *Process Safety and Environmental Protection*. 2021;155:108–121.

https://doi.org/10.1016/j.psep.2021.09.013

[6] Al-Baldawi IA, Abdullah SRS, Anuar N, Suja F, Mushrifah I. Phytodegradation of total petroleum hydrocarbon (TPH) in diesel-contaminated water using Scirpus grossus. *Ecological Engineering*. 2015;74:463–473. https://doi.org/10.1016/j.ecoleng.2014.11.007

[7] Allamin IA, Halmi MIE,Yasid NA, Ahmad SA, Abdullah SRS, Shukor Y. Rhizodegradation of Petroleum Oily Sludge-contaminated Soil Using Cajanus cajan Increases the Diversity of Soil Microbial Community. *Scientific Reports*. 2020;10(1). https://doi.org/10.1038/s41598-020-60668-1

[8] Sonowal S, Nava AR, Joshi SJ, Borah SN, Islam NF, Pandit S, Prasad R, Sarma H. Biosurfactant-assisted phytoremediation of potentially toxic elements in soil: Green technology for meeting the United Nations Sustainable Development Goals. *Pedosphere*, 2022;*32*(1):198–210. https://doi.org/10.1016/S1002-0160(21)60067-X

[9] Lal S, Ratna S, Said OB, Kumar R. Biosurfactant and exopolysaccharide-assisted rhizobacterial technique for the remediation of heavy metal contaminated soil: An advancement in metal phytoremediation technology. In *Environmental Technology and Innovation*. 2018;10:243–263. https://doi.org/10.1016/j.eti.2018.02.011

[10] Almansoory AF, Hasan HA, Abdullah SRS, Idris M, Anuar N, Al-Adiwish WM. Biosurfactant produced by the hydrocarbon-degrading bacteria: Characterization, activity and applications in removing TPH from contaminated soil. *Environmental Technology and Innovation*. 2019;14. https://doi.org/10.1016/j.eti.2019.100347

[11] Sharuddin SSN, Abdullah SRS, Hasan HA, Othman AR. Comparative tolerance and survival of scirpus grossus and lepironia articulata in real crude oil sludge. In *International Journal of Engineering & Technology*. 2019; 8(1). www.sciencepubco.com/index.php/IJET

[12] Fatima K, Imran A, Amin I, Khan QM, Afzal M. Successful phytoremediation of crude-oil contaminated soil at an oil exploration and production company by plants-bacterial synergism. *International Journal of Phytoremediation*. 2018;20(7):675–681. https://doi.org/10.1080/15226514.2017.1413331

[13] Liduino VS, Servulo EFC, Oliveira FJS. Biosurfactant-assisted phytoremediation of multi-contaminated industrial soil using sunflower (Helianthus annuus L.). *Journal of Environmental Science and Health. Part A Toxic/Hazardous Substances and Environmental Engineering*. 2018; *53*(7):609–616. https://doi.org/10.1080/10934529.2018.1429726

[14] Abdullah SRS, Al-Baldawi IA, Almansoory AF, Purwanti IF, Al-Sbani NH, Sharuddin SSN. Plant-assisted remediation of hydrocarbons in water and soil: Application, mechanisms, challenges, and opportunities. In *Chemosphere*. 2020;247.https://doi.org/10.1016/j.chemosphere.2020.125932

[15] Palomino SY, Vilchez GL, Matencios CC, Cornejo TJ, Camargo HS, Canales GV. Systematic Review of the Efficiency of Aquatic Plants in the Wastewater Treatment. *IOP Conference Series: Earth and Environmental Science*. 2022;1009(1). https://doi.org/10.1088/1755-1315/1009/1/012004

[16] Xianagang M, Iyobosa E, Jun NH, Fang S, Zhennan W. Biodegradation of petroleum

hydrocarbon polluted soil. *Indian Journal of Microbiology Research*. 2020;*7*(2):104–112. https://doi.org/10.18231/j.ijmr.2020.022

B.2

Phytotoxicity of *Phragmites karka* in petroleum hydrocarbon exposure towards different plant densities

Nuratiqah, M., Siti Rozaimah, S.A., Hassimi, A.H. and Ahmad Razi, O.

Department of Chemical and Process Engineering, Faculty of Engineering and Built Environment, National University of Malaysia, 43600, Bangi, Selangor, Malaysia

Correspondence should be addressed to Nuratiqah, M.; E-mail address: nuratiqahmarsidi@gmail.com

Abstract

Phytoremediation method has become a promising technology to treat various type of pollutants using plants. In this experiment, *Phragmites karka,* a perrenial plant was selected to degrade total petroleum hydrocarbons (TPH) inside petroleum sludge at different number of plant densities (five plants (P5), seven plants (P7), and nine plants (P9)). Thirteen plastic crates filled with 30 kg of petroleum sludge at 100% concentration were used for this phytotoxicity test, with three replicates for each different plant density. Another three crates served as plant control (PC), while one crate was used as a control contaminant (CC) without plant. The crate that has the highest TPH elimination at the end of the exposure will be used for the following study. It was observed that 39.7%, 54.0%, and 94.5% removal were achieved at P5, P7, and P9 crates, respectively. Based on the results obtained, it was suggested to use 9 plants in P9 crate as the TPH removal was highest throughout 28 days of exposure.

Keywords: Phytotoxicity, Phragmites karka, total petroleum hydrocarbon (TPH), numbers of plant, phytoremediation

1. Introduction

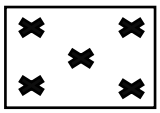
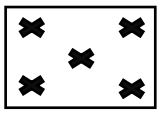
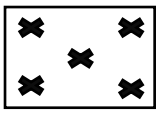
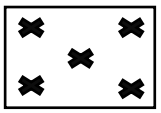
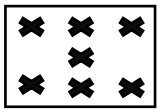
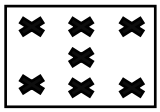
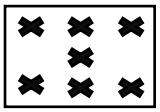
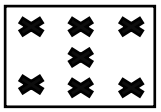
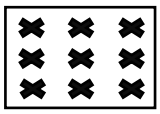
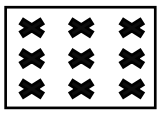
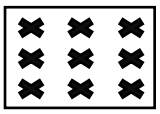
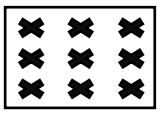
Petroleum is a purified form of crude oil which consist of hydrocarbon combination that is ready to be distilled fractionally to give out a petroleum product. Crude oil exits as a liquid in natural subterranean reservoirs and stays liquid when brought to the surface [1]. Crude oil-based hydrocarbons are responsible for many global environmental contaminants [2]. Processing activities in the hydrocarbon oil sector release hazardous aromatic organic chemicals into the environment, including polyaromatic hydrocarbons (PAHs), phenolic substances that are hardly degradable by nature, chlorophenols, and cresols toxins [3, 4, 5]. Many reports from throughout the world have documented the negative impacts of petroleum hydrocarbons in air, water, and soil on people's health, including psychological issues, respiratory tract irritation, skin, and renal difficulties, and blood profiling disturbances [6,7].

Due to the fact of phytotoxicity, this study explores the phytoremediation technique to treat hydrocarbon contaminants from petroleum sludge using a native plant. This method has long been regarded as a reliable, cost-effective, and ecologically friendly approach for removing hydrocarbon from petroleum contaminated soils [8,9,10]. Different plants such as *Scirpus grossus* [10], *Festuca arundinacea L.* [11, 12], *Lepironia articulata* [13], *Impatiens balsamina L.* & *Crotalaria retusa L.* [14], and *Rhizophora mangle L.* [15] have been used to remediate petroleum hydrocarbon contaminant. However, native plant, *Phragmites karka* has not been extensively studied to degrade total petroleum hydrocarbon in petroleum sludge. The aim of this study was to use *P. karka* as a plant remediator and to determine the effect of plant densities during phytotoxicity test of petroleum sludge. Hydrocarbon degradation and toxicity decrease were tracked. This experiment's findings will be used in further analysis to create plant-mycorrhizae synergy for effective remediation of petroleum hydrocarbon contaminated soil.

2. Materials and Methods

Phytotoxicity study was run for 28 days in a greenhouse crate in order to determine the tolerance of *P. karka* when the petroleum sludge was introduced. Experiment was done in 13 plastic crates, each with dimension of 60 cm x 30 cm x 30 cm (L x W x D). It was filled with 30 kg of 100% concentration of petroleum sludge containing hydrocarbon. The numbers of *P. karka* plant used were 5, 7, and 9 in triplicates (R1, R2, R3), while another three crates served as plant control (PC) (without petroleum sludge contaminant). Only one crate acted as a control contaminant (CC). The design of phytotoxicity test at different numbers of plant was shown in Table 1. During the study, plants were watered on alternate days with no additional fertilizer was added.

Table 1. Experimental setup during phytotoxicity test

Number of plant	Petroleum sludge with *P.karka*			Plant control (PC)	Control contaminant (CC)
	Replicate 1 (R1)	Replicate 2 (R2)	Replicate 3 (R3)		
5					
7					
9					

To determine the TPH concentration in sludge, sampling was done on day 0, day 7, day 14, day 21, and day 28. TPH concentration in sludge can be determine as in Eq. (2) below:

$$\textbf{TPH degradation}(\%) = \frac{TPH_0 - TPH_t}{TPH_0} \times 100 \quad (1)$$

with, TPH_0 is the total petroleum hydrocarbon on day 0 and TPH_t is the total petroleum hydrocarbon on each sampling time.

3. Results and Discussion

The finding from the GC-FID analysis for TPH concentration in sludge when exposed to different plant numbers were shown as in Figure 3. Initially, TPH concentration on Day 0 was 37892 mg/kg in each treatment crate while the ratio of TPH to plant mass were 18.9, 13.5, and 10.5 for P5, P7, and P9 respectively. After 7 days of sampling, it was recorded that all the treatment crates including control contaminant are experienced lower TPH concentration which was 24277 mg/kg, 11687 mg/kg, 16841 mg/kg, and 7006 mg/kg with removal of 35.9%, 69.2%, 55.6%, and 81.5% in CC, P5, P7, and P9, respectively. The reduction in TPH content indicates the potential of plants for removal of hydrocarbons from the contaminated soil [16]. This finding agrees with the previous works which use native plants such as *Salvinia molesta* [17], *Ludwigia octovalvis* [8], and *Lepironia articulata* [18] to remove the hydrocarbon. The TPH concentration was then shifted to increase on Day 14 at all crates and remain increased until Day 21. The TPH concentration shows the lowest removal of 7.9% at P7 compared to P5 (27.3%) and P9 (50.6%) at Day 21. At this stage, some plants experienced withered stem and leaves and some plants died due to hydrocarbon toxicity and because the complexity of oily sludge composition of hydrocarbon, it is highly resistant and less bioavailable for microbial degradation [19]. At the end of exposure, all crates show TPH concentration of 12120 mg/kg, 22840 mg/kg, 17433 mg/kg, and 2090 mg/kg giving removal of 68.0%, 39.7%, 54.0%, and 94.5% in CC, P5, P7, and P9, respectively. The highest TPH removal was recorded at P9 with 94.5% compared to P5 and P7 with a minimum ratio of TPH mass to plant mass of 10.5.

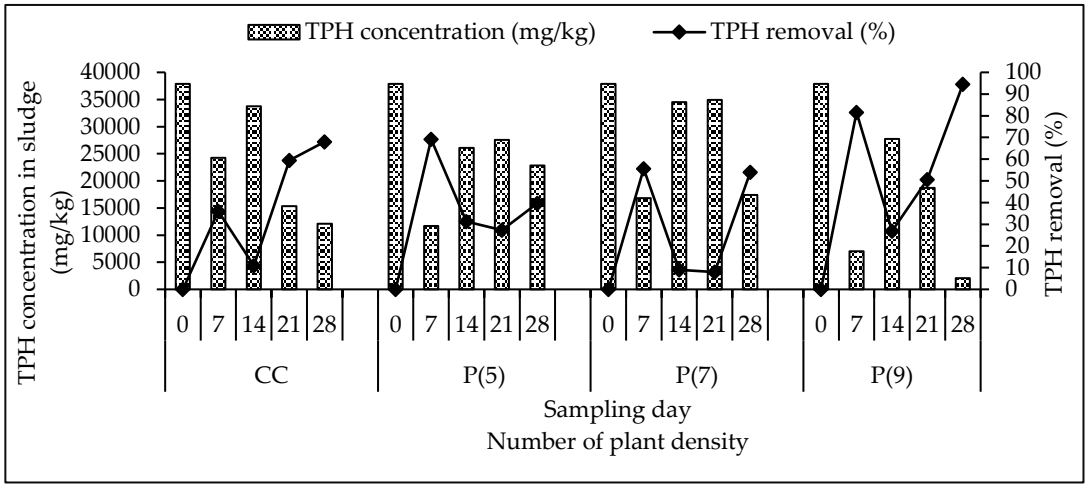


Figure 3. TPH concentration and TPH removal in sludge

4. Conclusion

The results showed that as the number of plants were increased, the TPH concentration decreased resulting to higher TPH removal. *P.karka* has the ability to survive in 100% concentration of petroleum sludge with 94.5% removal and minimum ratio of 10.5 of TPH mass to plant mass. The possibilities of toxic effects to plants increase if the ratio of TPH mass to plant mass was higher. Therefore, a maximum of 9 numbers of plant were selected as a basis to determine TPH concentration in the next application.

Acknowledgement

This research was financially supported by the Malaysia Ministry of Higher Education and Universiti Kebangsaan Malaysia through grant number FRGS-1-2019-TK02-UKM-01-1.

References

[1] Islam, B. Petroleum sludge, its treatment and disposal: A review. International Journal of Chemical Sciences. 2015; 13(4): 1584–1602.

[2] Ławniczak Ł, Woźniak-Karczewska M, Loibner AP, Heipieper HJ, Chrzanowski Ł. Microbial degradation of hydrocarbons—basic principles for bioremediation: A Review. Molecules. 2020; 25(4): 856. DOI: 10.3390/molecules25040856

[3] Al-Khalid T, El-Naas MH. Organic contaminants in refinery wastewater: characterization and novel approaches for biotreatment. Recent Insights in Petroleum Science Engineering. 2018; 371. DOI: 10.5772/intechopen.72206

[4] Varjani SJ. Microbial degradation of petroleum hydrocarbons. Bioresource Technology. 2017; 223: 277–286. DOI: 10.1016/j.biortech.2016.10.037

[5] Zhang B, Matchinski EJ, Chen B, Ye X, Jing L, Lee K. Marine oil spills—Oil pollution, sources and effects. In: heppard C, editor. World Seas: An Environmental Evaluation Volume III: Ecological Issues and Environmental Impacts. 2nd ed. Warwick: Academic Press; 2019; 391–406. DOI: 10.1016/C2015-0-04336-2

[6] da Silva BM, Maranho LT. Petroleum-contaminated sites: Decision framework for selecting remediation technologies. Journal of Hazardous Materials. 2019; 15: 378. DOI: 10.1016/j.jhazmat.2019.05.115

[7] Jeevanantham S, Saravanan A, Hemavathy RV, Kumar PS, Yaashikaa PR, Yuvaraj D. Removal of toxic pollutants from water environment by phytoremediation: A survey on application and future prospects. Environmental Technology & Innovation. 2019; 13: 264–276. DOI: 10.1016/j.eti.2018.12.007

[8] Alanbary SRN, Abdullah SRS, Al-Baldawi IAW, Hassan HA, Anuar N, Othman AR, Suja F. Phytotoxicity of contaminated sand containing crude oil sludge on *Ludwigia octovalvis*. Journal of Ecological Engineering. 2019; 20(11): 246–255. DOI: 10.12911/22998993/113189

[9] Fatima K, Imran A, Amin I, Khan QM, Afzal M. Successful phytoremediation of crude-oil contaminated soil at an oil exploration and production company by plants-bacterial synergism. International Journal of Phytoremediation. 2018; 20(7): 675–681. DOI: 10.1080/15226514.2017.1413331

[10] Sharuddin SSN, Abdullah SRS, Hasan HA, Othman AR. Comparative tolerance and survival of *Scirpus grossus* and *Lepironia articulata* in real crude oil sludge. Progress in Color, Colorants and Coatings. 2018; 8(1): 8–15. DOI: 10.14419/ijet.v8i1.12337

[11] Hou J, Wang Q, Liu W, Zhong D, Ge Y, Christie P, Luo Y. Soil microbial community and association network shift induced by several tall fescue cultivars during the phytoremediation of a petroleum hydrocarbon-contaminated soil. Science of the Total Environment. 2021; 792: 148411. DOI: 10.1016/j.scitotenv.2021.148411

[12] Steliga T, Kluk D. Application of *Festuca arundinacea* in phytoremediation of soils contaminated with Pb, Ni, Cd and petroleum hydrocarbons. Ecotoxicology and Environmental Safety. 2020; 194: 110409. DOI: 10.1016/j.ecoenv.2020.110409

[13] Sbania NHAL, Abdullah SRS, Idris M, Jehawi OH, Ismail NI. Preliminary test of hydrocarbon exposure on *Lepironia articulate* in phytoremediation process. Applied Mechanics and Materials. 2015; 773–774: 1121–1126. DOI: 10.4028/www.scientific.net/AMM.773-774.1121

[14] Gamage SSW, Masakorala K, Brown MT, Gamage SMKW. Comparative phytoremediation potentials of *Impatiens balsamina L.* and *Crotalaria retusa L.* for soil contaminated with used

lubricating oil. Environmental Advances. 2021; 5: 100095. DOI: 10.1016/j.envadv.2021.100095

[15] Moreira ITA, Oliveira OMC, Triguis JA, dos Santos AMP, Queiroz AFS, Martins CMS, Silva CS, Jesus RS. Phytoremediation using Rizophora mangle L. in mangrove sediments contaminated by persistent total petroleum hydrocarbons (TPH's). Microchemical Journal. 2011; 99(2): 376–382. DOI: 10.1016/j.microc.2011.06.011

[16] Chakravarty P, Deka H. Enzymatic defense of *Cyperus brevifolius* in hydrocarbons stress environment and changes in soil properties. Scientific Reports. 2021; 11(718). DOI: 10.1038%2Fs41598-020-80854-5

[17] Al-Baldawi IA, Abdullah SRS, Ismail NI, Almansoory AF, Jasim SS. Phytotoxicity of *Salvinia molesta* in diesel exposure. Al-Khwarizmi Engineering Journal. 2021. 17(3): 13-21. DOI: 10.22153/kej.2021.09.001

[18] Al-Sbani NH, Abdullah SRS, Idris M, Hasan HA, Jehawi OH, Ismail NI. Toxicity test of hydrocarbon exposure on *Lepironia articulata* in phytoremediation process. Asian Journal of Chemistry. 2016; 28(1): 30-34. DOI: 10.14233/ajchem.2016.19188

[19] Allamin IA, Halmi MIE, Yasid NA, Ahmad SA, Abdullah SRS, Shukor Y. Rhizodegradation of petroleum oily sludge-contaminated soil using *Cajanus cajan* increases the diversity of soil microbial community. Scientific Reports. 2020; 10(4094). DOI: 10.1038%2Fs41598-020-60668-1

B.3

Feasibility of sludge from aquaculture effluent as bio-fertilizer

Ku Zhi Wei, Zul Aidil Fitri Mohd Ghazali, Faridatun Najiha Mohd Jaafar, Nur Emylia Ezanna Mazlan, Wong Whui Dhong, Siti Rozaimah Sheikh Abdullah, Nur 'Izzati Ismail*

Department of Chemical and Process Engineering, Faculty of Engineering and Built Environment, Universiti Kebangsaan Malaysia, 43600 UKM Bangi, Selangor, Malaysia

*Corresponding author: nurezatyismail@ukm.edu.my

Abstract

The present study was conducted to determine the potential of aquaculture sludge produced after biocoagulation-flocculation process of nutrient-rich effluent for biofertilizer. The sludge resulting from this process has a high potential to be used as biofertilizer considering no chemical coagulant-flocculant was employed during the whole treatment plus the existing nutrients in the aquaculture effluent. A total of five pots (48 × 19 ×17 cm: L × W × H) were prepared with different composition of plant medium (5 kg): 100% garden soil, 100% sand and 92% sand + 8% sludge. Five tomato saplings were planted in each pot. The growth of the plants (physical observation, the numbers of leaves and the height of plants) was monitored for 8 weeks. At the end of exposure period, plants in sand with sludge recorded the highest average number of leaves and average plant height of 91 leaves and 67 cm. These values are slightly higher from plants in garden soil (average number of leaves is 77, average plant height is 63 cm). The utilisation of sludge from aquaculture treatment effluents has additional value as bio-fertilizer in plant systems.

Keywords: Sludge management; nutrient recovery; biofertilizer; circular economy.

1. Introduction

Recently, researchers from all over the globe are throwing their attention towards the utilisation of coagulants-flocculants from natural resources rather than metal-based coagulants-flocculants in water and wastewater treatment. This is due to the toxicity of metal-based coagulants-flocculants which will affect human health and usually linked to Alzheimer's disease and senile dementia [1, 2]. In addition, the employment of metal-based coagulants-flocculants makes it unsuitable to be recovered and to be converted into fertilizer even if the effluent having adequate nutrients content. The fate of sludge after coagulation-flocculation using metal-based compounds requires special handling to be secured landfilled.

Therefore, in order to recover the sludge produced after coagulation-flocculation process of nutrient-rich effluent and to adopt zero waste discharge, natural resources from plants: *Moringa oleifera* [3,4] and okra [5, 6], microorganisms: *Bacillus enclensis* [7], *Serratia marcescens* [8] and *B. velezenis* [9] and animal: shellfish [10], shrimp shell [11], and periwinkle shell [12] were used in previous studies. These natural resources for natural coagulants in treating nutrient-rich effluent such as aquaculture, palm oil mill and sago mill effluent exhibited good performance in removing turbidity, TSS, colour and COD. According to studies conducted by [13], [14] and [15], the capability of available nutrients in effluent to become biofertilizer is high. Therefore, based on the performances from past studies, it was chosen to study the effect of existing nutrients in sludge produced after coagulation-flocculation process of aquaculture effluent towards the growth of tomato plant (*Solanum lycopercium* L.).

2. Materials and Method

2.1. Pot experiment

Pot experiment was carried out in a greenhouse condition. Tomato plants was exposed to different composition of planting medium (100% garden soil, 100% sand and 92% sand + 8% sludge) to determine the growth of the plants. Pot with dimension of 48 cm long × 19 cm wide × 17 cm high was used and each pot was filled with 5 kg of planting medium. In each pot, 5 saplings were planted. The effect of sludge on the growth of tomato plants planted in sand mixed with sludge was compared with the growth of tomato plants planted in garden soil and sand.

2.2. Plant growth monitoring

Monitoring on physical observation of plants was done every two weeks for two months (week 0, 2, 4, 6 and 8). Visual monitoring of plants condition and three physical characteristics related to the number of leaves, height and plant condition were monitored.

3. Results and Discussion

3.1. Physical observation of plant

At the end of exposure period, all plants in pot with 100% soil and 100% sand remained healthy. In contrast, two plants in pot of sand with sludge died at the end of exposure period leaving only three plants in that pot. Visual symptoms of chlorosis of the dead plants confirmed the presence of phytotoxicity due to excess of nutrients [16]. It is suggested that in a future study, the sand will be mixed with sludge using a mixer to reduce inconsistency of the nutrient distribution in the sand-mixture [17]. The condition of remaining plants was healthy. On Week 8, the height of plants planted in the sand was shorter that the ones planted in garden soil and also in the sand with sludge. This might be due to the minimum nutrients available in sand.

According to Ismail et al. [18] and Titah et al. [19], only minimum content of nitrate, potassium, sulfate, calcium, magnesium, chlorine, iron, zinc and manganese were available in sand.

3.2. Plant growth

Throughout the exposure period, the number of leaves and the height of the plant experienced an increasing trend for all pots (Figure 1). Pot with sand as planting medium showed the lowest increment in height and also number of leaves. Both the number of leaves and height of plant showed substantially increase for pot with garden soil and sand with sludge. On Week 8, the number of leaves and the height of plants planted in sand is the least (18 leaves and 12.5 cm, respectively) compared to plants planted in garden soil (77 leaves and 63.4 cm, respectively) and in sand with sludge (91 leaves and 67.8 cm, respectively). These results are in accord with a recent study [20] indicating that NPK available in bio-organic fertilizer from agricultural solid waste had increased the growth of plant, giving evidence that nutrients available in waste plays an important role for enhancing plant growth. The current study found that the sludge after biocoagulation-flocculation process of nutrient-rich effluent turn out to be a great potential as bio-fertilizer. Despite these promising results, questions remain. Further investigations will be carried out to determine macronutrients and micronutrients presence in the sludge and relate to the plant growth.

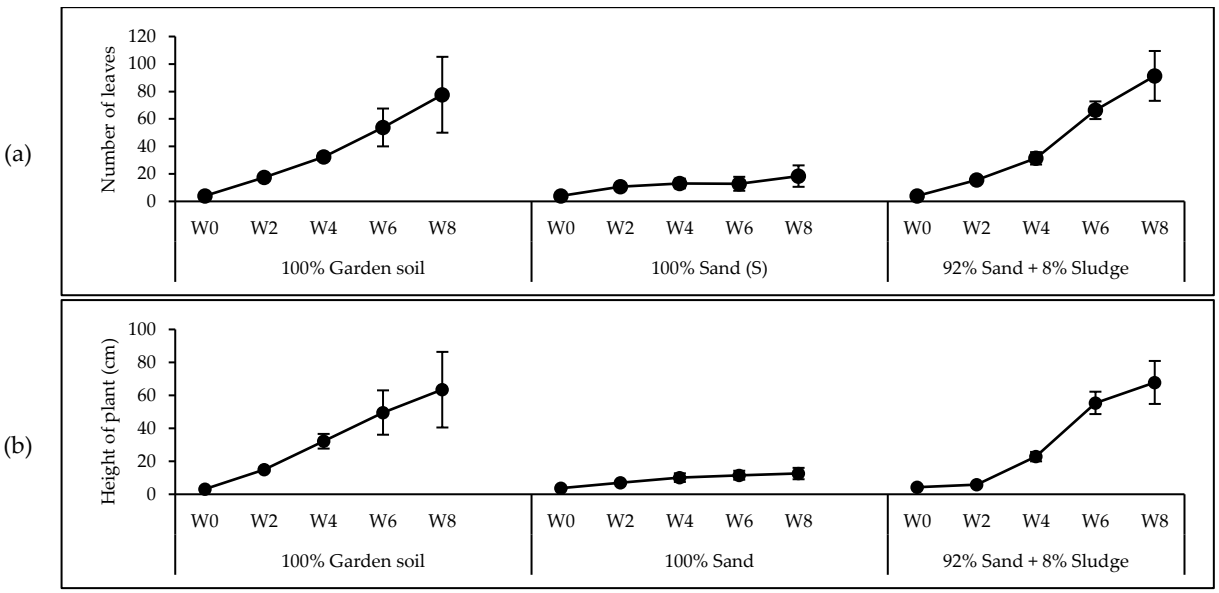


Figure 1. Agronomic parameters all through exposure period (a) number of leaves and (b) height of plant.

4. Conclusions

The results of this study showed that the application of sludge recovered after coagulation-flocculation of aquaculture effluent using natural coagulants-flocculants has a positive effect on the tomato growth. The growth of tomato plant in sand with sludge is in agreement with the growth of tomato plants in the garden soil. The addition of sludge in the sand led to plants with more leaves and taller compared to sand only. The current research showed that the additional of sludge in sand contains nutrients compounds which had a beneficial effect on plant.

Acknowledgements

The authors would like to extend their gratitude to Universiti Kebangsaan Malaysia for funding this research (GUP-2022-022).

References

[1] Kurniawan SB, Abdullah SRS, Imron MF, Said NSM, Ismail NI, Hasan HA, Othman AR, Purwanti IF. Challenges and opportunities of biocoagulant/bioflocculant application for drinking water and wastewater treatment and its potential for sludge recovery. International Journal of Environmental Research and Public Health 2020;17:1-33. DOI: 10.3390/ijerph17249312.

[2] Purwanti IF, Kurniawan SB, Ismail NI, Imron MF, Abdullah SRS. Aluminium removal and recovery from wastewater and soil using isolated indigenous bacteria. Journal of Environmental Management. 2019;249:109412. DOI: 10.1016/j.jenvman.2019.109412.

[3] Camacho FP, Sousa VS, Bergamasco R, Teixeira MR. The use of *Moringa oleifera* as a natural coagulant in surface water treatment. Chemical Engineering Journal. 2017;313:226-237. DOI: 10.1016/j.cej.2016.12.031.

[4] Gandiwa B, Moyo L.B, Ncube S, Mamvura TA, Mguni LL, Hlabangana N. Optimisation of using a blend of plant based natural and synthetic coagulants for water treatment: (*Moringa Oleifera*-*Cactus Opuntia*-alum blend). South African Journal of Chemical Engineering. 2020;34:158-164. DOI: 10.1016/j.sajce.2020.07.005.

[5] Lanan FABM, Sethu ASV, Arumugasamy SK. Utilisation of natural plant based fenugreek (*Trigonella foenum-graecum*) coagulant and okra (*Abelmoschus escluentus*) flocculant for palm oil mill effluent (POME) treatment. Journal of Environmental Chemical Engineering. 2020.;9(1):104667. DOI: 10.1016/j.jece.2020.104667.

[6] Muniz GL, Borges AC, da Silva TCF. Performance of natural coagulants obtained from agro-industrial wastes in dairy wastewater treatment using dissolved air flotation. Journal of Water Process Engineering. 2020;37:113. DOI: 10.1016/j.jwpe.2020.101453.

[7] Shukri ZNA, Chik CENCE, Hossain S, Othman R, Endut A, Lananan F, Terkula IB, Kamaruzzan AS, Rahim AIA, Draman AS, Kasan NA. A novel study on the effectiveness of bioflocculant-producing bacteria *Bacillus enclensis*, isolated from biofloc-based system as a biodegrader in microplastic pollution. Chemosphere 2022;08:136410. DOI:10.1016/j.chemosphere.2022.136410.

[8] Kurniawan SB, Abdullah SRS, Othman AR, Purwanti IF, Imron MF, Ismail NI, Ahmad A, Hasan HA. Isolation and characterisation of bioflocculant-producing bacteria from aquaculture effluent and its performance in treating high turbid water. Journal of Water Process Engineering. 2021;42:102194. DOI: 10.1016/j.jwpe.2021.102194.

[9] Hasan HA, Hafiz RE, Muhamad MH, Abdullah SRS. Bioflocculant production using palm oil mill and sago mill effluent as a fermentation feedstock: Characterization and mechanism of flocculation. Journal of Environmental Management. 2020;260:110046. DOI: 10.1016/j.jenvman.2019.110046.

[10] Lichtfouse E, Morin-Crini N, Fourmentin M, Zemmouri H, do Carmo Nascimento IO, Queiroz LM, Tadza MYM, Picos-Corrales LA, Pei H, Wilson LD, Crini G. Chitosan for direct bioflocculation of wastewater. Environmntal Chemistry Letters. 2019;17:1603-1621. DOI: 10.1007/s10311-019-00900-1.

[11] Hosny R, Fathy M, Ramzi M, Abdel Moghny T, Desouky SEM, Shama SA. Treatment of the oily produced water (OPW) using coagulant mixtures. Egyptian Journal of Petroleum. 2016;25:391-396. DOI: 10.1016/j.ejpe.2015.09.006.

[12] Menkiti MC, Ndaji CR, Ezemagu IG, Uddameri V. Application of periwinkle shell coagulant (psc) for the remediation of petroleum produced water (PPW) by coag-flocculation. Journal of Dispersion Science and Technology. 2016;37:760–774. DOI: 10.1080/01932691.2015.1060488.

[13] Cristina G, Camelin E, Tommasi T, Fino D, Pugliese M. Anaerobic digestates from sewage sludge used as fertilizer on a poor alkaline sandy soil and on a peat substrate: Effects on tomato plants growth and on soil properties. Journal of Environmental Management. 2020;269:110767. DOI: 10.1016/j.jenvman.2020.110767.

[14] Hernández-Arenas R, Beltrán-Sanahuja A, Navarro-Quirant P, Sanz-Lazaro C. The effect of sewage sludge containing microplastics on growth and fruit development of tomato plants. Environmental Pollution. 2021;268:115779. DOI: 10.1016/j.envpol.2020.115779.

[15] Velli P, Manolikaki I, Diamadopoulos E. Effect of biochar produced from sewage sludge on tomato (*Solanum lycopersicum* L.) growth, soil chemical properties and heavy metal concentrations. Journal of Environmental Management. 2021;297:113325. DOI: 10.1016/j.jenvman.2021.113325.

[16] Caspersen S, Oskarsson C, Asp H. Nutrient challenges with solid-phase anaerobic digestate as a peat substitute – Storage decreased ammonium toxicity but increased phosphorus availability. Waste Management. 2023;165;128-139. DOI: 10.1016/j.wasman.2023.04.032.

[17] Sharuddin SSN, Abdullah SRS, Hasan HA, Othman AR, Ismail NI. Potential bifunctional rhizobacteria from crude oil sludge for hydrocarbon degradation and biosurfactant production. Process Safety and Environmental Protection. 2021;155;108-121. DOI: 10.1016/j.psep.2021.09.013.

[18] Ismail NI, Abdullah SRS, Idris M, Hasan HA, Halmi MIE, Al Sbani NH, Jehawi OH. Simultaneous bioaccumulation and translocation of iron and aluminium from mining wastewater by *Scirpus grossus*. Desalination Water Treatment. 2019;163;133–142. DOI: 10.5004/dwt.2019.24201.

[19] Titah HS, Abdullah SRS, Idris M, Anuar N, Basri H, Mukhlisin M. Phytotoxicity and uptake of arsenic by *Ludwigia octovalvis* in a pilot reed bed system. Environmental Engineering Scence. 2014;1;71–79. DOI:10.1089/ees.2013.0207.

[20] Majee S, SarkarKK, Sarkhel R, Halder G, Mandal DD, Rathinam NK, Mandal T. Bio-organic fertilizer production from industrial waste and insightful analysis on release kinetics. Journal of Environmental Management. 2023;325;116378. DOI: 10.1016/j.jenvman.2022.116378.

B.4

Potential of Local Plant as Natural Coagulating Agent in Treating Coffee Industrial Effluent

Radhiatul Atiqah Ramli Shah*, Siti Rozaimah Sheikh Abdullah, Hassimi Abu Hasan, Ahmad Razi Othman & Nur 'Izzati Ismail

Department of Chemical and Process Engineering, Faculty of Engineering and Built Environment, Universiti Kebangsaan Malaysia, 43600 UKM Bangi, Selangor, Malaysia.

Correspondence should be addressed to Radhiatul Atiqah Ramli Shah; E-mail address: radhiatul97@gmail.com

Abstract

The diversity of plants in Malaysia can be utilized as biocagulant to remove suspended solids from industrial effluent. This study aims to determine the potential of local plants as biocoagulant agents in treating industrial wastewater of coffee industrial effluent. Cassava leaves (*Manihot esculenta*) are selected for treating coffee industrial effluent that contains high total suspended solids (TSS) which exceeds the acceptable limits under Malaysia Environmental Quality Regulations (Industrial Effluent) 2009. These suspended solids can be removed through a liquid-solid separation treatment process known as the coagulation-flocculation method after determining the protein and polysaccharides content in the selected plant. To achieve the objective, the experiment was carried out by extracting protein and polysaccharide from local plant (M. *esculenta*) and analyzed using Bradford assay and phenol-sulfuric acid method, respectively at the range of 5 to 100 g/L. M. *esculenta* have shown to have the highest protein content at 6.15 g/L. Then, the experiment of the mass ratio of biocoagulant (mg)/ mass of TSS (mg) on TSS removal with different pH conditions was studied. The operational conditions of the experiment were fixed at 300 rpm and 5 min for rapid mixing, 180 rpm and 30 min for slow mixing and a settling time of 30 min. The results showed a TSS removal of 30.1% at an optimum ratio of 0.01 mg coagulant/mg TSS and biocoagulant dosage of 0.5 g/L. The findings give evidence that M. *esculenta* leaves are a potential biocoagulant to flocculate suspended solids from coffee industrial effluent.

Keywords: plant, natural coagulating agent, coffee industrial effluent, total suspended solid.

1. Introduction

In Malaysia, there were approximately 2,000 local coffee producers, specifically Liberica and Robusta making up the majority of the coffee species cultivated in Malaysia, with the

temperature range of 18°C to 28°C, providing the best condition for growth [1]. The data from the Department of Agriculture Malaysia in 2021, about 2,220 hectares of Malaysia's coffee growing area, the cultivation area was 106 hectares more than in the year 2019. Where Johor and Sabah were the primary producers of the 4,241 tons of coffee produced in 2020 compared to the 3,559 tons produced in the previous year [1]. The demand for coffee in Malaysia is expected to rise, around 80,000 of 60kg bags of coffee would be consumed in 2022. This has led to an increase in the production of coffee in Malaysia to meet market needs [2].

Consequently, a lot of wastewater and solid byproducts known as effluent will be generated. This is because the wet processing method is being applied in producing high-quality coffee in order to recover a few quantities of pure green coffee beans. This effluent typically contains a lot of suspended solids and large amounts of organic matter and is also acidic. [3]. Due to the mass discharge of coffee pulp, husk, and effluents into arable land and surface water, the coffee-processing industries are generating environmental risks. [4]. Based on a study by [5], the solids waste produced causes an unpleasant odor and promotes insect population in the disposal region, while the fermentation of sugar solids also causes the effluent to have lower pH. In addition, during the pulping process, the mucilage layer that's released into the coffee wastewater lowered the dissolved oxygen ratio causing an anaerobic condition and generating a harmful environment for aquatic life.

Therefore, it is necessary for the effluent to have proper treatment before releasing it into surrounding. Coagulation-flocculation process is a method carried out to remove suspended solids from industrial effluent which usually utilizes chemicals such alum and polymers to flocculate particles for rapid settling. Disadvantageous of using chemical coagulant is the generation of toxic sludge resulting from the coagulation-flocculation process which requires special handling. Thus, nowadays research in this field has actively searched for more environmentally friendly coagulants/flocculants derived from natural resources such as plants, bacteria, and wastes. Utilization of natural coagulants will widen the opportunity to recover the resultant sludge and reused it as fertilizers.

Cassava or scientifically named as *Manihot esculenta*, a tropical root that belongs to the *Euphorbiaceae* family, is one of the tropical plants that can be easily found in tropics and subtropics countries. [6]. Moreover, M. *esculenta* is a multipurpose plant that can be used as food, feed, and raw materials in a wide range of industries due to its high concentration of carbohydrates and is easy to grow. [7]. Due to the abundance of organic compounds found in *M. esculenta,* some studies were utilized it in treating water and wastewater and coagulation-flocculation [8] and many more.

In this study, coagulation and flocculation method were selected for treating the coffee processing effluent by using a biocoagulant, *M. esculenta*. The objective of this study is to find

the optimum condition of coagulation-flocculation process in different pH conditions, settling time and dosage of biocoagulants.

2. Materials and Methods

2.1 Preparation of Biocoagulants from M. *esculenta*

Leaves of M. *esculenta* were dried under direct sunlight and left for 3 to 5 days. Then, the dried leaves were grinded and stored in a tightly sealed container in the chiller (4°C) to avoid the growth of fungus. Then, the M. *esculenta* leaf powder was weighed and prepared in different concentrations (5 g/L, 10 g/L, 20 g/L, 30 g/L, 40 g/L, 50 g/L, 60 g/L, 70 g/L, 80 g/L, 90 g/L and 100 g/L) by using distilled water as solvent.

Then, the protein content was determined by Bradford assay [5]. An amount of 0.5 mL sample was inserted into a different test tube before adding 2.5 mL of Bradford reagent and was then well stirred. Finally, the mixture was transferred into a 2.5 mL disposable cuvette to analyze the protein content using HACH DR 3900 (HACH Company, U.S.A) spectrophotometer at an absorbance of 595 nm.

Quantification of polysaccharides in *M. esculenta* leaf powder was adopted from [9]. 1 mL of samples was added into different test tubes and then added with 1 mL of 5% phenol and 5 mL of concentrated sulphuric acid (H_2SO_4). The mixture was transferred into a cuvette and analyzed using the HACH spectrophotometer at 488 nm.

Based on the study, 100 g of initial weight *M. esculenta* leaves were weighed separately to account for the yield of proteins and polysaccharides in g/g unit. After the drying and grinding process, the dry weight becomes 21.18 g. Then, M. *esculenta* leaf powder was extracted using distilled water. The yield of the protein and polysaccharide identified were 0.02 g/g and 0.04 g/g, respectively.

2.2 Source of coffee processing effluent

The coffee processing effluent was collected from one of the coffee industries in Malaysia and stored in a closed tank. The initial pH of the coffee effluent was 3.27 ± 0.09 and the total suspended solids was 503.33 ± 197.99 mg/L TSS, the turbidity recorded as 116.02 ± 49.94 NTU and the color of this effluent was 19,000 ± 707.11 ADMI. With these characteristics, the effluent need to be treated as it is above the limit provided by Malaysia Environmental Quality (Industrial Effluent) Regulations 2009 [10].

2.3 Design of Experiment for coagulation-flocculation

This study was conducted in different pH conditions (original pH of the effluent and pH 7) and the setup of the experiment is shown in Figure 1. The different pH conditions were applied to

determine the optimal pH of coagulation-flocculation under natural conditions. Six 1000 mL-beakers filled with coffee effluent and biocoagulant at the working volume of 500 mL were used to determine the optimum biocoagulant dose in treating coffee processing effluent Then, the dosage of biocoagulant in the beakers was prepared in the range of 0.5 g/L to 2.5 g/L. They were rapid mixed in 300 rpm for 5 minutes and followed by a slow mixing at 180 rpm in 30 minutes. After that, the suspension was let to settle for 60 minutes. The measurement of total suspended solids (TSS) were recorded.

Figure 1. The setup of the jar test

The dosage of biocoagulant applied in these two pH conditions was fixed from 500 mg/L to 2,500 mg/L with the stock solution of 80 g/L. This concentration was chosen because it has the highest protein content. The pH of the raw effluent was used directly in the jar test as the real pH condition. While for the pH 7, 1 M sodium hydroxide was added to the effluent.

3. Results and Discussion

3.1 Protein and polysaccharide content from M. *esculenta* leaves

Various concentrations of the solvent were tested to determine the maximum protein content that can be extracted from the *M. esculenta* leaves. Figure 2 shows that protein content increased gradually from 5 g/L up to 70 g/L. When the solvent concentration increased to 80 g/L, the protein content starts to decrease by 0.32 g/L from the previous concentration. It continues decreasing drastically when the solvent concentration increases to 100 g/L. Theoretically, the higher the concentration of solvent will give a large amount of protein being extracted. The result shows that starting from a concentration of 80 g/L, the solvent becomes saturated. So, the highly concentrated solvents are metastable, which means they can withstand minor perturbations but become unstable when with significant ones. [11].

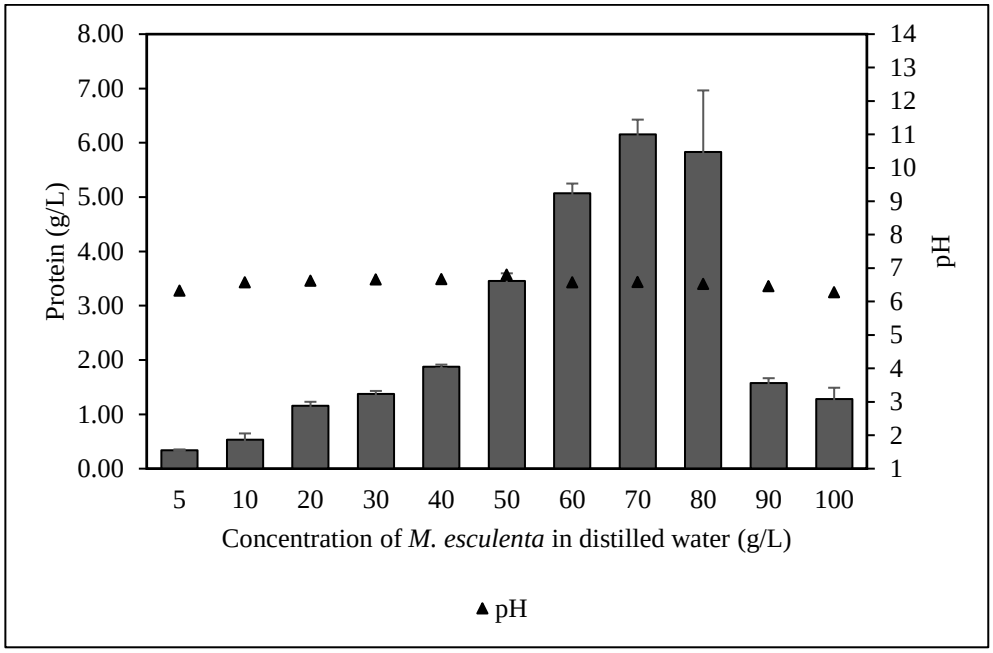


Figure 2. The protein concentration of M. *esculenta* leaf powder

Polysaccharides are one of the main components in coagulation activity besides protein [12]. The coagulation process is enhanced by the presence of hydroxyl functional groups because of the hydrogen interaction between the functional group of the effluent and the hydroxyl groups. [12]. Therefore, it is practical to account for the optimum polysaccharides that can be extracted before preparing the natural plant-based coagulant. Figure 3 illustrates the results of the extraction of polysaccharides in M. *esculenta* leaf powder by using distilled water. The concentration of polysaccharides increased with the increasing concentration of solvent from 5 g/L to 40 g/L. Then, it slightly decreases to 1.42 g/L polysaccharides extracted at the concentration of 50 g/L of M. *esculenta* leaf powder. The quantity of polysaccharides continues increasing gradually up to 2.84 g/L at a concentration of solvent of 80 g/L. The concentrations of 90 g/L and 100 g/L show that the solvent becomes saturated due to the decrease in the number of total polysaccharides.

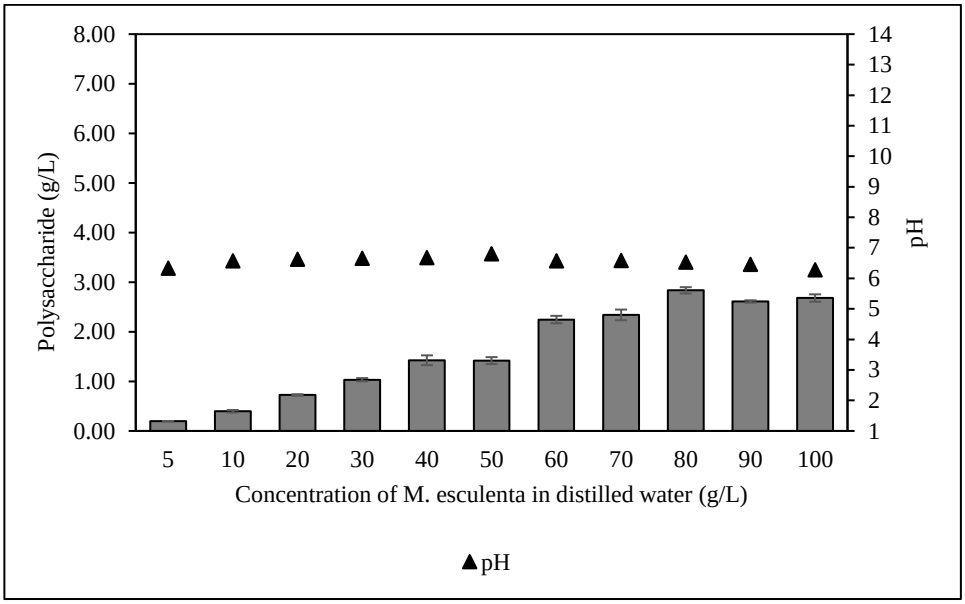


Figure 3. The polysaccharides concentration of M. *esculenta* leaf powder

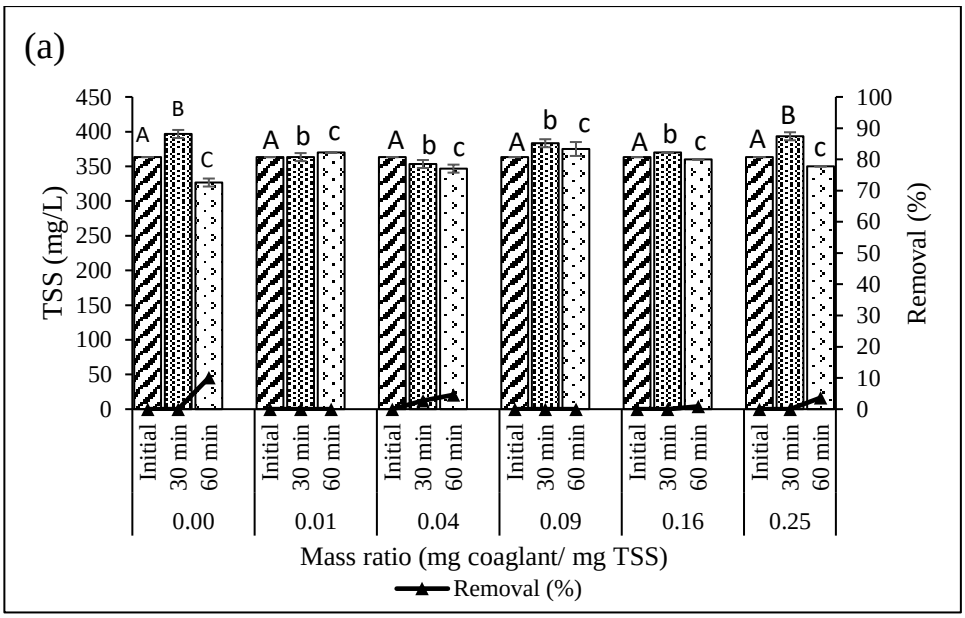
In this study, the water extraction method was used to determine the total protein and polysaccharides from M. *esculenta* leaves. According to studies by [12], the solubilization of the compound which binds with polysaccharides might enhance together with the increasing yield when performed at high temperatures (60°C to 80°C). Despite this, protein will denature due to high temperature because the protein was the most thermosensitive compound. The weakening of the electrostatic force leads the denatured protein to detach from the polysaccharide. [13].

3.2 Performance of Coagulation-flocculation by jar test

Coffee processing effluent contains high number of total suspended solids. [3]. In this study, the total suspended solids recorded were in the range of 363.33–1283.33 mg/L TSS. The acceptable limit under Malaysia Environmental Quality Regulations (Industrial Effluent) 2009 is in the range of 50 -100 mg/L TSS, therefore, the TSS of this coffee processing effluent needs to be treated and reduced to this standard limits before being discharged to the environment.

The jar test procedure was conducted at two different pH conditions: the real pH of the coffee processing effluent and pH 7. The results show a difference between these two conditions. Figure 4 demonstrates the performance of the jar test on TSS removal at two different pH conditions. Based on Figure 4(a), the highest TSS removal was achieved after adding M. *esculenta* leaf powder at a mass ratio of 0.25 mg coagulant/mg TSS after 60 minutes of settling time, resulting in 3.7% removal. On the other hand, at a mass ratio of 0.04 mg coagulant/mg TSS, the TSS removal percentage was 2.8% after 30 minutes of settling and increased to 4.6% after 60 minutes

of settling. However, the results demonstrate that the highest TSS removal was achieved at the natural pH condition without addition of biocoagulant with 10.1% removal. In comparison to Figure 4(b), the percentage of removal was higher, even though the initial TSS of the coffee effluent recorded was 643.33 mg/L.



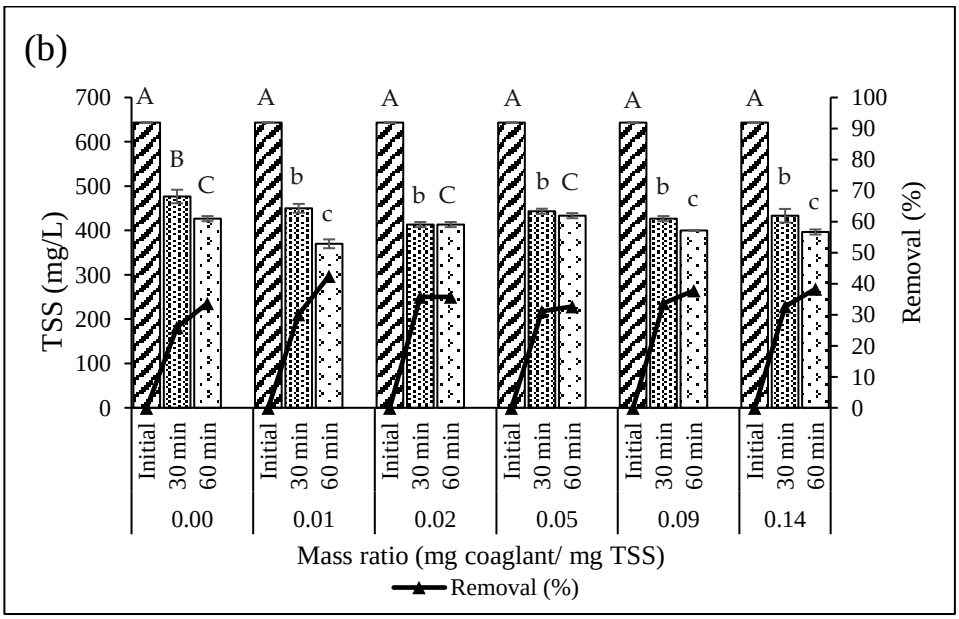


Figure 4. The TSS removal under (a) original pH coffee processing effluent and (b) condition of pH 7. Letter 'A-a', 'B-b' and 'C-c' show significance difference of removal between the mass ratios and the control (0.00 mg coagulant/mg TSS) for initial, 30 minutes and 60 minutes of settling

Statistical analysis was carried out on both pH conditions using SPSS software. The findings revealed that pH 7 was the optimal condition with an optimum mass ratio of 0.01 mg coagulant/mg TSS. The study also showed a significant difference (p<0.05) in TSS removal at 30 min and 60 min settling time with the minimum dosage of biocoagulant. When the pH was increased to neutral pH, TSS removal was much higher compared to the real pH condition. This is because the hydrophilic and hydrophobic equilibrium in the jar test, which is influenced by the pH, affecting the protein solubility of the active compounds found in *M. esculenta*. These results are consistent with a previous study [14] who employed chickpeas as a biocoagulant to treat palm oil mill effluent and demonstrated excellent TSS removal at higher pH. Because the coagulant released more cations as the positive charge increased, therefore, more flocks formed and reduces the number of TSS. [15].

4. Conclusions

In conclusion, distilled water was successfully used to extract the protein and polysaccharides in M. esculenta leaves. The highest amount of protein extracted was 6.15 g/L at 70 g/L of M. esculenta in distilled water with an amount of polysaccharide of 2.34 g/L. This concentration (70 g/L) was chosen as a stock solution for biocoagulant agent in treating the real coffee processing effluent by implementing a coagulation-flocculation treatment approach. According to the results, 0.5 g/L of biocoagulant at a mass ratio of 0.01 mg coagulant/mg TSS could remove up to 30.1% of the TSS at neutral pH condition. Furthermore, the removal of TSS shows a significant difference (p<0.05) when conducting the jar test at the original pH of the effluent. Thus, M. *esculenta* has a high potential as a biocoagulant.

Acknowledgments

The authors would like to thank Universiti Kebangsaan Malaysia (UKM) for granting this project through DIP-2021-002.

References

[1] Bernama. Agriculture Dept ready to assist coffee entrepreneurs in Malaysia, says deputy D-G | Malay Mail. Malay Mail, Putrajaya. Available https://www.malaymail.com/news/money/2021/10/01/agriculture-dept-ready-to-assist-coffee-entrepreneurs-in-malaysia-says-depu/2010018. [Accessed: 2022-11-07]

[2] Hirschmann R., Total Coffee Consumption in Malaysia 2013-2022. Jul. 23, 2021. Available

from: https://www.statista.com/statistics/877125/malaysia-total-coffee-consumption/#statisticContainer [Accessed: 2022-01-25]

[3] Ashenafi Hailemariam F., Velmurugan P., Selvaraj S. K., Treatment of wastewater from coffee (coffea arebica) industries using mixed culture Pseudomonas florescence and Escherichia coli bacteria. In Materials Today: Proceedings. 2021.Vol. 46, p. 7396–7401, DOI: 10.1016/j.matpr.2020.12.1124.

[4] Dadi D., Daba G., Beyene A., Luis P., Van der Bruggen B., Composting and co-composting of coffee husk and pulp with source-separated municipal solid waste: a breakthrough in valorization of coffee waste. International Journal of Recycling of Organic Waste in Agriculture. 2019. Vol. 8. no. 3. p. 263–277. DOI: 10.1007/s40093-019-0256-8

[5] Ancy J. A., Vasanthy M., Thamarai selvi C., Ravindran B., Chung W. J., Chang S. W., Treatment of coffee cherry pulping wastewater by using lectin protein isolated from Ricinus communis L. seed. Journal of Water Process Engineering. 2020. Vol. 39. 11 p. DOI: 10.1016/j.jwpe.2020.101742

[6] Hasmadi M., Harlina L., Jau-Shya L., Mansoor A. H., Jahurul M. H. A., Zainol M. K. Physicochemical and functional properties of cassava flour grown in different locations in Sabah, Malaysia. Food Research . 2020. Vol. 4. no. 4. p. 991–999. DOI: 10.26656/fr.2017.4(4).405

[7] Setiawan A., Rosmayati, Rahmawati N., Identification of the Morphophysiological Characteristics of Cassava (Manihot esculenta Crantz) Based on Altitude. IOP Conference Series: Earth Environmental Science.2019. AEFS 2018. Vol. 260. no. 1. p. 1. DOI: 10.1088/1755-1315/260/1/012125

[8] Rahmadyanti E., Wiyono A., Aritonang N., Combination of Phytocoagulant Moringa Oleifera Seeds and Constructed Wetland for Coffee Processing Wastewater Treatment. Journal of Engineering Science and Technology. 2020. Vol 15, no. 1. p 728-745

[9] Bhatti M., Kamboj A., Saluja A. K., Spectrophotometric estimation of total polysaccharides in Kalanchoe pinnatum and Kalanchoe crenata. International Journal of Pharmacy and Pharmaceutical Sciences. 2013. Vol. 5, no. 2, p. 40–41

[10] Department of Environment. 2002. Available from: https://www.doe.gov.my/en/national-policy-of-the-environment/ [Accessed: 2022-11-06]

[11] Bioprocess Engineering Principles. 2nd ed. Elsevier Ltd; 2013. 457p

[12] Ang W. L., Mohammad A. W. State of the art and sustainability of natural coagulants in water and wastewater treatment. Journal of Clean Production. 2020. Vol. 262, p. 121267. DOI: 10.1016/j.jclepro.2020.121267

[13] Bealer E. J., Onissema-karimu S., Rivera-galletti A., Francis M., Wilkowski J.,Cruz D. S., Hu X., Protein – Polysaccharide Composite Materials. Polymers (Basel). 2020. p. 1–28

[14] Chong Lek B. L., Peter A. P., Qi Chong K. H., Ragu P., Sethu V., Selvarajoo A., Arumugasamy S. K. Treatment of palm oil mill effluent (POME) using chickpea (Cicer arietinum) as a natural coagulant and flocculant: Evaluation, process optimization and characterization of chickpea powder. Journal of Environmental Chemical Engineering. 2018. Vol. 6. no. 5. p. 6243–6255. DOI: 10.1016/j.jece.2018.09.038

[15] I. Aditya and S. Kartohardjono. Effect of pH of Coagulant on the Treatment of Wastewater from Tofu Industry. E3S Web Conferences (3rd i-TREC'18). Vol. 67. p. 3–6, 2018. DOI: 10.1051/e3sconf/20186704006

B.5

The potential of *Praxelis clematidea* extract as a bioherbicide

Inas Nuha Afifa[1], Trimurti Hesti Wardini[1], Rizkita Rachmi Esyanti[1], and Hidayat Pujisiswanto[2]

[1] Biology, School of Life Sciences and Technology, Bandung Institute of Technology, Bandung, Indonesia
[2] Agronomy and Horticulture, Faculty of Agriculture, Lampung University, Bandar Lampung, Indonesia

Correspondence should be addressed to Trimurti Hesti Wardini; E-mail address: trimurti@office.itb.ac.id

Abstract

Weed control is one of the important maintenance activities in plant cultivation to stabilize the quantity and quality of crop yields. Unfortunately, spraying synthetic herbicide is still the main choice to solve this risk. Improper use can increase environmental damage. Therefore, the development of bioproducts from plants by utilizing their toxic properties is considered to be able to act as an eco-friendly biocontrol. This study aims to determine the potential of *Praxelis clematidea* extract of different plant parts on the seed germination, seed mean germination time (MGT), and sprout morphology of *Asystasia gangetica* weed. The study was conducted by bioassay test using a completely randomized design (CRD) in germination room to explore the effect of *Praxelis* water extract from the aerial part (including stems; leaves; and flowers), non-aerial part (including basal stems and roots), and whole plant. Whole plant extract suppressed germination and MGT higher than extract derived from aerial and non-aerial parts. The result was also supported by results of color changes observation, which showed browning in the roots, cotyledons, and hypocotyls of *Asystasia* treated seedling. Thus, *Praxelis* extract using all parts of the plant is the optimal formula for bioherbicide against *Asystasia,* as weeds in Palm oil plantation.

Keywords: invasive alien plant, weed control, herbicide, extract source, efficacy

1. Introduction

Weeds are unwanted plant which grow in an agro-ecosystem. It has the potential to harm the environment and human interests because it grows rapidly covering the cultivated area, has

high adaptability [1, 2] and compete with cultivated plants for resources such as water, nutrient, sun shine which can reduce the quantity and quality of crop yields [2, 3].

Chemical weed control management practices, such as by using inorganic synthetic herbicides, are dominantly chosen by farmers because they have been proven to be effective in suppressing weeds and reducing costs, so as to optimize crop productivity [4]. But recently, many environmental issues are connected to the use of synthetic herbicides, such as weed resistance to herbicides and pollution in water and soil [5].

Allelopathic activity in plants can be an alternative solution for chemical weed control by utilizing plant toxicity. Biological solutions aimed to weed management practices that minimize the harmful effects of synthetic herbicides [6]. *Praxelis clematidea* is an invasive plant that has a potency as a candidate for biocontrol. Several previous studies have proven the allelochemical performance of *Praxelis* on aerial parts separately and combined aerial parts in inhibiting the seed germination [8–10]. One of the factors that affect the efficacy of a plant extract, namely the allelochemistry contained [11, 12]. The development of new herbicides derived from various compounds is a good candidate because the extract has multitarget-site activity [12]. Therefore, this study aimed to determine the effect of *Praxelis clematidea* extract with different combinations of plant parts on the seed germination, seed MGT, and sprout morphology of *Asystasia gangetica* weed, so can optimize the efficacy of the product.

2. Materials And Methods

2.1 Materials

Praxelis clematidea plants and *Asystasia gangetica* seeds were obtained from oil plam plantations in Lampung Province, Indonesia. *Praxelis* plant parts were separated into two parts, namely the aerial and non-aerial. The aerial part included the stem, leaves, and flowers. The non-aerial part included the roots and basal stems with a cutting distance ±2 cm from the root base. The samples were washed and oven-dried at 80°C for 48 hours. The dried samples were ground using a grinder and stored in sealed containers [8, 9, 14]. Seeds were separated from the seed coat, dried in the sun for 1 hour, and stored in a sealed container.

2.2 Methods

2.2.1 Preparation of *Praxelis* Extracts

Each fine powder was macerated with distilled water at a ratio of 1:10 (g/mL) which was carried out 3 times in a row for 3 days (3 x 24 hours). Each extract solution was filtered using 2 pieces of cloth and the filtrate was evaporated with a vacuum rotary evaporator at 40°C until the weight of concentrated extract was constant. Each stock solution was made with a 100% concentration and dilution was carried out to obtain 50% concentration. The ratio of the whole plant extract

was 1:3 (non-aerial and aerial parts) [8, 9, 15].

2.2.2 Preparation of Seeds

The seeds were soaked in 2.5% NaOCl for 1 minute and washed with water. After that, the seeds were soaked in warm water at 40°C until the water temperature turned cold, followed by drying the seeds with tissues [8, 16].

2.2.3 Bioassay Test

Each 9 cm diameter petri dish was covered with cotton and 20 seeds were placed. The extract solution was dripped as much as 2 mL in 20 mL distilled water and poured into each petri dish. The petri dishes were closed and stored in the germinator at 28°C under light conditions (12 hours photoperiod). Seeds are declared to germinate when radicles have appeared with a length of at least 2 mm [8, 9]. The control treatment was done in the same method and only given 20 mL distilled water. The study was conducted for 3 weeks of observation. Parameters observed were seed germination, seed MGT, and sprout morphology of *Asystasia gangetica* weed.

2.2.4 Statistical Analyses

The transformed bioassay data were analyzed by simple linear regression test and T-test on SPSS Statistics 26 program. Interpretation of correlation values refers to Elva *et al*. [16].

3. Results and Discussion

The *Praxelis* extracts showed different effect on seed germination, both between sources and control. The average value of the weed (*Asystasia*) seed germination from highest to lowest, namely the control (67,5%), non-aerial extract (20%), aerial extract (12,5%), and whole plant extract (0%), consecutively. Extract from whole plant can suppress seed germination better than the aerial and non-aerial parts which might be due to more complicated metabolites compositions or its concentrations (Figure 1).

Based on the simple linear regression analysis (Figure 2), it showed that there was a very strong correlation (R = 0,899) between *Praxelis* extract sources and *Asystasia* seed germination. The effect was 81% (R2 = 0,808). Then, the relationship between extract sources and germination was negative (Unstandardized Coefficients = -1,293), which means that the seed viability is lowest in whole plant treated extract. Extract sources had a significant effect on seed germination ($0.002 < 0.05$). Whole plant extract only had different effect with control ($0.017 < 0.05$). Meanwhile, the other formulas were not significantly different with control or between extract sources.

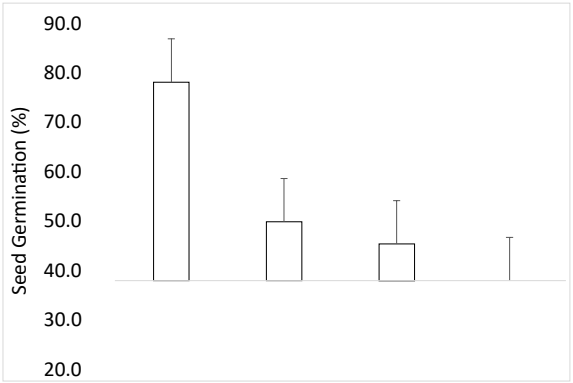


Figure 1. Effect of *Praxelis* extract source on *Asystasia* seed germination.

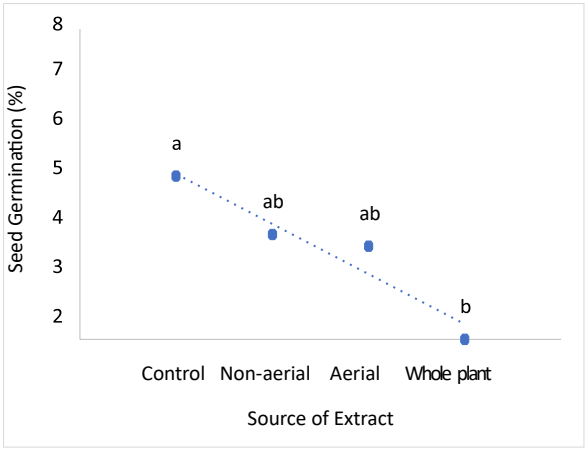


Figure 2. Correlation of *Praxelis* extract source on *Asystasia* seed germination.

All sources of *Praxelis* extract showed different effects on the MGT, both between sources and control. The average value of weed seed MGT from highest to lowest was aerial extract (18,8 days), non-aerial extract (10,6 days), control (7,6 days) and whole plant extract (0 days). All sources can inhibit the MGT. Extract derived from whole plant had the ability to delay seed germination longer than the aerial and non-aerial parts because germination did not occur for 3 weeks observation time (Figure 3).

Based on the simple linear regression analysis (Figure 4), it showed that there was a strong correlation (R = 0,713) between *Praxelis* extract sources and *Asystasia* seed MGT. The effect was 51% (R2 = 0,509). The relationship between extract sources and MGT was positive

(Unstandardized Coefficients = 0,310), which means that seed germination time will be longest in the whole plant extract treatment. Extract sources had a significant effect on seed germination (0,047 < 0,05). Extract formulas which had significantly different with control were the aerial and whole plant parts.

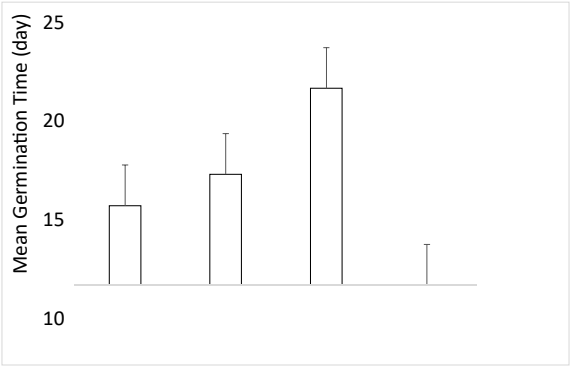


Figure 3. Effect of *Praxelis* extract source on *Asystasia* seed MGT.

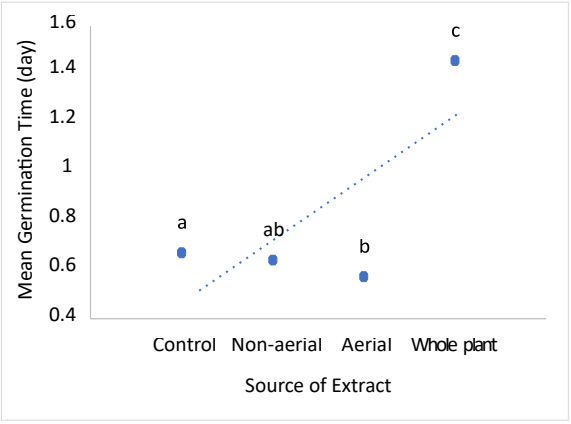


Figure 4. Correlation of *Praxelis* extract source on *Asystasia* seed MGT.

Based on morphological observations, treatment with the aerial and non-aerial extracts resulted in roots, cotyledons, and hypocotyls discoloration (Figure 5). All seeds turned black and failed to germinate on the weed treated with whole plant extract. Treatment with the aerial extract in the sprouts caused the roots, cotyledons, and hypocotyls that initially fresh become wilted, followed with a color change from white-green to yellow-brown. Meanwhile, the use of non-aerial extract on sprout made the roots, cotyledons, and hypocotyls that initially fresh become rotten with a color change from white-green to brownish.

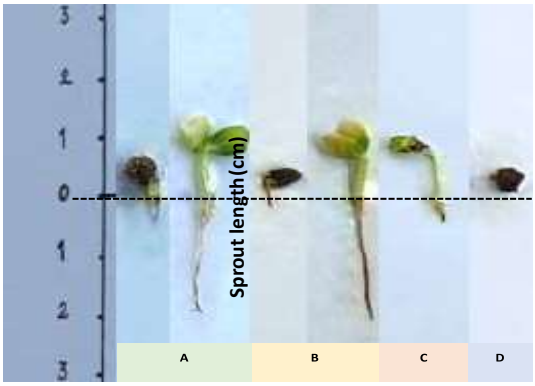


Figure 5. Effect of *Praxelis* extract source on sprout morphology of *Asystasia*, [A] control; [B] non-aerial extract; [C] aerial extract; and [D] whole plant extract.

Praxelis extracts can inhibit germination and MGT, as well as inducing changes in sprout morphology due to allelopathic activity. Allelopathic activity depends on the allelochemicals contained in the extract. Allelochemicals can be found in every part of the plant with different levels and types of composition, resulting in different mechanisms of action and effectiveness [10]. Several previous studies showed that the aerial *Praxelis* extract was toxic to seed germination and caused discoloration of the root cap [8, 9]. However, research in a bioassay test study related to the use of non-aerial and whole plant extracts in *Praxelis* were not available.

Praxelis plants extracts can suppress, and delay seed germination as attributed by its allelochemical. Allelochemicals that have been studied in this plant and have been applied through a seed germination bioassay test are terpenoids such as monoterpene and sesquiterpene groups in a form of essential oils extract from aerial part [9]. One of them is *Caryophyllene* compound with a relatively high content (30.34%) through GC-MS analysis on *Praxelis* flowers [17]. Previous studies showed that *Caryophyllene* can inhibit and delay *Arabidopsis* seed germination which is thought to have the same mechanism of action as terpenoids in general, such as by inhibiting the works of germination enzymes (e.g a- and b-amylase) and affect hormones activity (e.g ABA) [18].

4. Conclusion

This study exemplified different effects of *Praxelis* extract on the seed germination and MGT, both between sources and control. All sources of *Praxelis* extract showed allelopathic activity against weed seeds. Extract formula produced by whole plant extract had a higher toxicity on the *Asystasia* weed, showed by inhibition activity and discoloration of the roots, cotyledons, and

hypocotyls that were also observed. Therefore, utilization of whole *Praxelis* plant is recommended as an extract formulation for biocontrol because it obtains the best efficacy.

Acknowledgments

Thanks to Bandung Institute of Technology and Lampung University for the facilities during this research.

References

[1] Hasan B. J. *Dasar-dasar Agronomi*. Jakarta: Raja Grafindo Persada, 2008; 250p.

[2] Dad R. J. S. *Gulma dan pengelolaannya*. Yogyakarta: Graha Ilmu, 2010; 168p.

[3] Kiki K., Marulam M. T. S., Danner S., Astri A., Riyanto R., Dewi M., Sri R. F. P., Dewi S., and Ismi P. R. *Ekologi Pertanian*. Medan: Yayasan Kita Menulis, 2021; 130p.

[4] Dadang D., Denny K., Nanik S., Budiawan B., Asep N. A., Dedi B. H., Soekisman T., Edison P., Lita S. S., and Tomy P. *Profil Keamanan dan Penggunaan Herbisida Parakuat Diklorida di Indonesia*. Bogor: IPB Press, 2020.

[5] Mahmudul H., Anis S. M., Khairil M., Zulkarami B., Adam M. R., Hafizuddin H., Mst M., and Muhammad S. A. H. Physiological and biochemical responses of selected weed and crop species to the plant-based bioherbicide WeedLock. *Sci. Rep.* 2022; 12(1): 19602. DOI: 10.1038/s41598-022-24144-2.

[6] Tran D. X., Tawata S., Tran D. K., and Ill M. C. Biological control of weeds and plant pathogens in paddy rice by exploiting plant allelopathy: an overview. *Crop Prot.* 2005; 24(3): 197–206. DOI: 10.1016/j.cropro.2004.08.004.

[7] Suphannika I., Buntoon W., and Carol A. M. S. Seed morphology and allelopathy of invasive Praxelis clematidea. *Not. Bot. Horti Agrobot. Cluj-Napoca*. 2020; 48(1): 261–272. DOI:10.15835/nbha48111831.

[8] Tamonwan T. and Suphannika I. Total phenolics, flavonoids, antioxidant activity, and allelopathic potential of praxelis. *J. Curr. Sci. Technol.* 2020; 10(1): 59–65. DOI: 10.14456/jcst.2020.6.

[9] Z. H. Wang, P. Christie, Q. B. Chen, X. X. Liu, L. L. Xie, C. J. Bai, and X. L. Lie. Allelopathic potential and chemical constituents of volatile oil from Praxelis clematidea. *Allelopath. J.* 2006; 18(2): 225–235.

[10] Marcelo P. G., Queila S. G., Leilane C. B., Lucia P. S. P., Miele T. M., and Cleber C. F. Allelopathy: An overview from micro- to macroscopic organisms, from cells to environments, and the perspectives in a climate-changing world. *Biol.* 2017; 72(2): 113–129. DOI: 10.1515/biolog-2017-0019.

[11] Tamonwan T., Manatchaya P., and Suphannika I. Total phenolic content and allelopathic effect of soil incorporation of Praxelis clematidea on emergence and growth of plants. *Khon Kaen Agric. J.* 2019; 47(1): 73–78.

[12] Mercedes V., Adela M. S. M., and Fabrizio A. Phytotoxic Effects and Mechanism of Action of Essential Oils and Terpenoids. *Plants*. 2020; 9(11): 1571. DOI: 10.3390/plants9111571.

[13] Gabriela L. de A. M., Vivyanne dos S. F. S., Pedro G. V. A., Joao X. de A. J., Josean F. T., Marcelo S. da S., Luis C. R., Jose P. de S. J., Jose M. B. F. Flavonoids from Praxelis clematidea R.M. King and Robinson modulate bacterial drug resistance. *Molecules*. 2011; 16(6): 4828-4835. DOI: 10.3390/molecules16064828.

[14] Heloina de S. F. Gabriela L. de A. M., Flavia B., Helio K., Thiago M. M., Clelia A. H. L., Christiane T., AndersonL. F., Alba R. B., Souza M., Maria de F. A., Jose M. B. F., and Leonia B. Gastroprotective mechanisms of the chloroform and ethyl acetate phases of Praxelis clematidea (Griseb.) R.M.King & H.Robinson (Asteraceae). *J. Nat. Med.* 2013; 67(3): 480–491. DOI: 10.1007/s11418-012-0705-4.

[15] Jerry M. B. and Carol C. B. A classification system for seed dormancy. *Seed Sci. Res.* 2004; 14 (1): 1–16. DOI: 10.1079/SSR2003150.

[16] Elva S., Nurjanna L., Laila Q., Vera S. A., Muhammad S., and Faula A. *Buku Ajar Statistika untuk Perguruan Tinggi*. Indramayu: Adab, 2021; 118p.

[17] Wang Q., Liu Y., Li S., Zhao Y., and Wang W. Chemical composition of essential oil of the invasive plant Praxelis clematidea and its repellence and lethality to Diaphorina citri. *J. Entomol. Terap. Cina*. 2018; 55(1): 117–125. DOI: 10.7679/j.issn.2095- 1353.2018.018.

[18] Fabrizio A., Adela M. S. M., Elisa Graña, Manuel J. R., and Maria R. A. Terpenoid trans-caryophyllene inhibits weed germination and induces plant water status alteration and oxidative damage in adult Arabidopsis. *Plant Biol.* 2017; 19(1): 79– 89. DOI: 10.1111/plb.12471.

B.6

Response of Cavendish Banana (*Musa acuminata* Cavendish AAA) Induced by Chitosan Elicitor against *Fusarium oxysporum* f.sp. *cubense* Tropical Race 4 Infection

Shinta Fitriannisa, Trimurti Hesti Wardini, Rizkita Rachmi Esyanti

School of Life Sciences and Technology, Institut Teknologi Bandung (ITB)
Jalan Ganesha No. 10 Bandung 40132 Indonesia

Correspondence should be addressed to Trimurti Hesti Wardini; E-mail address: trimurti@itb.ac.id

Abstract

Fusarium wilt disease caused by *Fusarium oxysporum* f.sp. *cubense* Tropical Race 4 (Foc TR4) resulted in the greatest damage to banana commodity in Indonesia. Chitosan is reported to be an elicitor capable of increasing resistance to Fusarium wilt, but further research is needed to determine the response of Cavendish Banana plants to chitosan elicitation and its optimum concentration to fight Foc infection. The experiments were carried out by giving banana plants with chitosan at different concentrations (40, 60, and 80 ppm), then treated them with Foc inoculation. There were two controls used, negative control (no chitosan elicitation and no Foc inoculated-banana plants) and positive control (no chitosan elicitacion but Foc inoculated-banana plants). The resistance parameter used was disease severity index (DSI). The results showed that all plants with chitosan elicitation categorized as susceptible and they were better than the positive control which categorized as highly susceptible. Based on DSI parameters, 80 ppm chitosan is an optimum concentration which increase the resistance of the Cavendish Banana plant against Foc infection.

Keywords: Cavendish banana, Fusarium wilt, chitosan, elicitor, defense

1. Introduction

Cavendish banana (*Musa acuminata* Cavendish AAA) is the fourth most consumed fruit commodities in Indonesia [1]. However, banana production is threatened by many pathogens and unfavorable environmental conditions. The fungi *Fusarium oxysporum* Schlecht f.sp. *cubense* (Foc) is a pathogen that causes Panama disease or Fusarium wilt disease [2] which lead to greatest damage to banana commodity in Indonesia and Asia, Fusarium wilt is mostly caused

by Foc tropical race 4 (TR4). It infects the roots, enters the xylem vessel network and causing blockage of the xylem lumen by the accumulation of mycelia.[3].

One of the efforts to increase the resistance of banana plants to pathogens is by using elicitor, a molecule that trigger plant defense responses [4]. Chitosan, a biopolymer produced from deacetylated chitin, and largest constituent of the arthropod exoskeleton and cell wall, is known as an elicitor capable of inducing resistance to disease through various mechanisms. Besides being safe, natural, and inexpensive, chitosan is known to increase plant tolerance to various soil and leaf pathogens. Chitosan is also known to have antimicrobial effects and has a broad spectrum of fungicidal activity by inhibiting fungal development at various stages of its life cycle [5].

Plant cells are known to have chitosan receptors in the form of chitosan-binding protein which belongs to the glycoprotein family of lectins [6]. After molecular recognition by the receptor, signal transduction occurs to induce a physiological response. Several studies have stated that the signal transduction process in this chitosan-mediated signaling pathway involves molecules such as reactive oxygen species (ROS), $Ca^{2+,}$ nitric oxide (NO), and several hormones [7]. This signal transduction is carried out both in the plant defenses and the synthesis of secondary metabolites such as polyphenols, lignin, flavonoids, and phytoalexins that function to increase the defense response to abiotic and biotic stress. In addition, chitosan is known to directly affect gene expression by interacting with chromatin or binding to its receptors [5].

Plants that have been elicited with chitosan are known to have higher resistance than control plants when infected with pathogens. Medjoub-Trabelsi *et al.* (2019) showed that potato plants infected with *Fusarium* spp. that had been treated with chitosan had a lower disease severity parameter than plants without chitosan elicitation [8]. However, further research is needed on the response of the chitosan-elicitated Cavendish Banana plants to Foc infection. In addition, the concentration of chitosan as an elicitor that provides the best resistance also needs to be investigated further. Therefore, this research was conducted with the aim of determining the response of the Cavendish Banana plant to chitosan elicitation and determining the best chitosan concentration as an elicitor to defend Foc infection with disease severity index (DSI) as a resistance parameter.

2. Methods

The research was carried out from November 2021 to July 2022, in the Culture Room, Bioprocess Laboratory II, Natural Materials Analysis Laboratory and Eastern Instructional Laboratory located at Labtek XI SITH ITB. The experiment was carried out with 4 repetitions.

2.1 Material and Methods

A total of 20 banana plantlets were divided into negative control which were plants without elicitation and without Foc inoculation, positive control groups which were plants infected with Foc without elicitation, and chitosan treatments of 40, 60, and 80 ppm

2.2 Elicitor Preparation

Chitosan was prepared by dissolving 0.5 g of chitosan into 100 mL of acetic acid. When the chitosan was completely dissolved, the pH of the solution was adjusted to 5.6. Different concentrations of chitosan were made by diluting prepared chitosan solution with water. All chitosan solutions were autoclaved at 121 ºC and 1 atm pressure for 15 min [9].

2.3 Acclimatization in Soil

The acclimatization process of plantlets was carried out in a sterile condition. Plantlets were removed from Erlenmeyer containing liquid media and each plant was planted in 200 g of sterilized planting media in a polypropylene plastic bag which used as a pot. The planting medium was a mixture of husk charcoal, coco peat and Malang sand with a ratio of 2:2:1. The growing medium was then added with 100 mL of an elicitor solution with concentration of 40, 60, and 80 ppm. As control, the growing medium was added 100 mL of sterile distilled water. Plantlet was stored in culture room under light for 12 hours and dark for 12 hours. Acclimatization was carried out for 110 days.

2.4 Identification and Preparation of Foc TR4 Inoculum

Fusarium oxysporum f.sp. *cubense* TR4 culture was isolated from PDA (Potato Dextrose Agar) medium of 1x1 cm^2 using a spatula and attached to the new PDA medium aseptically, then incubated at room temperature in the dark for 14 days. Culture was identified both macroscopically and microscopically. Macroscopic observations were carried out by observing the morphology of the culture on the PDA while microscopic observations were carried out under light microscope using Lactophenol Cotton Blue (LCB) dye.

Conidia suspension for inoculum was prepared by adding sterile 0.85% NaCl into a petri dish containing 14 days old fungal cultures. The conidia were then collected with an oose. The suspension was then homogenized and transferred to a sterile bottle. The number of conidia was then calculated by haemocytometer, and the calculated number of conidia is converted to conidia/mL units (Eq. (1)). Conidia suspension can be diluted by adding 0.85% NaCl to obtain a concentration of $2x10^6$ conidia/mL [10].

$$\text{Conidia/mL} = \frac{number\ of\ conidia\ in\ 5\ squares}{5\ x\ 4\ x\ 10{-}6} \quad (1)$$

2.5 Foc TR4 Inoculation

The polypropylene plastic bag containing banana plants that had been acclimatized for 110 days was perforated at the bottom and placed on a larger plastic bag containing sterile growing media which had been inoculated with 3 mL of Foc TR4 conidia suspension containing $2x10^6$

conidia/mL. The roots of the plants are cut about 1-2 cm to create a wound and hence inducing infection [11].

2.6 Disease Severity Index Assessment

Assessment of disease severity was carried out on the last day of infection using the Leaf Symptom Index (LSI) and Rhizome Discoloration Index (RDI) table guidelines (Table 1 and 2) [12].

Table 1. Leaf Symptom Index Scales

Scores	Description
1	No streaking or yellowing leaves.
2	Slight streaking and/or yellowing of lower leaf.
3	Streaking and/or yellowing of most of the lower leaves.
4	Extensive streaking and/or yellowing on most or all of the leaves.
5	Dead plant.

Table 2. Rhizome Discoloration Index Scales

Scores	Description
1	No discoloration of tissue of stellar region of rhizome or surrounding tissue.
2	No discoloration of stellar region of rhizome; discoloration at junction of root and rhizome.
3	Trace to 5% of stellar region of discoloured.
4	6-20% of stellar region discoloration.
5	21-50% of stellar region discoloration.
6	>50% of stellar region discoloured.
7	Discoloration of the entire rhizome stele.
8	Dead plant.

The LSI and RDI scale values were then used to obtain the DSI (Disease Severity Index) (Eq. (2)). The DSI was then matched with the translation in Table 3 [12].

$$\text{DSI} = \frac{\Sigma\ (\text{number on scale}\ \text{x number of seedlings in that scale})}{\Sigma\ \text{number of treated seedlings}} \quad (2)$$

Table 3. Translation of DSI Scales

DSI Scales for LSI	DSI Scales for RDI	Translation
1	1	Resistant
1.1 – 2	1.1 – 3	Tolerant
2.1 – 3	3.1 – 5	Susceptible
3.1 - 4	5.1 – 8	Highly Susceptible

3. Results and Discussion

3.1 Identification and Preparation of Foc TR4 Inoculum

Colony of the fungus showed that the colonies had fibrous textured like cotton with purple color and wavy margin (Figure 1). Under microscope, fungus shows the presence of hyphae, macroconidia, microconidia, chlamydiospores, and phialid structures. The observed fungal hyphae have septate characteristics. In addition, it was also observed that chlamydiospores formed on hyphae and phialids (Figure 2a). The length of macroconidia is ±29.74 m with a straight and oval-shaped, while microconidia is ±9.71 m long (Figure 2b).

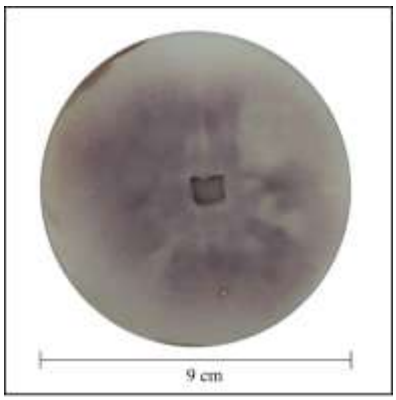


Figure 1. Macroscopic image of Foc TR4

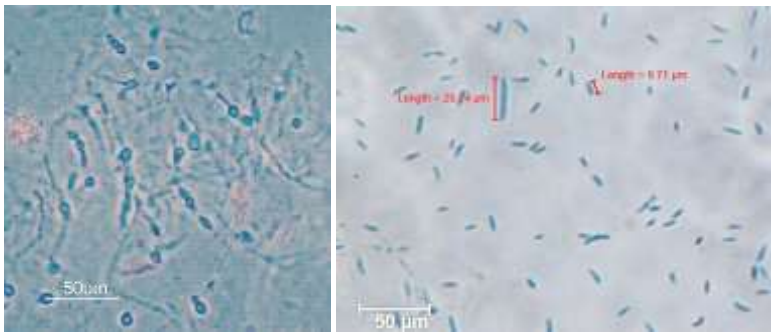


Figure 2. Microscopic image of Foc TR4: (a) structures of hyphae, chlamydiospores, and phialid; (b) macroconidia and microconidia

These results consistent with Ploetz's (2006) statement that *Fusarium oxysporum* f.sp *cubense* colony had a purplish white color with a fibrous and wavy margin, septate hyphae, straight or curved oval macroconidia with thin walls with a size of 27-55 x 3.3-5.5 m, while the microconidia of Foc TR4 are oval, elliptical, or elliptical, kidney-like shape, and usually not septate with a size of 5-16 x 2.4-3.5 m [14]. In Foc, chlamydiospores can form in hyphae or on conidia, can form chains or single, and have a diameter of about 7-11 m.

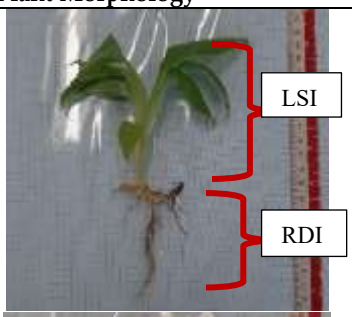
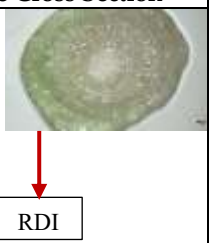
3.2 Determination of the Severity of Disease

It is an external symptom found on assessed leaves (Leaf Severity Index - LSI) and on rhizome (Rhizome Discoloration Index - RDI). The higher LSI and RDI values indicate the more severe disease symptoms in plants [12]. The external symptoms of plants after an incubation period of 35 days are shown in Table 5.

Table 4. Banana plant's disease severity index after 35 days of infection

Treatment	LSI	RDI	Category
Negative Control	1	1	Resistant
Positive Control	4	6.5	Highly Susceptible
Chi 40 ppm	2.5	3.75	Susceptible
Chi 60 ppm	2.5	3.5	Susceptible
Chi 80 ppm	2.25	3.5	Susceptible

Table 5. The condition of banana leaves and rhizomes after 35 days of infection

Treatment	Plant Morphology	Rhizome Cross Section
Negative Control		
Positive Control		
Chitosan 40 ppm		
Chitosan 60 ppm		

Chitosan 80 ppm		

Negative control plants look healthy with green leaves and whitish green rhizome. After 35 days of infection, positive control plants showed symptoms of yellowing in old and young leaves and based on Table 4 they had an LSI index of 4. The rhizome showed a brownish color change of more than 50%, with RDI index of 6.5 (Table 5). Based on these symptoms positive control plants can be assume as highly susceptible to Fusarium wilt disease.

Chitosan-treated plants that had been infected with Foc for 35 days showed slightly greener leaves than the positive control plants. Although the older leaves were yellowish in color. Chitosan-treated rhizome exhibited less discoloration than the positive control. The discoloration area was about 5-50% in cross section of the rhizome. The symptoms shown in the chitosan-treated plants was less severe as the concentration of chitosan increased (Table 5). Based on the LSI and RDI scores obtained on the 35th day of infection, the chitosan-treated plants showed an increase in resistance compared to positive control plants

Higher DSI value indicates the severity of the disease symptoms in plants. Symptoms showed by positive control plants are in line with the results of Ploetz's (2016). The author reported that Foc-infected plants will show symptoms of chlorosis and necrosis starting from the oldest leaves to younger leaves, as well as rhizome discoloration that occurs due to tissue damage and accumulation of phenol defense compounds [13]. The lower DSI value in chitosan-elicitated plants indicated an increase in plant defense against Foc.

The increased resistance of chitosan-treated plants can be caused by the ability of chitosan as an antifungal, by inhibiting sporulation and spore germination [14]. Experiment conducted by Moya (2019) showed that Chitosan can also alter gene expression in several species of chitosan-sensitive fungi to trigger the production of ROS and fungal cell death [15].In addition, chitosan can increase resistance in plant by influencing gene expression, either by interacting with chromatin or binding to receptors, resulting in signal transduction. This process leads to physiological and chemical responses such as the synthesis of various secondary metabolites including various phenolic compounds, flavonoids, phytoalexins and various plant defense enzymes like polyphenol oxidase (PPO) enzymes, peroxidases (POD), and phenylalanine ammonia-lyase (PAL) [5]. These effects of chitosan on fungi and plants are thought to be the

reason why chitosan-elicitated plants show less severe symptoms of leaves chlorosis and tissue damage.

4. Conclusion

Based on this study, it can be concluded that all chitosan concentrations (40, 60, and 80 ppm) used in this study can increase plant resistance against Fusarium wilt with 80 ppm chitosan gave the highest resistance. After 35 days infection, DSI values showed a change from highly susceptible to susceptible Cavendish banana plant against Foc infection.

Acknowledgments

The authors would like to acknowledge PPMI Institut Teknologi Bandung 2022 for funding this research.

References

[1] Nurjanah, S. Dampak ekspor pisang Indonesia ke Jepang dalam rangka Indonesia-Japan Economic Partnership Agreement (IJEPA). Jom Fisip. 2018;5:1-13. DOI: 1401112006

[2] Dita, M., Barquero, M., Heck, D., Mizubuti, E. S., & Staver, C. P. Fusarium wilt of banana: current knowledge on epidemiology and research needs toward sustainable disease management. Frontiers in Plant Science. 2018;9:1-21. DOI: 10.3389/fpls.2018.01468

[3] Yadeta, K., & Thomma, B. The xylem as battleground for plant hosts and vascular wilt pathogens. Frontiers in Plant Science. 2013;4: 1-12. DOI: 10.3389/fpls.2013.00097

[4] Thakur, M., Sohal B.S. Role of elicitors in inducing resistance in plants against pathogen infection: a review. ISRN Biochemistry. 2013; 1:1-10. DOI: 10.1155/2013/762412

[5] Malerba, M., Cerana, R. Chitosan effects on plant systems. International Journal of Molecular Sciences. 2016; 17:1-15. DOI:10.3390/ijms17070996

[6] Chen, H., & Xu, L. Isolation and characterization of a novel chitosan-binding protein from non-heading Chinese cabbage leaves. J.Integr. Plant Biol. 2005;47:452-456. DOI: 10.1111/j.1744-7909.2005.00022.x

[7] Ali, W., Rajendran, S., Joshi, M. Synthesis and characterization of chitosan and silver loaded chitosan nanoparticles for bioactive polyester. Carbohydrate Polymeres. 2011; 83:438-446. DOI: 10.1016/j.carbpol.2010.08.004

[8] Medjoub-Trabelsi, B., Touihri, S., Ammar, N., Riahi, A., Daami-Remadi, M. Effect of chitosan for the control of potato diseases caused by Fusarium species. Journal of Phytopathology. 2019; 168:1-10. DOI: 10.1111/jph.12847

[9] Romanazzi, G., Gabler, F.M., Margosan, D., Mackey, B.E., Smilanick, J.L. Effect of chitosan dissolved in different acids on its ability to control postharvest gray mold of table grape. Phytopathology. 2009; 99:1028-36. DOI: 20.1094/PHYTO-99-9-1028

[10] Paz-Lago, D., Guiterrez, B., Borges, A., Cabrera, G., Ramirez, A., Falccon, A. Tomato-Fusarium oxysporum resistance against Fol in young tomato plants. Cultivos Tropicals. 2000; 21:17-20.

[11] Fortunato, A., Rodrigues, F., Baroni, J., Soares, G., Rodriguez, M., Pereira, O. Silicon suppresses Fusarium wilt development in banana plants. Journal of Phytopathology. 2012; 120:11-12. DOI: 10.1111/jph.12005

[12] Food and Agriculture Organization of the United Nations. Early Screening Technique for Fusarium With Resistance in Banana Micropropagated Plants [Internet]. 2014. Available from: https://www.fao.org/3/ae216e/ae216e0k.htm#bm20 {Accessed: 2022-11-29]

[13] Ploetz, R. C. Fusarium Wilt of Banana Is Caused by Several Pathogens Referred to as Fusarium oxysporum f.sp. cubense. Phytopathology. 2006;96:653–656. DOI: 10.1094/PHYTO-96-0653.

[14] Atay, H. (2019). Antibacterial Activity of Chitosan-Based Systems. *Functional Chitosan. 2020;6:457-489*. DOI: 10.1007/978-981-15-0263-7_15

[15] Lopez-Moya, F., Suarez-Fernandez, M., & Lopez-Llorca, L. Molecular mechanisms of chitosan interactions with fungi and plants. International Journal of Molecular Sciences. 2019;20:1-15. DOI: 10.3390/ijms20020332

B.7

Review on *Hatompul* (*Artocarpus rigidus* Blume), An Underutilized Species Collected from Dolok Sibual-buali Nature Reserve, Sipirok, North Sumatra

Arifin Surya Dwipa Irsyam[1], Rina Ratnasih Irwanto[2]*, Arni Rahmawati Fahmi Sholihah[2], Popi Septiani[2], Magdalena Lenny Situmorang[2], Rizkita Rachmi Esyanti[2], Imaduddien Raihan Budiyanto[3]

[1]Herbarium Bandungense (FIPIA), School of Life Sciences and Technology, Institut Teknologi Bandung, Jl. Let. Jen. Purn. Dr (HC) Mashudi No. 1, Jatinangor, Sumedang Regency, West Java

[2]School of Life Sciences and Technology, Institut Teknologi Bandung, Jl. Ganesha No. 10, Bandung, West Java

[3]Biomanagement Magister Program, School of Life Sciences and Technology, Institut Teknologi Bandung, Jl. Ganesha No. 10, Bandung, West Java

Correspondence should be addressed to Rina Ratnasih Purnamahati; E-mail address: rina@sith.itb.ac.id

Abstract

Artocarpus J. R. Forst. & G. Forst. is the third largest genus in the Moraceae, and it comprises approximately 70 species distributed from India to the Solomon Islands. During our biodiversity exploration, an underutilized species of *Artocarpus*, namely *A. rigidus* Blume, was collected in the Dolok Sibual-buali Nature Reserve, Sipirok, North Sumatra. The species has been known as *Hatompul* by the Batak Ethnic Group. The species is not cultivated, and the local people collect it from the forest or its borders. Its juicy arillus is freshly consumed as a fruit dessert, and the seeds are often roasted. *Artocarpus rigidus* has not been used optimally by the people around the forest. Previous studies have reported that the flavonoids and xanthone extracted from *A. rigidus* have antimalarial activity against *Plasmodium falciparum* and cytotoxic properties, demonstrating its potential as a medicinal plant in the future.

Keywords: *Artocarpus*, Fruit, Medicine, Moraceae, Sipirok

1. Introduction

Dolok Sibual-buali Nature Reserve area is administratively located in the subdistricts of Sipirok, Batang Toru, and Padang Sidempuan, South Tapanuli Regency, North Sumatra. The government of Indonesia established the Dolok Sibual-buali Nature Reserve on December 27, 1982, by Letter Decree of the Minister of Agriculture No 923/Kpts/Um/12/1982, with the function of biodiversity protection. Dolok Sibual-buali Nature Reserve has also been designated for the hydrological system protector [1].

The vegetation cover in Dolok Sibual Buali Nature Reserve was classified as safe, with the secondary forest area covering 4624.84 hectares, or approximately 92.26% of the total area [2].

Other vegetation types include mixed farms, dry land farms, shrubs, and rice fields [2]. This nature reserve is a habitat for several endangered animals, such as sumatran tiger (*Panthera tigris sumatrae*), Tapanuli orangutan (*Pongo tapanuliensis*), and Sumatran serow (*Capricornis sumatraensis*). Some commercials tree species can be found in the nature reserve, for example, meranti (*Shorea leprosula* Miq.), kayu manis (*Cinnamomum* spp.), and gaharu (*Aquilaria malaccensis* Lam.) [3, 4]

Dolok Sibual-buali Nature Reserve has excellent natural resources for further development. However, bioprospecting research in this area is limited. Thus, information on its biological potential needs to be explored. Therefore, this study is focused on discovering biodiversity and bioprospecting in the Dolok Sibual-buali Nature Reserve. *Artocarpus rigidus* Blume, as a non-timber forest product, is one of the potential plants collected from the nature reserve area. Review of the species in this paper includes its potential for medicine and food in the future.

2. Materials and Methods

A biodiversity survey was conducted in Dolok Sibual-buali Nature Reserve, Sipirok Subdistrict, South Tapanuli Regency, North Sumatra, from July to August 2022. The field study was carried out using the exploration methods [5]. Plant materials were collected according to van Balgooy's guideline [6], and specimen preservation was done at Herbarium Bandungense, School of Life Sciences and Technology, ITB Jatinangor campus, Sumedang Regency. The taxonomical examination was conducted to confirm the identity of the collected specimens by images from ANDA gbif.org/dataset/10f8ba9a-e298-4256-88b0-997205d66a30), L (bioportal.naturalis.nl), and JSTOR Global Plants (plants.jstor.org).

3. Result and Discussion

A total of 120 plant species were collected from Dolok Sibual-buali Nature Reserve. One unique and potential species is Artocarpus rigidus Blume, which belongs to the Moraceae family. Taxonomically, the species is a wild relative of jackfruit (*A. heterophyllus* Lam.) and breadfruit (*Artocarpus altilis* (Parkinson) Fosberg). It is commonly known as *Hatompul* by the Batak ethnic group and has a common name as monkey jackfruit. The botanical description, photograph, and a brief discussion of its potential are presented here.

3.1 Botany and Ecology

Artocarpus rigidus Blume, Bijdr. Fl. Ned. Ind.: 482 (1825).

A large tree, up to 30 m tall, evergreen, produces milky latex. Branchlets covered by densely strigillose hairs, scabrous, lenticellata. Stipules lanceolate, 9–12 mm long, caducous. Leaves spirally arranged; petiole 1.1–2 cm long; lamina is obovate to elliptic, 9.5–19.5 × 4–9.5 cm, base cuneate, margin entire, apex shortly acuminate, coriaceous; adaxial surface puberulous to strigillose; abaxial surface strigillose on the main veins, scabrous; lateral veins 14–20 pairs. Staminate inflorescences axillary, solitary; peduncle 4–6 mm long; head ovoid to subglobose; perianth tubular; stamen 1 mm long, anther capitate. Pistillate inflorescences axillary, solitary; peduncle up to 3 cm long; head ellipsoid to subglobose; perianth tubular, apex convex; stigma

simple, filiform. Infructescences subglobose, covered by straight, cylindrical apices of perianths, yellow; arils yellowish orange; fruits ellipsoid, 1–1.5 cm long. – Figure 1.

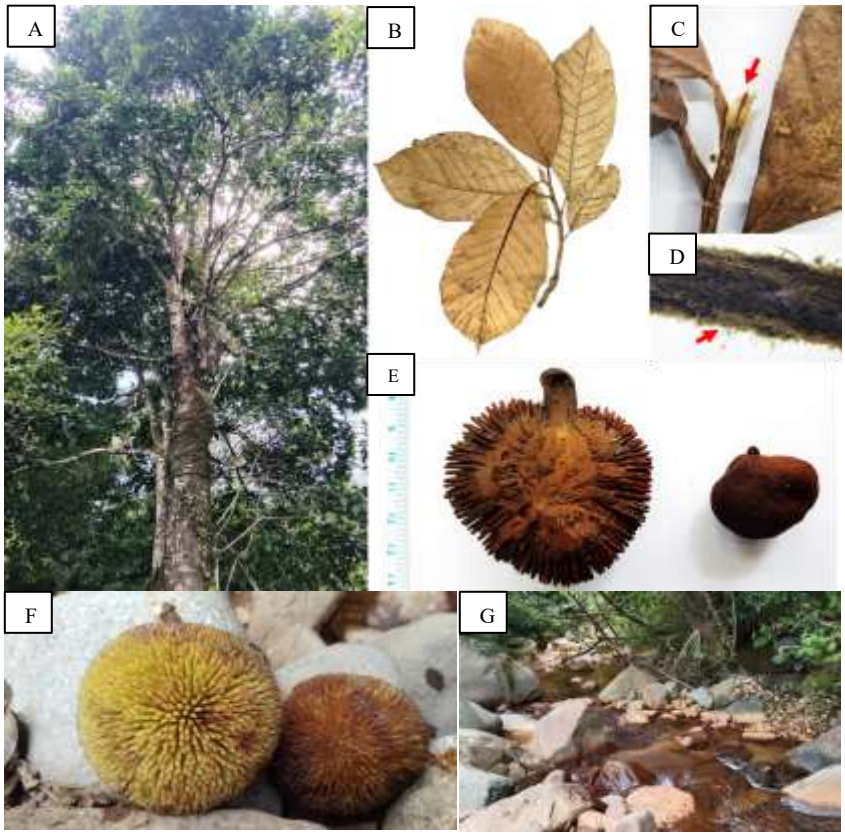


Figure 1. *Hatompul* (*Artocarpus rigidus* Blume). A. Habit; B. Leafy twig; C. Stipule (arrow); D. Hairs on the branchlet (arrow); E. Immature infructescence (left) and staminate inflorescence (right); F. The fresh materials of immature infructescences; G. Habitat.

The distributional range of this species is Myanmar, Thailand, Peninsular Malaya, Singapore, Sumatra, Borneo, Java, and the Lesser Sunda Islands [7, 8, 9]. In the Dolok Sibual-buali Nature Reserve, the species can be found in riversides, forest borders, and brinks at 1000–1100 m asl. *Artocarpus rigidus* is a pre-disturbance remnant tree species in the forest borders of Dolok Sibual-buali. Animals play an important role as seed dispersers because the fruit is eaten by the Tapanuli orangutan (*Pongo tapanuliensis*) and birds.

3.2 Traditional uses

Artocarpus rigidus is a minor fruit tree in South East Asia. When compared to jackfruit and breadfruit, it has a low economic value. Traditionally, local communities in the Malay Peninsula use timber for furniture and house beams. Wood is also often used for making a boat in Sumatra [10]. The Sakai tribe of Riau, Sumatra, frequently utilize natural fibers extracted from the bark for clothing [11] The milky latex is mixed with wax for calico dyeing and used as a veterinary medicine for wounds by the Javanese. The decoction of root roots and bark is applied for stomach aches by the Bunong people in Cambodia [10].

In our study, *A. rigidus* has not been cultivated by the local communities around Dolok Sibual-buali Nature Reserve. The locals sometimes collect it from the forest or its borders. Fleshy perianth surrounding the seed is freshly eaten as a dessert fruit, and local communities often roast the seeds. It has a similar taste to jackfruit. The utilization of *A. rigidus* is limited to traditional snacks, and other uses are unknown.

3.3 Phytochemistry

Previous studies demonstrated that *Artocarpus* is rich in flavonoids and xanthone [12, 13, 14, 15, 16, 17, 18, 19]. In South East Asia, some of the *Artocarpus* species have a long history in treating abscesses, diarrhoea, dysentery, hypertension, inflammation, liver cirrhosis, malaria, tuberculosis, and ulcers [20, 21]. Several pharmacological studies have also been conducted on the biological activities of flavonoids extracted from *A. rigidus*.

Roots of *A. rigidus* were rich in phenolic compounds such as 7-demethylartonol flavonoid E (1) and the chromone artorigidusin (2). The root extract also contains four phenolic compounds, namely artonol B (3), artonin F (4), and cycloartobiloxanthone (5), as well as xanthone C artoindonesianin (6) [13]. The flavonoids 1, 4, and 5 isolated from the root bark exhibited antiplasmodial activity against *Plasmodium falciparum*, while all six compounds have antimycobacterial activity [13, 22]. Compound 4 was reported as the most active antimycobacterial agent. Moreover, compounds 1, 3, 5, and 6 were also cytotoxic. Compounds 5 and 6 were active against KB tumour cells (a sub-line of the keratin-forming tumour cell line HeLa), whereas compounds 2, 5, and 6 were toxic to BC (breast cancer) cells in various degrees. In the NCI-H187 (human small cell lung cancer) cytotoxicity assay, compounds 1-3, 5, and 6 were active to inhibit this cell line [13]. In 2016, Artonin O was isolated from the root, and the compound has antibacterial activity against *Bacillus subtilis* [15].

Other phenolic compounds isolated from the root bark are artocarpols G–H, rubraflavone C and trans-stilbene-2,4,3',S'-tetrol [23]). The latter compound demonstrated an anti-inflammatory activity. The compound strongly inhibited the release of beta-glucuronidase and histamine from mast cell degranulation in a concentration-dependent manner. It also inhibited formyl-peptide-stimulated superoxide anion formation in neutrophils [23].

The stem bark contains artorigidinone A–C and artorixanthone, along with artonol B, artonin B, artonin O, cycloartobiloxanthone, artobiloxanthone, c-geranylapigenin and artonin E [18]. Compound 10 exhibited cytotoxicity to a fibroblast-like cell line (SW1353) (IC_{50} < 0.32 μg/mL) [18]. The wood extract shows the presence of 4-chromenones artocarmins G–M, norartocarpetin, artocarpin, artogomezianone, artochamin A, pyranocycloartobiloxanthone A, cycloartobiloxanthone, artocarpone B, artonin J, artonol A, and p-hydroxybenzoic acid [17, 19]. Norartocarpetin had the most significant effect in the tyrosinase inhibitory activity test, with an IC_{50} value of 0.023 μM [17].

The twigs of *A. rigidus* have also been screened to determine their chemical properties. This vegetative part contains artorigidin A–C, artonin O, 3-hydroxy4,3′,5′-trimethoxy-trans-stilbene, artonin K, artonin N, artonin G, betulinic acid, cyclorigidol, artoristilbene, artobiloxanthone, cycloartobiloxanthone, and ursolic acid [24]. Several of the compounds obtained were cytotoxic to HT-29 human colon cancer cells, with artorigidin B being the most potent [24].

4. Further research

The species is a promising future source of medicinal products. Crude extracts and chemical compounds isolated from *A. rigidus* have antibacterial, anticancer, antitumour, anti-inflammatory, antimalarial, antimycobacterial, and tyrosinase inhibitor properties, as discussed here. Further bioassay analysis on other activities can be carried out in the future.

Artocarpus rigidus is an underutilized species that has the potential to be used as an ingredient of seed-based crackers. This idea is supported by the results of previous studies that jackfruit seeds can be processed into crackers [25, 26]. The information on the nutritional value of *A. rigidus* is lacking. Thus, we can refer to jackfruit as the close sister. Fresh jackfruit seeds contain an adequate amount of nutrients, including crude protein (6.6 g), fat (0.4 g), carbohydrates (38.4 g), fibre (1.5 g), ash (1.25-1.50 g), and moisture (51.6-57.77 g) [27]. Jackfruit seeds have a high starch content and can be used as an alternative source of starch [28], as well as *A. rigidus*. Because *A. rigidus* is a wild plant, its nutritional components could be smaller than jackfruit.

Acknowledgments

This research is supported by the School of Life Sciences and Technology, ITB. We also thank BKSDA Sumatera Utara (Balai Konservasi Sumber Daya Alam) for helping us during the field exploration.

References

[1] Balai Konservasi Sumberdaya Alam Sumatera Utara. Buku Panduan Kawasan Cagar Alam Dolok Sibual-buali. Medan: Departemen Kehutanan; 2004.

[2] Gurusinga WLB, Latifah S, Chasanatun, SN. Analisis profil resort cagar alam dolok sibual-buali sebagai penentu strategi pengelolaan Kawasan. Jurnal Agrotek Lestari. 2018;4(1):20-35.

[3] Kuswanda W, Sugiarti S. Potensi Habitat dan Pendugaan Populasi Orangutan (*Pongo abelii* Lesson 1827) di Cagar Alam Dolok Sibual-Buali Sumatera Utara. Jurnal Penelitian Hutan dan Konservasi Alam. 2005;2(6):555-556.

[4] Kuswanda W, Sukmana A. Kesesuaian jenis untuk pengayaan habitat orangutan terdegradasi di daerah penyangga Cagar Alam Dolok Sibual-Buali. Jurnal Penelitian Hutan dan Konservasi Alam. 2009;6(2):125-139.

[5] Rugayah, Windadri FI, Hidayat A. Pengumpulan Data Taksonomi. In: Rugayah, Widjaja EA, Praptiwi, editors. Pedoman Pengumpulan Data Keanekaragaman Flora. Bogor: LIPI; 2004. p. 5-24.

[6] van Balgooy MMJ. Collecting. In: de Vogel EF, editor. Manual of Herbarium Taxonomy Theory and Practice. Jakarta: UNESCO; 1987. p. 14-19

[7] Berg CC, Corner EJH, Jarrett FM. Moraceae genera other than Ficus. Flora Malesiana - Series 1, Spermatophyta. 2006;17(1):1-146.

[8] Williams EW, Gardner EM, Harris III R, Chaveerach A, Pereira JT, Zerega NJ. Out of Borneo: biogeography, phylogeny and divergence date estimates of *Artocarpus* (Moraceae). Annals of Botany. 2017;119(4):611-627. DOI: 10.1093/aob/mcw249

[9] Gardner EM, Zerega NJC. Taxonomic updates to *Artocarpus* subgenus *Artocarpus* (Moraceae) and allied taxa with a particular focus on the species native to Singapore. Gardens' Bulletin Singapore. 2021;73(2):309-374. DOI: 10.26492/gbs73(2).2021-08

[10] Lim TK. *Artocarpus rigidus*. In: Lim TK, editor. Edible Medicinal and Non-Medicinal Plants. Dordrecht: Springer; 2012. p. 348-350. DOI: 10.1007/978-94-007-2534-8

[11] Minza LQ. Eksplorasi Material Kulit Kayu Pudu (*Artocarpus rigidus*) untuk Produk Fashion dengan Inspirasi Busana Suku Sakai [undergraduate thesis]. Bandung: Faculty of Arts and Design, ITB; 2021.

[12] Hakim EH, Achmad SA, Juliawaty LD, Makmur L, Syah YM, Aimi N, Kitajima M, Takayama H, Ghisalberti EL. Prenylated flavonoids and related compounds of the Indonesian *Artocarpus* (Moraceae). Journal of Natural Medicines. 2006;60(3):161-184. DOI: 10.1007/s11418-006-0048-0

[13] Namdaung U, Aroonrerk N, Suksamrarn S, Danwisetkanjana K, Saenboonrueng J, Arjchomphu W, Suksamrarn A. Bioactive constituents of the root bark of *Artocarpus rigidus* subsp. *rigidus*. Chemical and Pharmaceutical Bulletin. 2006;54(10):1433-1436. DOI: 10.1248/cpb.54.1433

[14] Jagtap UB, Bapat VA. *Artocarpus*: A review of its traditional uses, phytochemistry and pharmacology. Journal of Ethnopharmacology. 2010;129(2):142-166. DOI: 10.1016/j.jep.2010.03.031

[15] Suhartati T, Hadi S. Artonin O, a Xanthone Compound from Root Wood of *Artocarpus rigida*. Oriental Journal of Chemistry. 2016;32(5):2777-2784. DOI: 10.13005/ojc/320552

[16] Ramli F, Rahmani M, Ismail IS, Sukari MA, Rahman MA, Zajmi A, Akim AM, Hashim NM, Go R. A new bioactive secondary metabolite from *Artocarpus elasticus*. Natural Product

Communications. 2016;11(8):1934578X1601100818. DOI: 10.1177/1934578X1601100818

[17] Nguyen MT, Le TH, Nguyen HX, Dang PH, Do TN, Abe M, Takagi R, Nguyen NT. Artocarmins G–M, prenylated 4-chromenones from the stems of *Artocarpus rigida* and their tyrosinase inhibitory activities. Journal of natural products. 2017;80(12):3172-3178. DOI: 10.1021/acs.jnatprod.7b00453

[18] Rattanaburi S, Sriklung K, Watanapokasin R, Mahabusarakam W. New flavonoids and xanthone from the stem bark of *Artocarpus rigidus* blume and cytotoxicity. Natural Product Research. 2021;35(21):4010-4017. DOI: 10.1080/14786419.2020.1753734

[19] Hawari D, Mutakin M, Wilar G, Levita J. Flavonoids of *Morus*, *Ficus*, and *Artocarpus* (Moraceae): A review on their antioxidant activity and the influence of climate on their biosynthesis. Journal of Applied Pharmaceutical Science. 2021;11(12):045-64. DOI: 10.7324/JAPS.2021.1101203

[20] Perry LM. Medicinal plants of East and Southeast Asia: attributed properties and uses. Cambridge: MIT Press; 1980. 620 p.

[21] Heyne K. Tumbuhan Berguna Indonesia. Badan Litbang Kehutanan; 1987. 2521 p.

[22] Soré H, Sanon S, Hilou A. Antiplasmodial properties of plants isolated flavonoids and their derivatives. International Journal of Herbal Medicine. 2018;6(5):43-56.

[23] Lu YH, Lin CN, Ko HH, Yang SZ, Tsao LT, Wang JP. Two novel and anti-inflammatory constituents of *Artocarpus rigida*. Helvetica Chimica Acta. 2002;85(6):1626-1632. DOI: 10.1002/1522-2675(200206)85

[24] Ren Y, Kardono LB, Riswan S, Chai H, Farnsworth NR, Soejarto DD, Carcache de Blanco EJ, Kinghorn AD. Cytotoxic and NF-κB inhibitory constituents of *Artocarpus rigida*. Journal of Natural Products. 2010;73(5):949-955. DOI: 10.1021/np1002065

[25] Hastuti ND. Karakteristik organoleptik pada produk olahan stik biji nangka. Teknologi Pangan: Media Informasi Dan Komunikasi Ilmiah Teknologi Pertanian. 2020;11(1):66-71.

[26] Astuti P, Zumar HA, Fathonah S, Ansori M. Differences in preferences, water content, and dietary fibre of jackfruit seed crackers enriched with red beetroot (*Beta vulgaris*). In: IOP Conference Series: Earth and Environmental Science 2022 (Vol. 969, No. 1, p. 012009). IOP Publishing. DOI: 10.1088/1755-1315/969/1/012009

[27] Madruga MS, de Albuquerque FS, Silva IR, do Amaral DS, Magnani M, Neto VQ. Chemical, morphological and functional properties of Brazilian jackfruit (*Artocarpus heterophyllus* L.) seeds starch. Food chemistry. 2014;143:440-445. DOI: 10.1016/j.foodchem.2013.08.003

[28] Swami SB, Kalse SB. Jackfruit (*Artocarpus heterophyllus*): Biodiversity, Nutritional Contents, and Health. In: Mérillon JM, Ramawat KG, editors. Bioactive Molecules in Food. Switzerland: Springer Cham; 2019. p. 2237-2259. DOI: 10.1007/978-3-319-78030-6

B.8

Enhanced Biomass and Astaxanthin Accumulation in Spirogyra sp. using Organic-Carbon Enriched Growth Medium

I Putu Ikrar Satyadharma, Erly Marwani, Khairul Hadi Burhan,
Sekolah Ilmu dan Teknologi Hayati, Institut Teknologi Bandung, Jalan Ganesha No. 10 Bandung 40132 Indonesia

Correspondence should be addressed to I Putu Ikrar Satyadharma; E-mail address: ikrarsatyad@gmail.com

Abstract

Astaxanthin is a carotenoid compound with antioxidant activity higher than other compounds. Astaxanthin is produced by a variety of autotrophic organism, such as *Spirogyra* sp., the freshwater green algae, in limited quantity. Astaxanthin production in green algae is suspected to increase with addition of inorganic and organic carbon sources into the cultivation media. In this study, we add the organic sources from acetic acid (CH_3COOH) with addition of C/N parameters on growth and astaxanthin production in *Spirogyra* sp. culture. *Spirogyra* sp. was cultivated in 25% BG-11 medium with variation of CH_3COOH addition that adjusted to C/N ratio of 1:1; 2:1; 3:1; 4:1; and 5:1. Each variation was repeated three times. The nitrogen source ($NaNO_3$) was derived from the composition of the standard 25% BG-11 medium. Cultivation was carried out for 15 days with destructive harvesting every 3 days. Extract of *Spirogyra* sp. dry biomass was analyzed for astaxanthin using High Performance Liquid Chromatography. Results showed an increase in the yield of biomass with the addition of CH_3COOH. The highest maximum biomass of 373.4 ± 61.2 mg/L was obtained at 1:1 C/N ratio on the 15th day, 1.8 times higher than control. CH_3COOH addition was also found to increase astaxanthin production with the highest astaxanthin concentration of 77.1 ± 35.1 μg/g dry weight and highest astaxanthin yield of 26.8 μg/L. The highest concentration and yield of astaxanthin was obtained at 1:1 C/N ratio cultivation on the 9th day.

Keywords: Astaxanthin, Biomass, *Spirogyra* sp., Organic-Carbon, Yield.

1. Introduction

Photoautotrophic organisms, such as plants, algae, and photosynthetic bacteria, are naturally exposed to reactive-oxygen-rich environment [1]. Under this stress, organisms tend to produce

highly-valuable antioxidant compounds, such as astaxanthin [2, 3]. Green algae are known to produce astaxanthin as defense mechanism under unfavorable living conditions [4].

Astaxanthin is a ketocarotenoid compound with antioxidant activity 10-times of β-carotene and is 500-times more effective than α-tocopherol [5]. Astaxanthin is widely used in cosmetics, nutraceutical, and pharmaceutical industry [6]. The structure of this compound allows it to move freely in and out of cells, making it extra effective against a wide range of oxidants [7]. Currently, 95% of astaxanthin sold in the world market consists of synthetic astaxanthin because of its cheaper production cost, using petrochemicals as raw materials [8]. Synthetic astaxanthin itself is not often used for human consumption, for it is known to have different stereochemical properties from its natural counterpart [9]. Therefore, a reliable and sustainable means of producing natural astaxanthin that is safe for human consumption is needed.

Spirogyra sp. is a species of filamentous green macroalgae, commonly found in freshwater bodies, such as ponds, rivers, paddy fields, and irrigation systems, especially on the Island of Java [10]. *Spirogyra* sp. can produce astaxanthin up-to 0.07% of it's dry weight [4], which is higher than some astaxanthin-producing-green-algae, such as *C. zofingiensis* (0.001%$_{\text{dry weight}}$), *U. lactuca* (0.01%$_{\text{dry weight}}$), and *Catenella repens* (0,02%$_{\text{dry weight}}$) [2]. Even so, *Spirogyra* sp. biomass is mostly, strictly, used as fish food and/or bait [1].

Biomass and astaxanthin production in algae can be enhanced through manipulating a wide range of factors, either physically (light, aeration) and/or chemically. Manipulation of carbon source and concentration in the growth-media is one example of chemical manipulation. Addition of extra carbon sources, either in the form of inorganic (sodium bicarbonate) or organic carbon sources (acetic acid and acetate salts), in green algae growth-media has shown to increase astaxanthin productivity [3]. Other researches has also shown an ability to utilize organic carbon sources in *Spirogyra* sp., in both heterotrophic and mixotrophic conditions [4, 9].

Addition of organic carbon sources, in the form of acids and salts, within a certain concentration range has been reported to increase carotenoids and astaxanthin production within *Haematococcus pluvialis* [12, 13] and *Nannochloropsis salina* [14]. Addition of acetate as a carbon source has shown to increase astaxanthin production, due to its easily utilized structure in Acetyl-CoA synthesis, to be used as precursor in astaxanthin production through Mevalonate (MVA) pathway [12]. Even so, high-levels of acetate, especially in the form of acetic acid, can induce accumulation toxic anions; reducing the cell's efficiency in metabolizing the acid and simultaneously disrupting the cell's internal structure [15]. Therefore, it is crucial to determine the optimum acetate addition in respect to the algae growth and astaxanthin production.

In order to improve astaxanthin production whilst retaining an optimal biomass growth, it is necessary to find the optimum acetic acid concentration. This study focused on determining the effects of various acetic acid concentration, in respect to C/N ratio within the growth media, on *Spirogyra* sp. growth and astaxanthin production under mixotrophic conditions.

2. Methodology

2.1 Materials

Wild *Spirogyra* sp. strain was collected from rice fields in Majalaya District, Bandung Regency, West Java. *Spirogyra* sp. is cultivated in BG-11 25% medium, comprised of $NaNO_3$ 375 mg/L; $CaCl_2.2H_2O$ 9 mg/L; $(NH_4)_5[Fe(C_6H_4O_7)_2]$ 1,5 mg/L; Citric acid 1,5 mg/L; Na_2EDTA 2,5 mg/L; K_2HPO_4 10 mg/L; $MgSO_4$ 18,75 mg/L; Na_2CO_3 5 mg/L; and also *Trace Elements* 0,25 mL/L, containing H_3BO_3 2,86 mg/L; $MnCl_2$ 1,81 mg/L; $ZnSO_4$ 0,22 mg/L; Na_2MoO_4 0,39 mg/L; $CuSO_4$ 0,08 mg/L; and $Co(NO_3)_2$ 0,05 mg/L. BG-11 composition is adapted from Hong et al. [16]. All of the above chemicals are a product of Merck Germany. Pure astaxanthin for HPLC use is a product of Sigma-Aldrich Co., St. Louis, USA. CH_3COOH (Acetic Acid) is a product of PT. Pudak Scientific.

2.2 Methods

2.2.1 Analysis of rice-field water composition

Analysis of rice-field water taken from Majalaya District with Liquid Organic Fertilizer parameters was outsourced through Laboratory of Soil Chemistry and Plant Nutrition, Faculty of Agriculture, Padjajaran University.

2.2.2 Cultivation of algae

Before cultivation, *Spirogyra* sp. was acclimated in BG-11 25% medium for 7 days, with pH level maintained within 7,5 ± 0,3 range. Acclimated *Spirogyra* sp. inoculums, weighing 6-grams each, were cultivated in a transparent plastic container, measuring 15×15×7,5 cm, with a working volume of 1-L growth media. Culture was grown in a dark-room with temperature maintained within 24 ± 4°C range [17] under light intensity of 23-30 μmolphoton/$m^{-2}s^{-1}$ [18] from *Phillips Cool White* TL-LED 16W with continuous illumination (24:0) for 15 days. Culture is grown in six different conditions, including standard BG-11 25% medium (control), and a variety of acetic acid addition treatment (Table 1); every variation was cultured with triplicate.

Table 1. Acetic acid addition per C/N ratio variation

Variation	CH_3COOH conc. (mg/L)	Carbon conc. (mg/L)
1:1	141,7	61,8
2:1	288,7	123,5
3:1	435,8	185,3
4:1	582,8	247,1
5:1	729,9	308,8

Air was continuously supplied through small pumps at the rate of 0,2 vvm. pH levels are checked every 2 days and maintained within 7,5 ± 0,3 range [17] using METTLER TOLEDO Five Easy pH/mV Portable. A total of 15 containers per variation is prepared.

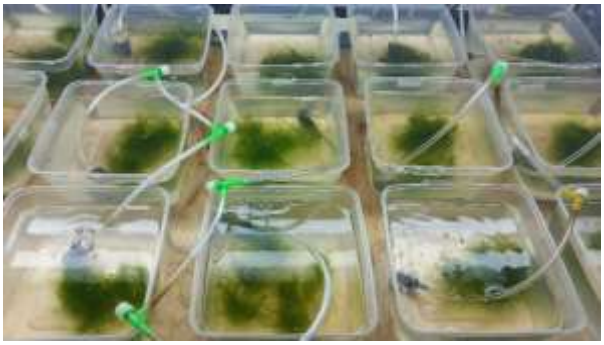


Figure 1. *Spirogyra* sp. cultivation

2.2.3 Harvesting and measurement of dry weight

Spirogyra sp. samples were harvested on the 3rd, 6th, 12th, and 15th day. Samples were sieved through mesh 60 and then weighted for wet weight. Samples are then placed inside a container made of parchment paper and alumunium foil with a known weight (m_1). Samples were then dried inside an oven on 50°C [19] for 24 hours. Dry samples were then weighted along with the container (m_2). Dry weight (m_{dw}) was determined from the difference between m_2 and m_1.

$$m_{dw} = m_2 - m_1 \tag{1}$$

2.2.4 Estimation of Biomass Growth-Rate

Dry weight was averaged and standard deviation was calculated. Dry weight of each variation was then plotted against its corresponding day and exponential growth phase was then identified. Specific growth rate is determined using Equation 2 [21].

$$\mu = \frac{\ln\left(\frac{x_2}{x_1}\right)}{\left(t_2 - t_1\right)} \tag{2}$$

With x_2 and x_1 are dry weight on the end and start of exponential phase, and $t_2 - t_1$ as the time interval of the exponential phase.

2.2.5 Measurement of nitrate concentration

Nitrate concentration was measured using spectrophotometry every three days with triplicates [20]. A standard curve was produced prior to sampling (Equation 1). Sample of 2-5 mL medium was taken and transferred into a 250 mL flask. Sample was diluted with aquadest until volume reaches 50 mL. 1 mL of HCl was then added and mixed using vortex. The mixture was then

incubated for 30 minutes. Absorbance was measured using λ = 220 nm and λ = 275 nm. Absorbance value was then fitted into the standard curve equation below.

$$[N] = 20{,}048 \times (\Delta A) - 0{,}2186 \quad (3)$$

With *[N]* as nitrate concentration in mg/L, dan ΔA is the difference between λ_{220nm} and $\lambda_{275nm.}$absorbance.

2.2.6 Extraction of Astaxanthin

Dried biomass was grinded using mortar and pestle. Extraction is done through maceration [22]. The 0,1 grams of dried biomass powder is macerated inside 5 mL of acetone for 30 minutes. Maceration was done inside and incubator shaker with a temperature of 30ºC and agitation speed of 100 rpm. Extract was then filtered through fat-free cotton. Leftover extracts were washed using 5 mL acetone. The filtered extracts were then evaporated on evaporation dishes in room temperature and redissolved in HPLC-grade methanol for HPLC analysis.

2.2.7 HPLC Analysis of Astaxanthin Extract

Before analysis, methanol-dissolved extract was filtered using a 0,22 µm PTFE (Polytetrafluoroethylene) syringe filter into an 1,5 mL HPLC vial. Sample is then inserted into the autosampler chamber (Shimadzu Autosampler SIL-20AC), equipped with a photodiode array (PDA) detector. The analysis was carried out using a C-18 reverse phase column with a mixture of HPLC-grade methanol:aqubidest (95:5 v/v) as its mobile phase. During the analysis, flow rate was set on 1 mL/min and temperature was sset on 25ºC. Detection was done using UV 482 nm [23, 24].

Astaxanthin concentration within the sample was then quantified using the equation derived from the standard curve (Equation 4), while astaxanthin content within the dry biomass was calculated using Equation 5.

$$[Ast] = (A) \times 10^{-4} - 0{,}0934 \quad (4)$$

With *[Ast]* as astaxanthin concentration in mg/L, and A is the peak area

$$Ast = \frac{[Ast] \times V}{m_{sample}} \quad (5)$$

Ast is astaxanthin content (mg/g_{dw}), V is solvent volume (L), and m_{sample} is the mass of dry *Spirogyra* sp. used for extraction

Estimation of Astaxanthin Yield

Astaxanthin yield is calculated using Equation 5.

$$Y_{ast} = \frac{Ast \times m_{dw}}{V_{medium}} \quad (5)$$

With V_{medium} as the volume of medium used during cultivation (1 L).

2.9 Statistical Analysis

One-way ANOVA test was done to determine the significance of the treatments on biomass accumulation and astaxanthin content. Post-hoc Tukey test was also done to determine the significance between groups of treatments. In addition, Pearson Correlation Coefficient (r) was also determined to test the correlation between parameters. The statistical tests were done using IBM SPSS Statistics ver. 25 and 28 software.

3. Results and Discussion

3.1 Rice-field water composition

According to the results received, rice-field water taken from Majalaya District contains 0,06% (0,6 g/L) Nitrogen and 9,69% (96,9 g/L) Organic Carbon. Compared to the content of BG-11 25% medium, rice-field water contains 10-times the amount of nitrogen. Even so, *Spirogyra* sp. was still able to grow on BG-11 25% medium. This phenomenon is possible due to the algae's ability and tendency to grow on low-nutrient stagnant water [25], much like the conditions of cultivation in this study.

The C/N ratio obtained from this test has a fairly high value of 161,5:1. In an effort to simulate the natural conditions of the culture's habitat, BG-11 25% medium was modified to contain C/N ratios of 100:1; 20:1; 10:1; and 2:1 with acetic acid as an organic carbon source. The *Spirogyra* sp. cultivated in these treatments, as a preliminary study, does not show an increase in biomass accumulation (Figure 2).

Based on statistical analysis, samples taken from C/N ratio 20:1 and 100:1 treatment shows no significant biomass yield between each sampling. Biomass samples taken from C/N ratio of 2:1 and 10:1 shows a downward trend over time, with a slight increase towards the end of the cultivation. The sudden change in growth conditions, might have been too significant, causing the algae's growth to be inhibited [26]. The high concentration of toxic anions (CH_3COO^-), from the actic acid, in the C/N ratio 20:1 and 100:1 treatment may also inhibit the metabolism of acetic acid to Acetyl-CoA [15].

From this preliminary study, it is decided that wild algae culture was to be acclimated for 7 days in BG-11 25% growth medium, prior to cultivation. C/N ratio for the treatments was also changed to a lower C/N ratio range to prevent toxic anion accumulation

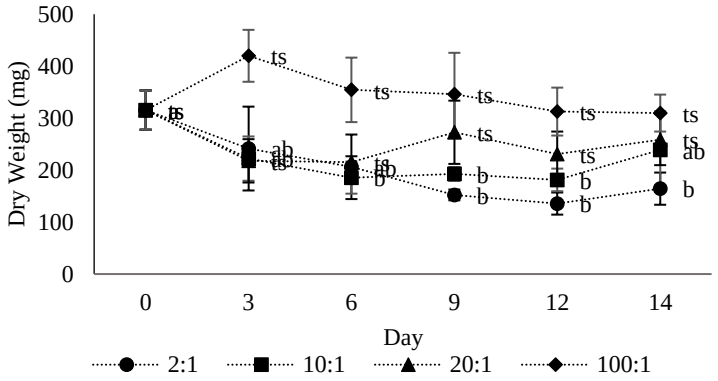


Figure 2. *Spirogyra* sp. preliminary biomass growth

Notes: Difference in notations (a, b) express a significant difference ($p < 0.05$) in one variation of treatment, while ts expresses no significant difference.

3.2 Effect of acetic acid addition on biomass growth

Highest biomass accumulation was obtained from treatment with a C/N ratio of 1:1 (373.4 ± 61.2 mg) and is then followed by 2:1 (324.6 ± 27.8 mg), 3:1 (270.8 ± 59.4 mg), control (205.6 ± 17.3 mg), 4:1 (201.2 ± 71.5 mg) and lastly 5:1 (182.2 ± 41.7 mg). Tukey post-hoc analysis shows that acetic acid addition into the growth medium has significant effects on *Spirogyra* sp. biomass accumulation (Figure 3). Pearson's coefficient correlation test shows a strong enough negative-relationship (-0,538) between biomass accumulation and C/N ratio.

In this study, optimum C/N ratio for biomass accumulation is 1:1 with 141.7 mg/L acetic acid addition. This concentration is within the range of acetic acid concentration, 71 – 207 mg/L, that was reported to enhance biomass accumulation in a variety of algae, whereas acetic acid addition outside of said range shows inhibitive effect on algae biomass growth [15]. The increase of biomass growth in a specific acetic acid concentration range is due to algae's ability that directly convert acetate ions into Acetyl-CoA through a single-step process with the help one ATP molecule and Acetyl-CoA synthetase (ACS2) [27]. However, exorbitant amount of acetic acid will lead to toxic anion accumulation, which inhibits the cell's ability to metabolize acetic acid, and also damages the cell [23].

Algae growth inhibition in high C/N ratio and/or high acetate concentration was also reported. Dittamart et al. reported that *Scenedesmus* sp. exhibited maximum growth on 0,01 M sodium acetate enriched medium, compared to the control, 0.02 and 0.05 M sodium acetate enriched medium [28], showing the same trend. Lu et al. has also reported the same trend on *H. pluvialis*, with maxium cell productivity reached on C/N ratio of 10:1; with a C/N ratio treatment range of 0:1 to 50:1 [29].

Table 2. Specific growth rate of *Spirogyra* sp. in this study

Treatment	μ_m (day^{-1})
Control	0,058
C/N 1:1	0,102
C/N 2:1	0,051
C/N 3:1	0,037
C/N 4:1	0,027
C/N 5:1	0,012

Spirogyra sp. biomass growth rate exhibited from the control treatment (Table 2) shows a similarity to *Spirogyra* sp. biomass growth rate reported on a previous study, 0.06 day^{-1} [30], higher than another green macroalge's, *Oedogonium* sp., growth rate of 0,047 day^{-1} [31].

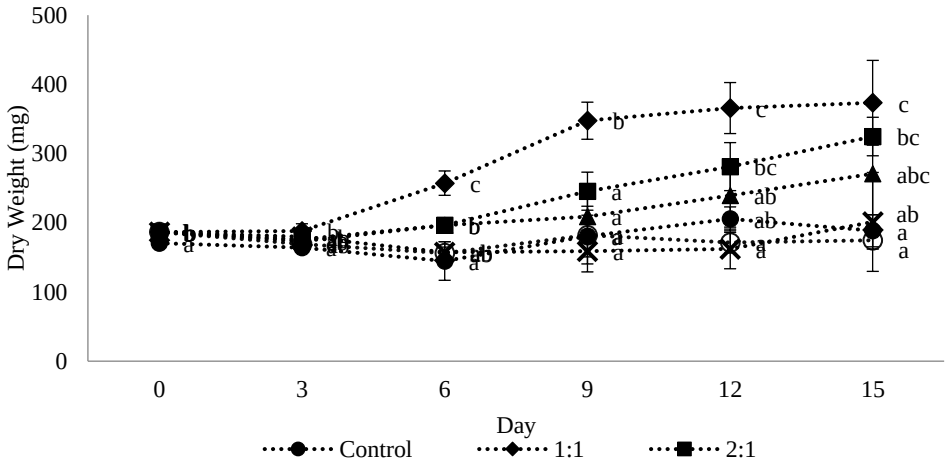


Figure 3. *Spirogyra* sp. biomass growth in modified BG-11 25% medium with acetic acid addition, in respect to C/N ratio of 1:1; 2:1; 3:1; 4:1; dan 5:1

Notes: Difference in notations (a, b, c, d, and e) express a significant difference ($p < 0.05$) on the same day of sampling

3.3 Effect of acetic acid on nitrate consumption

Highest nitrate consumption was observed on C/N ratio 5:1, whereas the lowest was on C/N ratio 1:1 (Figure 4). Tukey post-hoc analysis also shows real effect of acetic acid addition on

nitrate consumption. Pearson correlation test shown a high negative correlation between C/N ratio and final nitrate concentration, with a value of -0,889. This shows that nitrate consumption increased as C/N ratio increased.

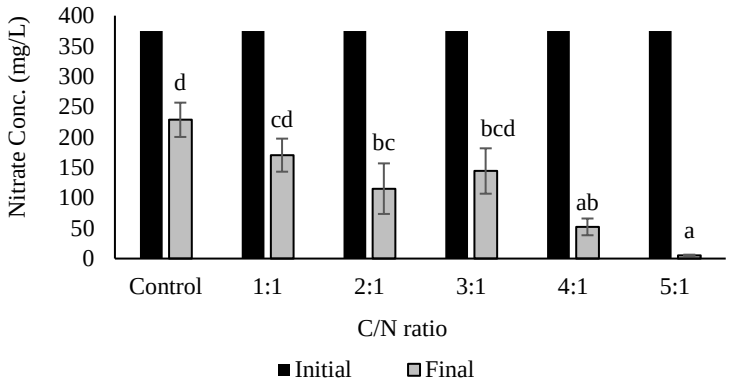


Figure 4. Initial and final nitrate concentration

Notes: Difference in notations (a, b, c, d, and e) express a significant difference ($p < 0.05$)

Spirogyra sp. was reported to also exhibit an increase in nitrate consumption along with the increase in glucose concentration in medium [11]. The same nitrate consumption trend was also reported on other green algae, *C. pyrenoidosa*, cultivation on modified medium with a C/N ratio range of 3:1 – 15:1 [32].

Significant nitrate consumption happened on the range of the first 6 days of cultivation (Figure 5). This is caused by a high surface area-to-volume ratio [33] and the presence of two protein transporter structure lining the cell wall [34], leading to a high nitrate absorption efficiency and effectivity. Between the 6th and 15th day, nitrate concentration tends to have a fluctuating trend. This could be caused by the release of nitrate from the dead cells of *Spirogyra* sp. [11], and also the inhibition of nitrate absorption due to an accumulation of ammonium inside the cell [34].

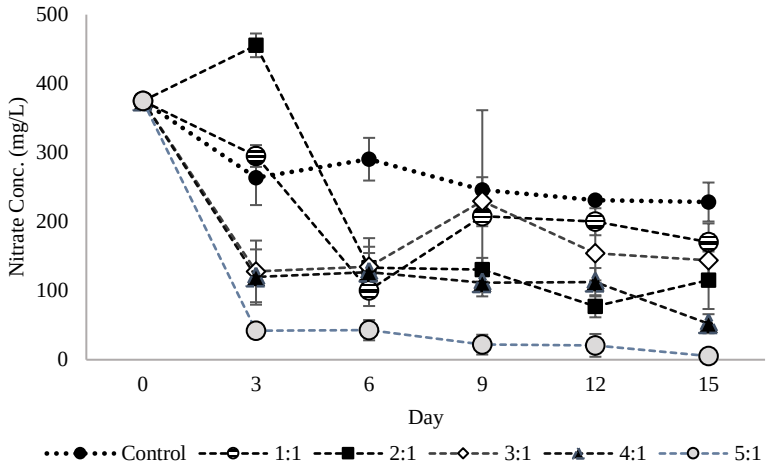


Figure 5. Nitrate concentration within growth-medium

3.4 Effect of acetic acid addition on astaxanthin content

HPLC analysis of dried biomass extract shows the highest astaxanthin content was obtained from *Spirogyra* sp. biomass that was cultivated with C/N ratio 1:1 treatment and harvested on the 9th day (Table 3). Post-hoc analysis shows acetic acid addition only have significant effect between each treatment on the 3rd, 6th, and 9th day. This result was obtained due to the high standard deviation of the mean astaxanthin levels on the 12th day.

Analysis of the obtained Pearson correlation coefficient shows a high correlation between C/N ratio and astaxanthin content on the 3rd day, and a quite strong correlation on the 6th and 9th day (Table 4). Correlation on the 3rd and 6th day shows a positive relationship; meaning astaxanthin content will increase along with the increase of C/N ratio. The same trend was reported on *H. pluvialis* cultivated in modified mediums with C/N ratio ranging from 0:1 to 50:1 [29]. Another study on *H. pluvialis* reported that the highest astaxanthin production was achieved in a treatment with the highest potassium acetate addition (100 mM) [3].

Table 3. Astaxanthin content in *Spirogyra* sp. biomass

Day	Astaxanthin content (μg/g)					
	Control	1:1	2:1	3:1	4:1	5:1
3	0.0^{a}	16.4 ± 9.1^{abc}	10.6 ± 7.6^{ab}	8.3 ± 5.7^{a}	**55.3 ± 17.5^{c}**	**50.5 ± 19.5^{bc}**
6	0.0^{a}	0.0^{a}	12.3 ± 11.0^{ab}	13.2 ± 4.2^{ab}	10.4 ± 4.2^{ab}	18.4 ± 3.8^{b}
9	0.0a	**77.1 ± 35.1^{b}**	$23.8^{t} \pm 12.9^{ab}$	0.0^{a}	27.1 ± 31.5^{a}	11.8 ± 2.5^{ab}
12	0.0^{ts}	20.8 ± 9.7^{ts}	**56.9 ± 34.8^{ts}**	**47.5 ± 46.5^{ts}**	23.8 ± 17.9^{ts}	50.3 ± 12.7^{ts}

Notes: Difference in notations (a, b, c) on the same line express a significant difference ($p < 0.05$), while ts expresses no significant difference.

The increase in astaxanthin production is because acetate, as a carbon source, can also be utilized in astaxanthin production as the precursor to Acetyl-CoA [27], the precursor to astaxanthin production through the Mevalonate (MVA) pathway [12]. However, based on the highest average astaxanthin-content data obtained from each treatment (Table 3), maximum astaxanthin content tends to decrease along with the increase of C/N ratio. This is because astaxanthin production is bound to biomass growth [35]. This phenomenon is reflected in the trend of biomass growth which tends to decrease along with the increase in the C/N ratio (Figure 3).

Table 4. Correlation analysis between C/N ratio and astaxanthin content

Day	Parameter	Value
3	Pearson Correlation	0,659**
	Significance (2-tailed)	0,008
6	Pearson Correlation	0,587*
	Significance (2-tailed)	0,021
9	Pearson Correlation	-0,525*
	Significance (2-tailed)	0,045
12	Pearson Correlation	0,115
	Significance (2-tailed)	0,682

Notes: ** Correlation is significant at the 0,01 level (*2-tailed*)
* Correlation is significant at the 0,05 level (*2-tailed*)

3.5 Effect of Acetic Acid on Astaxanthin Yield

Astaxanthin yield from each treatment was calculated using the data on average *Spirogyra* sp. dry weight yield and astaxanthin content on each sampling day (Figure 6).

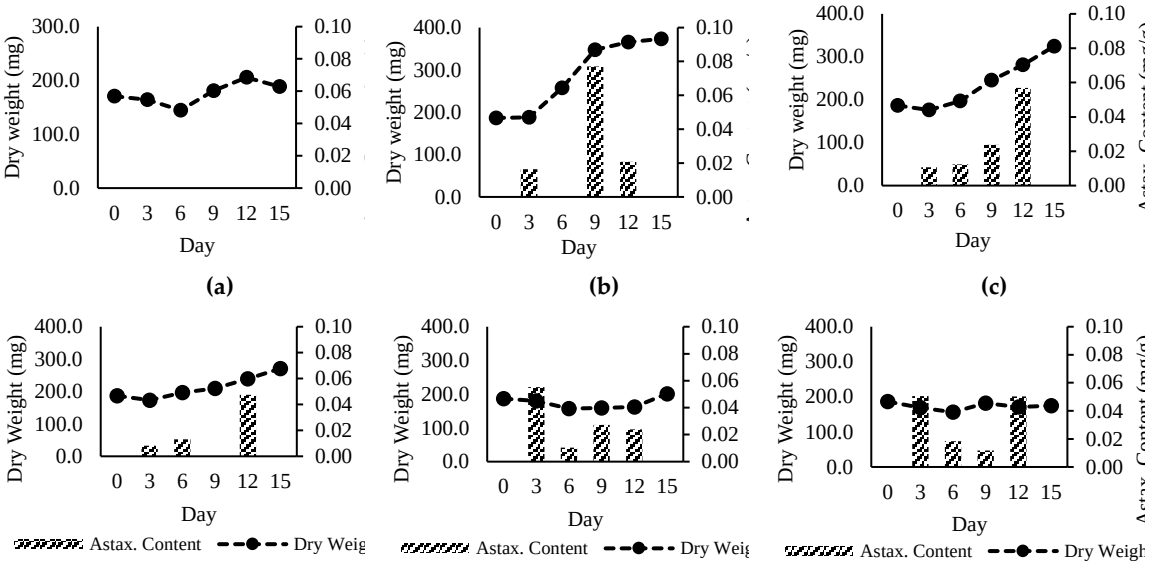


Figure 6. *Spirogyra* sp. dry weight and astaxanthin content across every treatment; (a) Control, (b) C/N ratio 1:1,
(c) C/N ratio 2:1, (d) C/N ratio 3:1, (e) C/N ratio 4:1, (f) C/N ratio 5:1

Astaxanthin yield trend obtained from the C/N ratio 1:1, 2:1, and 3:1 treatment shows a tendency to increase, while astaxanthin yield from the C/N ratio 4:1 and 5:1 treatment showed to be stagnant and even decreasing as time passed, resulting from the Although the results from astaxanthin content in the previous section shows a promising result for the top two highest C/N ratio treatment (4:1 and 5:1), once the biomass yield from each treatments are added into the equation, the highest astaxanthin yield in this study was obtained from *Spirogyra* sp. grown in C/N ratio 1:1 treated medium, with a yield of 26.8 μg/L on the 9th day and an average yield of 9.37 μg/L (Figure 7). biomass growth trend obtained from the two treatments (Figure 3).

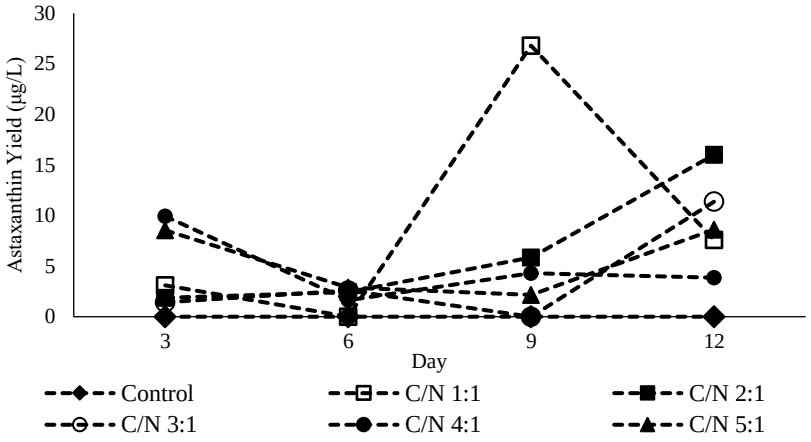


Figure 7. Estimated *Spirogyra* sp. astaxanthin yield

4. Conclusion

While acetic acid addition can increase biomass growth, once the concentration of acetic acid exceeds a certain limit, it has a tendency to negatively affect biomass growth. Highest biomass accumulation obtained from *Spirogyra* sp. grown in 141.7 mg of acetic acid added medium (C/N ratio 1:1) was 1.8 times the biomass accumulated in the control medium. Acetic acid addition in general was also found to cause an increase in nitrate consumption and astaxanthin content within *Spirogyra* sp. biomass, with the highest astaxanthin content reached 77.1 ± 35.1 $\mu g/g_{dw}$. The optimum acetic acid concentration for biomass and astaxanthin production was 141.7 mg/L, which corresponds to a C/N ratio of 1:1.

Acknowledgments

We thank Ela Kamelia Saleh, A.Md (Institut Teknologi Bandung) for the assistance regarding HPLC sample preparation and analysis

References

[1] Sari, L., Utami, E., & Rosalina, D. (2015). Pengaruh Umpan Alga (Spirogyra sp.) Terhadap Hasil Tangkapan Bubu Dasar di Perairan Perlang Kabupaten Bangka Tengah. *AKUATIK – Jurnal Sumberdaya Perairan, 9*(2), 37-45.

[2] Ambati, R., Phang, S.-M., Ravi, S., & Aswathanarayana, R. (2014). *Astaxanthin: Sources, Extraction, Stability, Biological Activities and Its Commercial Applications—A Review. Marine Drugs, 12(1), 128–152.* doi:10.3390/md12010128

[3] Pan-utai, W., Parakulsuksatid, P., & Phomkaivon, N. (2017). Effect of inducing agents on growth and astaxanthin production in Haematococcus Pluvialis: Organic and inorganic.

Biocatalysis and Agricultural Biotechnology, *12*, 152–158. https://doi.org/10.1016/j.bcab.2017.10.004

[4] Salim, M.A. (2012) Biomass and lipid content of heterotrophic Spirogyra sp by using cassava starch hydrolysate. International Journal of Scientific and Engineering Research, 15, 21–26.

[5] Schaechter, M. (2009). *Encyclopedia of microbiology* (3rd ed.). Elsevier/Academic Press.

[6] Kumar, S., Kumar, R., Kumari, A., & Panwar, A. (2021). Astaxanthin: A super antioxidant from microalgae and its therapeutic potential. *Journal of Basic Microbiology. Wiley*. https://doi.org/10.1002/jobm.202100391

[7] Fakhri, S., Nouri, Z., Moradi, S. Z., & Farzaei, M. H. (2020). Astaxanthin, COVID -19 and immune response: Focus on oxidative stress, apoptosis and autophagy. *Phytotherapy Research* (Vol. 34, Issue 11, pp. 2790–2792). Wiley. https://doi.org/10.1002/ptr.6797

[8] Panis, G., & Carreon, J. R. (2016). Commercial astaxanthin production derived by green alga *Haematococcus pluvialis*: A microalgae process model and a techno-economic assessment all through production line. *Algal Research* 18:175–190. Elsevier BV. https://doi.org/10.1016/j.algal.2016.06.007

[9] Villaró, S., Ciardi, M., Morillas-España, A., Sánchez-Zurano, A., Acién-Fernández, G., & Lafarga, T. (2021). Microalgae derived astaxanthin: Research and consumer trends and industrial use as food. *Foods, 10*(10), 2303. https://doi.org/10.3390/foods10102303

[10] Desmawati, I., Ameivia, A., & Ardayanti, L. B. (2020). Studi Pendahuluan Kelimpahan Plankton di Perairan Darat Surabaya Dan Malang. *Rekayasa, 13*(1), 61–66. https://doi.org/10.21107/rekayasa.v13i1.5918

[11] Amiri, R., & Ahmadi, M. (2019). Treatment of wastewater in sewer by *spirogyra sp.* green algae: Effects of light and Carbon Sources. *Water and Environment Journal*, *34*(3), 311–321. https://doi.org/10.1111/wej.12463

[12] Liyanaarachchi, V. C., Premaratne, M., Viraj Nimarshana, P. H., & Udayangani Ariyadasa, T. (2020). Investigation of the effect of organic and inorganic carbon on biomass production and astaxanthin accumulation of the microalga haematococcus pluvialis using artificial neural network. *2020 IEEE 17th India Council International Conference (INDICON)*. https://doi.org/10.1109/indicon49873.2020.9342373

[13] Lu, Z., Zheng, L., Liu, J., Dai, J., & Song, L. (2019). A novel fed-batch strategy to boost cyst cells production based on the understanding of intracellular carbon and nitrogen metabolism in haematococcus pluvialis. *Bioresource Technology*, *289*, 121744. https://doi.org/10.1016/j.biortech.2019.121744

[14] Kumar, S. S., & Saramma, A. V. (2017). Effect of organic carbon compounds on the growth and pigment composition of Microalga - Nannochloropsis salina. *International Journal of Applied Environmental Sciences*, *12*(9), 1707. https://doi.org/10.37622/ijaes/12.9.2017.1707-1719

[15] Lacroux, J., Trably, E., Bernet, N., Steyer, J.-P., & van Lis, R. (2020). Mixotrophic growth of microalgae on volatile fatty acids is determined by their undissociated form. *Algal Research*, *47*, 101870. https://doi.org/10.1016/j.algal.2020.101870

[16] Hong, J. W., Kim, O. H., Jo, S.-W., Kim, H., Jeong, M. R., Park, K. M., Lee, K. I., & Yoon, H.-S. (2016). Biochemical composition of a Korean Domestic Microalga Chlorella vulgaris knua027. *Microbiology and Biotechnology Letters*, *44*(3), 400–407. https://doi.org/10.4014/mbl.1512.12008

[17] Auyo, M. I., Abdullahi, S., & Ahmed, A. (2021). EXTRACTION AND QUANTIFICATION OF INTRACELLULAR LIPID AS POTENTIAL BIOFUEL FROM ALGAL BIOMASS. *International Journal of Biology*, *2*(1), 8–17. https://doi.org/10.47604/ijb.1222

[18] Vogel, V., & Bergmann, P. (2017). Culture of SPIROGYRA sp. in a flat-panel AIRLIFT PHOTOBIOREACTOR. *3 Biotech*, *8*(1). doi:10.1007/s13205-017-1026-9

[19] Cong, X.-Y., Miao, J.-K., Zhang, H.-Z., Sun, W.-H., Xing, L.-H., Sun, L.-R., Zu, L., Gao, Y., & Leng, K.-L. (2019). Effects of drying methods on the content, structural isomers, and composition of astaxanthin in Antarctic Krill. *ACS Omega*, *4*(19), 17972–17980. https://doi.org/10.1021/acsomega.9b01294

[20] Kurniawati, P., Gusrianti, R., Dwisiwi, B. B., Purbaningtias, T. E., & Wiyantoko, B. (2017). Verification of spectrophotometric method for nitrate analysis in water samples. *AIP Conference Proceedings*. https://doi.org/10.1063/1.5016005

[21] Muloiwa, M., Nyende-Byakika, S., & Dinka, M. (2020). Comparison of unstructured kinetic bacterial growth models. *South African Journal of Chemical Engineering*, *33*, 141–150. https://doi.org/10.1016/j.sajce.2020.07.006

[22] Ruen-ngam, D., Shotipruk, A., & Pavasant, P. (2010). Comparison of Extraction Methods for Recovery of Astaxanthin from Haematococcus pluvialis. Informa UK Limited. https://doi.org/10.1080/01496395.2010.493546

[23] Régnier, P., Bastias, J., Rodriguez-Ruiz, V., Caballero-Casero, N., Caballo, C., Sicilia, D., Fuentes, A., Maire, M., Crepin, M., Letourneur, D., Gueguen, V., Rubio, S., & Pavon-Djavid, G. (2015). Astaxanthin from haematococcus pluvialis prevents oxidative stress on human endothelial cells without toxicity. *Marine Drugs*, *13*(5), 2857–2874. https://doi.org/10.3390/md13052857

[24] Karnila, R., Hasan, B., Ilza, M., Leksono, T., & Ahmad, M. A. (2021). Astaxanthin extraction of Vanname shrimp (litopenaeus vanname) using palm oil. *IOP Conference Series: Earth and Environmental Science*, *695*(1), 012056. https://doi.org/10.1088/1755-1315/695/1/012056

[25] Bellinger, E. G., & Sigee, D. C. (Eds.). (2015). *Freshwater Algae.* doi:10.1002/9781118917152

[26] Japar, A. S., Takriff, M. S., & Mohd Yasin, N. H. (2021). Microalgae acclimatization in industrial wastewater and its effect on growth and primary metabolite composition. *Algal Research*, *53*, 102163. https://doi.org/10.1016/j.algal.2020.102163

[27] Avidan, O., & Pick, U. (2015). Acetyl-COA Synthetase is activated as part of the PDH-bypass in the oleaginous green alga*chlorella desiccata*. *Journal of Experimental Botany*, *66*(22), 7287–7298. https://doi.org/10.1093/jxb/erv424

[28] Dittamart, D., Pumas, C., Pekkoh, J., & Peerapornpisal, Y. (2014). Effects of organic carbon source and light-dark period on growth and lipid accumulation of Scenedesmus sp. AARL G022. *Maejo Int. J. Sci. Technol., 8*(2). http://dx.doi.org/10.14456/mijst.2014.28

[29] Lu, Z., Zheng, L., Liu, J., Dai, J., & Song, L. (2019). A novel fed-batch strategy to boost cyst cells production based on the understanding of intracellular carbon and nitrogen metabolism in haematococcus pluvialis. *Bioresource Technology*, *289*, 121744. https://doi.org/10.1016/j.biortech.2019.121744

[30] Munir, N., Imtiaz, A., Sharif, N., & Naz, S. (2015). Optimization of Growth Conditions of Different Algal Strains and Determination of Their Lipid Contents. *The Journal of Animal & Plant Sciences, 25*(2), 546-553

[31] Pratiwi, N. T. M., Hasani, Q., Muhtadi, A., & Kautsari, N. (2020). Pertumbuhan dan Produktivitas *Oedogonium* sp. pada Intensitas Cahaya yang Berbeda. *Berita Biologi, 19*(3A). Doi: 10.14203/beritabiologi.v19i3.3890

[32] Tan, X.-B., Meng, J., Tang, Z., Yang, L.-B., & Zhang, W.-W. (2020). Optimization of algae mixotrophic culture for nutrients recycling and biomass/lipids production in anaerobically digested waste sludge by various organic acids addition. *Chemosphere*, *244*, 125509. https://doi.org/10.1016/j.chemosphere.2019.125509

[33] Wang, C., Lei, A., Zhou, K., Hu, Z., Hao, W., & Yang, J. (2014). Growth and nitrogen uptake characteristics reveal outbreak mechanism of the opportunistic Macroalga Gracilaria tenuistipitata. *PLoS ONE*, *9*(10). https://doi.org/10.1371/journal.pone.0108980

[34] Sanz-Luque, E., Chamizo-Ampudia, A., Llamas, A., Galvan, A., & Fernandez, E. (2015). Understanding nitrate assimilation and its regulation in microalgae. *Frontiers in Plant Science*, *6*. https://doi.org/10.3389/fpls.2015.00899

[35] Liaqat, F., Khazi, M. I., Bahadar, A., He, L., Aslam, A., Liaquat, R., Agathos, S. N., & Li, J. (2022). Mixotrophic cultivation of microalgae for carotenoid production. *Reviews in Aquaculture*. https://doi.org/10.1111/raq.12700

B.9

Biomass and Protein Analysis from *Chlorella* sp. and *Nannochloropsis* sp. on Single and Mixed Culture with Anaerobically Digested Dairy Manure Wastewater (ADDMW) Using Open Raceway Pond

Muhammad Farhan Aidira, Taufik Taufikurahman, Lili Melani
Bandung Institute of Technology, Bandung, Indonesia
Corresponding Author: taufik@itb.ac.id

Abstract

Microalgae has a very good potential in producing protein. In addition, microalgae also have the ability to reduce waste levels in certain pollutants. The provision of synthetic media is often used as a problem in microalgae cultivation. ADDMW waste is considered to have good enough potential to be used as a growth medium for microalgae because the nutrient content is high enough to support microalgae growth. This study aims to determine the effect of microalgae cultivation, both monoculture and mixed using *Chlorella* sp. and Nannochloropsis sp. on the growth rate, biomass produced, protein production, and the efficiency of adsorption of ADDMW waste in an open raceway pond bioreactor. Cultivation was carried out using *Chlorella* sp. and *Nannochloropsis* sp. with an initial volume ratio of 1:1 with a cultivation time of 7 days. The dilution of ADDMW waste used is 25 times. The values of specific growth rate, biomass gain, and the highest biomass productivity were obtained in mixed culture variations, namely 1.47 day-1, 0.95 g.L-1, and 0.28 g.L-1 day-1, respectively. The highest protein content was obtained in the mixed culture variation, which was 56.25% (dry biomass), whereas in the single culture *Chlorella* sp. and *Nannochloropsis* sp. respectively are 40.42% and 40.11%. Mixed culture is superior based on its ability to produce biomass and protein production when compared to the single cultured microalgae.

Keywords: *Chlorella* sp., micoralgae, mixed culture, *Nannochloropsis* sp., protein

1. Introduction

Currently, there is an increase in the human population around the world. The population growth rate in Indonesia in 2021 is 0.98% of the total population in the previous year [1]. Along with the increase in human population, food and nutritional needs must also be optimized. In addition to increasing efficiency and effectiveness in the sector, other solutions also need to be implemented, such as the use of alternative biomass and relying on diet as a food source. Specifically, protein is one of the macronutrients that will experience scarcity in the near future.

Substitute or alternative protein sources and more efficient production techniques are developed and developed to meet global demand.

Microalgae are considered to be one of the ideal and potential organisms to be used as raw materials for bioenergy production [2]. There are various types of microalgae that have the potential to fulfill nutrition for humans, especially protein. *Chlorella* sp. and *Nannochloropsis* sp. is an example of microalgae species with a high protein content of 45-65% of their dry weight [3].

The effectiveness of the production of metabolites in microalgae cannot be separated from the quality and quantity of the growing medium used, such as nitrogen and phosphate content. This study used growth media derived from biogas processing waste or anaerobically digested dairy manure wastewater (ADDMW). ADDMW has high levels of nitrogen and phosphate so it has excellent potential to be used as a growth medium for microalgae [4].

Microalgae are also known as biological agents that are able to degrade waste well. According to Bashi et al. (2019) [5], microalgae are able to absorb harmful pollutants, it also has the potential as a source of biomass for the production of a lot of protein and lipids and can grow in areas that are less than ideal, such as areas with limited light intensity, nutritional limitations, and unfavorable temperature conditions. stable [6].

Microalgae propagation using waste medium is often constrained by the presence of contaminants that can cause microalgae cultures to die. One solution that can be used is to combine two types of microalgae in the same cultivation system or also known as mixed culture [7]. Under the right nutritional and environmental conditions, mixed cultures can grow with high biomass productivity. In addition, mixed cultures are also considered to be able to carry out waste degradation better because they are more stable and resistant to contamination [8].

In general, microalgae cultivation can be carried out using two types of bioreactors, namely photobioreactors and open raceway ponds. ORP is considered more economical than photobioreactor in terms of maintenance and energy requirements [9].

This study aims to compare the effect of microalgae cultivation either in monoculture or in a mixture using the microalgae *Chlorella* sp. and *Nannochloropsis* sp. on the growth rate, protein production, and efficiency of adsorption of ADDMW waste in open raceway ponds.

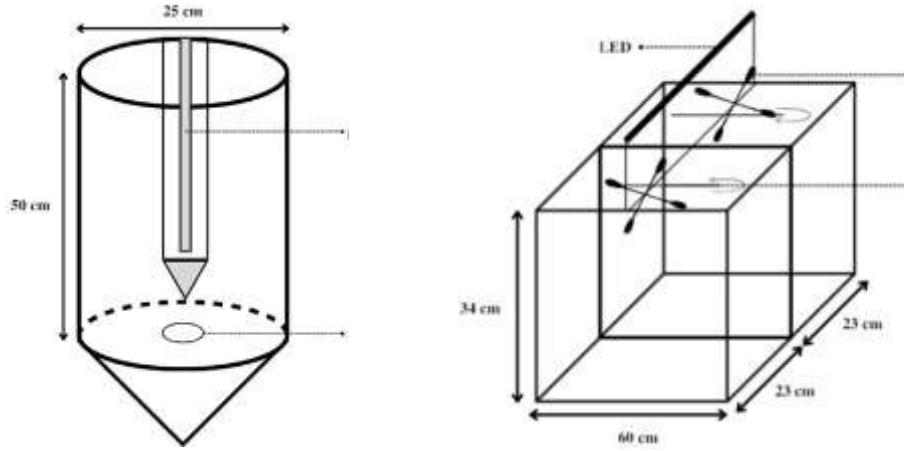
2. Material and Method

2.1 Tools and Materials

The bioreactors used in this research are Vertical Tubular Reactor (VTR) and open raceway pond (ORP) made of acrylic material. The VTR bioreactor with a capacity of 25 L with dimensions of 25 cm × 50 cm (diameter × height) consists of a lamp storage section, a vacuum chamber, a culture compartment, a sparger, and an air intake chamber. The ORP bioreactor with dimensions of 23

cm × 34 cm × 60 cm (width × length × height) has a capacity of up to 38 L and is equipped with an agitator in the form of a paddle wheel with a rotating speed of 38 rpm. Lighting during cultivation uses LED lamps with a power of 8 Watts and air aeration is provided through a pump with a power of 10 Watts with an air aeration rate of 3L/minute.

This research utilizes analytical balance, hemacytometer, binocular microscope, UV-VIS spectrophotometer, centrifuge, and autoclave which are facilities of the School of Biological Sciences and Technology (SITH) Bandung Institute of Technology Jatinangor Campus. Inoculum *Chlorella* sp. and *Nannochloropsis* sp. were obtained from purchases through Sneaky Store. ADDMW waste was obtained from the Haurgombong SITH Educational Garden, Bandung Institute of Technology, Jatinangor Campus. The chemicals used in this study were obtained from the SITH chemical warehouse, Bandung Institute of Technology, and suppliers from Sneaky Stores.



(a) (b)

Figure 1. (a) Vertical Tubular Reactor scheme, (b) Open Raceway Pond scheme

2.2 Microalgae Cultivation

Cultivation of microalgae inoculum was carried out in three stages. The first stage is cultivation on a culture bottle scale using Walne medium in a glass bottle with a working volume of 800 mL. Next, the culture was transferred to a VTR bioreactor with a ratio of inoculum volume: media = 1:7 (v/v), and then the acclimatization process was carried out for 7 days using ADDMW waste which had been sterilized with a ratio of inoculum: media solution = 1:1 (v/v). The sterile ADDMW media used was diluted 25 times. During cultivation, the culture is aerated. The lighting used is white fluorescent for 16 hours/day.

The variations given are single culture and mixed culture. The mixed culture was prepared with the ratio of inoculum volume: medium = 1: 4 with the ratio of the culture volume of *Chlorella* sp.: *Nannochloropsis* sp. = 1:1. The cultivation medium used in this study was ADDMW waste.

2.3 Cell and Biomass Production Calculation

Determination of the number of cells was carried out using a hemacytometer and a microscope. The biomass was obtained by comparing the weight of the biomass obtained with the initial volume of the culture. Biomass productivity was determined by comparing the highest biomass density with the time when the highest biomass density was obtained.

2.4 Protein Content Determination

Protein extraction was carried out by the extraction method according to Rausch, 3 mL of 0.5 N NaOH was added to the falcon tube containing the biomass. The falcon tube containing biomass and 0.5 N NaOH was then incubated in a water bath at 80°C for 10 minutes to allow the extraction process to proceed. The extracted samples were then centrifuged using a centrifuge at a speed of 5000 rpm for 10 minutes. The supernatant was separated in a test tube, then the extraction was repeated with the same procedure once.

Determination of protein content was carried out using the Bradford method. A total of 100 mg of Coomassie Blue G250 was dissolved in 50 mL of 96% ethanol. The solution was then mixed with 100 mL of 85% phosphoric acid and dissolved with distilled water until the volume reached 1 L. The solution was then filtered using Whatman No. filter paper. 1. The resulting filtrate is used as a test reagent for protein content.

3. Result and Discussion

3.1 Cell Growth and Production of Biomass

In this study, there were three types of microalgae cultivation variations, namely single culture of *Chlorella* sp., single culture of *Nannochloropsis* sp., and mixed culture of *Chlorella* sp. and *Nannochloropsis* sp. with a ratio of 1:1 (v/v). The growth of the microalgae cells was analyzed based on the number of cells. The microalgae growth curve for each variation of cultivation treatment can be seen in Figure 2 as follows.

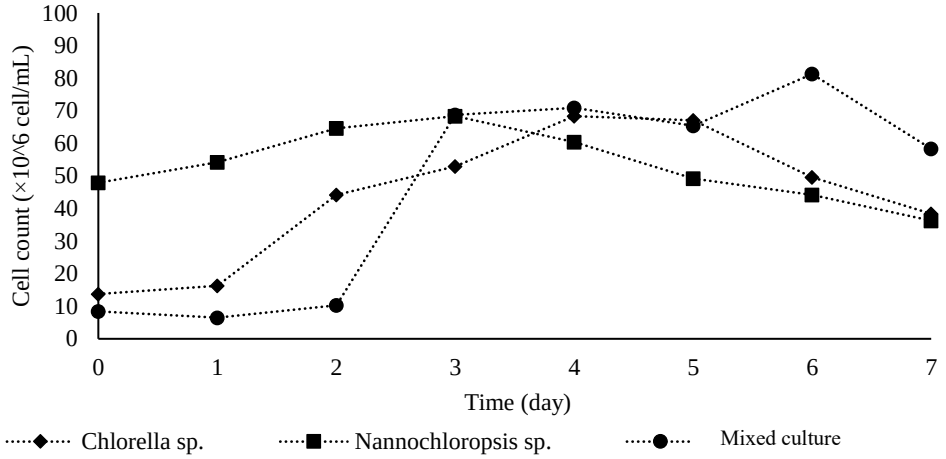


Figure 2. Cell growth curve on each cultivation variation

There are differences in the life phase of each variation of cultivation treatment, either from the lag phase, exponential phase or log phase, past stationary phase, and death phase (Figure 2). In the single culture variation of *Chlorella* sp., the exponential phase started on day 1 and continued to grow exponentially until day 4. This phenomenon is in accordance with the research conducted by Purkan et al. (2019) [10], exponential phase of *Chlorella* sp. starting from day 1 and can last up to day 6 using BG-11 media which has more nitrogen content than ADDMW media.

In the monoculture variation of *Nannochloropsis* sp. the exponential phase can be observed starting on day 1 to day 3. This is in accordance with the research conducted by Metsoviti et al. (2019) [11], who cultivated *Nannochloropsis* sp. in an open raceway pond bioreactor by offering a variety of cultivation temperatures.

Table 1. Microalgae growth parameter

Microalgae Species	Medium	Specific Growth Rate (day^{-1})	Biomass Yield ($g\ L^{-1}$)	Biomass Productivity ($g\ L^{-1}\ day^{-1}$)	Reference
Chlorella sp.	ADDMW	$0,78\pm0,11^{a}$	$0,34\pm0,02^{A}$	$0,14\pm0,05^{\alpha}$	This study
Chlorella sp.	Medium BG-11	0,35	-	0,118	[12]
Nannochloropsis sp.	ADDMW	$0,74\pm0,53^{a}$	$0,53\pm0,32^{A}$	$0,11\pm0,06^{\alpha}$	This study
Nannochloropsis sp.	Medium BBM	-	0,75	0,194	[11]

Chlorella sp. and *Nannochloropsis* sp.	ADDMW	1,47±1,21[b]	0,95±0,15[B]	0,28±0,6[β]	This study
Chlorella sp. and *Nannochloropsis* sp.	Fish farm wastewater	0,309	0,75	0,19	[13]

In mixed culture variations of *Chlorella* sp. and *Nannochloropsis* sp. It can be seen that the exponential phase is on day 2 to day 3. The biomass recovery value of this mixed culture variation tends to be higher than monoculture as can be seen in table 1. This is in line with research conducted by Militao et al. (2019) [14], who showed that microalgae with mixed culture treatment had a biochemical composition with a high degree of stability so that they were able to grow well in open raceway pond bioreactors. According to Rashid et al. (2018) [15], the main reason why mixed culture treatment can increase biomass productivity is due to good exhaust gas capitalization and the reuse of organic carbon in cultivation systems. In addition, mixed microalgae cultures allow for different tolerances of each species used so that they can maintain cultures in undesirable conditions, for example, extreme pH and temperature, contaminants, and invasion of microalgae or foreign bacteria [16].

3.2 Protein Production from Microalgae

In this study, the calculation of the protein content in microalgae was carried out using the Bradford method [17]. This method uses Bradford reagent as an indicator of the presence of protein in the sample. Protein levels in microalgae for each cultivation treatment can be seen in Table 2 as follows.

Table 2. Protein content on third day of cultivation from variety microalgae

Species	Medium	Reactor	Protein content (%)	Reference
Chlorella sp.	ADDMW	ORP	40,42[a]	This study
Chlorella beijerinck	Medium BG-11	Erlenmeyer	43,22	[10]
Nannochloropsis sp.	ADDMW	ORP	40,11[a]	This study
Nannochloropsis sp.	*Enriched sea water*	Photobioreactor	40,00	[18]
Chlorella sp. and *Nannochloropsis* sp.	ADDMW	ORP	56,25[a]	This study
Chlorella sp. and *Nannochloropsis* sp.	Fish farm wastewater	VTR	45,58	[13]

In this study, protein content was calculated on the 3rd day of cultivation. It was seen that the difference in protein content values in each cultivation treatment variation was not significantly different ($p > 0.05$). Based on Table 2, it can be seen that there are differences in the value of protein content in each variation of cultivation treatment. The maximum protein content of *Chlorella* sp. monoculture. *Nannochloropsis* sp., and their mixed cultures were 40.42%, 40.11%, and 56.25% of their dry weight, respectively. In the monoculture variation of *Chlorella* sp., the protein content obtained was lower than the results obtained by Phukan et al. (2011) [19]. This can happen because there are several differences in cultivation conditions between this study and the literature such as the medium, bioreactor, and cultivation temperature. Furthermore, in the monoculture variation of *Nannochloropsis* sp., the levels obtained were relatively the same as the literature research conducted by Zhu et al. (2013) [18].

Protein content in mixed culture cultivation variation has the highest value compared to other monoculture treatment variations. This is in accordance with research conducted by Annisa (2022). In this study, it was explained that in mixed culture conditions, microalgae would tend to have better culture stability than monocultures. In another study, it was also explained that mixed culture cultivation was able to produce higher protein than single culture cultivation [14].

Another factor that can increase protein production in microalgae biomass is increasing the total nitrogen content in cultivation nutrients [20]. This can happen because proteins are composed of strands of amino acids whose main constituent is nitrogen. So that the nitrogen content in the medium directly affects the protein content in microalgae. The higher the available nitrogen content, the higher the protein content of microalgae that can be produced [21]. The protein content in this study had a higher value than the research conducted by Zhu et al. [18]. This is because the nitrogen content of the ADDMW medium is higher than the fishery waste medium so that the ability of microalgae to produce protein compounds will also be better.

The formation of protein is included in the growth-related product which means that the production of its compounds takes place simultaneously with the formation of its biomass [22]. This is evidenced by the high yield value and productivity of mixed culture biomass compared to single culture variations. Likewise with the value of the maximum protein content possessed by the mixed culture compared to the single culture.

4. Conclusion

This study demonstrated mixed culture of *Chlorella* sp. and *Nannochloropsis* sp. can increase the value of the specific growth rate, biomass gain, and biomass productivity compared to the single culture of each species. The highest values for specific growth rate, biomass gain, and biomass productivity were obtained in mixed culture variations, namely 1.47 day-1, 0.95 g L-1, and 0.28

g. L-1 day-1 respectively. The highest protein content was also obtained in the mixed culture, which was 56.25% of the dry weight.

Therefore, the microalgae cultivation strategy using mixed culture techniques is considered capable of maximizing the potential of microalgae in the process of growth and production of its metabolites.

Acknowledgments

We would like to thank PPMI ITB in 2021 for funding this research.

References

[1] United Nations, Department of Economic and Social Affairs, Population Division. *World Population Prospects 2022, Online Edition Nannochloropsis Rev. 1;* 2022.

[2] Barsanti, L., & Gualtieri, P. Algal Culturing. *Algae: anatomy, biochemistry, and biotechnology*, 1-15.; 2006.

[3] Chen, P., Min, M., Chen, Y., Wang, L., Li, Y., Chen, Q., ... & Ruan, R. Review of biological and engineering aspects of algae to fuels approach. *International Journal of Agricultural and Biological Engineering*, 2(4); 2010. 1-30.

[4] Taufikurahman, T., & Istiqomah, I. N. Phycoremediation of anaerobic digested dairy manure wastewater using *Chlorella* vulgaris. In *IOP Conference Series: Earth and Environmental Science* (Vol. 299, No. 1). IOP Publishing; 2019. 012013 p.

[5] Bashi, Z., McCullough, R., Ong, L., & Ramirez, M. Alternative proteins: The race for market share is on. *McKinsey & Company: Denver, CO, USA*; 2019. 1-11.

[6] Padmaperuma, G., Kapoore, R. V., Gilmour, D. J., & Vaidyanathan, S. Microbial consortia: a critical look at microalgae co-cultures for enhanced biomanufacturing. *Critical reviews in biotechnology*, *38*(5), 690-703; 2018.

[7] Guccione, A., Biondi, N., Sampietro, G., Rodolfi, L., Bassi, N., & Tredici, M. R. *Chlorella* for protein and biofuels: from strain selection to outdoor cultivation in a Green Wall Panel photobioreactor. *Biotechnology for biofuels*, 7(1); 2014. 1-12.

[8] Azizi, Soroush, et al. Nitrate and phosphate removal from treated wastewater by Chlorella vulgaris under various light regimes within membrane flat plate photo bioreactor. *Environmental Progress & Sustainable Energy*; 2021. 40.2: e13519.

[9] Jorquera, Orlando, et al. Comparative energy life-cycle analyses of microalgal biomass production in open ponds and photobioreactors. *Bioresource technology*; 2010, 101.4: 1406-1413.

[10] Purkan, Purkan, et al. Biodiesel production by lipids from Indonesian strain of microalgae Chlorella vulgaris. *Open Chemistry*; 2019, 17.1: 919-926.

[11] Metsoviti, Maria N., et al. Comparison of growth rate and nutrient content of five microalgae species cultivated in greenhouses. *Plants*; 2019, 8.8: 279.

[12] Sofiyah, E. S., Septiariva, I. Y., & Suryawan, I. W. K. Domestic wastewater treatment using various microalgae for lipid production. *Annals of the Romanian Society for Cell Biology*; 2021. 15156-15163.

[13] Annisa, Isna, Taufikurahman, & Meliani, L. Produksi Protein dan Biomassa dari Kultivasi *Chlorella vulgaris* dan *Nannochloropsis* sp dalam Medium Limbah Cair Budidaya Ikan dengan Variasi Perlakuan *Mixed culture;* 2022.

[14] Militão, F. P., Fernandes, V. D. O., Bastos, K. V., Martins, A. P., Colepicolo, P., & Machado, L. P. Nutritional value changes in response to temperature, microalgae mono and mixed cultures. *Acta Limnologica Brasiliensia*, *31*; 2019.

[15] Rashid, Naim, et al. Co-cultivation of two freshwater microalgae species to improve biomass productivity and biodiesel production. *Energy Conversion and Management*; 2019, 196: 640-648.

[16] Tate, John J., et al. The effects of plant growth substances and mixed cultures on growth and metabolite production of green algae Chlorella sp.: a review. *Journal of plant growth regulation*; 2013, 32.2: 417-428.

[17] Kruger, N. J. The Bradford method for protein quantitation. *The protein protocols handbook*; 2009.17-24.

[18] Zhu, Y., & Dunford, N. T. (2013). Growth and biomass characteristics of Picochlorum oklahomensis and Nannochloropsis oculata. Journal of the American Oil Chemists' Society, 90, 841-849.

[19] Phukan, Mayur M., et al. Microalgae Chlorella as a potential bio-energy feedstock. *Applied Energy*; 2011, 88.10: 3307-3312.

[20] Makareviciene, V., Andrulevičiūtė, V., Skorupskaitė, V., & Kasperovičienė, J. Cultivation of microalgae Chlorella sp. and Scenedesmus sp. as a potentional biofuel

feedstock. *Environmental Research, Engineering and Management*, *57*(3); 2011. 21-27.

[21] Hulatt, C. J., Wijffels, R. H., Bolla, S., & Kiron, V. Production of fatty acids and protein by Nannochloropsis in flat-plate photobioreactors. PloS one, 12(1); 2017. e0170440.

[22] de Carvalho, J. C., Sydney, E. B., Tessari, L. F. A., & Soccol, C. R. Culture media for mass production of microalgae. In *Biofuels from algae*. Elsevier; 2019. 35 p.

B.10

Protein and Biomass Analysis from Monoculture and Mixed Culture of *Spirulina sp.* and *Chlorella sp.* Using *Anaerobically Digested Dairy Manure Wastewater* (ADDMW) in *Open Raceway Pond*

Rizky Pradyantama[1], Muhammad Farhan Aidira[1], Rachma Anisa Maulani[1], Taufik Taufikurahman[1*], Lili Melani[1]
School of Life Sciences and Technology, Bandung Institute of Technology, Ganesha Street No. 10 Bandung 40132 Indonesia
E-mail address: taufik@.itb.ac.id

Abstract

Microalgae have the potential as an alternative protein source apart from animals and plants. However, one of the problems faced in microalgae cultivation on an industrial scale is the cost of providing synthetic media as a growth medium. ADDMW can be used as an alternative as a medium for growing microalgae because the nutrient content is high enough to support the growth of microalgae. This study aimed to determine the effect of single culture of *Spirulina sp.*, single culture of *Chlorella sp.*, and mixed culture of *Spirulina sp.* and *Chlorella sp.* on the recovery of biomass, protein, and remediation ability of ADDMW. In this study, ADDMW was diluted 25 times. The ratio of culture volume to waste used is 1:4 (v/v). In mixed culture the ratio of *Spirulina sp.* : *Chlorella sp.* is 1:1 (v/v) with a cultivation time of 7 days. The mixed culture variants produced the maximum yield of biomass and specific growth rate with 0.87 g L^{-1} (dry weight) and 1.46 day^{-1} respectively. When *Spirulina sp.* and *Chlorella sp.* were grown separately, their protein contents were only 40% and 44%, respectively, while the maximum protein level was attained in mixed culture at 66.75% (dry weight).

Keywords: *Spirulina sp.*, *Chlorella sp.*, ADDMW, mixed culture, protein

1. Introduction

One of the problems for developing countries such as Indonesia, is the fulfillment of energy, nutrition, and food needs for its people. This is very common and important problem to be resolved so that it becomes one of the important points in the sustainable development goals (SDGs) formulated by the United Nations (UN). One of the goals in the SDGs is food security. In Indonesia, food security is still a problem because there are still many Indonesian people

whose nutritional needs have not been fulfilled. Protein is one of the nutrients needed by humans. Based on data obtained from Marketwatch [1], more people consume protein substitutes to maintain healthier diets and reduce their environmental effects. The amount of world protein needs is expected to continue to increase from year to year.

Microalgae is a microscopic-algae and can be found in freshwater and seawater. Microalgae can also be grown in wastewater and act as a remediator. Microalgae culture on a large scale with the aim of commercialization has been carried out about 30 years ago and continues to grow until now [3]. Microalgae culture is carried out to produce biomass and various primary and secondary metabolites that can be used in various fields. One of them is protein that can be used as food supplement from *Spirulina platensis*, lipids that can be used as biofuel from *Botyrococcus braunii*, and β-carotene from *Dunaliella salina* which can be used as a dietary supplement as well [2]. *Spirulina platensis* and *Chlorella vulgaris* have high protein content, namely 68% and 51% respectively [4-5]. The composition of the media used in the cultivation process can affect protein and lipid yields. Media with high nitrogen content will increase protein production, while low nitrogen content will increase lipid production from microalgae biomass [6-7].

Protein production with microalgae has several advantages when compared to animal protein production. In the production process, microalgae cultivation requires a much smaller area than animal husbandry. Microalgae only need less than 2.5 m^2 of land to produce 1 kg of protein. The land area is much smaller when compared to protein production from chickens and cows which require 42-52 m^2 and 144-258 m^2 of land [8, 9]. In addition to the fact that microalgae can be grown in a narrower area, the protein content of microalgae is 670% higher than the protein content of tofu, 180% higher than the protein content of milk, 5.100% higher than spinach's iron content, and 3.100% higher than carrots' beta-carotene content. [10].

Lab-scale microalgae cultivation is mostly done using synthetic media such as Bold Basal Medium (BBM), Zarrouk, and Walne [11-13]. On a small scale, microalgae cultivation is usually carried out in erlenmeyer flasks or culture bottles, while on a large scale it can be carried out in open raceway ponds [12, 14]. Microalgae cultivation is mostly done for the production of microalgae biomass, primary metabolites (proteins or lipids), or secondary metabolites (such as phycocyanin or astaxanthin) [14-16].

By using synthetic media, large-scale microalgae cultivation will be less economical because of the high cost of purchasing synthetic media. Therefore, other cheaper media alternatives are needed for microalgae cultivation. Due to its high organic content, organic waste is one of the greatest solutions for growing microalgae. One of the wastes that is often used is livestock waste. One of the livestock wastes that can be used as a medium for microalgae cultivation is anaerobically digested dairy manure wastewater (ADDMW) which is waste from the biogas processing process from livestock manure [17]. ADDMW contains high concentrations of nitrate and phosphate compounds [18]. The high nitrate and phosphate content in ADDMW will be dangerous if it is directly discharged into the environment [17]. One method to remediate waste

is the phycoremediation method or the waste remediation method using microalgae [11]. Besides being able to act as phycoremediation agents, these microalgae can utilize the inorganic components of the ADDMW and convert them into biomass and metabolites that have high selling value, one of which is protein.

In the microalgae cultivation process, the cultivation process is often constrained by contamination. One solution to this problem is mixed culture cultivation [19]. In this study, we reviewed mixed cultures of microalgae *Spirulina sp.* and *Chlorella sp.* cultured in ADDMW to be used for the production of dietary supplements. On the other hand, microalgae cultivation in a vertical tubular reactor (VTR) or in a closed system has some limitations on a large scale. This is because it requires higher construction and operating costs than microalgae cultivation in an open raceway pond (ORP) [20]. Biomass production using an open system (ORP) has a more economical value than microalgae cultivation in a closed system (VTR) [21].

The aim of this study was to compare the effect of single and mixed cultures between *Spirulina sp.* and *Chlorella sp.* on protein production using ADDMW media at ORP. Through this research, we can observe the potential of mixed culture in microalgae culture for the development of the production of various bioproducts from microalgae cultivated on ORP. As a result, a production system with a higher protein productivity and lower cost can be created to produce bioproducts at a specific scale.

2. Materials and Methods

2.1 Material

Open raceway ponds (ORP) and vertical tubular reactors (VTR) with acrylic bases were the bioreactors used in this study. Both single and mixed *Spirulina sp.* and *Chlorella sp.* cultures were used. Figures 1 and 2 show the design of the bioreactor. The open raceway pond (ORP) and vertical tubular reactor (VTR) used have a total volume of 47 L and 25 L, respectively. LED-based lighting is used, with a power demand of 8 Watts and a light intensity of 25 mol m^{-2} s^{-1}. Dimensions of the employed VTR are 25 cm x 50 cm (diameter x height). The ORP in use is 23 cm by 60 cm by 33.9 cm in size (width, length, height). A spurger at the bottom of the VTR is used to aerate air in the culture at a rate of 3 L/min, which controlled by a flowmeter. The paddle wheel in the middle of the ORP rotates at 38 rpm to circulate the microalgae culture from the bottom to the surface of the medium.

The study utilized equipment from the School of Biological Sciences and Technology (SITH) at the Bandung Institute of Technology Jatinangor Campus, including an analytical scale, oven, UV-VIS spectrophotometer, centrifuge, autoclave, vortex, water bath, hemacytometer, and digital microscope. Inoculums of *Spirulina sp.* and *Chlorella sp.* were obtained from suppliers from Klaten, Central Java. ADDMW was obtained from the SITH ITB Haurngombong

Educational Garden, Jatinangor. All chemicals used in this study were obtained from the SITH Chemical Warehouse, Bandung Institute of Technology and purchased through suppliers.

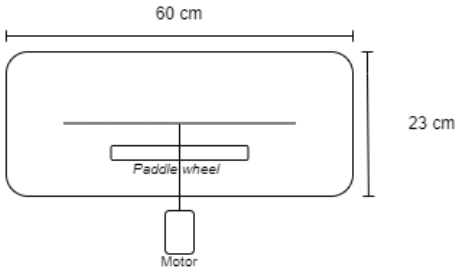


Figure 1. Open raceway pond scheme (ORP)

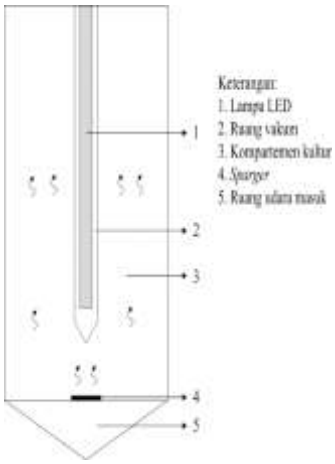


Figure 2. Vertical tubular reactor scheme (VTR)

2.2 Methods

2.2.1 Culture Medium Preparation

The cultivation medium used in this study was Walne synthetic media which was used as much as 1 mL for each addition of 1 liter of cultivation water [22]. The ADDMW media was first filtered 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. The sterile ADDMW is ready to be used as a cultivation medium.

2.2.2 Microalgae Inoculum Preparation

Cultivation of each microalgae culture was carried out in stages. First, microalgae cultivated in 1000 mL culture bottle with 800 mL working volume in walne synthetic media. After that, microalgae cultivated in 20.000 mL VTR with 7.600 mL working volume in walne synthetic media. Cultivation was carried out with a ratio of inoculum volume:media solution = 1:7. Cultivation in bottles and VTR was carried out for 10 days, then acclimatization was carried out for both cultures with ADDMW growth media with an inoculum: media solution ratio of 1:1. The microalgae acclimatization process was carried out for 7 days. Cultivation was carried out under conditions of room temperature (23-28^{o}C) and photoperiodism of 16:8.

Figure 3. Microalgae cultivation in culture bottle

Figure 4. Microalgae cultivation in VTR

2.2.3 Microalgae Cultivation in ORP 38 L and Mixed Culture Variations

Microalgae cultivation was carried out in an open raceway pond (ORP) made of acrylic with a total working volume of 38 L. The variations given were single culture and mixed culture. Mixed cultures were made with the ratio of inoculum volume:media = 1:4 with the ratio of culture

volume of *Spirulina sp.*:*Chlorella sp.* = 1:1 (3,8 L:3,8 L). The cultivation medium used in this study was ADDMW. Microalgae cultivation was carried out for 8 days. All cultivation was carried out at room temperature (~25°C). This research was conducted at ITB Jatinangor Campus, Sumedang.

Figure 5. Microalgae cultivation in ORP

2.2.4 Sampling Procedure

Sampling of each treatment was carried out by isolating the culture in 600 mL plastic bottles. Sampling was carried out once a day.

2.2.5 Cell Number Quantification

Determination of the number of cells was carried out using a hemacytometer. The sample is applied on the hemacytometer using a dropper to fill the center of the hemacytometer. The hemocytometer was then covered with a cover glass and then observed using a microscope. The number of cells of each microalgae species contained in the four main grids of the hemacytometer was calculated, then the average of the four values of the number of cells was determined. The number of cells in the sample is determined by the following equation.

$$N = \bar{n} \; x \; 10^4 \; x \; d \ldots\ldots\ldots\ldots\ldots\ldots \quad (1)$$

Where N is the number of cell density, n is the average number of cells in each grid, d is the dilution factor of the solution. The calculation of the number of cells was carried out in triples. Cell growth curves are created based on data on the number of cells at each time point. The specific growth rate is then determined based on the gradient of the cell growth curve.

2.2.6 Biomass Yield and Productivity

A total of 10 mL of culture in a falcon tube was centrifuged using a centrifuge with a rotation speed of 5.000 rpm for 10 minutes. The supernatant was discarded and then the wet weight of

the microalgae biomass was determined by calculating the difference between the weight of the falcon tube containing the centrifuged biomass and the weight of the empty falcon tube. The yield of biomass was determined by comparing the weight of the obtained biomass with the initial volume of the culture. Biomass productivity was determined by comparing the highest biomass density with the time when the highest biomass density was obtained.

2.2.7 Protein Extraction

Protein extraction was carried out by the extraction method according to Rausch [23]. A total of 3 mL of 0,5 N NaOH was added to the falcon tube containing the biomass. Falcon tube containing biomass and 0,5 N NaOH then incubated in a water bath at 80°C for 10 minutes so that the extraction process can run. The extracted samples were then centrifuged using a centrifuge at a speed of 5.000 rpm for 10 minutes. The supernatant was separated in a test tube, then the extraction was repeated with the same procedure once.

2.2.8 Protein Content Assay

Determination of protein content was carried out according to the Bradford method according to Kruger [24]. A total of 100 mg of Coomassie Blue G250 was dissolved in 50 mL of 96% ethanol. The solution was then mixed with 100 mL of 85% phosphoric acid and dissolved with distilled water until the volume reached 1 L. The solution was then filtered using Whatman No. filter paper. 1. The resulting filtrate is used as a test reagent for protein content.

The protein content of the extracted sample was determined by mixing 1 mL of the extracted supernatant with 5 mL of Bradford's reagent [23]. The mixture was then determined for absorbance at a wavelength of 595 nm using a UV-VIS spectrophotometer. The absorbance value obtained was converted into protein concentration using a standard curve equation that had been made using bovine serum albumin (BSA) solution in a concentration range of 0-50 ppm. The protein weight obtained was determined by multiplying the protein concentration by the volume of the supernatant. The protein content of dry biomass was determined by comparing the protein mass to the dry mass of microalgae biomass in percent (%).

2.2.9 Analysis and Data Interpretation

All parameters measured in this study were analyzed using one-way analysis of variance (one-way ANOVA) followed by Duncan's multiple range test to determine whether there were significant differences between the parameter values in each treatment. Parameters are considered to have a significant difference if the p value < 0.05.

3. Result and Discussion

3.1 Cell Number and Biomass

In *Spirulina sp.* single culture, the microalgae entered the exponential phase between day 2 to day 7, but in the single culture of *Chlorella sp.*, the microalgae entered the exponential phase in day 1 and the death phase day 6. However in mixed culture, the culture reached the exponential phase at day 0 and the death phase day 6 (See Figure 6).

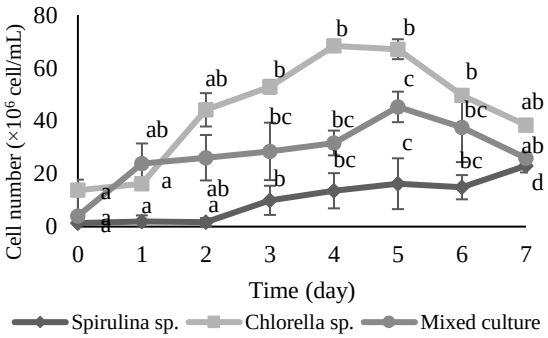


Figure 6. Growth curve in various culture

Note: Differences in letter notation (a, b, c, d, and e) indicate a significant difference ($p < 0.05$) in each variation of cultivation treatment

From the growth curve it can be observed that a single culture of *Spirulina sp.* has a longer log phase than a single culture of *Chlorella sp.*. This is in accordance with a study conducted by Cheunbarn & Peerapornpisal [24] who cultivated *Spirulina sp.* In the Anaerobic Swine Wastewater medium, the log phase was observed for 3 to 4 days and the exponential phase could last up to 8 or 10 days of treatment. In the mixed culture growth curve, it was found that the maximum number of cells at the end of the exponential phase was slightly lower than that of the single culture of *Chlorella sp.*. Ammonium is a very important nutrient for the growth of microalgae. Ammonium levels that are too low can inhibit the growth of microalgae, while ammonium levels that are too high will be toxic to microalgae [25]. The lag phase in mixed culture variations was much faster when compared to single cultures of Spirulina sp. and *Chlorella sp.* ie takes place at t = 0 days. With this phenomenon, it can be concluded that the lag phase can be shortened by performing a mixed culture of microalgae [23] (Figure 5).

In mixed culture variations, *Chlorella sp.* dominate the culture (Figure 7). At the beginning of cultivation period, *Spirulina sp.* composed about 94% of the total culture, while on the next day the culture of *Chlorella sp.* dominate the culture up to about 80% of the total culture. This is due to the complex cell structure and thick cell wall, causing *Chlorella sp.* more resistant to parasites [26]. In addition, the cell size of *Chlorella sp.* which is smaller than *Spirulina sp.* caused the ratio of surface area to cell volume of *Chlorella sp.* larger than *Spirulina sp.* [27]. *Chlorella sp.* has higher

nutrition absorption as a result. However, on the sixth day, the dominance of *Chlorella sp.* reduced because of the life phase of *Chlorella sp.* has entered the death phase [28].

The growth performance varies depending on the culture variation (Table 1). The dry weight of the microalgae was used in determining the yield and biomass productivity, while the value of the specific growth rate was calculated from the growth curve of the microalgae cells. A single culture of *Spirulina sp.* and *Chlorella sp.* yielded specific growth rates of 0.71 and 0.78 days^{-1}, respectively. The growth rate value is higher than the reference, which had specific growth rates of 0.23 day^{-1} for single culture of *Spirulina sp.* and 0.35 day^{-1} for single culture of *Chlorella sp.* [29, 30]. The amount of nitrogen and total phosphate compounds present in each medium can have an impact on the specific growth rate variation [40].

In this study, it was discovered that mixed culture treatments exceeded single culture treatments in terms of specific growth rate, yield, and biomass productivity. These results are in line with Cai & Duan's research, which found that mixed culture treatment generally results in superior performance and growth patterns than monoculture cultivation, which increases the microalgae's biomass production. According to Brito et al. [32], the treatment of mixed cultures of microalgae is not just a combination of the two monocultures, but rather, the differences in growth characteristics, biochemical composition, nutritional composition, and other growth factors of mixed cultures that cause the productivity of the culture to increase. The yield value of the mixed culture variation biomass was higher than the single culture variation of *Spirulina sp.* and *Chlorella sp.* This finding is consistent with earlier research by Das et al. [33] who discovered that mixed culture treatment could improve the production of microalgae biomass and lipids.

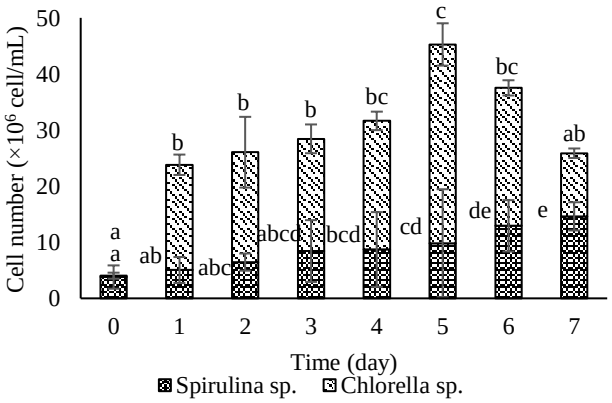


Figure 7. Comparison of cell numbers in mixed culture variations

Note: Differences in letter notation (a, b, c, d, and e) indicate a significant difference ($p < 0.05$) in each variation of cultivation treatmen

Table 1. Growth parameter in various culture

Culture	Medium	Reactor	Specific growth rate (day^{-1})	Biomass Yield ($g\ L^{-1}$)	Biomass Productivity ($g\ L^{-1}\ day^{-1}$)	Reference
Spirulina sp.	ADDMW	Open raceway pond	$0,71^{a}$	$0,67^{\alpha\beta}$	$0,35^{A}$	This research
	ADDMW + $NaHCO_3$ +NaCl	Culture bottle	0,23	-	0,10	[29]
Chlorella sp.	ADDMW	Open raceway pond	$0,78^{a}$	$0,34^{\alpha}$	$0,13^{A}$	This research
	Food waste compost	Erlenmeyer flask	0,35	0,63	0,12	[30]
Spirulina sp. and *Chlorella sp.*	ADDMW	Open raceway pond	$1,46^{b}$	$0,87^{\beta}$	$0,42^{A}$	This research
	Fishery liquid waste	Vertical tubular reactor	0,51	1,93	0,32	[34]

Note: Differences in letter notation (a, b, c, d, and e) indicate a significant difference ($p < 0.05$) in each variation of cultivation treatment.

3.2. Protein Production

Proteins produced by single culture of *Spirulina sp.*, single culture of *Chlorella sp.*, and mixed culture with a ratio of 1:1 (v/v) were 44.11%, 40.42% and 66.75%, respectively. Protein content has a value that varies in each cultivation variation with the highest value obtained in mixed cultures, which is 66.75%. These results were different from the single culture of *Spirulina sp.* and *Chlorella sp.* which has a protein content of 44.11% and 40.42%. However, the protein content for all variations did not differ significantly ($p \geq 0.05$). The results of obtaining protein levels for all cultivation variations can be seen in Table 2.

Protein is one of the growth link related compounds or related to the growth of microalgae. Protein will be formed along with the production of microalgae biomass. The higher the biomass produced, the amount of protein produced will also increase. In general, biomass has a high

protein content when the culture enters the exponential growth phase [35]. This is in accordance with the growth curve data which shows that on the third day of cultivation all cultures were in the exponential growth phase.The values of the specific growth rate, yield, and biomass productivity were correlated with the protein content. It is showed that the mixed culture variation had the highest protein content with higher specific growth rate, biomass yield, and biomass productivity when compared to single cultures of *Spirulina sp.* and *Chlorella sp.*

The protein content obtained in a single culture of *Spirulina sp.* and *Chlorella sp.* higher than the results of research conducted by Olguin et al. [36] and Zhao et al. [37]. This could be due to differences in the cultivation conditions used, such as the composition of the medium, the temperature of cultivation, and the photoperiodism of the culture. The mixed culture in this study also had a higher protein content than the previous study using fishery wastewater [34]. It is because ADDMW has better nutrient availability than fisheries wastewater. Greater protein production may result from better nutrient availability [40].

Protein is one of the primary metabolites produced by microalgae [38]. Therefore, an increase in protein production will occur when the microalgae culture enters the exponential phase. Proteins were extracted from the dry biomass of microalgae by the Rausch method [39]. In this study, the extraction process was carried out on the third day of cultivation. The culture is considered to be in its exponential phase based on the growth curve.

Table 2. Protein content in various culture

Culture	Medium	Reactor	Protein content (% dw)	Reference
Spirulina sp.	ADDMW	*Open raceway pond*	44,11[a]	This research
	Pig wastewater	*Open raceway pond*	43,05	[36]
Chlorella sp.	ADDMW	*Open raceway pond*	40,42 [a]	This research
	Wastewater	*Suspended polyethylene bag system*	38,96	[37]
Spirulina sp. and *Chlorella sp.*	ADDMW	*Open raceway pond*	66,75 [a]	This research

Fishery liquid waste	*Vertical tubular reactor*	9,39	[34]

Note: Differences in letter notation (a, b, c, d, and e) indicate a significant difference ($p < 0.05$) in each variation of cultivation treatment

Acknowledgments

We would like to thank PPMI ITB 2021 for funding this research.

References

[1] Murphy M. Beyond Meat soars 163% in biggest-popping US IPO since 2000. 2019.

[2] Chaiklahan R, Chirasuwan N, Loha V, Bunnag B. Lipid and fatty acids extraction from the cyanobacterium Spirulina. Science Asia. 2008;24:299-305. DOI: 10.2306/scienceasia1513-1874.2008.34.299

[3] Liang S, Liu X, Chen F, Chen Z. Current microalgal health food R & D activities in China. In Asian pacific phycology in the 21st century: Prospects and challenges. Dordrecht: Springer; 2004. 45-48 p. DOI: 10.1007/978-94-007-0944-7_7

[4] Aysun Y, Oznur S, Ceren D-D, Fatih B, Beraat O. Optimisation of ultrasound-assisted extraction of protein from Spirulina platensis using RSM. Czech Journal of Food Sciences. 2018;36:98–108. DOI: 10.17221/64/2017-CJFS

[5] Mohamed A G, Abo-El-Khair B E, Shalaby S M. Quality of novel healthy processed cheese analogue enhanced with marine microalgae Chlorella vulgaris biomass. World Applied Sciences Journal. 2013;23:914-925. DOI: 10.5829/idosi.wasj.2013.23.07.13122

[6] Dolganyuk V, Belova D, Babich O, Prosekov A, Ivanova S, Katserov D, ... Sukhikh S. 2020). Microalgae: A promising source of valuable bioproducts. Biomolecules. 2020;10:1153. DOI: 10.3390/biom10081153

[7] Rana M S, Prajapati S K. Stimulating effects of glycerol on the growth, phycoremediation and biofuel potential of Chlorella pyrenoidosa cultivated in wastewater. Environmental Technology & Innovation. 2021;24:102082. DOI: 10.1016/j.eti.2021.102082

[8] Van Krimpen M M, Bikker P, Van der Meer I M, Van der Peet-Schwering C M C, Vereijken J M. Cultivation, processing and nutritional aspects for pigs and poultry of European protein sources as alternatives for imported soybean products. Wageningen UR Livestock Research. 2013. 662.

[9] De Vries M, de Boer I J. Comparing environmental impacts for livestock products: A review of life cycle assessments. Livestock science. 2010;128:1-11. DOI: 10.1016/j.livsci.2009.11.007

[10] Capelli B, Cysewski G R. Potential health benefits of spirulina microalgae. Nutrafoods. 2010;9:19-26. DOI: 10.1007/BF03223332

[11] Taufikurahman T, Suyadi T A, Sa Susanti H. The Potency Of Microalgae (Chlorella Vulgaris) In Reducing The Level Of Ammonium And Orthophosphate In Bioslurry Media. Kocenin Serial Konferensi (E) ISSN: 2746-7112. 2020;1:3-13.

[12] Ilhamsyah D P A, Taufikurahman T. Biomass and Phycocyanin Production from Spirulina platensis cultivated in Anaerobically Digested Dairy Manure Waste (ADDMW) with Sodium Bicarbonate Addition. Joint Symposium Plant Sciences And Product. 2019.

[13] Arsad S, Kholifah S N, Prabawati E, Sari L A, Kadim M K, Kilawati Y. Effect of mercury on growth of several microalgae. Asian Journal of Water, Environment and Pollution. 2020;17:13-17. DOI: 10.3233/AJW200029

[14] Banu J R, Kannah R Y, Kavitha S, Ashikvivek A, Bhosale R R, Kumar G. Cost effective biomethanation via surfactant coupled ultrasonic liquefaction of mixed microalgal biomass harvested from open raceway pond. Bioresource technology. 2020;304:123021. DOI: 10.1016/j.biortech.2020.123021

[15] Janssen M, Wijffels R H, Barbosa M J. Microalgae based production of single-cell protein. Current Opinion in Biotechnology. 2022;75:102705. DOI: 10.1016/j.copbio.2022.102705

[16] Lu Q, Li H, Zou Y, Liu H, Yang L. Astaxanthin as a microalgal metabolite for aquaculture: A review on the synthetic mechanisms, production techniques, and practical application. Algal Research. 2021;54:102178. DOI: 10.1016/j.algal.2020.102178

[17] Taufikurahman T, Istiqomah I N. (2019, July). Phycoremediation of anaerobic digested dairy manure wastewater using Chlorella vulgaris. IOP Conference Series: Earth and Environmental Science; July, 2019; Indonesia: IOP Publishing; 2019. p. 012013

[18] Möller K, Müller T. Effects of anaerobic digestion on digestate nutrient availability and crop growth: A review. Engineering in Life Sciences. 2012;12:242-257. DOI: 10.1002/elsc.201100085

[19] Tate J J, Gutierrez-Wing M T, Rusch K A, Benton M G. The effects of plant growth substances and mixed cultures on growth and metabolite production of green algae Chlorella sp.: a review. Journal of plant growth regulation. 2013;32:417-428. DOI: 10.1007/s00344-012-9302-8

[20] De Vree J H, Bosma R, Janssen M, Barbosa M J, Wijffels R H. Comparison of four outdoor pilot-scale photobioreactors. Biotechnology for biofuels. 2015;8:1-12. DOI: 10.1186/s13068-015-0400-2

[21] Schipper K, Al-Jabri H M S, Wijffels R H, Barbosa M J. Techno-economics of algae production in the Arabian Peninsula. Bioresource Technology. 2021;331:125043. DOI: 10.1016/j.biortech.2021.125043

[22] Bashi Z, McCullough R, Ong L, Ramirez M. (2019). Alternative proteins: The race for market share is on. Denver: McKinsey & Company; 2019. 1-11 p.

[23] Rashid N, Ryu A J, Jeong K J, Lee B, Chang Y K. Co-cultivation of two freshwater microalgae species to improve biomass productivity and biodiesel production. Energy Conversion and Management. 2019;196:640-648. DOI: 10.1016/j.enconman.2019.05.106

[24] Cheunbarn S, Peerapornpisal Y. Cultivation of Spirulina platensis using anaerobically swine wastewater treatment effluent. Int. J. Agric. Biol. 2010;12:586-590.

[25] Van Vooren G, Le Grand F, Legrand J, Cuiné S, Peltier G, Pruvost J. Investigation of fatty acids accumulation in Nannochloropsis oculata for biodiesel application. Bioresource Technology. 2012;124:421-432. DOI: 10.1016/j.biortech.2012.08.009

[26] Darwish R, Gedi M A, Akepach P, Assaye H, Zaky A S, Gray D A. Chlamydomonas reinhardtii is a potential food supplement with the capacity to outperform Chlorella and Spirulina. Applied Sciences. 2020;10:6736. DOI: 10.3390/app10196736

[27] Fogg G E, Thake B. Algal cultures and phytoplankton ecology. Univ of Wisconsin Press. 1987.

[28] Borowitzka M A. Biology of microalgae. Amsterdam: Academic Press; 2018. DOI: 10.1016/B978-0-12-811405-6.00003-7

[29] Taufikurahman T, Ilhamsyah D P A, Rosanti S, Ardiansyah M A. (2020). Preliminary Design of Phycocyanin Production from Spirulina platensis Using Anaerobically Digested Dairy Manure Wastewater. In IOP Conference Series: Earth and Environmental Science (Vol. 520, No. 1); 21-23 August 2020; Changchun, China: IOP Publishing; 2020. p. 012007

[30] Chew K W, Chia S R, Show P L, Ling T C, Arya S S, Chang J S. Food waste compost as an organic nutrient source for the cultivation of Chlorella vulgaris. Bioresource technology. 2018;267:356-362.

[31] Cai Z, Duan S. Growth in polyculture of algae, Dunaliella salina and Platymonas subcordifomis. Fisheries Science. 2008;27:330-333.

[32] Brito D, Castro A, Guevara M, Gómez E, Villarroel A, Aron N. Biomass and pigments production of the mixed culture of microalgae (Hyaloraphidium contortum and Chlorella vulgaris) by cultivation in media based on commercial fertilizer. AUDJG-Food Technology. 2013; 37:85-97.

[33] Das P K, Rani J, Rawat S, Kumar S. Microalgal co-cultivation for biofuel production and bioremediation: Current status and benefits. BioEnergy Research. 2022;15:1-26. DOI: 10.1007/s12155-021-10254-8

[34] Prawira, M.Y., Taufikurahman, T., & Meliani, L. (2021). Production of Protein and Biomass from Cultivation of Spirulina platensis and Chlorella vulgaris by Utilizing Fishery Liquid Waste as Cultivation Medium and Variations in Mixed Culture Treatment**.** SITH-ITB Final Project Repository. 2021; 1.

[35] de Carvalho J C, Sydney E B, Tessari L F A, Soccol C R. Culture media for mass production of microalgae. In Biofuels from algae. Elsevier. 2019:33-50. DOI: 10.1016/B978-0-444-64192-2.00002-0

[36] Olguin E, Galicia S, Mercado G, Perez T. Annual productivity of Spirulina (Arthrospira) and nutrient removal in a pig wastewater recycling process under tropical conditions. Journal of Applied Phycology. 2003;15:249-257. DOI: 10.1023/A:1023856702544

[37] Zhao B, Ma J, Zhao Q, Laurens L, Jarvis E, Chen S, Frear C. Efficient anaerobic digestion of whole microalgae and lipid-extracted microalgae residues for methane energy production. Bioresource technology. 2014;161:423-430. DOI: 10.1016/j.biortech.2014.03.079

[38] Schulze C, Wetzel M, Reinhardt J, Schmidt M, Felten L, Mundt S. Screening of microalgae for primary metabolites including β-glucans and the influence of nitrate starvation and irradiance on β-glucan production. Journal of Applied Phycology. 2016;28:2719-2725. DOI: 10.1007/s10811-016-0812-9

[39] Rausch T. The estimation of microalgal protein content and its meaning to the evaluation of algal biomass: Comparison of methods for extracting protein. Hydrobiologia. 1981;78:237-251. DOI: 10.1007/BF00008520

[40] Metsoviti M.N., Katsoulas N., Karapanagiotidis I.T., Papapolymerou G. Effect of nitrogen concentration, two-stage and prolonged cultivation on growth rate, lipid and protein content of *Chlorella vulgaris*. *J. Chem. Technol. Biotechnol.* 2019;94:1466–1473. DOI: 10.1002/jctb.5899

B.11

Protein and Biomass Analysis of *Spirulina* sp. and *Nannochloropsis* sp. on Single and Mixed Cultures at Anaerobically Digested Dairy Manure Wastewater (ADDMW) Remediation in Open Raceway Pond

Rachma Anisa Maulani[1], Rizky Pradyantama[1], Muhammad Farhan Aidira[1], Lili Melani[1], Taufik Taufikurahman[1*]

[1] Sekolah Ilmu dan Teknologi Hayati, Institut Teknologi Bandung, Jalan Ganesha No. 10 Bandung 40132 Indonesia

[*] E-mail: taufik@itb.ac.id

Abstract

Microalgae have been widely considered as a source of protein because of their high protein content. For large-scale microalgae cultivation, synthetic media is not an economical alternative. The use of wastewater for microalgae cultivation is being developed to support sustainable microalgae cultivation. Due to the complexity and variability of wastewater, microalgae cultures used for remediation purposes are expected to have high stability and resistance. Therefore, in this study, single and mixed culture microalgae cultivation was carried out for 7 days on ADDMW waste media diluted 25 times with the ratio of microalgae culture and waste media is 1 : 4 (v/v), the ratio of *Spirulina* sp. and *Nannochloropsis* sp. in mixed culture is 1 : 1 (v/v), to see its effect on cell growth, biomass, and protein production on day 3. In biomass production, the highest values of specific growth rate, yield, and productivity were produced in the mixed culture of *Spirulina* sp. and *Nannochloropsis* sp., which are 3.14 day^{-1}, 3.33 g L^{-1}, and 0.40 g L^{-1} day^{-1}, respectively. The highest protein content on day 3 was obtained in a mixed culture variation. The mixed culture is superior based on its ability to produce biomass and protein.

Keywords: Mixed culture, *Spirulina* sp., *Nannochloropsis* sp., Anaerobically Digested Dairy Manure Wastewater, Open Raceway Pond.

1. Introduction

Indonesia's population growth in 2022 increases by 1% from 2021 and the average protein consumption of the Indonesian population in 2021 increases by 7.17 grams from 2015 to 62.28

grams [1, 2]. The 2030 Agenda for Sustainable Development adopted by UN member states in 2015 provides a common blueprint for peace and prosperity for people and the planet, and one of the goals is zero hunger. To be able to end hunger, achieve food security, better nutrition, and promote sustainable agriculture, it is necessary to have sustainable resources that can meet food needs, that in line with increasing population growth and increasing protein consumption.

Microalgae have been widely considered a source of protein because of their high protein content. Microalgae are photosynthetic microorganisms found in marine and freshwater environments with photosynthetic efficiency 10 times higher than terrestrial plants, achieving higher growth rates and biomass productivity, with some species doubling their biomass in just a few hours [3]. Microalgae *Spirulina* sp., for example, is one of the richest sources of protein in microorganisms, with protein content up to 70% [4]. In addition, other microalgae such as *Nannochloropsis* sp. has a protein content of about 50–55% under nutrient-filled media conditions [5]. Protein-rich biomass is the natural state of microalgae cultivation using culture media that usually have normal or excess nitrogen levels.

The keys to profitability and sustainability in microalgae cultivation are low operational costs including low energy input for agitation and low cultivation media costs [6]. There are many choices of microalgae cultivation media. However, most are developed for the maintenance or isolation of microalgae, not for mass production. For large-scale microalgae cultivation, synthetic media is not an economical alternative. Wastewater used for microalgae cultivation is a trend in the development of sustainable processes. This process utilizes wastewater that is rich in nutrients, inexpensive or at no cost, and available in large quantities. One of the nutrient-rich wastes that can be used for microalgae cultivation is anaerobically digested dairy manure wastewater (ADDMW).

ADDMW is a by-product of the anaerobic digestion process that still contains high total nitrogen and total phosphate [7]. Nutrients that are still contained in ADDMW can be used as a source of nutrients in microalgae growing media to be converted into various important metabolites. However, the high levels of ammonium and phosphate in the effluent must be reduced before being returned to the water body, so the use of waste as a medium for microalgae cultivation is the right solution, both in reducing the operational costs of microalgae cultivation and in waste treatment. In addition, microalgae are considered the most suitable repair agents for the removal/absorption of these compounds because they have higher biosorption capacity, have the adaptability to grow both autotrophs and heterotrophs, have high surface-to-volume ratios, and have the potential to be genetically manipulated [8].

Due to the complexity and variability of wastewater, microalgae cultures used for remediation purposes are expected to have high stability, resilience, and self-sustainability [9]. Monoculture cultivation is often unrealistic for wastewater treatment because contamination is a major consequence of microalgae cultivation in waste media [10]. To overcome the limitations of monoculture cultivation, it can be considered the use of mixed cultures consisting of several

species of microalgae. If properly designed and operated, mixed cultures have the potential to achieve increased biomass productivity, improve wastewater treatment efficiency, enable better light absorption, and have more effective use of nutrients in wastewater [11]. Mixed culture of microalgae allows for different tolerances of each species used so as to maintain the culture in undesirable conditions, such as extreme pH, temperature, the presence of contaminants, and invasion of microalgae or foreign bacteria [11].

Algae cultivation systems are mostly carried out on open raceway ponds (ORP) and tubular photobioreactors. The technique used for microalgae cultivation determines the economic feasibility of the process. ORP requires lower capital costs, operating costs, and energy requirements. Researchers have determined that ORP is economically preferable because it provides better energy use efficiency than tubular photobioreactors [12]. In this study, we reviewed the use of the single culture of *Spirulina* sp., the single culture of *Nannochloropsis* sp., and the mixed culture of both species on protein and biomass production cultured with ADDMW in ORP. Through this study, it was observed how the mixed culture of microalgae can affect the production of protein and microalgae biomass.

2. Materials and Methods

2.1 Materials

The bioreactors used for this study were vertical tubular reactor (VTR), with a diameter of 25 cm and a height of 50 cm with a total volume of 25 L, and open raceway pond (ORP) with a size of 23 cm × 34 cm × 60 cm with a total volume of 47 L of acrylic material. This research used autoclave, oven, analytical balance, water bath, digital microscope, hemacytometer, UV-Vis spectrophotometer, centrifuge, and vortex provided by the School of Life Sciences and Technology (SITH), Bandung Institute of Technology (ITB). Anaerobically digested dairy manure wastewater (ADDMW) was obtained from the SITH ITB Haurngombong Educational Garden. All chemicals used in this study were obtained from the SITH chemical warehouse, Bandung Institute of Technology.

2.2 Method

2.2.1 Cultivation Media Preparation

Walne synthetic medium was used for microalgae inoculum cultivation. 1 mL Walne medium was added to 1 L water. The composition of the synthetic Walne media is presented in Table 1 as follows.

Table 1. Walne synthetic media composition [13]

Compound	Concentration (L^{-1})
$NaNO_3$	100 g
H_3BO_3	33.6 g
Na_2EDTA	45 g
$NaH_2PO_4.H_2O$	20 g
$FeCl_3.6H_2O$	1.3 g
$MnCl_2.4H_2O$	0.36 g
Trace element solution	1 mL
Vitamin solution	100 µL
Trace elements:	
$ZnCl_2$	21 g
$CoCl_2.6H_2O$	20 g
$(NH_4)_6Mo_7O_{24}.4H_2O$	9 g
$CuSO_4.5H_2O$	20 g
Vitamin:	
Thiamine.HCl (B1)	1 g
Cyanocobalamin (B12)	0.05 g

ADDMW was filtered using T180 filter cloth with a pore size of 210 microns to separate solid and liquid fractions. The liquid waste fraction was put into a glass bottle and then sterilized using an autoclave at 121 °C and 1.5 bar for 15 minutes. ADDMW which has been sterilized is ready to be used as a cultivation medium. The composition of ADDMW is presented in Table 2 as follows.

Table 2. ADDMW media composition

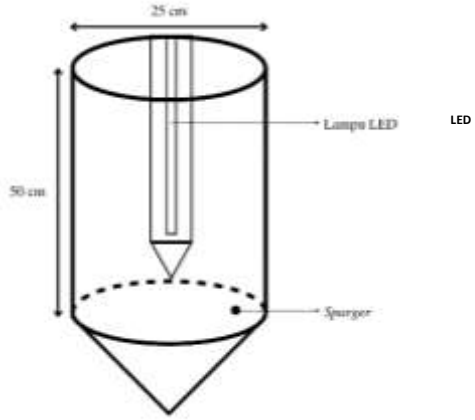
Compound	Concentration (mg L^{-1})
Nitrate	19.53
Ammonium	15.75
Orthophosphate	1606
COD	34.79
BOD	9.98

2.2.2 Microalgae Inoculum Cultivation

Single culture *Spirulina* sp. and *Nannochloropsis* sp. was cultivated using Walne synthetic media in glass bottles (Figure 1) with a working volume of 800 mL and VTR (Figure 2) with a working volume of 7,600 mL. Cultivation was carried out with the ratio of inoculum volume : media solution is 1 : 7. Cultivation on culture bottles was carried out for 7 days and on VTR for 3 days. Sterile ADDMW was diluted 25 times to be used as a medium in the acclimatization process with an inoculum : media solution ratio of 1 : 1 (v/v). The need to liquefy the anaerobic effluent generated from animal manure was carried out to avoid toxicity and excess turbidity [27]. Microalgae acclimatization was carried out for 7 days. Cultivation was carried out at room temperature (25 ± 4 °C), 16 : 8 photoperiodism [49], using an 8-watt LED lamp, and an aeration rate of 3 L min^{-1} regulated by flowmeter. Cultivation was carried out at ITB Jatinangor Campus.

Figure 1. Microalgae cultivation in culture bottles

(a)

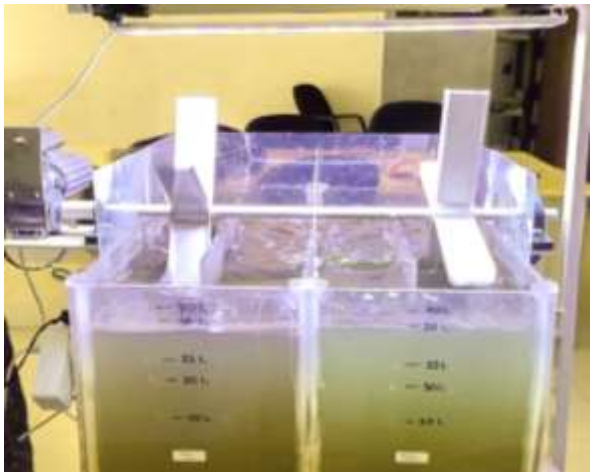


(b)

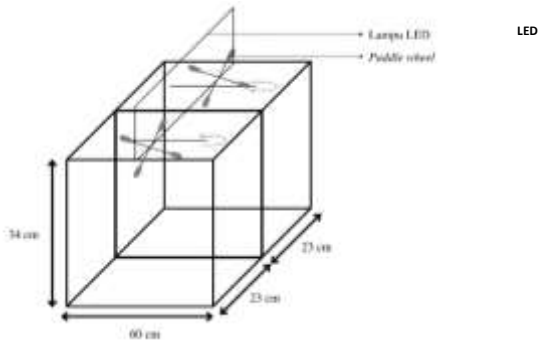
Figure 2. (a) Cultivation of microalgae on VTR. (b) VTR scheme

2.2.3 Microalgae Cultivation at Open Raceway Pond

The single culture of *Spirulina* sp., the single culture of *Nannochloropsis* sp., and 1 : 1 (v/v) mixed culture (3.8 L each) were carried out on ORP (Figure 3) with a working volume of 38 L. Sterile ADDMW was diluted 25 times to be used as a medium in this cultivation process, with a ratio of inoculum : media solution of 1 : 4. Microalgae were cultivated for 7 days with paddle wheel rate of 38 rpm, room temperature (25 ± 4 °C), and photoperiodism L : D = 16 : 8 using an 8-watt LED lamp.



(a)



(b)

Figure 3. (a) Cultivation of microalgae on ORP. (b) ORP scheme

2.2.4 Sampling

Sampling was done by storing samples in 300 mL bottles for each treatment variation. Sampling was carried out once a day for eight days (day 0 to day 7) at ORP.

2.2.5 Determination of the Number of Cells

Determination of the number of cells was done with a hemacytometer. The sample was dripped onto the hemacytometer using a dropper to fill the center of the hemacytometer and then covered with a cover glass. The hemacytometer containing the sample is then observed using a microscope. The number of cells in the four main grids of the hemacytometer was counted and the average was calculated. The calculation of the number of cells was carried out three times. The number of cells was determined by multiplying the average number of cells by 10^4 as in Eq. (1) as follows.

$$N = \bar{n} \times 10^4 \times d \quad (1)$$

Where N is the cell density, $\bar{n}$ is the average number of cells, and d is the dilution factor. The data on the number of cells is then used to make a growth curve to determine the specific growth rate of microalgae through Eq. (2) as follows.

$$\mu = \frac{\ln X_m - \ln X_i}{t} \quad (2)$$

Where X_m is the maximum biomass concentration, X_i is the initial biomass concentration, and t is the cultivation time between X_i and X_m.

2.2.6 Determination of Yield and Productivity of Microalgae Biomass

Microalgae culture 10 mL in falcon tube was centrifuged with at 5,000 rpm for 10 minutes. The supernatant was removed and the weight of the microalgae biomass was determined by calculating the difference between the weight of the falcon tube containing the centrifuged

biomass and the weight of the empty falcon tube. The yield of biomass was determined by comparing the weight of the obtained biomass with the initial volume of the culture. Biomass productivity was determined by comparing the highest biomass density with the time when the highest biomass density was obtained.

2.2.7 Protein Extraction from Microalgae Biomass

Protein extraction from microalgae biomass on day 3 was carried out by the extraction method according to Rausch [14]. 3 mL of 0.5 N NaOH was added to the falcon tube containing the biomass. The extraction process was carried out on a falcon tube which was incubated in a water bath at 80 °C for 10 minutes, then centrifuged at 5,000 rpm for 10 minutes. The supernatant was separated into a test tube and the extraction process was repeated once again with the same steps.

2.2.8 Determination of Protein Concentration in Microalgae Biomass

Determination of protein content was carried out according to the Bradford method from Kruger [15]. Coomassie Blue G250 100 mg was dissolved in 50 mL of 96% ethanol, then mixed with 100 mL of 85% phosphoric acid and dissolved with distilled water until it reached a volume of 1 L. The solution was then filtered through Whatman No. 1. The resulting filtrate was used as a reagent for testing protein levels. The protein content of the extracted sample was determined by mixing 5 mL of Bradford's reagent with 1 mL of the extracted supernatant. The absorbance of this solution was determined at 595 nm using UV-Vis spectrophotometer. The absorbance value obtained was converted into protein content using a standard curve equation made using bovine serum albumin (BSA) solution at concentration of 0–50 ppm. The protein weight obtained was determined by multiplying the protein content by the volume of the supernatant. The protein content of dry biomass was determined by comparing protein mass and dry mass of microalgae biomass in percent (%).

2.2.9 Data Analysis and Interpretation

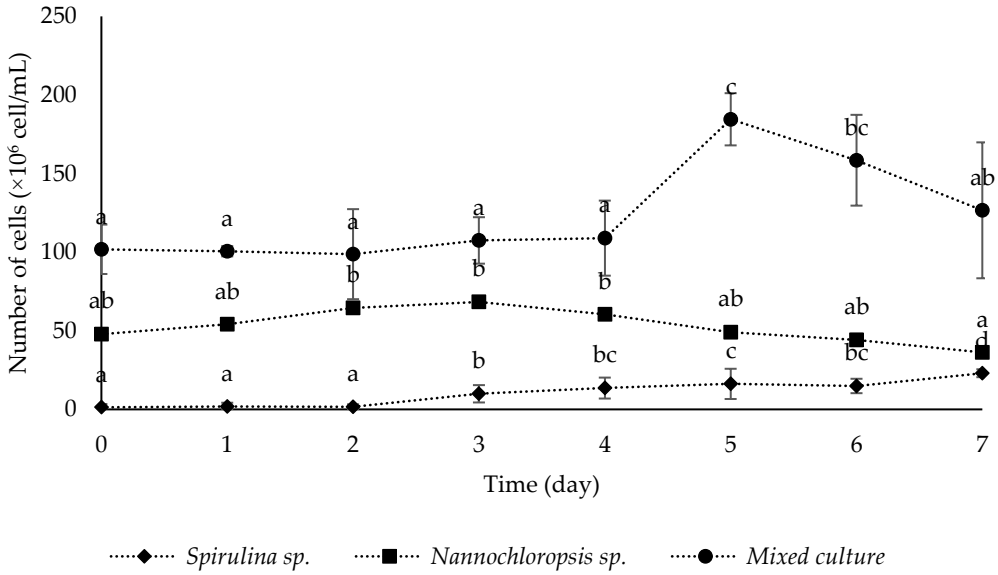
The parameters measured in this study were analyzed using one-way analysis of variance (one-way ANOVA) and followed by Duncan's multiple range test to determine whether there were significant differences between the parameter values in each treatment. Parameters considered to have a significant difference if the p-value <0.05.

3. Results and Discussion

3.1 Cell Growth and Microalgae Biomass

The lag phase in the culture of *Spirulina* sp. lasted for 2 days, the exponential phase started on day 2 while the death phase was not observed for 7 days of cultivation (Figure 4). This is in

accordance with the literature that cultivates *Spirulina* sp. on ORP using Zarrouk's medium with an exponential phase starting on day 2 and no death phase was observed, even for 20 days of cultivation [16].



Note: Differences in letter notation (a, b, and c) indicate a significant difference ($p < 0.05$) in each variation of cultivation treatment

Figure 4. Growth curve for each variation of cultivation treatment

The culture of *Nannochloropsis* sp. entered the exponential phase without any lag phase and entered the death phase on day 4. This is quite in accordance with the literature that cultivates *Nannochloropsis* sp. on palm oil mill effluent (POME) media which immediately entered the exponential phase without a lag phase first, and the death phase started around day 5 [17].

The mixed culture had a longer lag phase than the single culture of *Spirulina* sp. and *Nannochloropsis* sp.. The exponential phase of mixed culture started on the 4th day while the death phase started on the 6th day. This is different from the literature that cultivates mixed cultures of *Nannochloropsis* sp. and *Tetraselmis* sp. in Conway's medium with an exponential phase which started immediately on the first day without any lag phase, and the death phase started on day 8 [18]. This difference is due to the mixed culture in this study requiring a longer time to adapt to the ADDMW media than when cultivating on synthetic media.

The growth of microalgae that took place in a short time in this study is due to the fact that the available nutrients were no longer sufficient to support growth, so it is necessary to add nutrients

to increase the growth of microalgae [35]. In addition, the slowly decreasing biomass can also be caused by the accumulation of toxic compounds in the media.

In mixed culture, *Nannochloropsis* sp. made up about 87% while *Spirulina* sp. made up about 13% of the total cell count at the beginning of cultivation (Figure 5). The culture of *Nannochloropsis* sp. continued to dominate until the end of cultivation. Although the culture mixing ratio is 1 : 1 (v/v), the cell count of *Nannochloropsis* sp. in this study is more than the number of *Spirulina* sp. cells. This is due to differences in cell size. *Spirulina* sp. has a larger cell size, 6–12 m, while the cell size of *Nannochloropsis* sp is 2–4 m [19, 20]. Smaller microalgae cells can grow faster than large cells because the larger surface or volume ratio of smaller cells can facilitate nutrient assimilation at a relatively faster rate [21]. In this mixed culture, both cultures grew in their respective portion ranges, *Spirulina* sp. in the range of 6–24% and *Nannochloropsis* sp. in the range of 76–94%, so that one culture did not suppress the growth of the other.

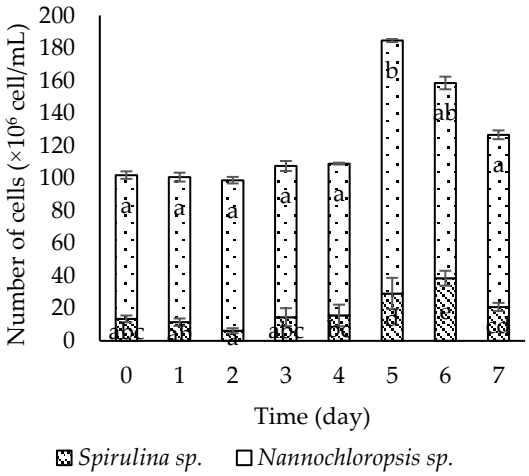


Figure 5. The average ratio of the cell number in the mixed cultivation of *Spirulina* sp. and *Nannochloropsis* sp.

Note: Differences in letter notation (a, b, c, d, and e) indicate a significant difference ($p < 0.05$) in each variation of cultivation treatment

The specific growth rate in the mixed culture was significantly different from the single culture of *Spirulina* sp. and the single culture of *Nannochloropsis* sp. ($p < 0.05$). The specific growth rate obtained in this study is still in the range of results in other studies. The specific growth rate of *Spirulina* sp. can reach about 0.67 day^{-1} [23] and *Nannochloropsis* sp. approximately 0.15–0.98 day^{-1} [24, 25].

The yield of biomass in each cultivation treatment was significantly different ($p < 0.05$). The yield of biomass in this study is still in the range of other studies. The biomass yield of *Spirulina* sp.

can reach about 0.2–0.9 g L^{-1} day^{-1} [47, 48], and *Nannochloropsis* sp. approximately 1.27 g L^{-1} day^{-1} [44].

Biomass productivity in mixed culture was significantly different from the single culture of *Nannochloropsis* sp. ($p < 0.05$) but not significantly different from single cultures of *Spirulina* sp. ($p > 0.05$). Biomass productivity in this study is still in the range in other studies. The productivity of *Spirulina* sp. can reach about 0.1–0.4 g L^{-1} day^{-1} [16, 46] and *Nannochloropsis* sp. approximately 0.02–0.2 g L^{-1} day^{-1} [17, 25].

Mixed culture of *Spirulina* sp. and *Nannochloropsis* sp. cultivated on ORP in this study were known to have a higher specific growth rate, yield, and productivity compared to mixed cultures of *Spirulina* sp. and *Nannochloropsis* sp. cultivated on VTR [37]. Mixed culture had the highest specific growth rate, followed by single-culture *Nannochloropsis* sp. and single-culture of *Spirulina* sp., respectively (Table 3). This is consistent with the literature cultivating mixed cultures of *Nannochloropsis* sp. and *Tetraselmis* sp., that mixed culture had the highest specific growth rate compared to single cultures [18].

Mixed culture yielded the highest biomass yield, followed by *Nannochloropsis* sp. and *Spirulina* sp., respectively. In addition, the mixed culture also produced the highest biomass productivity, followed by the single culture biomass productivity of *Spirulina* sp. and single culture of *Nannochloropsis* sp., respectively (Table 3). This is in accordance with the literature which shows that mixed culture has the potential to facilitate better growth when compared to single culture so it can increase the productivity of microalgae [22]. Mixed cultures have the potential to increase productivity and improve wastewater treatment efficiency, enable better light absorption, and more effective use of nutrients in wastewater [11]. Mixed culture of microalgae allows for different tolerances of each species used to maintain the culture in undesirable conditions, such as extreme pH and temperature, contaminants, and invasion of microalgae or foreign bacteria [11].

Table 3. Comparison of microalgae growth parameters

Species	Media	Bioreactor	Specific growth rate (day^{-1})	Biomass Yield (g L^{-1})	Productivity (g L^{-1} day^{-1})	Reference
Spirulina sp.	ADDMW	ORP	0.71[a]	0.67[a]	0.35[b]	This research
S. platensis	Domestic wastewater	VTR	0.67	-	-	[23]
S. platensis	Zarrouk	ORP	0.20	-	0.40	[16]
Nannochloropsis sp.	ADDMW	ORP	0.75[a]	0.93[b]	0.11[a]	This research

Nannochloropsis sp.	Raw plant substrate	ORP	0.98	-	-	[24]
Nannochloropsis salina	AD effluent	ORP	0.15	-	0.20	[25]
Spirulina sp. and *Nannochloropsis* sp.	ADDMW	ORP	3.14[b]	3.33[c]	0.40[b]	This research
S. platensis and *Nannochloropsis* sp.	Fishery liquid waste	VTR	0.33	0.96	0.16	[37]

Note: Differences in letter notation (a, b, and c) indicate a significant difference ($p < 0.05$) in each variation of cultivation treatment

3.2 Protein Production by Microalgae

The highest protein content on day 3 was obtained in mixed culture, followed by single culture of *Spirulina* sp. and *Nannochloropsis* sp., respectively (Table 4). Each protein content of *Spirulina* sp., *Nannochloropsis* sp, and mixed culture was not significantly different ($p > 0.05$). The protein content obtained in this study was still within the protein levels in other studies. The protein content of *Spirulina* sp. can reach about 43–70% [4, 27] and *Nannochloropsis* sp. about 40–55% [5, 28] with the moisture content in the dry biomass of microalgae around 62%. The mixed culture of *Spirulina* sp. and *Nannochloropsis* sp. cultivated on ORP in this study were known to have a higher protein content than the mixed cultures of *Spirulina* sp. and *Nannochloropsis* sp. cultivated on VTR [37].

Based on this study, mixed culture with a culture ratio of 1 : 1 (v/v) can increase protein content when compared to single-culture cultivation. This is in accordance with the literature that cultivates *Ettlia* sp. and *Chlorella* sp., that mixed culture cultivation was able to produce higher protein than single cultures [26]. In addition, the protein content in mixed cultures of *Nannochloropsis* sp. and *Tetraselmis* sp. and mixed cultures of *Dunaliella salina* and *Phaeodactylum tricornutum* were higher than the single cultures [18, 38].

Based on cell and biomass growth in this study, mixed culture has a higher specific growth rate, yield, and productivity compared to single culture. Because protein is a growth-linked product that is formed along with the production of microalgae biomass, the high yield of microalgae biomass will be proportional to the high protein content. This is consistent with this study because the highest protein content in mixed cultures was followed by high biomass yield in mixed cultures. Biomass with high protein content is a natural state of exponential growth culture [6]. Protein is a primary metabolite produced by microalgae so protein production will occur when the microalgae culture is in the exponential phase. In this study, protein extraction

is carried out on the 3rd day of microalgae culture which is estimated that the culture is in the exponential phase.

Table 4. Protein content in various microalgae cultivation

Species	Media	Bioreactor	Protein content (%)	Reference
Spirulina sp.	ADDMW	ORP	44[a]	This research
S. platensis	Pig wastewater	ORP	43,5	[27]
Nannochloropsis sp.	ADDMW	ORP	40,1[a]	This research
Nannochloropsis gaditana	Agricultural fertilizers	Culture bottle	40	[28]
Spirulina sp. and *Nannochloropsis* sp.	ADDMW	ORP	60[a]	This research
S. platensis and *Nannochloropsis oculata*	Seafood processing wastewater	-	33,4	[29]
S. platensis and *Nannochloropsis sp.*	Fishery liquid waste	VTR	22,2	[37]

Note: Differences in letter notation (a, b, and c) indicate a significant difference ($p < 0.05$) in each variation of cultivation treatment

4. Conclusion

This study showed that the mixed culture of microalgae *Spirulina* sp. and *Nannochloropsis* sp. can increase the growth rate, yield, and productivity of biomass, as well as its protein content compared to the single cultures. The highest value of the specific growth rate, yield, and productivity in this study is in the mixed culture of *Spirulina* sp. and *Nannochloropsis* sp., which are 3.14 day^{-1}, 3.33 g L^{-1}, and 0.4 g L^{-1} day^{-1}, respectively. The highest protein content is obtained in the mixed culture of *Spirulina* sp. and *Nannochloropsis* sp. compared to the single cultures. The mixed culture is superior based on its ability to produce biomass and protein.

Acknowledgments

This project was funded by PPMI Institut Teknologi Bandung 2021.

References

[1] United Nations, Department of Economic and Social Affairs. Population Division. Online

Edition Rev. 1. World Population Prospects; 2022

[2] BPS. Konsumsi Kalori dan Protein Penduduk Indonesia dan Provinsi. 2021;1979-6250.

[3] Santos FM, Gonçalves AL, Pires JC. Negative emission technologies. InBioenergy with Carbon Capture and Storage. Academic Press. 2019;1-13. DOI: 10.1016/B978-0-12-816229-3.00001-6

[4] Pelizer LH, de Carvalho JC, de Oliveira Moraes I. Protein production by Arthrospira (Spirulina) platensis in solid state cultivation using sugarcane bagasse as support. Biotechnology Reports. 2015;1;5:70-6. DOI: 10.1016/j.btre.2014.12.006

[5] Hulatt CJ, Wijffels RH, Bolla S, Kiron V. Production of fatty acids and protein by Nannochloropsis in flat-plate photobioreactors. PloS one. 2017;19;12(1). DOI: 10.1371/journal.pone.0170440

[6] de Carvalho JC, Sydney EB, Tessari LF, Soccol CR. Culture media for mass production of microalgae. InBiofuels from algae. Elsevier. 2019;33-50. DOI: 10.1016/B978-0-444-64192-2.00002-0

[7] Möller K, Müller T. Effects of anaerobic digestion on digestate nutrient availability and crop growth: A review. Engineering in life sciences. 2012;12(3):242-57. DOI: 10.1002/elsc.201100085

[8] Laraib N, Hussain A, Javid A, Hafeez-ur-Rehman M, Bukhari SM, Rashid M, Ali W. Recent advancements in microalgal-induced remediation of wastewaters. InMicroorganisms for sustainable environment and health. Elsevier. 2020;205-217. DOI: 10.1016/B978-0-12-819001-2.00010-3

[9] Padmaperuma G, Kapoore RV, Gilmour DJ, Vaidyanathan S. Microbial consortia: a critical look at microalgae co-cultures for enhanced biomanufacturing. Critical reviews in biotechnology. 2018;4;38(5):690-703. DOI: 10.1080/07388551.2017.1390728

[10] Santiago AF, Calijuri ML, Assemany PP, Calijuri MD, Reis AJ. Algal biomass production and wastewater treatment in high rate algal ponds receiving disinfected effluent. Environmental technology. 2013;34(13-14):1877-85. DOI: 10.1080/09593330.2013.812670

[11] Luo Y, Le-Clech P, Henderson RK. Characterisation of microalgae-based monocultures and mixed cultures for biomass production and wastewater treatment. Algal Research. 2020;49:101963. DOI: 10.1016/j.algal.2020.101963

[12] Yadav K, Vasistha S, Rai MP. Algal physiology and cultivation. InHandbook of Algal Biofuels. Elsevier. 2022;79-96. DOI: 10.1016/B978-0-12-823764-9.00016-9

[13] Walne PR. Experiments in the large-scale culture of the larvae of Ostrea edulis L. 1966

[14] Rausch T. The estimation of micro-algal protein content and its meaning to the evaluation of algal biomass I. Comparison of methods for extracting protein. Hydrobiologia. 198;78(3):237-51. DOI: 10.1007/BF00008520

[15] Kruger NJ. The Bradford method for protein quantitation. The protein protocols handbook. 2009:17-24. DOI: 10.1007/978-1-59745-198-7_4

[16] Göksan T, Zekeriyaoğlu A, Ak İ. The growth of Spirulina platensis in different culture systems under greenhouse condition. Turkish journal of biology. 2007;31(1):47-52.

[17] Hadiyanto H, Soetrinanto D, Silviana S, Mahdi MZ, Titisari YN. Evaluation of growth and biomass productivity of marine microalga Nannochloropsis sp. cultured in palm oil mill effluent (POME). Philippine Journal of Science. 2017;146(4);355-360.

[18] Arkronrat W, Deemark P, Oniam V. Growth performance and proximate composition of mixed cultures of marine microalgae (Nannochloropsis sp. & Tetraselmis sp.) with monocultures. Songklanakarin Journal of Science and Technology. 2016;38(1):1-5.

[19] Pelosi E, Margheri MC, Tomaselli L. Characteristics and significance of Spirulina morphology. In Caryologia. 1982;157-158.

[20] Borowitzka MA. Biology of microalgae. In Microalgae in health and disease prevention. Academic Press. 2018;23-72

[21] Fogg GE, Thake B. Algal cultures and phytoplankton ecology. Univ of Wisconsin Press. 1987

[22] Cai ZP, Duan SS. Growth in polyculture of algae, Dunaliella salina and Platymonas subcordifomis. Fisheries Science. 2008;27(7);330-333

[23] Sofiyah ES, Septiariva IY, Suryawan IW. Domestic wastewater treatment using various microalgae for lipid production. Annals of the Romanian Society for Cell Biology. 2021;7:15156-63

[24] Schambach JY, Finck AM, Kitin P, Hunt CG, Hanschen ER, Vogler B, Starkenburg SR, Barry AN. Growth, total lipid, and omega-3 fatty acid production by Nannochloropsis spp. cultivated with raw plant substrate. Algal Research. 2020;51:102041. DOI: 10.1016/j.algal.2020.102041

[25] Sheets JP. Cultivation of Nannochloropsis Salina in diluted anaerobic digester effluent under simulated seasonal climatic conditions and in open raceway ponds [Doctoral dissertation]. The Ohio State University; 2013

[26] Rashid N, Ryu AJ, Jeong KJ, Lee B, Chang YK. Co-cultivation of two freshwater microalgae species to improve biomass productivity and biodiesel production. Energy Conversion and Management. 2019;15;196:640-8. DOI: 10.1016/j.enconman.2019.05.106

[27] Olguín EJ, Galicia S, Mercado G, Pérez T. Annual productivity of Spirulina (Arthrospira) and nutrient removal in a pig wastewater recycling process under tropical conditions. Journal of applied phycology. 2003;15(2):249-57. DOI: 10.1023/A:1023856702544

[28] Camacho-Rodríguez J, Cerón-García MC, González-López CV, Fernández-Sevilla JM, Contreras-Gómez A, Molina-Grima E. A low-cost culture medium for the production of Nannochloropsis gaditana biomass optimized for aquaculture. Bioresource technology. 2013;144:57-66. DOI: 10.1016/j.biortech.2013.06.083

[29] Duong HV. Study on cultivation of co-culture microalgae of spirulina platensis and nannochloropsis oculata for biomass production in wastewater [Doctoral dissertation]. International University HCMC Vietnam; 2014

[30] Sayadi MH, Ahmadpour N, Fallahi CM, Rezaei MR. Removal of nitrate and phosphate from aqueous solutions by microalgae: An experimental study. 2016

[31] Kim CW, Sung MG, Nam K, Moon M, Kwon JH, Yang JW. Effect of monochromatic

illumination on lipid accumulation of Nannochloropsis gaditana under continuous cultivation. Bioresource technology. 2014;1;159:30-5. DOI: 10.1016/j.biortech.2014.02.024

[32] Hii YS, Soo CL, Chuah TS, Mohd-Azmi A, Abol-Munafi AB. Uptake by Nannochloropsis sp. J. Sustain. Sci. Manag. 2011;6:60-8.

[33] Jia H, Yuan Q. Removal of nitrogen from wastewater using microalgae and microalgae–bacteria consortia. Cogent Environmental Science. 2016;2(1):1275089. DOI: 10.1080/23311843.2016.1275089

[34] Sankaran K, Premalatha M. Nutrients uptake from anaerobically digested distillery wastewater by Spirulina sp. under xenon lamp illumination. Journal of Water Process Engineering. 2018;25:295-300. DOI: 10.1016/j.jwpe.2018.08.014

[35] Cheunbarn S, Peerapornpisal Y. Cultivation of Spirulina platensis using anaerobically swine wastewater treatment effluent. Int. J. Agric. Biol. 2010;12(4):586-90.T

[36] Resdi R, Lim JS, Idris A. Batch kinetics of nutrients removal for palm oil mill effluent and recovery of lipid by Nannochloropsis sp. Journal of Water Process Engineering. 2021;1;40:101767. DOI: 10.1016/j.jwpe.2020.101767

[37] Assabiqi FA, Taufikurahman, Melani L. Produksi Protein dan Biomassa dari Kultivasi Spirulina platensis dan Nannochloropsis sp. dengan Memanfaatkan Limbah Cair Perikanan sebagai Medium Kultivasi dan Variasi Perlakuan Mixed Culture [thesis]. Bandung Institute of Technology; 2021

[38] Huang WW, Dong BZ, Cai ZP, Duan SS. Growth effects on mixed culture of Dunaliella salina and Phaeodactylum tricornutum under different inoculation densities and nitrogen concentrations. African Journal of Biotechnology. 2011;10(61):13164-74

[39] Caswell M, Zilberman D. Algol – culture. University of California at Berkeley. 2002;6:1-12

[40] Chuntapa B, Powtongsook S, Menasveta P. Water quality control using Spirulina platensis in shrimp culture tanks. Aquaculture. 2003;220(1-4):355-66. DOI: 10.1016/S0044-8486(02)00428-3

[41] Afifah AS, Suryawan IW, Sarwono A. Microalgae production using photo-bioreactor with intermittent aeration for municipal wastewater substrate and nutrient removal. Communications in Science and Technology. 2020;5(2):107-11. DOI: 10.21924/cst.5.2.2020.200

[42] Saiu G, Pistis A, Chindris A, Grosso M, Baroli M, Scano E. Study of the growth parameters of the Nannochloropsis oculata for the nitrogen and phosphorus removal from wastewater through design of experiment approach. Chemical Engineering Transactions. 2016;49:553-8. DOI: 10.3303/CET1649093

[43] Sofiyah ES, Suryawan IW. Cultivation of Spirulina platensis and Nannochloropsis oculata for nutrient removal from municipal wastewater. Rekayasa. 2021;14(1):93-7. DOI: 10.21107/rekayasa.v14i1.8882

[44] Emparan Q, Jye YS, Danquah MK, Harun R. Cultivation of Nannochloropsis sp. microalgae in palm oil mill effluent (POME) media for phycoremediation and biomass production: Effect of microalgae cells with and without beads. Journal of Water Process

Engineering. 202033:101043. DOI: 10.1016/j.jwpe.2019.101043

[45] APHA/AWWA/WPCF. Standard Methods for the Examination of Water and Wastewater. 22nd ed. 2012

[46] Taufikurahman T, Ilhamsyah DP, Rosanti S, Ardiansyah MA. Preliminary Design of Phycocyanin Production from Spirulina platensis Using Anaerobically Digested Dairy Manure Wastewater. InIOP Conference Series: Earth and Environmental Science 2020 Jun 1 (Vol. 520, No. 1, p. 012007). IOP Publishing. DOI: 10.1088/1755-1315/520/1/012007

[47] Nogueira SM, Souza Junior J, Maia HD, Saboya JP, Farias WR. Use of Spirulina platensis in treatment of fish farming wastewater. Revista Ciência Agronômica. 2018;49:599-606. DOI: 10.5935/1806-6690.20180068

[48] Farida H, Harahap PS, Sobari R, Gunawan R, Rahman DY, Susilaningsih D. Outdoor Closed System of Algal Mass Culture: In Sight of Comparison on Vertical and Horizontal Photobioreactor for Cultivating the Spirulina sp. Reaktor. 2019(2):54-61. DOI: 10.14710/reaktor.19.2.54-61

[49] Suyadi TA. Optimizing Photoperiodism, Growth Media Dilution, and Frequency of Inoculum Addition in Microalgae (Chlorella vulgaris) Culture Using Bioslurry (ADDMW) as Growth Media. Jurnal Matematika dan Sains. 2020;25(1):6-10

B.12

Biomonitoring of Air Quality on Three Types of Roads (Local, Collector, and Arterial) in the City of Bandung: Total Cu in Lichen as a Bioindicator

Robi Dandi [1], Hantoro Ilham Mahendra [1], Nathanael Wilbert[1], Taufik Taufikurahman[1]

[1)] School of Life Sciences and Technology, Institut Teknologi Bandung

Provide full correspondence details here including e-mail for the robidandi@students.itb.ac.id

Abstract

Bandung is experiencing a growing population, leading to increased vehicular traffic both within and outside the city. This surge in local motor vehicle activity is contributing to rising temperatures and local air pollution, resulting in emissions of heavy metals such as Pb, Cu, and Zn. Lichen, a symbiotic organism formed through the partnership of mycobiont and photobiont, is frequently employed to monitor and assess air quality levels. Lichens possess the unique ability to directly absorb water, gases, and nutrients, making them valuable indicators in environmental bio-assessment. This study specifically analyzed lichens growing on tree bark to assess their Cu (copper) concentration and species diversity. The research spanned a duration of three months, focusing on three distinct road locations within Bandung: a local road (Jl. Raden Patah), a collector road (Jl. Sulanjana), and an arterial road (Jl. Sukajadi). Lichen samples were collected ten times from each location, covering various cardinal directions within a squared plot measuring 25 cm x 25 cm. Cu concentrations were quantified using Atomic Absorption Spectroscopy at a wavelength of 324.8 nm, while species diversity was determined by the frequency of lichen species and its correlation with air quality scores. The findings revealed that the highest Cu concentration was observed on Jl. Sukajadi (6.89 ppm), followed by Jl. Sulanjana (6.30 ppm) and Jl. Raden Patah (6.16 ppm). In total, eight lichen species were successfully characterized at each observation point, with the highest diversity recorded on Jl. Raden Patah. Consequently, this study concludes that Jl. Raden Patah exhibits the best air quality among the selected locations, attributed to its rich diversity and abundance of lichen species, coupled with the lowest Cu concentrations.

Keywords: Air quality, bioindicator, copper, lichen, species diversity

1. Introduction

Clean air is fundamental to life. Unfortunately, with the rapid expansion of urban areas, industrial growth, and the widespread use of vehicles, air quality has undergone substantial deterioration due to pollution (Faroqi et al., 2016). The issue of inefficient urban transportation in large metropolises like Bandung City has exacerbated the reliance on private vehicles, consequently increasing traffic density and, in turn, raising air pollution levels (Putra et al., 2013). Air quality is vital for sustaining life. However, the rapid urban development, industrial expansion, and widespread use of motorized vehicles have led to significant air pollution, characterized by the presence of heavy metals such as copper (Cu), iron (Fe), zinc (Zn), lead (Pb), manganese (Mn), and chromium (Cr) in vehicle emissions (Faroqi et al., 2016). Copper, in particular, plays a crucial role in fuel gas distribution systems due to its cost-efficiency and ease of installation, making it a preferred material (Favaro et al., 2017). However, copper emissions contribute to air pollution and can potentially interfere with brain development (Scheiber et al., 2014).

Abnormal copper levels are associated with conditions like Wilson's disease, a genetic disorder that affects motor control (Bandmann et al., 2015). The deteriorating air quality, including copper contamination, necessitates ongoing monitoring, often involving the use of living organisms as bioindicators. Living organisms can react and adapt to environmental changes, making them valuable indicators of environmental quality (Abas, 2021). For example, the presence of catfish correlates positively with water quality, while ant activity correlates positively with soil moisture (Šestinová et al., 2019; Standen et al., 2017). Lichen is a well-established bioindicator for monitoring and assessing air quality levels, with a history dating back to 1866 in Luxembourg (Nylander, 1866). Lichens result from a mutualistic symbiosis between fungi (mycobiont) from the Ascomycetes group and green or blue algae (photobiont) from the Cyanobacteria group (Rancovic, 2007). They can be found in various environments, from leaves and bark to extreme conditions, contributing to soil formation and withstanding prolonged periods of drought. The morphology of lichen typically consists of four parts: the upper cortex for protection, the algal layer for photosynthesis, the medulla for structural support, and the lower cortex with rhizines to anchor the lichen (Yurnaliza, 2002; Nelsen et al., 2020).

Lichens exhibit diverse shapes, sizes, and colors, influenced by their constituents and environmental conditions. They are classified into categories based on their habitat and substrate, including saxicolous, corticolous, terricolous, musicolous, and follicolous (Murningsih, 2016). Thallus shapes include crustose, foliose, fruticose, and squamulose. Lichens are sensitive organisms, absorbing chemical substances from the air and rainwater due to their lack of a cuticle. They accumulate pollutants, including sulfur dioxide (SO2), which can affect human health, ecosystems, and even objects (Hadiyati, 2013).

Air pollutants result from natural and anthropogenic sources, including fossil fuel combustion, industrial emissions, and vehicle exhausts. These pollutants can deposit as dry particles or react with rainwater. Lichens, known for their sensitivity to airborne pollutants, serve as both active

and passive indicators, making them ideal for tracking pollutants (Panjaitan, Fitmawati, & Martina, 2012). Their advantages include widespread distribution, year-round pollutant accumulation monitoring, longevity, and vulnerability of all thallus parts to pollutants. A decrease in lichen species diversity often signifies pollution and degraded air quality (Smith & Baker, 2003)

2. Methodology

2.1. Determination of Research Sites

The research was conducted at three distinct road types: Jalan Raden Patah, Jalan Sulanjana, and Jalan Sukajadi, as illustrated in Figure 1. These roads were categorized as LO (Local roads), CO (Collector roads), and AR (Arterial roads). To ensure unbiased sampling, the selection of tree species for lichen sampling was carefully considered. In this study, the host tree species chosen was the mahogany tree (*Swietenia mahagoni* (L.) Jacq) due to its abundant population in the study area.

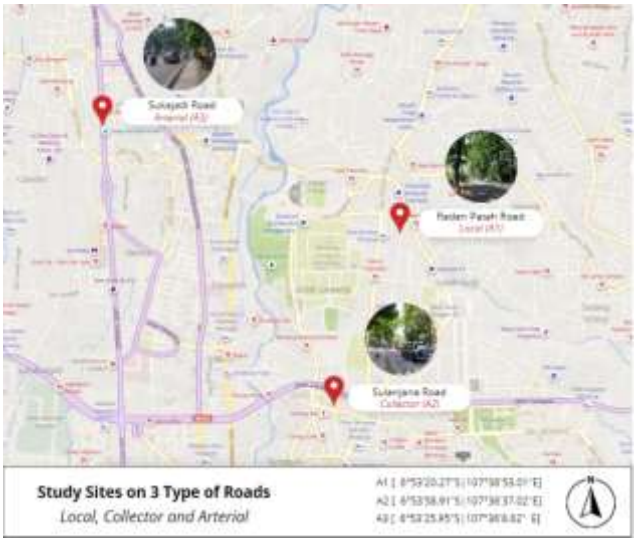


Figure 1. Study Sites Map

2.2. Lichen Diversity

The method used for measuring lichen diversity, involving the assessment of ten trees at each observation site. Measurements were conducted using a 25x25 cm squared plastic plot. Aftrewards, observations were made on all four sides of the tree, according to the wind direction. Data recorded including species names, species counts, and thallus types, followed by lichen colonies that outlined using a plastic sheet, and their shapes were marked with a marker. Once all data had been recorded, lichen diversity documentation was accomplished by capturing images using a camera or smartphone in macro mode ried out by taking pictures with a camera or smartphone using macro mode.

2.3. Total Copper in Lichen

The determination of total copper content in lichen samples was conducted through the method of Atomic Absorption Spectrometry (AAS) involving a destructive process. First, 10 grams of lichen were weighed and heated at 105°C for a duration of 1 hour. Afterthat, the samples were dried in a furnace set at 600°C for 1 hour. Then, the residue was transferred to an Erlenmeyer flask and mixed with 30 mL of distilled water and 5 mL of HNO_3. The mixture was heated on a hotplate until it reached a concentrated state.The mixture sample was filtered using Whatman No. 42 filter paper and compressed into a 25 mL flask. The copper (Cu) concentration was determined by measuring the sample's absorbance at a wavelength of 324.8nm through Atomic Absorption Spectrophotometry (AAS), with comparisons made against a Cu standard solution.

2.4. Data Analysis

In this study, the collected data was processed using Microsoft Excel Version 2112, Build 16.0.14729.20254, and OriginPro 2023 software, Version 10.0.0.154. The software facilitated the following data analyses comparison chart illustrating the frequency of lichen species across the three sampling locations, comparison chart displaying the number of lichen colonies recorded at each location, comparison chart depicting lichen species distribution at each location, calculation of diversity and dominance indices, frequency of lichen species categorized by cardinal directions at each location and, graphical representation of copper (Cu) content in lichen samples collected from each location.

3. Results and discussion

3.1. Lichen Diversity

At three data collection sites, eight lichen species were identified, consist of four crustose species and four foliose species, as illustrated in Figure 2. Figure 3 presents a comparative analysis of species distribution frequencies across the three data collection locations.

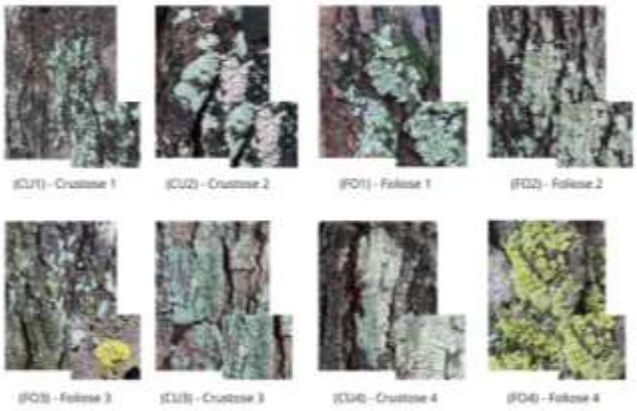


Figure 2. Species of lichen found in 3 types of roads.

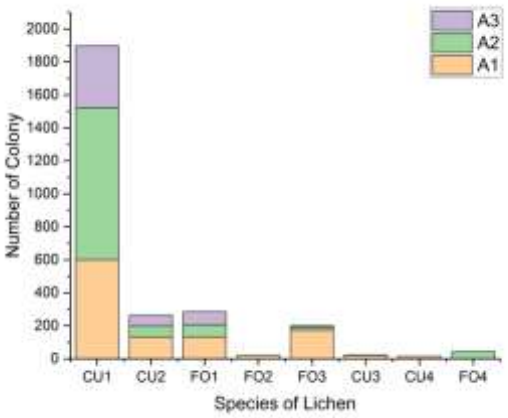


Figure 3. The comparison of the distribution frequency of the species from the 3 data collection sites.

It can be seen that species 1 (CU 1) predominates in all three locations. Among these eight species, several exhibit site-specific occurrences, highlighting the distinct environmental conditions characterized by varying temperature, humidity, and pollution levels in each area. This observation highlights the differential pollution resistance of various lichen types, especially based on thallus morphology, crustose lichen species demonstrate superior adaptability to polluted environments. Moreover, foliose-type lichen was present but in lower abundance, while fructicose-type lichen, typically indicating the clean air, was conspicuously absent in this study (Noer, 2004).

Table 1. Diversity Index and Dominance Index of 3 Types of Roads.

Index	A1	A2	A3
Diversity Index	1,342,312	0,702146	0,863554
Dominance Index	0,355501	0,682607	0,542154

The highest species richness is evident at location A1, indicating the good environmental conditions than the other two sites. Figure 4 provides a comprehensive overview of the cumulative lichen colonies observed across all three data collection sites.

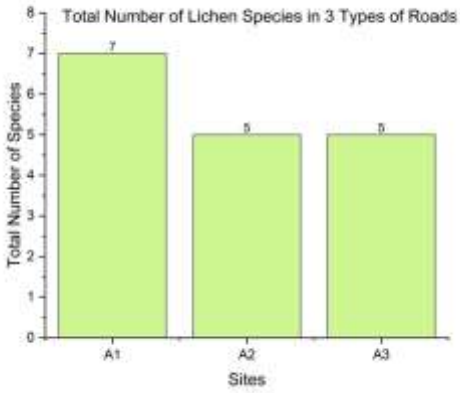


Figure 4. Total Number of Species in 3 Types of Roads.

The locations of A1 and A2 have a very different number of lichen colonies, proving that the environmental conditions are not much different. At the A3 site, a smaller number of lichen colonies was found, which proves that environmental conditions and pollution are worse compared to the other two locations (Policina & dela Cruz, 2020). While the graph depicting the number of lichen colonies (Figure 5) shows a higher count at location A2 compared to A1, the percentage of lichen cover observed at A1 surpasses that of A2. This finding further supports the notion that the environmental conditions at A1 are superior to the other two locations.

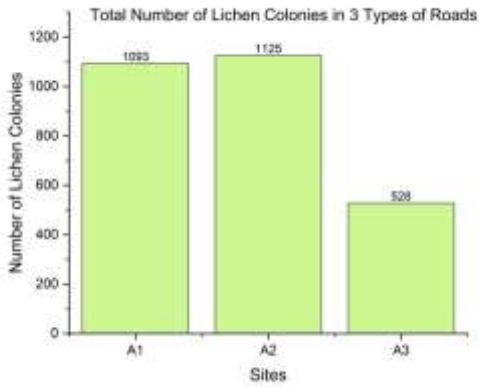


Figure 5. Total Number of Colonies in 3 Types of Roads.

This observation aligns with the statement made by Hardini (2010) that characteristics used to gauge the extent of air pollution in a location diversity, frequency, and the percentage of lichen cover (density). Diversity and dominance index were computed to assess the distribution of lichen species across the three data collection locations, as outlined in Table 1. The Shannon-Wiener diversity and dominance index were employed in this study. This statistical method simplifies the interpretation of population data for organisms and provides insights into the number of individuals for each species within a community (Krebs, 1989; Fachrul, 2007; Odum, 1993). As per the Shannon-Wiener diversity index, site A1 exhibited a moderate diversity level ($1 < H' < 3$), while both sites A2 and A3 fell into the low diversity category ($H' < 1$). The Dominance Index revealed that at site A2, species dominance was classified as high, whereas at sites A1 and A3, it fell within the medium category.

We also conducted an analysis of species frequency based on wind directions at data collection sites to observe species trends align with wind directions. Figure 5 illustrates the frequency analysis based on cardinal directions specifically for Jalan Raden Patah. It can be seen that species 1 (CU 1) is the most common in the four wind directions, while other species have almost the same frequency and do not predominate. Following the number of species found, only species 8 (FO 4) were not found on Jalan Raden Patah, while the remaining 7 species could be found, indicating that the air condition at that site was still relatively free from pollution. This is in line with the study by Policina & dela Cruz (2020), which states that the presence of lichens is one of the indicators that the air quality in the environment is relatively clean from pollution.

From the frequency analysis chart based on the cardinal directions on Jalan Sulanjana (**Figure 6**), it is evident that species 1 (CU 1) dominates compared to other species. Of the ten trees observed, only five lichen species could be found, whereas the other three species could not be found along Jalan Sulanjana.

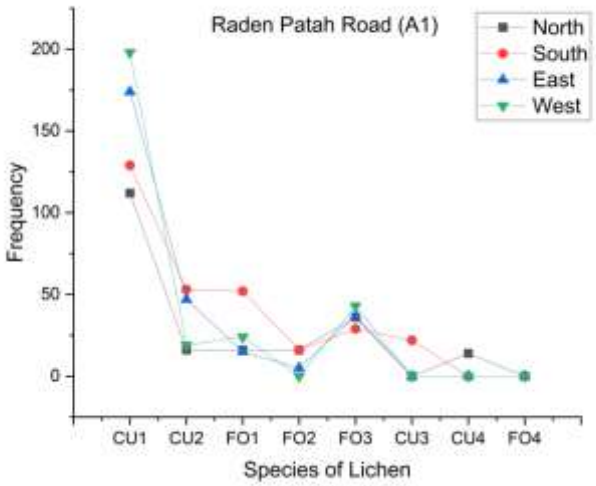


Figure 6. The frequency analysis based on the cardinal directions on Jalan Raden Patah

The frequency and number of lichen colonies found on Jalan Sulanjana are still very high, although the percentage of coverage is not as good as the previous sites. This proves that at this site, the air condition is still relatively good, although not as good as at Jalan Raden Patah (Stacey *et al*. 2018)

From the graph of frequency analysis based on cardinal directions on Jalan Sukajadi (**Figure 7**), it can be seen that species 1 (CU 1) also dominates compared to other species. Only five species of lichen could be found in the ten trees studied along the Sukajadi road. Both the frequency, the number of colonies, and the percent cover of lichens at Jalan Sukajadi were much lower than at the other two sites. This proves that the air condition at Jalan Sukajadi is much worse than at the other two sites (Pinho *et al*. 2004). From the three graphs, the distribution of lichen colonies does not correlate with the cardinal directions

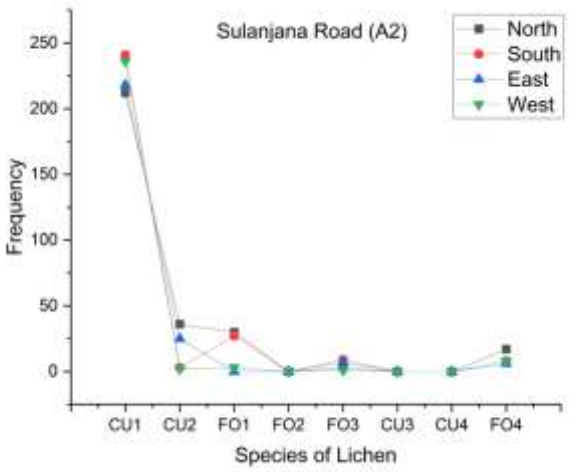


Figure 7. The frequency analysis based on the cardinal directions on Jalan Sulanjana

3.2. Phytochemical Content in Lichen

Based on the observations, it was obtained that there were differences in the value of Cu content at each observation location. The content of Cu in lichen respectively from Jalan Raden Patah, Jalan Sulanjana, and Jalan Sukajadi is 2,869 μM, 2,896 μM, and 2,939 μM. The visualization of the Cu content data on the lichen at each observation site is shown in (**Figure 7 and Table 2)**.

Table 2. Cu Level Measurement with AAS (Atomic Absorption Spectrophotometer) Method.

Location	Absorption (324.8 nm)	Concentration (μM)
Raden Patah (A1)	0.337 ± 0.4676	2.869 ± 3.458
Sulanjana Road (A2)	0.350 ± 0.480	2.896 ± 3.619
Sukajadi Road (A3)	0.380 ± 0.537	2.939 ± 3.558

The highest Cu content was on Jalan Sukajadi with 2,939 μM, then Jalan Sulanjana with 2,896 μM, and Jalan Teuku Umar with 2,869 μM. In terms of morphology and physiology, the lichen in Jalan Sukajadi has a crustose shape and a dark colour compared to lichen colonies in other sites. This happens because there is a physiological response carried out under stress in the form of the presence of Cu with a decrease in the level of sterols and total polyunsaturated fatty acids in lichens (Guschina & Hairwood, 2006). In terms of Cu content in lichen, compared to other studies by Klimek et al. (2015) at Mount Beskidy, Poland, a Cu level of 7.12 μM was obtained. Another studies by Abas & Din (2020) at Johor, Malaysia, a Cu level of 1.70 μM was obtained. Thus the Cu level at the study site was much lower when compared to that study.

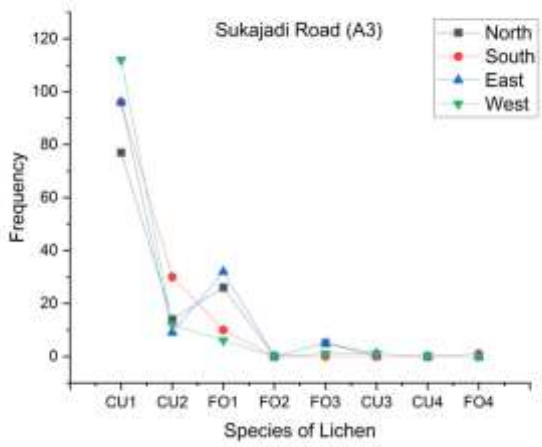


Figure 8. The frequency analysis based on the cardinal directions on Jalan Sukajadi

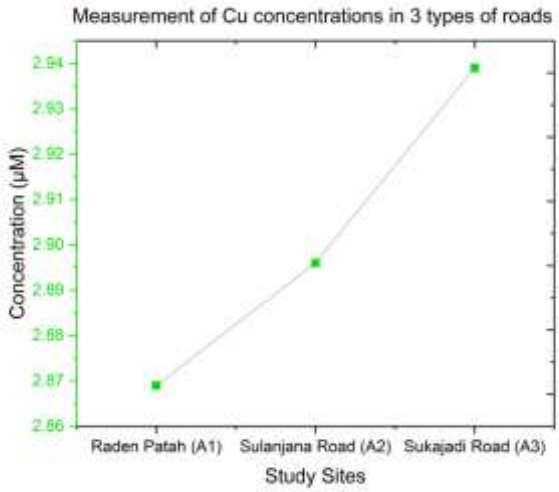


Figure 9. Cu Level Measurement with AAS (Atomic Absorption Spectrophotometer) Method

Compared to the three research sites, it was found that Jalan Sukajadi had a high level of pollution compared to the other two sites, namely Jalan Raden Patah and Jalan Sulanjana. This is influenced by the high level of mobility of motorized vehicles, especially two-wheeled vehicles that cross Jalan Sukajadi, especially during data collection, which takes place during peak hours (08.00 – 10.00 WIB).

4. Conclusion

Based on the research carried out, a total of 8 lichen species were found at the three sites. According to the Shannon Wiener diversity index, the diversity in site A1 is still in the moderate category (1<H'<3) while in sites A2 and A3 both are included to the low category (H'<1). From the acquisition of the Dominance Index, it can be seen that at site A2 the dominance of species is included in the high category, while A1 and A3 are in the medium category. Judging from the three sites, the highest dominance index is in A2. This proves that in A2, there are dominant species compared to the others.

The Cu content in lichen from Jalan Raden Patah, Jalan Sulanjana, and Jalan Sukajadi were 2,869 μM, 2,896 μM, and 2,939 μM. Based on the analysis, it can be concluded that the condition of the A1 road has a clean environment and A2 has a pristine condition but is classified as having little pollution, while A3 has a significant difference, namely the atmosphere is relatively clean but the pollution is also at a moderate level.

Acknowledgements

The authors would like to thank Dr. Taufik Taufikurahman as lecturer for Plants as Environmental Bioindicators Lector, School Life Sciences and Technology, Institut Teknologi Bandung, for the research funding.

References

[1] Abas, A., & Din, L. (2020). Heavy Metal Concentration Assessment Using Transplanted Lichen Usnea Misaminensis at Pasir Gudang, Johor. In IOP Conference Series: Earth and Environmental Science (Vol. 549, No. 1, p. 012063). IOP Publishing.

[2] Abas, A. (2021). A systematic review on biomonitoring using lichen as the biological indicator: A decade of practices, progress and challenges. Ecological indicators, 121, 107197. DOI: 10.1016/j.ecolind.2020.107197.

[3] Agung Laksono, "Identifikasi Jenis Lichen Sebagai Bioindikator Kualitas Udara Di Kampus Institut Agama Islam negeri Raden Intan Lampung. (Skripsi, FTK IAIN Raden Intan Lampung, Lampung,2011), hlm. 19-21

[4] Bandmann, O. , Weiss K. H., and Kaler S. G.. 2015. Wilson's disease and other neurological copper disorders. Lancet Neurol. 14:103–113.

[5] Boonpeng, C., Sriviboon, C., Polyiam, W., Sangiamdee, D., Watthana, S., Boonpragob, K., 2018. Assessing atmospheric pollution in a petrochemical industrial district using a lichen-air quality index (LiAQI). Ecol. Indic. 95, 589–594. DOI: 10.1016/ j.ecolind.2018.08.012

[6] Fachrul, Melati. (2007). Metode Sampling Bioekologi (Ed. I). Jakarta: Bumi Aksara.

[7] Faroqi, A., Hadisantoso, E. P., Halim, D. K., Ws, M. S., & Sains, F. (2016). Perancangan Alat Pendeteksi Kadar Polusi Udara Menggunakan Sensor Gas MQ-7 Dengan Teknologi Wireless HC-05 Latar Belakang. X(2), 33–47.

[8] Favaro M, Xiao H, Cheng T, Goddard WA 3rd, Yano J, Crumlin EJ. Subsurface oxide plays a critical role in CO2 activation by Cu (111) surfaces to form chemisorbed CO2, the first step in reduction of CO2. Proc Natl Acad Sci U S A. 2017 Jun 27;114(26):6706-6711. doi: 10.1073/pnas.1701405114. Epub 2017 Jun 12. PMID: 28607092; PMCID: PMC5495248.

[9] Guschina, I. A., & Harwood, J. L. (2006). Lead and copper effects on lipid metabolism in cultured lichen photobionts with different phosphorus status. Phytochemistry, 67(16), 1731–1739. doi:10.1016/j.phytochem.2006.01.023

[10] Hadiyanti. (2013). Kandungan Sulfur dan Kondisi thallus lichen Parmelia sp. dan Graphis sp. pada pohon peneduh jalan di Kecamatan Pontianak Utara. Probotiont, 12-17.

[11] Halpen-Felsher BL, Morrell HE. Preventing and reducing tobacco use. In: Berlan ED, Bravender T, editors. Adolescent medicine today: a guide to caring for the adolescent patient [Internet]. Singapore: World Scientific Publishing Co.; 2012 [cited 2012 Nov 3]. Chapter 18. Available from: http://www.worldscientific.com/doi/pdf/10.1142/9789814324496_0018

[12] Handoko, T. S. (2015). Keanekaragaman Lumut Kerak (Lichens) Sebagai Bioindikator Kualitas Udara di Kawasan Asrama Internasional IPB. Bogor: Departemen Konservasi Sumberdaya Hutan dan Ekowisata, Fakultas Kehutanan.

[13] Hardini, Y. (2010). Keanekaragaman Lichen di Denpasar Sebagai Bioindikator Pencemaran Udara. Seminar Nasionl Biologi Fakultas Biologi UGM (pp. 790-793). Jogjakarta: UGM Press.

[14] Jannah, M., Rahayu, D., Sari, M., & Untari, L. (2019). The Fruticose Lichens In The Forest Tahura (Taman Hutan Raya) R. Soeryo, Jawa Timur. Biotropika:Journal Of Tropical Biology, 7(2),63-66. doi: 10.21776/ub.biotropika.2019.007.02.3

[15] Kaasalainen, U., Kukwa, M., Rikkinen, J., & Schmidt, A. (2019). Crustose lichens with lichenicolous fungi from Paleogene amber. Scientific Reports, 9(1). doi: 10.1038/s41598-019-46692-w

[16] Kanneganti P, Harris JD, Brophy RH, Carey JL, Lattermann C, Flanigan DC. The effect of smoking on ligament and cartilage surgery in the knee: a systematic review. Am J Sports Med [Internet]. 2012 Dec [cited 2013 Feb 19];40(12):2872-8. Available from: http://ajs.sagepub.com/content/40/12/2872 DOI: 10.1177/0363546512458223

[17] Klimek, B., Tarasek, A., & Hajduk, J. (2015). Trace element concentrations in lichens collected in the Beskidy Mountains, the Outer Western Carpathians. Bulletin of environmental contamination and toxicology, 94(4), 532–536. doi: 0.1007/s00128-015-1478-8

[18] Krebs, C. J. (1989). Ecological methodology. New York, NY Harper and Row Publishers Inc., 654 p. - References - Scientific Research Publishing.

[19] Kristanto, P. (2006). Ekologi Industri. Yogyakarta: Universitas Kristen PETRA Surabaya.

[20] Murningsih, & Mafazaa, H. (2016). Jenis-Jenis Lichen di Kampus Undip Semarang. Bioma, 20-29.

[21] Nelsen, M., Lücking, R., Boyce, C., Lumbsch, H., & Ree, R. (2020). The macroevolutionary dynamics of symbiotic and phenotypic diversification in lichens. Proceedings Of The National Academy Of Sciences, 117(35), 21495-21503. doi: 10.1073/pnas.2001913117

[22] Noer, I. S. Bioindikator Sebagai Alat Untuk Menengarai Adanya Pencemaran Udara. Forum Komunikasi Lingkungan III. 2004, Kamojang, Bandung.

[23] Nylander, W., 1866. Les Lichen du Jardin du Luxembourg. Bull. Soc. Bot 13, 364–372.

[24] O'Campo P, Dunn JR, editors. Rethinking social epidemiology: towards a science of change. Dordrecht: Springer; 2012. 348 p.

[25] Odum, E.P. 1993. Dasar-dasar Ekologi. Terjemahan Tjahjono Samingan. Edisi Ketiga. Yogyakarta: Gadjah Mada University Press.

[26] Panjaitan, D. M., Fitmawati, & Martina, A. (2012). Keanekaragaman Lichen sebagai Bioindikator Pencemaran Udara di Kota Pekanbaru Provinsi Riau. Keanekaragaman Lichen sebagai Bioindikator Pencemaran, 1-17.

[27] Pinho, P., Augusto, S., Branquinho, C., Bio, A., Pereira, MJ, Soares, A., & Catarino, F. (2004). Mapping lichen diversity as a first step for air quality assessment. Journal of Atmospheric Chemistry, 49(1), 377-389

[28] Putra, D. P., Sulandari, E., & Said. (2013). Analisis Hubungan Antara Kemacetan dan Polusi Udara di Jalan Sultan Abdurahman Pontianak. 1–11.

[29] Policina, M. S. & dela Cruz TEE (2020). Influence of cardinal directions on corticolous myxomycetes associated with Swietenia macrophylla King. Karstenia, 58(2), 201-214.

[30] Rankovic B, Misic M, Sukdolak S (2007a) Antimicrobial activity of the lichens Cladonia furcata, Parmelia caperata, Parmelia pertusa, Hypogymnia physodes and Umbilicaria polyphylla. Br J Biomed Sci 64:143–148

[31] Rasyidah (2018). Kelimpahan Lumut Kerak (Lichenes) Sebagai Bioindikator Kualitas Udara Di Kawasan Perkotaan Kota Medani : UIN Sumatra Utara

[32] Schiraldi GR. Post-traumatic stress disorder sourcebook: a guide to healing, recovery, and growth [Internet]. New York: McGraw-Hill; 2000 [cited 2006 Nov 6]. 446 p. Available from: http://books.mcgraw-hill.com/getbook.php?isbn=0071393722&template=#toc DOI: 10.1036/0737302658

[33] Scheiber, I. F. , Mercer J. F., and Dringen R.. 2014. Metabolism and functions of copper in brain. Prog. Neurobiol. 116:33–57.

[34] Šestinová, O., Hančulák, J., & Špaldon, T. (2019). Earthworms as useful bioindicator of soils contamination around Košice city, Slovakia. Nova Biotechnologica et Chimica, 18(1), 10-17. DOI: 10.2478/nbec-2019-0002. S

[35] Smith, G. L., & Baker, T. R. (2003). Lichens as Bioindicators. Science Scope, 16–19.

[36] Stacey, V, Majetic CJ, & Carson W. 2018. Percentage cover of lichens and bryophytes on three host tree species in the Upper Peninsula of Michigan and Northern Wisconsin. The Great Lakes Botanist 57: 15–22.

[37] Standen, K., Chambers, P., Culp, J., 2017. Arrowhead (Sagittaria cuneata) as a bioindicator of nitrogen and phosphorus for prairie streams and wetlands. Wetl. Ecol. Manag. 26 (3), 331–343. DOI: 10.1007/s11273-017-9576-5.

[38] Stem cells in the brain [television broadcast]. Catalyst. Sydney: ABC; 2009 Jun 25.

[39] Stockhausen L, Turale S. An explorative study of Australian nursing scholars and contemporary scholarship. J Nurs Scholarsh [Internet]. 2011 Mar [cited 2013 Feb 19];43(1):89-96. Available from: http://search.proquest.com.ezproxy.lib.monash.edu.au/docview/858241255?accountid=12528

[40] Subbarao M. Tough cases in carotid stenting [DVD]. Woodbury (CT): Cine-Med, Inc.; 2003. 1 DVD: sound, color, 4 3/4 in.

[41] Yurnaliza. (2002). Lichenes (Karakteristik, Klasifikasi, dan Kegunaan). Universitas Sumatera Utara, 1-12.

B.13

Synchronization of cell division in root tips of *Oryza sativa* L. cv. Sertani 13 for cell cycle study using flow cytometry

Tessa Fauziah[1,2]; Dina Hermawaty[1]; Karlia Meitha[1]; Rizkita Rachmi Esyanti[1]; Muryanto[3], *Iriawati[1]

1. School of Life Sciences and Technology, Bandung Institute of Technology, Bandung, West Java 40132, Indonesia
2. Faculty of Agriculture, University of Singaperbangsa Karawang, Karawang, West Java 41361, Indonesia
3. PT East West Seed Indonesia

Correspondence should be addressed to Iriawati; E-mail address: iriawati@itb.ac.id

Abstract

Hydroxyurea (HU) for synchronizing cell cycle has been applied in several plant species, including in the root of rice (*Oryza sativa*). However, there is a considerable lack of data on cell cycle progression post-synchronization. The present work aims to establish a cell synchronization method for rice root tips using HU and analyze cell cycle kinetic data post-synchronization using flow cytometry. Six days old rice seedlings were immersed in different concentrations of HU solution (0, 0.5, 1, 1.5, 2, 2.5, 3, and 5 mM) for 18 hours at room temperature. Using the optimal HU concentration, root samples were collected every 3 hours for 18 hours after removal from the HU solution to observe cell cycle progression using flow cytometry. We found that cell cycle synchronization at S-phase was obtained after the root was exposed to 2 mM and 2.5 mM HU for 18 hours. The root tips cells start resuming cell cycle progression 3 hours after release from HU treatment. Fraction of cells at G2 and G1 phase were observed at the maximum at 3 and 15 hours post release from HU treatment, respectively. This study demonstrates the effectiveness of HU in synchronizing the cell cycle at the root tips of rice and the time spent for root tip cells to complete one cell cycle process. This synchronization system will be useful to elaborate on the effect of biotic or abiotic disturbance on the root cell cycle and its consequences on molecular regulation of cell cycle.

Keywords: Cell cycle, flow cytometry, HU, *Oryza sativa*, synchronization

1. Introduction

Duplication of cells in somatic tissues occurred via mitotic division. In this process, two daughter cells genetically identical to their parent cell are produced from a series of events known as the cell cycle [1]. The plant cell cycle consists of four distinct phases: G1 (postmitotic interphase), S (DNA synthesis phase), G2 (premitotic interphase), and M (mitosis/cytokinesis) [2]. Cells grow and expand in size during the G1 phase, while the RNA and protein required for DNA replication are synthesized in the S phase [3]. During the S phase, DNA is duplicated so that the two daughter cells inherit identical genetic material [4, 5]. In the G2 phase, the cell harbour double the number of chromosomes (4n) compared to the G1 phase (2n). Lastly in the M phase, two daughter cells with an identical composition of chromosomes are produced through cytokinesis [5].

Cell cycle progression in most somatic tissues is asynchronous, with only a fraction of cells cycling Dolezel et al. [1]. The root, for example, consists of root apical meristematic, elongation, and maturation zone, each with different cell cycle status [6]. Therefore, it is often complicated to analyze plant cell cycle progression. Synchronizing the cell cycle progression has made identifying the cell cycle machineries possible. Cell cycle synchronization is commonly achieved by applying chemical agents, such as HU, that affect (arrest) the progression of the cell cycle at a particular phase [7]. HU is an anti-proliferative chemical that synchronizes cells at the S-phase [8]. HU inhibit DNA synthesis by preventing the conversion of ribonucleotides into deoxyribonucleotides through ribonucleotide reductase (RNR) inactivation and formation of nitroxide-free radical that binds and inhibits the enzyme active site [9]. So thus, HU decreases the cellular dNTP levels, which causes slows replication forks and arrests the cell cycle in S phase [10]. The root architecture allows an easy separation between dividing cells (cells at the tip of the root) and differentiated (presence of root hair) and hence an excellent system for studying cell synchronization. The root tip has been used for studying synchronization in several plant species, including *Oryza sativa* [11]. However, there is a considerable lack of data on cell cycle progression post-synchronization. In this study, we conducted an analysis of cell cycle kinetic data post-synchronization using flow cytometry on control and HU-treated rice root tip.

2. Materials and Methods

2.1 Plant Material

A local Indonesian rice cultivar, Sertani 13, was obtained from a commercial seed supplier. The seed was imbibed for 24 hours in distilled water, followed by transfer to a 2 mm mesh installed on top of a water bucket (**Figure 1**). The imbibed seed was first grown in the dark for 24 hours before receiving natural light for 3-4 days until the root grew to 4-5 cm in length.

2.2 Methods

2.2.1 Cell cycle synchronization

Cell cycle synchronization was performed on seedlings with a 4–5 cm root length (approx. 6 days old). Grown seedlings on mesh were transferred to a bucket filled with distilled water supplemented with freshly added HU in different concentrations (0, 0.5, 1, 1.5, 2, 2.5, 3 and 5 mM). Seedlings were incubated with HU solution for 18 hours at room temperature. Following HU treatment, the root was washed three times by dipping it into distilled water. Root samples were collected every 3 hours after removal from the HU solution for 18 hours to observe cell cycle progression using flow cytometry.

2.2.2 Flow cytometry

Nuclei preparation and flow cytometry analysis followed the method described by Hermawaty et al. [12] with modifications. A pooled 1 cm root tip from 10 seedlings (approx. 30 mg) per replicate was chopped using a sharp razor blade in 1 mL cold nuclear isolation buffer (MgSO4.7H2O 10 mM, KCl 50 mM, HEPES, triton X-100 and PVP10 1% (w/v)) until fine fragments were obtained, and then subsequently passed through 40 μm nylon mesh filter (Biologix, USA, 15-1040) in 50 mL conical. RNase A (Thermo Scientific, Australia, EN0531) at a final concentration of 50 $\mu g.mL^{-1}$ were added, followed by 6 minutes of incubation at room temperature to remove RNA from nuclei suspension. RNase-treated nuclei suspension was divided equally into two parts: one for the stained sample and the other for unstained negative control. For stained nuclei, propidium iodide (PI; Sigma-Aldrich #P4170) was added to a final concentration of 20 $\mu g.mL^{-1}$. For each treatment group, three biological replicates were prepared. Nuclear DNA content was measured using a single-sample system Guava® easyCyte (Merk, Germany) flow cytometer emitting a 488 nm laser beam at 50 mW laser at 300 - 500 events/second flow rate. The PI fluorescence light was collected using a 664/20 nm filter, and data acquisitions were done using InCyte™ software. A linear forward scatter area (FCS-A), side scatter area (SSC-A), and PI-fluorescence pulse area (FL2-A) values were used to obtain the PI-positive nuclei. Up to 5000 single PI positive nuclei per sample was collected, used to generate a univariate FL2-A histogram, and analysed by Floreada.io cytometry analysis software (https://floreada.io/) to calculate the percentage of nuclei at each cell cycle phase.

2.2.3 Statistical Analysis

A completely randomized design was used with 3 replications for HU concentration and HU recovery experiment. One-way ANOVA tested the results at a 95% confidence level and Duncan's Multiple Range Test ($p< 0.05$) using IBM SPSS Statistics 22.0.

3. Results and Discussion

3.1 HU triggers synchronization of rice root tip at S-phase

HU acts as a DNA synthesis inhibitor, resulting in the accumulation of cells in the S phase. Therefore, we focused on HU concentration, which can accumulate cells in the S phase. The average percentage of cells in the S phase without HU treatment (control) was 18,14% (Figure 1). HU concentrations less than 2 mM and greater than 2,5 mM had a minor effect on the accumulation of cells in S phase. However, the largest proportion of cells in S phase was observed at 2 mM and 2,5 mM HU solutions, which had similar effectiveness (24,68% and 22,83, respectively). This result indicates that in *Oryza sativa* L. cv. Sertani 13, partial synchrony was achieved by applying 2 mM or 2.5 mM HU block for 18 hours. Therefore, 2.5 mM HU was used in the subsequent experiment. This result is consistent with a study conducted by Cajero et al. [6], reporting that application of HU at low concentrations resulted in a partial synchronization, but when used at high concentrations, it became toxic to cells. Musialek and Rybaczek [15] reported that HU can inhibit the activity of ribonucleotide reductase (RNR) that catalyzes the production of new deoxyribonucleotides (dNTPs) by disrupting the proton-coupled electron transfer. Therefore, HU prevents the synthesis of daughter strands of DNA and, as a result, causes cell cycle arrest and checkpoint activation. A higher dosage of HU or prolonged HU exposure may affect the integrity of the DNA.

The HU concentration and incubation time in synchronizing the cell cycle vary among species. Incubation at 1.25 mM HU for 10 hours can synchronize Chinese fir root tips over 30% [13]. In Vicia faba, 2.5 mM HU for 18 hours of incubation can synchronize the cell at the root tip Dolezel et al. [1]. The optimal HU treatment was 2 mM for 18 hours in *Saccharum* spp [14], as also observed in this research.

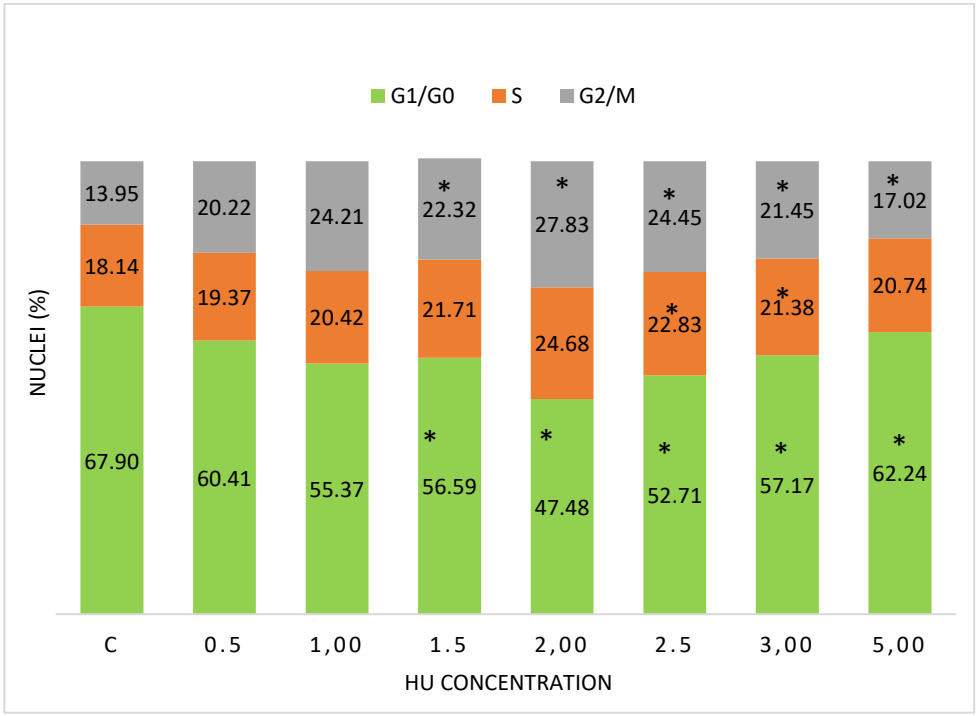


Figure 1 The proportion of cells at each cell cycle phase after 18 hours of 2 mM HU treatment in several concentrations at room temperature and without HU treatment (C, control). Asterisks indicate significant differences at $p < 0.05$ according to a Duncan Multiple Range Test ($n = 10$).

3.2 Recovery processing

Analysis of cell cycle progression post synchronization was conducted to get recovery process of root tip cells after HU removal. In the recovery stage, flow cytometry analysis was performed at 3 hours interval after HU removal for 18 hours to get incubation time in water (without HU) would yield the highest portion of cell in the G2/M phase. The result showed that the maximum proportion of G2/M phase cells was 37.54% after 3 hours of HU-free treatment (Figure 2). We concluded that rice roots resumed cell cycle progression 3 hours after the removal of 2.5 mM HU and that 2.5 mM HU is the optimal concentration for synchronizing root tip cells of *Oryza sativa*. Dolezel et al. [1] reported that when the concentration is higher than the minimum dose required to inhibit DNA synthesis, it may result in delayed recovery and poor synchrony. On the contrary, when HU concentration is below the optimal dose for S-phase syncronization, cells may escape the S-phase before HU is removed. After escaping HU treatment, the cell entered the G1 phase at 6 hours and continued until 15 hours after HU-free treatment. Then, cells enter G2 again after

18 hours after HU-free treatment. This result indicated that the cell completes its cycle in about 15 hours. Recovery time from HU was different across plant species. In *V. faba* root tips, the cells escaped synchronously from blockage about 2 hours after removal from an 18 hours 1.25 mM HU treatment Dolezel et al. [1]. In *Saccharum spp*., recovery time was obtained at 3.5, 2.5 and 1.5 hours after 2 mM HU treatment for 18 hours, at 25°C, 28°C, and 30°C, respectively Yang et al. [14].

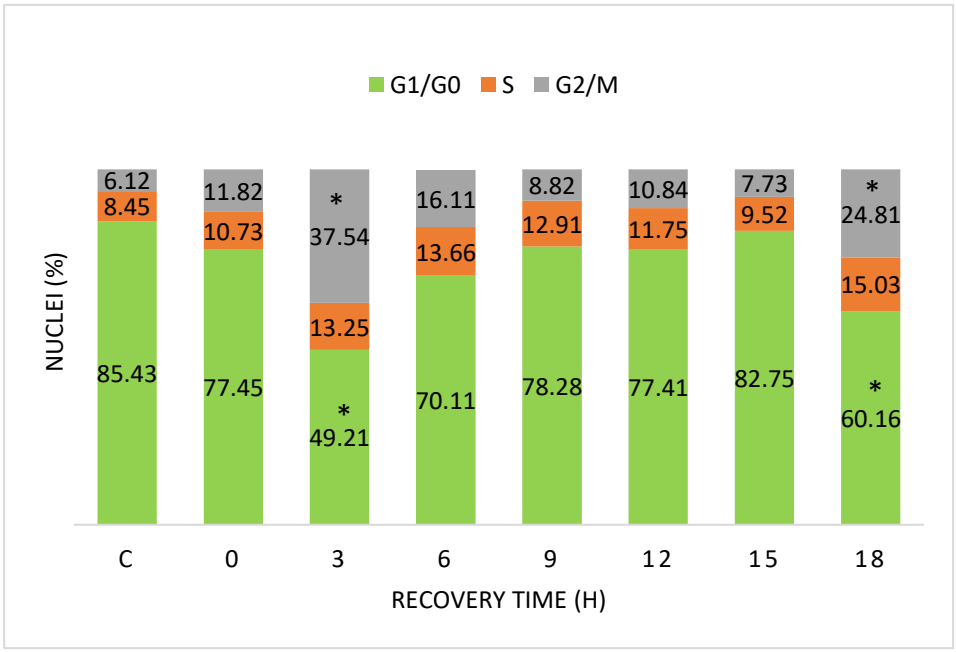


Figure 2. Proportion of cells in different phases after different recovery time treatments. ANOVA test results showed a p-Value <0.05. Asterisks indicate significant differences at $p < 0.05$ according to a Duncan Multiple Range Test ($n = 10$).

4. Conclusions

In the root tip of *Oryza sativa* L. cultivar MSP 13, cell cycle synchronization at S-phase was optimum after the root was exposed to 2 mM and 2.5 mM HU for 18 hours. At 2.5 mM of HU, cells at the root tip start resuming cell cycle progression 3 hours after the release from HU treatment and complete its cycle in about 15 hours. This synchronization system will be a valuable tool to study the effect of biotic or abiotic disturbance on the root cell cycle and its consequences on molecular regulation of cell cycle.

Acknowledgements

We would like to thank the Institute for Research and Community Services of ITB (LPPM-ITB) for the P3MI 2022 grant to the Plant Sciences and Biotechnology Research Group, and also the Biotechnology Laboratorium Core Staff at PT East West Seed Indonesia (Purwakarta, West Java, Indonesia) for their valuable assistance during the flow cytometry experiment.

References

[1] Dolezel, J., Cihalikova, J., Weiserova, J., & L.ucretti, S. Cell cycle syncnhronization in plant root meristems. *Methods in Cell Science*. **1999**, 21, 95–107.

[2] Qi, F., & Zhang, F. Cell cycle regulation in the plant response to stress. *Frontiers in Plant Science*. **2020**, 10, 1765.

[3] Lodish, H., Berk, A., Kaiser, C. A., Krieger, M., Bretscher, A., Ploegh, H., Amon, A., & Martin, K. C. *Molecular Cell Biology*. *8th ed*. New York: Latherine Ahr Parker; **2016**, 1166 p.

[4] Reece, J. B., Urry, L. A., Cain, M. L., Wasserman, S. A., Minorsky, P. v., & Jackson, R. B. *Biology. 10th ed.* New York: Pearson; **2014**, 1254 p.

[5] Han-shu, F., Ming-fei, L., & Jing, S. U. N. New methods for cell cycle analysis. *Chinese Journal of Analytical Chemistry*. **2019**, 47(9), 1293–1301.

[6] Cajero Sánchez, W., García-Ponce, B., Sánchez, M. de la P., Álvarez-Buylla, E. R., & Garay-Arroyo, A. Identifying the transition to the maturation zone in three ecotypes of *Arabidopsis thaliana* roots. *Communicative and Integrative Biology*. **2018**, 11(1), 1-7.

[7] Ligasová, A., & Koberna, K. Strengths and weaknesses of cell synchronization protocols based on inhibition of dna synthesis. *International Journal of Molecular Sciences.* **2021**, 22, 10759.

[8] Pan, W. H., Houben, A., & Schlegel, R. Highly effective cell synchronization in plant roots by HU and amiprophos-methyl or colchicine. *Genome*. **1993**, 36(2), 87–390.

[9] Tate, S., & Ko Ferrigno, P. Cell Cycle: Synchronization at various stages. *In eLS. Wiley*.

[10] Xu, Y. J., Singh, A., & Alter, G. M. HU induces cytokinesis arrest in cells expressing a mutated sterol-14α-demethylase in the ergosterol biosynthesis pathway. *Genetics***. 2016**, 204(3), 959-973.

[11] Kaeppler, H. F., Kaeppler, S. M., Lee, J.-H., & Arumuganathan, K. Synchronization of cell division in root tips ofseven major cereal species for high yields of metaphase chromosomes for flow-cytometric analysis and sorting. *Plant Molecular Biology Reporter*. **1997,** 5(2), 141-147.

[12] Hermawaty, D., Considine, J. A., & Considine, M. J. An evaluation of nuclei preparation of the dormant axillary bud of grapevine for cell cycle analysis by flow cytometry. *Frontiers in Plant Science*. **2022**, 13.

[13] Shen, J., Xu, J., Chen, J., Zheng, R., & Shi, J. Cell synchronization and isolation of chromosomes from chinese fir root tips for flow cytometric analysis. *Biotechnology Letters*. **2015**, 37(6), 1309-1314.

[14] Yang, S., Zeng, K., Luo, L., Qian, W., Wang, Z., Doležel, J., Zhang, M., Gao, X., & Deng, Z. A flow cytometry-based analysis to establish a cell cycle synchronization protocol for *Saccharum* spp. *Scientific Reports*. **2020**, 10, 5016.

[15] Musiałek, M.W., & Rybaczek, D. Hydroxyurea—the good, the bad and the ugly. *Genes*. **2021**, 12, 1096.

B.14

Analysis of the Auxin Response Factor Gene due to Drought Stress in Banana Shoot-tip in vitro Culture (*Musa acuminata* cv. Barangan)

Simon Duve, Husna Nugrahapraja, Erly Marwani, Sri Nanan B. Widiyanto*

School of Life Sciences and Technology, Institut Teknologi Bandung

*Corresponding author email: srinanan@sith.itb.ac.id

Abstract

Banana plant responses to environmental stresses are extremely complex and involve changes at the transcriptome, cellular and physiological levels to prevent damage and ensure survival. One of the defensive mechanisms of banana plants against drought stress is by regulating gene expression using transcription factors. Auxin response factors (ARFs) related genes have been identified to have increased expression in drought-stressed banana plants. ARFs are an important family of transcription factors involved in the exertion of auxin in plants and play a key role in regulating the growth and development of vegetative and reproductive organs such as roots, stems, leaves, flowers, fruits, and seeds. To date, however, their function and responses to drought stress in banana are rarely known. Analysis of MaARF gene needs to be done to determine its structural characteristics and function. The result then were compared to *Arabidopsis thaliana* database. The compaison were based on structure and physicochemical characteristics. Analysis of gene expression were done using qPCR. The analysis showed that ARF in *Musa acuminata* and *Arabidopsis thaliana* have a similar structure with different physicochemical characteristics. Results suggest that ARF in Musa and Arabidopsis have different domain binding DNA, although the function is the same that is to control auxin response genes. Our findings reveal a positive regulatory of ARF gene in banana responses to drought stresses.

Keywords: Auxin response factor, drought stress, Musa acuminata, transcription factor

1. Introduction

The *Musa acuminata* cv. Barangan, commonly known as Pisang Barangan, is a tropical plant that highly beneficial for the population of Indonesia. Its fruit, which is not only tasty but also easily accessible and cultivated, is often underestimated by farmers in terms of its business potential due to its easy growth. Many farmers grow bananas on what's known as marginal lands, leading to subpar yields. Such lands typically have clayey and arid soils, challenging the plant's water intake (Suharta, 2010). Consequently, banana production suffers, with many fruits going unsold. One of the main challenges for banana plants on these lands is the scarce capillary water, due to the compactness and small particle size of the soil. This scarcity affects the ion exchange in the

roots and disrupts the osmotic balance with the environment, causing the plants to release water through transpiration, ultimately leading to drought conditions (Santos et al., 2018). To survive in drought stress, plants activate the auxin response genes with the auxin response factor (ARF). The transcriptomic analysis already done in *Musa acuminata* exposed to drought stress and showed that ARF gene were upregulated (Widiyanto et al., 2019). However, the specific ARF type that responds to drought stress in banana plants is yet to be identified.

Auxin plays an important role in plant growth and development, including shoot elongation, lateral root formation, vascular tissue differentiation, apical margin patterning, and response to environmental stimuli (Liu et al., 2018). Auxin response factors (ARFs), a critical family of transcription factors in auxin-mediated pathway, encode transcriptional factors that bind specifically to the TGTCTC containing auxin response elements found in the promoters of primary/early auxin response genes that regulate plant development (Tiwari et al., 2003). Analysis of auxin response factor in *Musa acuminata* cv. Barangan needs to be done to determine its phylogenetics relationship, characteristics, structure predictions, and validate the expression level using qPCR as a study in understanding the defensive mechanism of banana plants against drought stress.

2. Materials and methods

2.1 Phylogenetics Analysis

Transcriptome data obtained from Plant Science and Biotechnology research group SITH ITB. The transcriptome data obtained from banana (*Musa acuminata* cv. Barangan) plantlet that were induced by drought stress. Drought stress induced by giving PEG 6000 solution (2.5%; 7.5%; and 10%) which is equivalent to drought conditions with a potential water of -0.14, -0.83, bar and -1.48 bar (Nugraheni, 2013). Phylogenetic analysis was carried out on the mRNA sequence at all type of auxin response factor in *Arabidopsis thaliana* and sequence that got from transcriptome data for *Musa acuminata* with actin as outgroup. The mRNA sequence of the auxin response factor protein was obtained from the NCBI website (https://www.ncbi.nlm.nih.gov/). The mRNA sequence used came from ARF 1 to ARF 23 in *Arabidopsis thaliana*. Alignment was carried out on all mRNA sequences using MEGA7 software to create phylogenetic trees based on the maximum likelihood method with bootstrap 100 replications. The distance in the phylogenetic tree is represented by a scale indicating evolutionary distance and the substitution number indicates the degree of confidence of the formed phylogenetic tree.

2.2 Protein Structure Analysis

Protein structure analysis was carried out by comparing the structure of the auxin response factor protein in *Musa acuminata* plant with *Arabidopsis thaliana* model plants. Analysis was performed by predicting secondary protein structures and binding site prediction with PredictProtein (https://open.predictprotein.org/). The tertiary structure of proteins was predicted with SwissModel (http://swissmodel.expasy.org/interactive). Analysis of

physicochemical properties consists of hydrophobicity, solubility, isoelectric point, and protein molecular weight. Protein hydrophobicity was analyzed by Kyte-Dollylte Hydropathy Plot (https://fasta.bioch.virginia.edu/fasta_www2/fasta_www.cgi? rm=misc1), protein solubility was analyzed by Protein Sol (https: //protein-sol.manchester.ac.uk/), and the isoelectric point and molecular weight were analyzed by Compute pi / Mw (http://web.expasy.org/ compute_pi/).

2.3 qPCR Analysis and Determination of Gene Expression Levels

For qPCR validation of gene expression levels, total RNA was isolated from the independent experiment. The RNA extracted from four samples with different drought stress treatment using CTAB methods described by (Diningrat et al., 2015). RNA quality and quantity was measures using Nanodrop™ Lite Spectrophotometer (Thermo Scientific, USA) and confirmed by gel electrophoresis in 1.5% agarose. The cDNA was reverse transcribed of total RNA using GoScriptTM reverse Transcription kit (Promega, USA). The cDNA synthesis reaction was done by incubation for 5 minutes at 25 °C, followed by incubation for 1h at 42 °C and deactivated of reverse transcriptase for 15 minutes at 70 °C. Auxin response factor gene and housekeeping gene (betatubulin) were designed using Primer3Plus (Table 1). Chosen parameters were product size range 100–250 bp, primer size 20–22 bp, primer Tm 57–60 °C (with maximum Tm difference = 2 °C) and GC content 45–60%. Validation of gene expression was performed by reverse transcriptase RT-qPCR using QuantStudio 1 (Thermo Scientific, USA). The qPCR was performed using GoTaq® qPCR Master Mix according to the manufacture manual (Promega, USA). The PCR reaction was done following procedure from Diningrat et al. (2015), with some optimization. The reaction program was started with predenaturation at 95 °C for 15 minutes, followed by 40 cycles of polymerization (15 s at 95 °C, 30 s at 58 °C and 30 s at 72 °C). Three technical replicates were performed in the RT-qPCR assay.

3. Results and discussion

3.1 Phylogenetic Analysis

The auxin response factor from *Arabidopsis thaliana* compared to *Musa acuminata* produced a phylogenetic tree showing the proximity of auxin response factor from *Musa acuminata* to ARF 10 in *Arabidopsis thaliana* (Figure 1). Overall, ARF grouped in three clades, while ARF in *Musa acuminata* grouped with ARF 10, ARF 16 and ARF 17. Phylogenetic analysis was used using maximum likelihood method with bootstrap 100 times. Maximum likelihood is an analysis based on character method where it's using all sequences, both informative and non-informative by calculating the probability of gene changes occurring in alignment (Xiong, 2016). This method will be effective because it can compare a family gene in one organism so ARFs can be classified based on their sequence.

ARF in *Arabidopsis thaliana* can be grouped in three classes base on their activator/repressor (Cancé et al., 2022). The class A ARFs, regarded as activators, carry MRs that are enriched in

glutamines, while MR in class B and C ARFs have a strong enrichment in serines, prolines, and threonines. This observation has not yet gone beyond a correlation, and it is unclear what mechanisms underlie activation and repression (Roosjen et al., 2018). The ARF 10, ARF16, and ARF17 in *Arabidopsis thaliana* were classified in class C with DNA binding affinity activator so ARF that found in *Musa acuminata* affected by drought stress could be have a same function base on phylogenetic analysis.

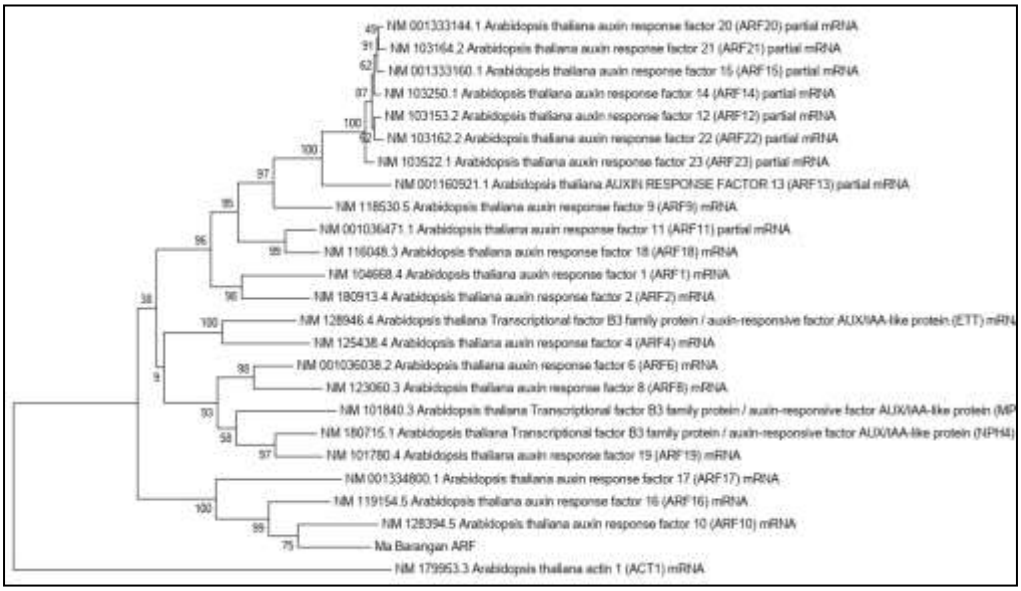


Figure 1. Phylogenetic trees were arranged by maximum likelihood method using actin as outgroups and bootstrap 100 times.

3.2 Protein Structure Analysis

The results of the auxin response factor protein analysis showed the prediction of differences in the DNA binding site, while the structure have a similar property. The secondary and tertiary structure showed that ARF in *Musa acuminata* and ARF10 in *Arabidopsis thaliana* have a similar structure and it's compatible with phylogenetic analysis. Analysis of physicochemical properties between the two ARFs showed hydrophobicity which was both hydrophobic (Table 1). The solubility level and the isoelectric point are higher in *Arabidopsis thaliana*, while the higher molecular weight is in *Musa acuminata*.

All ARFs possess at their N-terminus a conserved DNA binding domain (DBD), followed by a middle region (MR) and a C-terminal PB1 domain (formerly called domain III/IV) (Y. Liu et al., 2022). The specificity of a TF for DNA determines which genes are bound and thus, controlled by it. Therefore, ARF-DBDs and their DNA binding properties will be fundamental in deciding which genes are regulated, or not, by auxin (Cancé et al., 2022). In between the N-terminal DBD

and the C-terminal domain, ARFs exhibit a region of variable length named the middle region (MR) that determines the way they are regulated. Contrary to the high conservation observed for the DBD, ARF-MRs are very variable domains (Wu et al., 2011). Still, some general properties characterize the MRs from different ARF classes. The C-terminal ARF domain mediates interactions with Aux/IAA proteins and determine the activity of ARF based on auxin content. Regulation of auxin output is executed by ARFs. Under low auxin levels, the Aux/IAA transcriptional co-repressors prevent ARFs from controlling auxin-regulated genes (Roosjen et al., 2018). When auxin levels increase, auxin serves as 'molecular glue' between the TIR1/AFB receptor and the Aux/IAA protein. This leads to subsequent ubiquitination and degradation of the Aux/IAAs, releasing ARFs from inhibition.

Table 1. Comparison of the physicochemical properties of auxin response factor between *Arabidopsis thaliana* and *Musa acuminata* model plants.

Characteristics	*Arabidopsis thaliana*	*Musa acuminata*
Hydrophobicity	Hydrophobic	Hydrophobic
Solubility	0,187	0,184
Isoelectric point (Pi)	7,73	7,01
Molecular mass (mw)	76720,88	78541,02

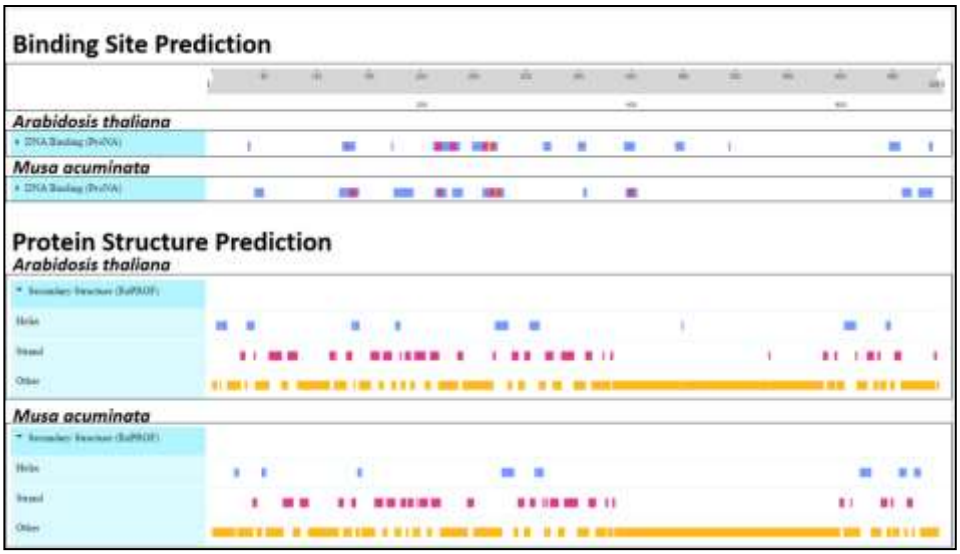


Figure 2. Prediction of protein structure and binding site of the auxin response factor.

3.3 qPCR Analysis and Determination of Gene Expression Levels

To confirm the accuracy and the robustness of analysis, ARF gene was validated using qPCR method. Therefore, an independent experiment was set up with three different PEG concentration and control treatment. In general, relative gene expression values were similar with mRNA-seq. ARF gene in every drought stress level was upregulated and showed that the sequence can be validated.

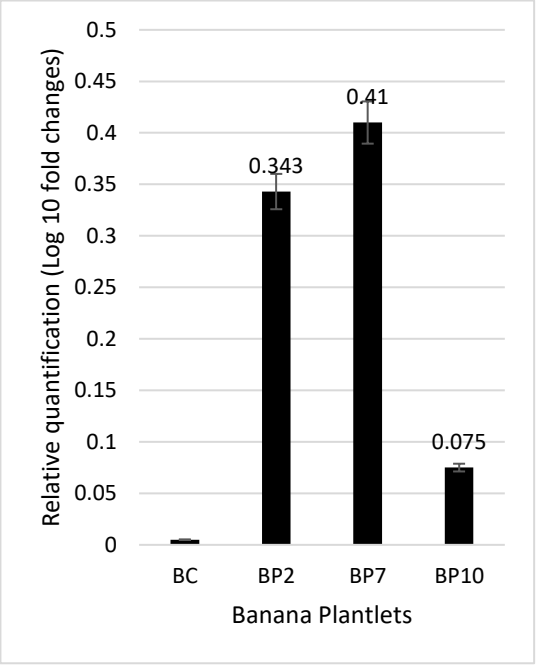


Figure 3. Auxin response factor gene in *Musa acuminata* were validated using qPCR with betatubulin as housekeeping genes (BC: Control; BP2: PEG 2.5%; BP7: PEG 7.5%; and BP 10: PEG 10%).

4. Conclusions

The auxin response factor in *Musa acuminata* belongs to the same clade as ARF10 in *Arabidopsis thaliana*. Differences in the structure of auxin response factor are at the DNA binding site. Different physicochemical characteristics exist in all aspects of the analysis but have the same characteristics. The ARF sequence in *Musa acuminata* can be validated using qPCR analysis and showed an upregulated properties in all drought stress level.

Acknowledgements

Thank you to the KK SBT SITH ITB, especially for Prof. Sri Nanan B. Widiyanto, Dr. Erly Marwani and Dr. Husna Nugrahapraja for their guidance. Thanks also to Ristekdikti in the PMDSU program for funding this research.

References

[1] Cancé, C., Martin-Arevalillo, R., Boubekeur, K., and Dumas, R. 2022. Auxin response factors are keys to the many auxin doors. *In New Phytologist*, **235**, 402 – 419.

[2] Diningrat, D.S., Widiyanto, S.M., Pancoro, A., Iriawati, and Shim, D. 2015. Identification of Terminal Flowering 1 (TFL1) genes associated with the teak (Tectona grandis) floral development regulation using RNA-seq. *Res. J. Bot.*, **10**, 1 – 13.

[3] Liu, N., Dong, L., Deng, X., Liu, D., Liu, Y., Li, M., Hu, Y., and Yan, Y. 2018. Genome-wide identification, molecular evolution, and expression analysis of auxin response factor (ARF) gene family in Brachypodium distachyon L. *BMC Plant Biology*, **18**, 1 – 15.

[4] Liu, Y., Zhang, J., Li, X., Zhu, L., Lian, Z., Fang, H., Lu, L., Lu, Y., Shi, J., Chen, J., Hao, Z., and Cheng, T. 2022. The identification and expression analysis of the *Nitraria sibirica* pall. auxin-response factor (ARF) gene family. *International Journal of Molecular Sciences*, **23**, 11122.

[5] Nugraheni, T. 2013. Pengaruh pemberian cekaman kekeringan terhadap pertumbuhan tunas aksiler pada kultur pucuk *in vitro Musa acuminata* cv mas dan cv barangan. Skripsi. Sekolah Ilmu dan Teknologi Hayati. Institut Teknologi Bandung.

[6] Roosjen, M., Paque, S., and Weijers, D. 2018. Auxin Response Factors: Output control in auxin biology. *In Journal of Experimental Botany*, **69**, 179 – 188.

[7] Santos, A.S., Edson, P.A., Claudia, F.F., and Carlos, P.P. 2018. Water stress in Musa spp.: a Systematic review. *Plos One*, **13**, 1 – 17.

[8] Suharta, N. 2010. Karakteristik dan permasalahan tanah marginal dari batuan sedimen masam di kalimantan. *Jurnal Litbang Pertanian*, **29**, 139 – 146.

[9] Tiwari, S. B., Hagen, G., and Guilfoyle, T. 2003. The roles of auxin response factor domains in auxin-responsive transcription. *Plant Cell*, **15**, 533 – 543.

[10] Wu, J., Wang, F., Cheng, L., Kong, F., Peng, Z., Liu, S., Yu, X., and Lu, G. 2011. Identification, isolation and expression analysis of auxin response factor (ARF) genes in Solanum lycopersicum. *Plant Cell Reports*, **30**, 2059 – 2073.

[11] Xiong, Jin. 2006. *Essential Bioinformatics*. Cambridge University Press: New York.

JSPSP

3rd Joint Symposium on Plant Sciences and Products

November 16th- 17th, 2022
ITB Ganesha Campus, Bandung

ISSN 3026-1589