Indonesia International Institute for Life Sciences

ENRICHMENT PROGRAM REPORT

Phycoremediation of Food Waste using Alginate-immobilized *Chlorella vulgaris* FSP-E Biosorbent and Reusing Treated Biomass for Biofertilizer Production

STUDY PROGRAM
Biotechnology

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RESEARCH REPORT

PHYCOREMEDIATION OF FOOD WASTE USING ALGINATE-IMMOBILIZED *CHLORELLA VULGARIS* FSP-E BIOSORBENT AND REUSING TREATED BIOMASS FOR BIOFERTILIZER PRODUCTION

By

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Biotechnology

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Approval Page

I hereby submit the final draft of EP Report as a requirement to participate in EP Final Presentation.

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ABSTRACT

Around 2.5 billion tonnes of food waste are generated globally every year, which is associated with many environmental issues (i.e. greenhouse gas emissions), economic issues, and ethical issues. Thus, a sustainable and innovative approach is implementing alginate-immobilized microalgae to remove contaminants from food waste. Sodium alginate is a gel matrix type used for microalgae encapsulation. This polysaccharide can form gel-like structures in the presence of multivalent cations such as Ca^{2+}. In this research project, *Chlorella vulgaris* FSP-E is encapsulated with sodium alginate and implemented in food waste for phycoremediation. After food waste treatment, the microalgae biomass is reused as biofertilizers, and its performance in assisting plant growth and development is analyzed. After food waste treatment, it is found that the alginate-microalgae beads cause alterations in food waste media color and performed the highest COD removal of 95.7%. However, the beads had alterations on their appearance and some had decreased on bead stability, speculated due to microorganism contamination. The biomass is also discovered to experience a decrease in carbohydrate and lipid composition due to limited nutrient supply. Additionally, the implementation of the biomass as biofertilizer is considered to be insignificant ($p > 0.05$) for lettuce growth mainly due to improper planting techniques of the lettuce.

Keywords: *Biofertilizer; Chlorella vulgaris FSP-E; Encapsulation; Food Waste; Sodium Alginate*

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LIST OF ABBREVIATIONS

BG-11	Blue-green 11
BOD	Biological oxygen demand
COD	Chemical oxygen demand
FW	Food waste
GHG	Global greenhouse gas
SMX	Sulfamethoxazole
TN	Total nitrogen
TOC	Total organic content
TP	Total phosphate
UNEP	United Environmental Programme

I. INTRODUCTION

1.1. Background

Every year, 2.5 billion tonnes of food are wasted globally from human activities. This is detrimental to the human population as this food mass is equivalent to 40% of the annual food production globally (World Fund for Nature, 2021). As stated by the United Environmental Programme (UNEP) Food Waste Journal Pre-proof Index Report (2024), the world's food loss amount correlates with 8% to 10% of the global greenhouse gas (GHG) emissions and ±30% of the world's agricultural land. Most food waste is generated throughout the household due to spoilage, plate leftovers, foods that cross their expiry date, and fresh foods with short shelf life (Pleissner, 2018; Richter and Bokelmann, 2018). If not treated, food waste is predicted to accumulate up to 138 billion by 2025, causing environmental, economic, and ethical issues (Paritosh et al., 2017; Patel et al., 2019). Traditionally, food waste treatment includes incineration, landfilling, composting, and anaerobic digestion (Shukla et al., 2024). However, these treatments are deemed not feasible in the long term due to their environmental pollution, as well as the high operational and capital costs. Thus, more sustainable approaches must be implemented to reduce the amount of food waste globally (Slorach et al., 2019).

Phycoremediation, defined as microalgae-based bioremediation, is a widely used strategy for treating wastewater including food waste. It is considered to be an effective approach as microalgae have the potential to be used as biosorbents, meaning they are able to remove pollutants from food waste through cellular structure assimilation. One of the parameters for food wastewater quality assessment is chemical oxygen demand (COD) removal which measures the COD reduction percentage after treatment. A research by Koutra et al. (2021) which used *Chlorella vulgaris* for agro-industrial and organic waste achieved the highest COD removal of 92%, along with total nitrogen (TN) removal of 77%, and total phosphorus (TP) removal efficiency of 94% that indicates microalgae's capability of assimilating organic matter, nitrogen, and phosphorus respectively. In return, food waste contains nutrients (i.e., carbohydrates, lipids, proteins, nitrogen, and phosphorus) that are also assimilated by microalgae for their proliferation (Phang et al., 2015). Aside from COD, this study thus measures carbohydrate and lipid composition in treated microalgae biomass. Additionally, *Chlorella vulgaris* FSP-E is featured due to its fast growth and ability to survive in a relatively wide range of environmental conditions (Chen et al., 2016).

Microalgal cells can be encapsulated with carrier material or gel matrix that immobilizes them during cultivation. Encapsulation protects the cells from unfavorable environmental conditions (i.e., extreme

temperature and pH, salinity, and toxic pollutants), facilitates pollutant removal from wastewater, increases cell viability, and eases algal biomass recovery compared to suspension-based cultivation (Eroglu et al., 2015; Han et al., 2022). Various gel matrix materials such as natural polymers (e.g., carrageenan, chitosan, alginate, cellulose, and pectin) and synthetic polymers (e.g., polyacrylamide, polyvinyl, polyurethane, and polypropylene) have been incorporated in microalgae encapsulation (Borin et al., 2018; Manzano et al., 2019). These polymer materials must be hydrophilic to allow the wastewater diffusion through the gel matrix. Although synthetic polymers are more stable, natural polymers are preferable due to their higher nutrient and product diffusion rates and environmentally friendly properties (de Jesus et al., 2019).

Among many natural polymers, sodium alginate is a gel matrix type regularly used in microalgae encapsulation. This hydrogel can form bead-like structures when exposed to multivalent cations like Ca^{2+} ions. Alginate is a polysaccharide comprising covalently linked α-L-guluronic (G) acid and β-D-mannuronic (M) acid residues. This anionic polysaccharide is extracted from various brown algae species such as *Sargassum* sp., *Laminaria* sp., and *Durvillaea* sp. (Limrujiwat et al., 2022). The advantages of using alginate matrices for microalgae encapsulation include non-toxicity, transparency, high cell viability retention, cost-effectiveness, and reversibility (de Jesus et al., 2019; Hasnain & Nayak, 2019). Most previous studies implement alginate-encapsulated microalgae in wastewater from the food industry. A research by Anagnostopoulou et al. (2024) which used alginate-encapsulated *Chlorella vulgaris* for brewery wastewater, expired orange juice, and cheese whey treatment reported COD and total organic content (TOC) removal efficiency of 31% and 23% respectively. Another study by Limrujiwat et al. (2022) who utilized alginate-encapsulated cyanobacteria *Synechocystis* sp. for shrimp wastewater treatment exhibited nitrate removal of 90.44% and phosphate removal of 99.35%. This provides a gap for plate leftover food waste which will be the focus of this research.

Aside from food waste phycoremediation utilization, the microalgae biomass could also be used as biofertilizers due to its relatively high nutrient content obtained from the waste, serving as a macronutrient for plant growth and development (Braun & Cola, 2023). A research by Roshidi et al. (2021) which incorporated encapsulated *Scenedesmus* sp. as biofertilizers for *Abelmoschus esculentus* plant managed to show significant plant growth within 2 weeks in terms of height (7.17 + 1.04 cm compared to the control 5.17 + 0.35 cm), leaf size (3.67 ± 0.32 cm compared to the control 2.67 ± 0.20 cm), and leaf number (4 leaves compared to the control 3 leaves). As not many previous studies have evaluated the implementation of the microalgae biomass from food waste treatment as

plant biofertilizer (i.e., lettuce), this study will incorporate similar approaches where the biomass of encapsulated *Chlorella vulgaris* FSP-E beads are reused as biofertilizers for lettuce (*Lactuca sativa*) plants.

1.2. Objectives

This research aims to:

1. Determining if cultivating alginate-encapsulated microalgae beads (*Chlorella vulgaris* FSP-E) in food waste media causes any changes in the appearance of food waste media.
2. Analyzing the impact of varying food waste concentrations (20%, 40%, 60%, 80%, and 100%) on the COD removal performed by alginate-microalgae beads.
3. Assessing the impact of varying food waste concentrations on the appearance of alginate-microalgae beads by comparing the beads' color before and after the treatment.
4. Assessing the impact of varying food waste concentrations on the bead stability of alginate-microalgae beads.
5. Evaluating the nutrient composition (lipid and carbohydrate) of harvested microalgae biomass after treatment in varying food waste concentrations.
6. Analyzing the significance of implementing treated microalgae biomass towards lettuce growth.

1.3. Hypothesis

Based on the objectives, the formulated hypotheses are:

1. **H_0**: Alginate-encapsulated microalgae beads do not cause changes in food waste appearance.
   H_1: Alginate-encapsulated microalgae beads cause changes in food waste appearance.

2. **H_0**: Higher food waste concentrations do not lead to higher COD removal percentages by alginate-microalgae beads.
   H_1: Higher food waste concentrations lead to higher COD removal percentages by alginate-microalgae beads.

3. **H_0**: Alginate-microalgae beads cultivated in higher food waste concentrations do not show color changes compared to lower concentrations.
   H_1: Alginate-microalgae beads cultivated in higher food waste concentrations show color changes compared to lower concentrations.

4. **H_0**: Cultivation in food waste media do not affect bead stability.
   H_1: Cultivation in food waste media affects bead stability.

5. **H_0**: Cultivation in food waste media do not affect microalgae nutrient composition.
   H_1: Cultivation in food waste media affects microalgae nutrient composition.

6. **H_0**: Treated microalgae biomass does not significantly affect lettuce growth.
   H_1: Treated microalgae biomass significantly affects lettuce growth.

1.4. Scope of Research

1. Cultivating microalgae in BG-11 medium prior to encapsulation.
2. Collecting and pre-treating food waste to be used as cultivation media.
3. Mixing alginate with microalgae biomass and dripping the mixture into a calcium chloride solution to obtain microalgae-alginate beads.
4. Cultivate the alginate-encapsulated microalgae (*Chlorella vulgaris*) in different concentrations of food waste (20%, 40%, 60%, 80%, and 100%).
5. Analyzing food waste appearance, COD removal, bead appearance, bead stability, and biochemical composition (lipid and carbohydrate) of the food waste before and after treatment with microalgae-alginate beads.
6. Harvesting treated microalgae biomass, implementing them as biofertilizers, and analyzing their ability to assist lettuce growth.

II. LITERATURE REVIEW

2.1 Food Waste Media for Microalgae Cultivation

2.1.1. Food Waste Growth Conditions and Impact

Food wastewater contains various nutrient substances including carbohydrates (41 - 62%), lipids (13 - 30%), proteins (15 - 25%), nitrogen (0.1 - 1.5%), ammonium (0.01 - 0.5%), and phosphate (0.01 - 0.5%) (Slopiecka et al., 2022; Rautiainen et al., 2023; Zhou et al., 2023). Despite their abundant food waste nutrient content, their contained organic matter also correlates with relatively high amounts of organic matter and chemical oxygen demand (COD) value. COD is an indicator of water quality that measures how much oxygen is consumed in organic matter-degradation reactions by microorganisms. This implies that higher COD values correspond to higher oxygen requirements and organic matter. COD value of food wastewater varies depending on the food waste source: meat industry wastewater is 2780 – 6720 mg/L, slaughterhouse wastewater is 4200 – 8500 mg/L, and food and vegetable wastewater is up to 30000 mg/L. These wastewater are deemed to be highly concentrated as their COD values exceed 2000 mg/l (León-Becerril et al., 2016; Xing et al., 2024). Due to its elevated COD value, food waste can cause dissolved oxygen (DO) depletion if exposed to water bodies, which is detrimental to aquatic organisms (Gana et al., 2022). As reducing the COD value of wastewater is crucial to reducing its environmental impact, COD removal is one of the main food waste treatment parameters assessed in this research.

2.1.2. Pre-treatment of Food Waste

Although food waste contains the necessary nutrients for microalgae growth, these nutrients are still in the form of complex molecules that need to be converted into simpler ones. These can be achieved by subjecting the food waste to pre-treatment to ease nutrient bio-accessibility to be utilized for the metabolism of microalgae (Ramandani et al., 2024). In this section, the discussed pre-treatment approaches are the ones incorporated for this research, including physical pre-treatment (i.e., mechanical pretreatment and thermal pretreatment) and chemical pre-treatment (i.e, acid pre-treatment).

2.1.2.1. Mechanical Pre-treatment

Mechanical pre-treatment of food waste effectively reduces the size of the food waste particles to increase their surface area (Gallego-García et al., 2023). Several techniques that are involved in processing solid food materials into smaller sizes include grinding, milling, and chopping (Feng et al.,

2018). Grinding utilizes mechanical force to break down food materials. This technique is widely used to grind meat (Tool: meat grinder), grains (Tool: food grinding machine), spice (Tool: food grinding machine, mortar and pestle, and spice grinders), and beans (Tool: food grinding machine) (Saravacos et al., 2016; Margasahayam & Balraj, 2018). If the solid food material is mixed with a liquid (e.g., water and broth), blendering can be used for homogenization which results in sludge (Tang et al., 2019). Meanwhile, milling is a process that dehulls and ground grains and beans. Milling tools include ball mills, hammer mills, and roller mills (Jamali et al., 2024). Lastl,y chopping involves cutting food into smaller pieces by using knives, choppers, and other cutting equipment (Raseeta et al., 2022).

2.1.2.2. Thermal Pre-treatment

Thermal pre-treatment involves subjecting the food waste to heat. The operational temperature is considered to be low in temperature ≤ 100^{o}C, while considered to be high in temperature ≥ 100^{o}C (Kavitha et al., 2017; Kannah et al., 2018). Thermal pre-treatment can be also used to hydrolyze food compounds to improve their bioavailability (Ravindran & Jaiswal, 2016; Scherzinger & Kaltschmitt, 2021). Additionally, thermal pre-treatment is also considered to be a sterilization method to remove contaminating microorganisms contained inside the food waste (Shyam & Palaniappan, 2023). Autoclave and pasteurizers are examples of equipment used for thermal pre-treatment (Pagliaccia et al., 2019).

2.1.2.3. Acid Pre-treatment

Acid pre-treatment utilizes acidic chemicals such as hydrochloric acid (HCl), sulfuric acid (H_2SO_4), and acetic acid (CH_3COOH) to hydrolyze food waste materials. This treatment is commonly combined with thermal pre-treatment and the combination depends on the usage of concentrated acid or diluted acid. A review article by Peguero et al. (2022) mentioned that the usage of concentrated acid [≥ 30% (w/v)] is usually combined with lower heat temperatures (≥ 100^{o}C) for several hours. On the other hand, the diluted acid [0.5 - 5% (w/v)] is combined with high temperatures (120 – 215°C) and a few minutes of heating time. Because high acid concentrations often lead to corrosiveness, using diluted is more recommended and implemented in this research.

2.2. *Chlorella vulgaris*

2.2.1. Phenotype of *C. vulgaris*

Chlorella vulgaris is a freshwater unicellular green microalgae in the *Chlorophyta* family. It is characterized by its spherical cell shape with size ranges from 1 - 10 µm (Coronado-Reyes et al., 2020). *C. vulgaris* has a green color as its pigment is dominated by chlorophyll. Specifically, it contains

chlorophyll a (± 2.5 - 3.5% of dry weight; ± 8.45 µg/ml), chlorophyll b (± 0.5 - 1% of dry weight; ± 4.33 µg/ml), and carotenoids (± 0.1 - 0.2% of dry weight) (Oo et al., 2017).

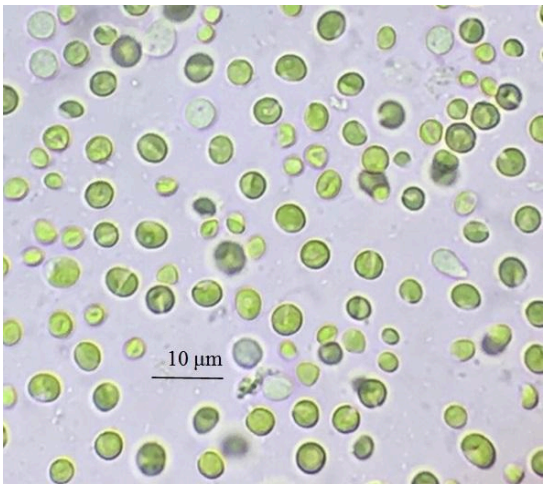


Figure 1 Morphology of *Chlorella vulgaris* (Ramaraj et al., 2016)

2.2.2. *C. vulgaris* role in Food Wastewater Phycoremediation

Due to its ability to metabolize and assimilate pollutants with their cellular structure, *Chlorella vulgaris* have been used in many implementations of wastewater bioremediation. Previous research by Koutra et al. (2021) used *Chlorella vulgaris* for different kinds of digestates (agro-industrial waste digestate, cheese whey, and digestate municipal organic waste) achieved the highest chemical oxygen demand (COD), total nitrogen (TN), and total phosphorus (TP) removal efficiency of 92%, 77%, and 94%, respectively. Another study by Hussain et al. (2024) that incorporated *Chlorella vulgaris* into food industry wastewater managed to obtain COD removal of 80.57%, biological oxygen demand (BOD) of 81.13%, and TN of 55.88%, respectively in 100% food wastewater. These proofs imply that microalgae are a potential biosorbent as they are capable of removing pollutants from food waste through cellular structure assimilation.

2.3. Sodium Alginate

Sodium alginate is an anionic polysaccharide extracted from brown algae (e.g., *Sargassum* sp., *Laminaria* sp., and *Durvillaea* sp.,). It is a type of hydrogel, meaning it consists of intertwining hydrophilic polymer chains that form sparse networks, enabling it to retain relatively large amounts of water (Frent et al., 2022).

2.3.1. Properties of Sodium Alginate

2.3.1.1. Molecular Structure

Sodium alginate [Chemical formula: $(C_6H_7NaO_6)_n$] is a polyanionic co-polymer made of linearly linked β-D-mannuronic acids (M) and α-L-glucuronic acids (G) by 1-4 glycosidic bonds (Frent et al., 2022). The proportions of and sequential arrangements of these two uronic acids might vary depending on the brown algae species from which the alginate is extracted (Hariadi & Islam, 2020).

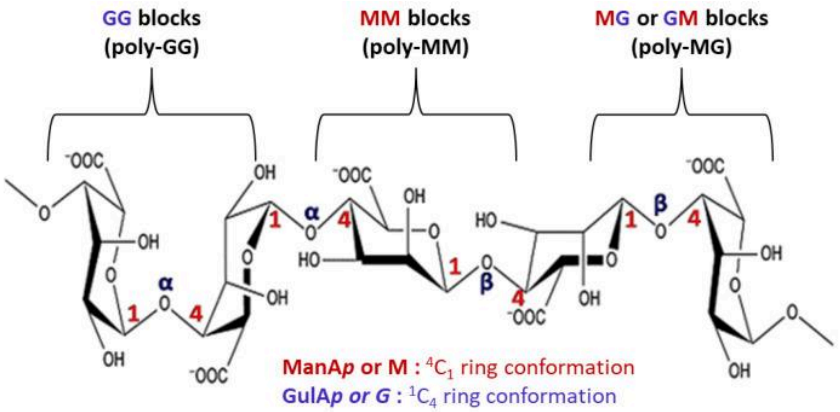


Figure 2 Chemical structure of alginate (Laurienzo, 2022)

2.3.1.2. Physical and Chemical Properties

Sodium alginate is seen physically as a solid powder with white to slightly yellowish color. Its form is influenced by its natural source where the alginate is extracted (i.e., species of brown algae and geographical location of the algae), as well as the extraction method and chemicals used (King, 2019). Alginate has an average molecular weight of 216.121 g/mol (Manaila et al., 2022). It is relatively easy to dissolve in water and soluble in relatively higher temperature but solubilizes slower in cold water and forms a more viscous solution. The viscosity is influenced by several factors such as pH, concentration, and molecular weight (Batista et al., 2019).

A unique property of alginate is that it can form gel-like structures when exposed to divalent ions (e.g., Ca^{2+}, Sr^{2+}, and Cu^{2+}). As stated by Malektaj et al. (2023), these cations react with the anionic alginate polymeric chains and occupy the space between them. Known as the “egg-box model”, the alginate chains have multiple guluronate units that coordinate with each of the present cations, forming a stable three-dimensional network as these chains are linked together. The properties of the gels depend on the type of divalent ion being used, in which the affinity of the alginate and the cations decrease in the following order: $Pb^{2+} > Cu^{2+} > Cd^{2+} > Ba^{2+} > Sr^{2+} > Ca^{2+} > Co^{2+} = Ni^{2+} = Zn^{2+} > Mn^{2+}$ (Wang et al., 2022). The stronger the affinity, the more stable the structure.

2.3.2. Alginate-microalgae Bead Implementation to Wastewater

Alginate has been commonly used in microalgae encapsulation – due to its gel-forming and non-toxic properties – for pollutant removal implementation in wastewater. A recent study by Han et al. (2024) which exposed both alginate-microencapsulated and unencapsulated *Chlamydomonas* sp. JSC4 to sulfamethoxazole (SMX)-containing wastewater as environmental stress reported that encapsulated microalgal cells have 99.62% – 99.72% NH^{4+}–N recovery efficiency and 100% for NO^{3-}–N and $PO4^{3-}$ recovery efficiencies. The research also found that the biomass of encapsulated microalgae is not significantly affected at low and high SMX concentrations, although the antibiotic is known to cause cellular oxidative stress response. On the other hand, the biomass of unencapsulated systems displayed significant differences according to SMX amount, as high SMX concentrations (5 mg/l and 10 mg/l) resulted in much lower biomass than lower ones (1 mg/l).

Another study by Solé & Matamoros (2016) that utilized a microalgae population made from *Chlorella* sp. and *Nitzschia acicularis* for wastewater treatment reported higher concentration removal of NH_4-N and phosphorus by alginate-encapsulated microalgae compared to microalgae with encapsulation (90% compared to 64% for NH_4-N removal; 97% compared to 89% for phosphorus removal) after 10 days of cultivation. Additionally, an experiment by Qin et al. (2020) on alginate-encapsulated *Chlorella pyrenoidosa* for industrial wastewater treatment demonstrated a COD removal efficiency of 62.23% and an ammonia nitrogen (NH_3-N) removal efficiency of 97.38%. Furthermore, Anagnostopoulou et al. (2024) that implemented alginate-encapsulated *Chlorella vulgaris* in food industry wastewater exhibited 31% COD and 23% TOC removal after 5 days of cultivation. Although these studies successfully provide evidence that alginate-encapsulated microalgae have a high potential to treat wastewater, similar approaches in food wastewater (particularly from plate leftovers) has yet to be discovered and will be the main focus of this study.

2.4. Implementation of Microalgae Biomass as Biofertilizer

2.4.1. Nutrient Content of Food Waste Treated C. vulgaris Biomass

C. vulgaris contains varying amounts of nutrients in their biomass – depending on their growth environmental conditions – that are essential for plant growth. Their protein content can range from ± 37.61% to 51% of dry weight, carbohydrate content ± 13.4 - 51.16% of dry weight, and lipid content ± 0.48 - 12.1% of dry weight (Ratomski & Hawrot-Paw, 2021; El Sayed et al., 2023; Jui et al., 2024). Additionally, their elemental composition includes carbon (C) ± 45 - 55% of dry weight, hydrogen (H)

± 6 - 7% of dry weight, nitrogen (N) ± 6 - 8% of dry weight, and phosphorus (P) ± 0.5 - 1% of dry weight (Dineshkumar et al., 2017; Adakamis et al., 2018).

Several previous studies have explored the nutrient content of *C. vulgaris* after food waste treatment. An experiment by Ramandani et al. (2024) which cultivated *C. vulgaris* FSP-E in various compositions of food waste and BG-11 medium exhibited the highest and lowest lipid composition of 3.18 mg/g and 9.65 mg/g respectively [Control (BG-11): 13.66 mg/g]; the lowest and highest carbohydrate composition of 226.72 mg/g and 396.06 mg/g respectively [Control (BG-11): 322.71 mg/g]; and the lowest and highest protein composition of 406.23 mg/g and 659.02 mg/g respectively [Control (BG-11): 692.12 mg/g]. Chew et al. (2018) also reported the lowest and highest lipid composition of 85.8 mg/g and 219.7 mg/g respectively [Control (BG-11): 199.5 mg/g]; with the lowest and highest carbohydrate composition of 197.2 mg/g and 346.5 mg/g respectively [Control (BG-11): 245.2 mg/g]; and the lowest and highest protein composition of 70.3 mg/g and 128.4 mg/g respectively [Control (BG-11): 122.3 mg/g] through the cultivation of *C. vulgaris* FSP-E in various compositions of food waste compost medium and BG-11 medium. Based on these findings, it is inferred that the nutrient composition of *C. vulgaris* biomass after treatment is highly dependent on the nutrient composition of the utilized medium.

2.4.2. Effects of Wastewater Treated Microalgae Biomass on Plant Growth

Due to their nutrient content, *Chlorella vulgaris* and other microalgae species biomass has been highly considered to be potential as plant biofertilizers. Many previous studies have investigated the effect of applying microalgae as a biofertilizer or biostimulant after wastewater treatment on plant growth. A research by Amaya-Santos et al. (2022) that used *Chlorella vulgaris* UAL-1 treated in urban wastewater as plant biostimulant achieved significant increase ($p < 0.05$) of germination index in watercress seeds (*Lepidium sativum* L.), a significant increase ($p < 0.05$) of adventitious root formation in soybean (*Glycine max L.*), and significant increase ($p < 0.05$) of cotyledon expansion in cucumber (*Cucumis sativus L.*). Another research by Loganathan et al. (2020) that incorporated *Chlorella variabilis* and *Scenedesmus obliquus* consortia after synthetic dairy wastewater treatment demonstrated 70.7% increase in shoot dry mass, 51.8% increase in root dry weight, 15.8% increase in plant height, 9.5% increase in number of leaves, and 36.9% increase in leaf area compared to the control group for corn (*Zea mays*) applied with 40% algal consortia. Meanwhile, soybean (*Glycine max*) applied with 40% algal consortia is reported to have 6.6% increase in number of leaves, 9.5% increase in plant height, 17.1% increase in root dry mass, 20.0% increase in shoot dry mass, 5.1% increase in chlorophyll content, and a 9.7% increase in leaf area compared to the control group.

III. METHODOLOGY

3.1. Research Location, Time, and Overall Design

This research was conducted from August 2024 to December 2024 under the Department of Chemical Engineering and Material Sciences at Yuan Ze University, Taoyuan, Taiwan. **Figure 1** below displays the project's overview methodology.

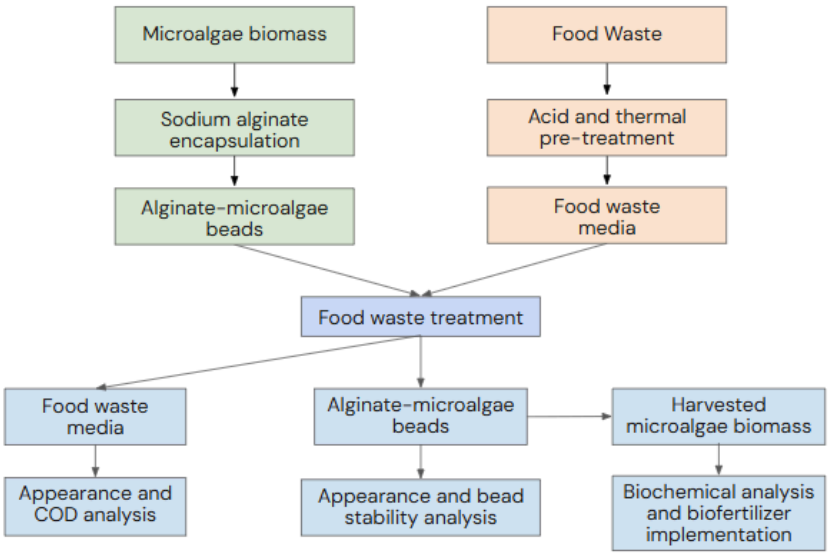


Figure 3 Project's overview methodology. Green-colored boxes signify the processing steps of microalgae biomass prior to food waste treatment, yellow-colored boxes signify the processing steps of food waste prior to food waste treatment, while blue-colored boxes signify the steps from food waste treatment onwards

3.2. Research Methodology

3.2.1. Microalgae Biomass Cultivation

To obtain *Chlorella vulgaris* FSP-E biomass, the microalgae obtained from National Cheng Kung University, Tainan (Taiwan) is cultivated in a premade BG-11 media inside a 1000 ml bottle (DURAN®, Germany). The cultivation apparatus components and BG-11 media were autoclaved for 20 minutes at 121°C for sterilization. Next, the microalgae inoculation is done under the biosafety cabinet (BSC). In each apparatus, 100 ml of microalgae mother stock culture was added to 900 ml BG-11 media for a 10% (v/v) culture concentration, followed by setting up the apparatus. The set microalgae culture was placed beside two-sided LED lights with a light intensity of ±1650 lux from the front side and ±1810 lux on the back side. A constant air supply was also given from an air pump (ALITA INDUSTRIES, Taiwan) with a pumping speed of 15 l/min. After 15 days of cultivation, the biomass was

harvested by centrifugation at 7000 rpm for 10 minutes. The obtained pellet was the desired microalgae biomass, while the supernatant was the remaining BG-11 media that could be reused for the next cultivation. As the biomass was not used directly, it was stored inside the refrigerator at 4^{o}C.

3.2.2. Food Waste Pre-treatment

The food waste was plate leftovers collected from a university food court in Taoyuan, Taiwan. It appears in solid and liquid form. The solid food waste included cabbage, carrots, meat, chicken, tofu, and noodles, while the liquid food waste was mostly spicy broth. The obtained food waste was then blended with a solid : liquid ratio of 2 : 3. This created a sludge that was filtered with a 300 µl-sized filter. The sludge filtrate was then mixed with liquid food waste with sludge : liquid ratio of 1 : 2. Next, the mixture was placed in the fridge for sedimentation. After 36 hours, the formed liquid phase was centrifuged at 7000 rpm for 10 minutes. The supernatant was then diluted with distilled water according to the treatment concentrations of 20%, 40%, 60%, 80%, and 100%. Furthermore, each differently concentrated food waste was treated with 1% (v/v) of 0.37 M HCl and autoclaved (YTM, Cylindraceous Steam Sterilizer, Taiwan) at 121^{o}C for 20 minutes. When the media had cooled down, the pH was adjusted to 7 - 7.5 with 10 M NaOH and a pH meter (Ultrabasic UB-10, Denver Instrument Co., USA). The food waste media was finally left overnight, followed by rechecking its pH before being added to the microalgae. 10 ml of each attained food waste concentration underwent initial chemical oxygen demand measurement (Day 0).

3.2.3. Alginate-microalgae Beads Encapsulation

Sodium alginate solution with a concentration of 20 g/l was prepared by adding alginate powder (Sigma-Aldrich, Norway) to distilled water and mixing until there were no clumps of alginate powder. The alginate solution was then added with 30 g/l of wet microalgae biomass and mixed homogeneously. Before being added to the alginate solution, the wet biomass was pre-washed once by adding distilled water and vortexed until mixed well. The homogenized solution was centrifuged at 7000 rpm for 10 minutes, in which the pellet was the re-attained wet biomass.

Next, the obtained alginate-microalgae solution was dripped slowly using a peristaltic pump (YZ1515x, Shenchen Pump, China) with the speed of 0.82 ml/min to 28 g/l concentrated $CaCl_2$ (Sigma-Aldrich, Japan) solution. The form alginate-microalgae beads were let in the $CaCl_2$ solution to let the alginate polymerize. After 60 minutes, the microalgae was rinsed with distilled water 2 times. The process was done and repeated separately with six different 500 ml bottles.

Furthermore, the diameters of ten beads from each treatment were measured using a digital vernier caliper (ACCUD Digital Caliper, China) for bead stability analysis and returned to the respective bottles after measurement. As the beads were not used directly, they were put inside 28 g/l of $CaCl_2$ solution and stored inside the refrigerator at 4^{o}C.

3.2.4. Food Waste Treatment

The obtained beads were used for five different concentrations of food waste as the treatment groups, specifically 20%, 40%, 60%, 80%, and 100%, denoted as FW 20%, FW 40%, FW 60%, FW 80%, and FW 100% respectively. For the control treatment, BG-11 media was used instead of food waste during the cultivation.

The alginate-microalgae beads were cultivated in varying food waste media concentrations inside 500 ml bottles and received a constant air supply and light source. After autoclaving the cultivation apparatus components, the bead inoculation procedure was done under the BSC. In each apparatus, 60 ml of alginate-microalgae beads were put into ±200 ml of food waste, followed by adding more food waste media until the total volume reached 300 ml. The set microalgae culture was placed beside LED lights with a light intensity of ±3600 lux and a constant air supply from a 220 l/min speed air pump (Aquarium Air Pump, Giant Electric Co) for 15 days of cultivation.

After cultivation, the beads and food waste media were separated. Subsequently, the beads were washed 2 times with distilled water to remove food waste remnants. The diameters of the beads were re-measured for bead stability analysis, while the final COD value of the food waste (Day 15) was measured for COD analysis. The appearance of food waste and beads was also analyzed before and after treatment.

3.2.5. Chemical Oxygen Demand (COD) Removal Analysis of Food Waste Media

To obtain the value of COD removal, the COD values of the initial (Day 0) and final (Day 15) food waste samples were measured. Before the procedure, the collected samples were centrifuged at 7000 rpm for 10 minutes to remove relatively big-sized organic particles. The refined samples were then diluted to ensure the read COD values were within the given interval range of the COD reagent (20 - 1500 mg/l). The dilution factor is correlated with the food waste concentration within the sample, as a higher concentration implies a higher dilution factor. As the factors were determined through trial and error, the subsequent heating process was conducted towards one vial at a time. **Table 1** below shows the dilution factor performed on each food waste sample.

Table 1 Dilution factors of tested samples before and after food waste treatment

Food Waste Concentration (%)	Dilution Factor Before Treatment (Day 0)	Dilution Factor After Treatment (Day 15)
FW 20%	40	10
FW 40%	40	20
FW 60%	60	20
FW 80%	80	20
FW 100%	100	25

To obtain the actual COD value, these dilution factors were then inputted into the following equation:

Actual COD value (mg/l) = Measured COD value (mg/l) x Dilution factor

After dilution, 2 ml of the diluted sample was transferred to the high-range COD reagent vial (CAT No. 2125915-TW; 150–1500 mg/l, USA). The vial was then digested inside a CR25 reactor (Rocker, Taiwan) to heat at 150^{o}C for 2 hours. When the heating was done, the vial was left to cool for ±30 minutes before being analyzed using a multiparameter colorimeter device to determine the sample's COD value (in mg/l). Distilled water was used as a blank. The measurements were performed in triplicates for each sample, and the obtained values were inputted into the following equation:

$$\text{COD removal (\%)} = \frac{COD0 - COD15}{COD15} \text{ x 100\%, where:}$$

COD_{15} = Final COD value on day 15

COD_{0} = Initial COD value on day 0

3.2.6. Bead Stability Analysis of Alginate-microalgae Beads

The bead stability analysis is conducted based on Limrujiwat et al. (2022) with modifications. For each treatment, ten beads were taken for their diameters to be measured using a digital vernier caliper. Similar to the COD value, the bead measurements were conducted before and after the food waste treatment. As bead stability is determined based on the number of stable beads, the beads after the treatment were classified as unstable when more than 20% of the average initial bead diameter was lost. The bead stability value is calculated using the following formula:

Bead stability (%) = $\frac{B15}{B0}$ x 100%, where:

B_{15} = Final number of stable beads in each treatment (Day 15)

B_0 = Initial number of stable beads in each treatment (Day 0)

3.2.7. Alginate Dissolution for Biomass Separation

The microalgae-alginate beads were dissolved based on a modified method of Murujew et al. (2021). Firstly, the microalgae-alginate beads were added to 0.5 M sodium citrate solution with a 1:1 volume ratio. The mixture was then stirred for 1 hour. After homogenization, the solution was centrifuged at 7000 rpm for 15 minutes to separate microalgae biomass (pellet) from the alginate (supernatant). The biomass was washed once by adding distilled water and then followed by centrifugation at 7000 rpm for 15 minutes. While the supernatant was discarded, the attained biomass was dried inside the oven for 2.5 days to remove the moisture. After drying, the dried biomass was crushed using a mortar and pestle until it was in powder form, which will be further processed for biochemical composition analysis (section 3.2.8) and biofertilizer implementation (section 3.2.9.2).

3.2.8. Nutrient Composition Analysis of Microalgae Biomass

3.2.8.1. Lipid Quantification

The lipid was initially extracted from the dry microalgae biomass using a modified Bligh and Dyer (1959) method. 0.02 g of dry microalgae biomass was suspended in a 7.6 ml mixture of chloroform, methanol, and water (ratio of 1:2:0.8 (v/v)). The mixture was then sonicated at 43 kHz for 5 minutes in an ultrasonic water bath (D150H, Delta Ultrasonic Cleaner, Taiwan). After sonication, 2 ml chloroform was added to the mixture, followed by another sonication for 5 minutes. Next, the mixture was homogenized using a vortex for 30 seconds and centrifuged at 3000 rpm for 5 minutes. The formed chloroform bottom layer was transferred to a pre-weighed aluminum weighing dish and dried in the oven (XUE058, France Etuves, China) at 80^{o}C for 30 minutes. After drying, the dish was put in a desiccator filled with silica gel (Honeywell Fluka, Germany) and let to cool down at room temperature. The cooled sample was finally weighed in triplicates to measure its final weight.

The lipid composition within the dry biomass was calculated using the following equation:

Lipid composition (mg/g) = $\frac{Final\ weight\ (mg) - Initial\ weight\ (mg)}{Dry\ microalgae\ biomass\ mass\ (gr)}$

$$= \frac{Final\ weight\ (mg) - Initial\ weight\ (mg)}{0.02\ gr}$$

3.2.8.2. Carbohydrate Quantification

The carbohydrate was extracted from the dry microalgae biomass and quantified using a modified Phenol-sulfuric (Pleissner et al., 2013) method. 0.04 g of dry microalgae biomass was added to 500 µl of 18 M sulfuric acid (Honeywell Fluka, Germany) and let to dissolve. After 30 minutes of reaction time, the mixture was added to 4.5 ml of distilled water, and the solution was put in the autoclave at 121°C for 30 minutes to complete hydrolysis. The hydrolyzed solution was centrifuged at 5000 rpm for 10 minutes. 10 µl of supernatant was then mixed with 1 ml of 18 M sulfuric acid and 200 µl of 4% (v/v) phenol (Sigma-Aldrich, USA) and left for 10 minutes reaction time. Next, the sample's OD value was measured with a UV-vis spectrophotometer at 490 nm wavelength in triplicates. To determine the carbohydrate concentration of the sample in mg/ml, a standard curve based on starch (Thermo Scientific, USA) with known concentrations ranging from 2 mg/ml to 10 mg/ml was plotted and the value was calculated based on the obtained linear equation.

The carbohydrate amount was determined through the calculation below:

Carbohydrate amount (mg) = Carbohydrate concentration (mg/ml) x Sample volume

= Carbohydrate concentration (mg/ml) x 5 ml

The carbohydrate composition within the dry biomass was calculated using the following equation:

$$\text{Carbohydrate content (mg/g)} = \frac{Carbohydrate\ amount\ (mg)}{Dry\ biomass\ mass\ (gr)}$$

$$= \frac{Carbohydrate\ amount\ (mg)}{0.04\ gr}$$

3.2.9. Implementation of Reused Alginate-microalgae Biomass as Lettuce Biofertilizers

3.2.9.1. Lettuce Germination and Growth

The lettuce seed was planted in cocopeat as the planting medium; two seeds were planted in each made planting set-up. The seeds were then let to germinate and grow for 14 days, under 16 hours per day photoperiod with the light intensity of ±3600 lux and watered with distilled water 1 - 2 times daily before biofertilizer implementation. After 10 days, one of two grown lettuce seedlings in each container was trimmed to ensure each planting set up had only one plant until the end of observation.

3.2.9.2. Biofertilizer Preparation

The dried microalgae biomass was dissolved in distilled water with a concentration of 5 g/l to attain the biofertilizer solution. To obtain enough microalgae biomass, an approximately 1.5-time scale-up was performed by repeating the procedures elaborated in sections 3.2.3 and 3.2.4.

3.2.9.3. Biofertilizer Implementation

In this experiment, five treatment groups and one control group are tested. For the treatment group, the microalgae biomass was obtained from beads used to treat food waste concentrations of 20%, 40%, 60%, 80%, and 100% (Five treatments). Distilled water was implemented instead of biomass for the control treatment. Each treatment was conducted on two plants (in duplicate).

After 14 days of lettuce germination (section 3.2.9.1), the microalgal biofertilizer solution was given to each treatment plant daily within 24 days of the observation period. The biofertilizer was applied through the roots (root drenching method). 1 ml of biofertilizer was provided for observation day 0 until day 10, while 2 ml of biofertilizer was provided for observation day 11 until day 24.
During observation, measurements were done using a digital vernier caliper with six-day intervals. The assisted parameters were stem length (in cm), leaf number, leaf length (in cm), and leaf width (in cm). The stem length and leaf number were measured five times from observation day 0 to observation day 24. Meanwhile, two leaves from every plant were measured for their leaf length and leaf width, with a total of two times of measurement (observation day 18 and day 24) when the true leaves have developed.

3.2.9.4 Statistical Analysis

The obtained data for lettuce growth was analyzed using paired t-tests with SigmaPlot software (Version 12.0) to determine their significance with $p \leq 0.05$.

IV. RESULTS AND DISCUSSION

4.1. Food Waste Treatment Analysis

4.1.1. Food Waste Media Observation

4.1.1.1. Appearance of Food Waste Media

After encapsulation, the alginate-microalgae beads were inoculated into the pre-treated food waste media and placed in the cultivation apparatus, as shown in **Figure 4a** below. As illustrated, the color of the food waste culture media ranged from dark brown to almost black. An explanation lies in the pre-treatment of the food waste media that incorporates acid treatment and thermal treatment. This combination efficiently breaks down the waste's organic compounds, i.e., hydrolysis of carbohydrates into reducing sugars and hydrolysis of protein into amino acids (Rawindran et al., 2024). Then, the sugar molecules react with the amino group of amino acids in a non-enzymatic reaction known as the Maillard reaction, when heat is incorporated from the thermal treatment. This creates dark-brown colored compounds called melanoidin, causing dark-colored food waste media (Croguennec, 2016; Sacchetti et al., 2016).

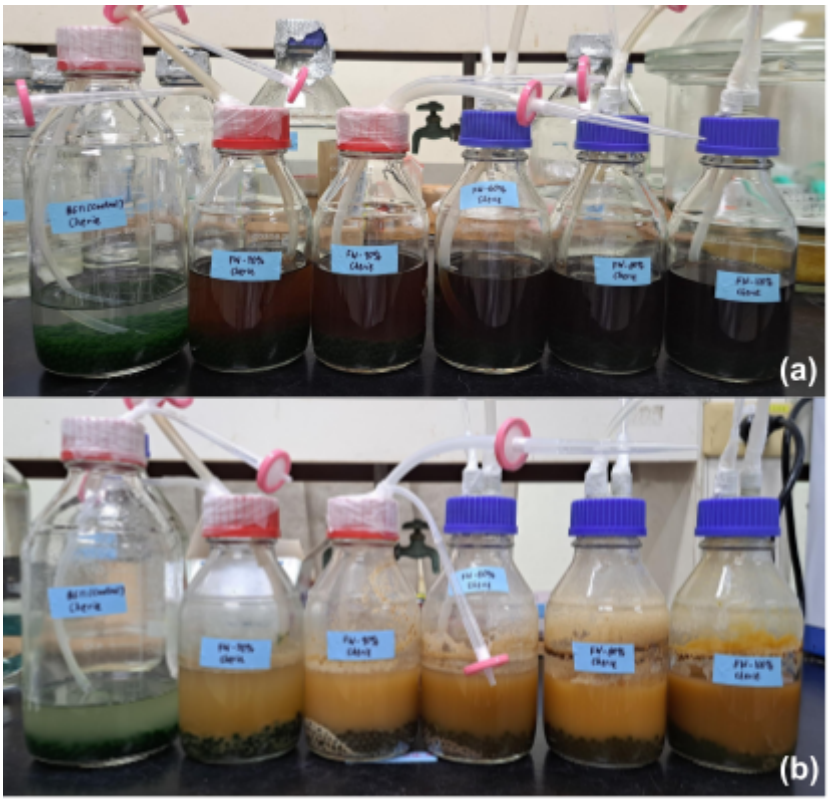


Figure 4 Appearance of microalgae beads cultivation set-up before (a) and after (b) food waste treatment [Left to right: Control (BG-11), FW 20%, FW 40%, FW 60%, FW 80%, and FW 100%]. The alginate-microalgae beads were inoculated to pre-treated food waste media inside 500 ml DURAN® bottles (1000 ml DURAN® bottles for

the control group). The bottles were sealed with plastic caps and given input and output hoses for air supply, with each hose equipped with a 22 µm filter

Figure 4b above displays the condition of the treated culture after 15 days of cultivation. The food waste media's color has turned lighter compared to the media prior to the treatment. According to a review by Kumar and Chandra (2019), microalgae produces substances including hydrogen peroxide (H_2O_2), hydroxyl, active oxygen radicals, and perhydroxyl that degrades melanoidin, leading to food waste decolorization. The photosynthetic organism also performs enzymatic reactions with glucose oxidase, manganese-independent peroxidases (MIP), manganese-dependent peroxidase, and glucose oxidase that maximize the production of H_2O_2. A research by Chaijak et al. (2024) that used *Chlorella* sp. BP01 to degrade melanoidin contained in palm oil mill effluent (POME) reported the highest melanoidin degradation of 79.54 ± 0.45%, confirming that microalgae are capable of removing melanoidin and causing lighter color of food waste media after treatment.

Aside from the color, it was also discovered that the food waste media changed from translucent to cloudy after treatment. It is speculated that fungi were present in the food waste media before cultivation and multiplied during the process, causing the media to become cloudy. Some fungi species, such as *Paecilomyces variotii*, *Talaromyces macrosporus*, and *Byssochlamys fulva* have exhibited resistance and resilience to high-temperature environments exhibited by the performed thermal pre-treatment at 121^{o}C as their spores can withstand those conditions (van den Brule et al., 2020; Piecková et al., 2020). Hence, these spores could survive and thrive during cultivation. While some of the grown fungi were suspended in the food waste media, the rest formed white sediments at the bottom of the cultivation apparatus. These precipitates are mostly conspicuous in FW 40% and FW 60% treatments as seen in **Figure 4b**.

Additionally, the cultivation strategy used to cultivate the alginate-microalgae beads was mixotrophic which provides light, inorganic carbon, and organic carbon sources to the culture to support the microalgae's metabolism (Pang et al., 2019; Gao et al., 2022).. In this case, the air pump is used to supply inorganic carbon sources like oxygen (O_2) and carbon dioxide (CO_2) for microalgae respiration and photosynthesis, respectively. Besides microalgae, oxygen also allows fungi to grow in the food waste media, as many fungal species such as *Aspergillus fumigatus* that are obligate aerobes (Misslinger et al., 2018). Therefore, the first alternative hypothesis (H_1) is accepted as alginate-encapsulated microalgae beads cause changes in food waste appearance.

The treated food waste media was separated from the beads and centrifuged at 7000 rpm for 10 minutes to remove the fungi and other contaminants. After being separated from the fungi, the food

waste appeared translucent. Comparing it to the food waste before the treatment re-confirms that the food waste media becomes lighter after treatment using alginate-microalgae beads (**Figure 5**).

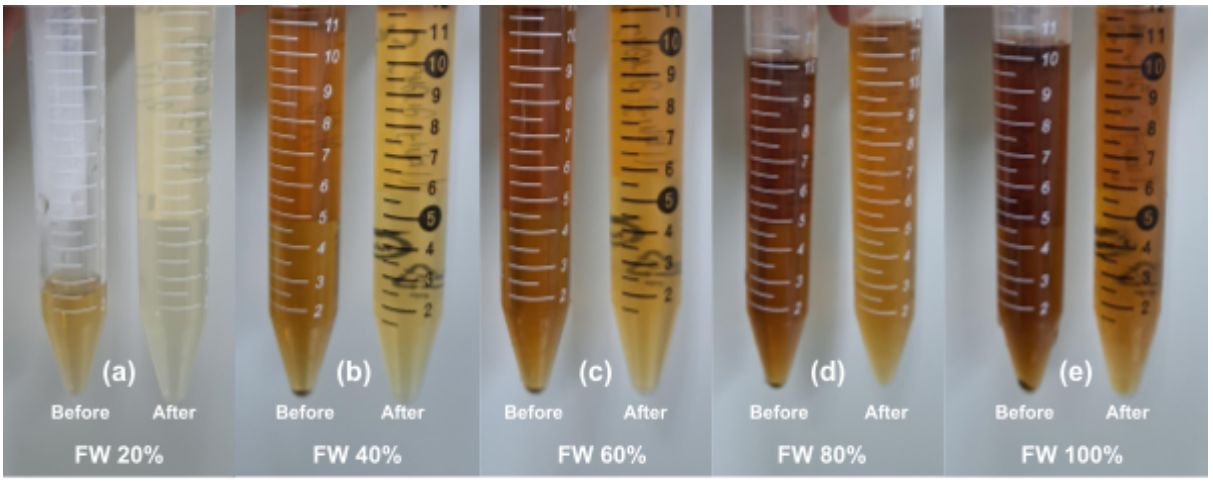


Figure 5 Appearance of food waste media before treatment (left tube in each figure) and after treatment (right tube in each figure). The post-treated food waste media was centrifuged beforehand to remove fungi and other remainings [Left to right figures: FW 20% (a), FW 40% (b), FW 60% (c), FW 80% (d), and FW 100%(e)]

4.1.1.2. COD Removal of Food Waste Media

The untreated and treated food waste media, as showcased in **Figure 5** above, were subjected to COD analysis, and the COD removal percentage was calculated based on the obtained COD values (**Table 2; Appendix B**). COD removal indicates the decrease of oxygen requirement to break down the organic matter in food waste samples, meaning a higher COD removal value implies more organic matter material being broken down by encapsulated microalgae. As shown in **Figure 6** below, the highest COD removal value of 95.7% was achieved in FW 60%, followed by FW 40% and FW 20%, with a COD removal value of 93.7% and 90.2%, respectively. This indicates the alginate-encapsulated microalgae performed the highest treatment efficiency in FW 60%.

Table 2 COD values of food waste media before and after treatment

Treatment	COD Before Treatment (mg/l)	COD After Treatment (mg/l)
FW 20%	17385.33	1697.67
FW 40%	34658.67	2197.33
FW 60%	54808	2345.33
FW 80%	73568	14418
FW 100%	82926.67	21535.83

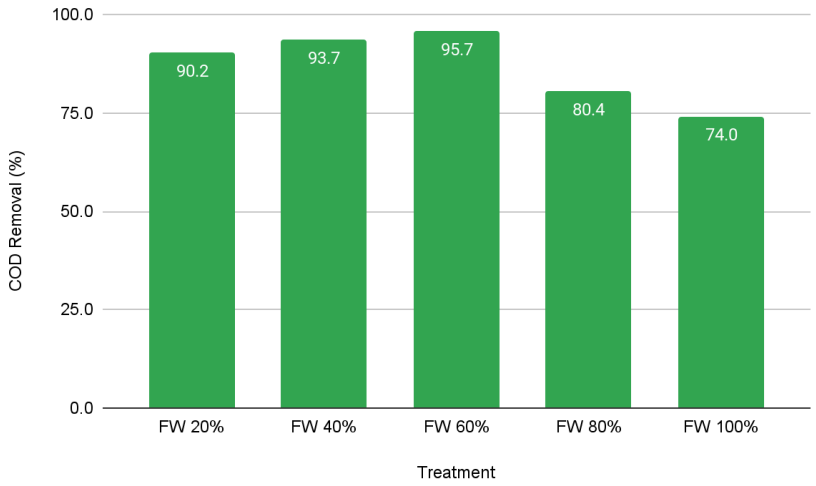


Figure 6 COD removal percentages in food waste media by alginate-microalgae beads based on the obtained COD values before and after food waste treatment

At food waste concentrations higher than 60%, the COD removal efficiency decreased to less than 90%, with COD removal of 80.4% for FW 80% and 74% for FW 100% (**Figure 6**). This indicates that COD removal is affected by the initial COD value of the food waste, as FW 80% and FW 100% had initial COD removal values of 73568 mg/ml and 82926.67 mg/ml respectively (**Table 2**). Some studies confirm that organic matter removal by *Chlorella* spp tends to decrease when the medium reaches certain COD values. For instance, Gupta et al. (2017) reported 61% - 66% of COD removal by *Chlorella pyrenoidosa* after 6 days of cultivation in synthetic wastewater with a 1000 mg/ml and 3000 mg/ml initial COD value. The COD removal decreased during microalgae cultivation in wastewater with higher initial COD values of 5000 mg/ml, with TOC removal value of 43%.

It is reported in section 4.1.1.1 that there are microorganisms present within the food waste media. These microbes produce enzymes such as pectinase and protease that break down organic matter in a process called decomposition (Chandrasekaran et al., 2016; Okonji et al., 2019). Not only does this reaction break down food waste material, but it also consumes oxygen as oxygen supply controls decomposition rate (Nguyen et al., 2022). In addition, waste decomposition produces toxic compounds such as ammonia (NH_3) and hydrogen sulfide (H_2S) that could hinder microalgae growth (Li & Qiao, 2015). As decomposition happens more in higher food waste concentrations due to higher organic matter amount, this explains why high initial COD levels correspond to oxygen depletion which induces stress toward the microalgae due to reduced respiration capability. This condition can affect their viability and performance in breaking down the organic materials contained in food

waste, resulting in lower COD removal. Thus, the second null hypothesis (H_0) is accepted as higher food waste concentrations had lower COD removal percentages compared to the lower ones.

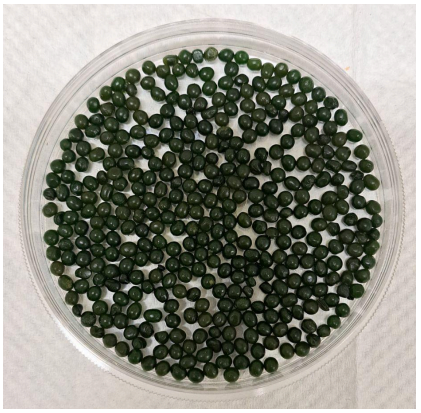
As the existence of microbes might compromise the microalgae viability, it is recommended that food waste media without alginate-microalgae beads be put in the same cultivation settings to enable COD comparison between treated and untreated food waste as a control group. This allows researchers to determine the significance of incorporating beads towards COD removal. A solution to minimize fungi growth is immediately using the obtained liquid food waste after pre-treatment without prolonged storage to prevent the thriving of fungi and other microorganisms. Another way to prevent fungi contamination is to centrifuge the food waste media at certain intervals during the cultivation period (similar to microalgae harvesting methods), as fungal cells form pellets after centrifugation to separate them from the food waste media (Chen et al., 2018; Pei et al., 2021). Furthermore, adding antifungal compounds extracted from *Chlorella vulgaris*, such as Diethyl Ether Extract (DEE), methanol, and acetone, would also be an effective strategy against fungal species like *Fusarium* spp. and *Aspergillus* spp without compromising microalgae growth (Perveen et al., 2022; Sultan & Marrez, 2022; Jokel et al., 2023).

4.1.2. Alginate-microalgae Beads Condition

4.1.2.1. Appearance of Alginate-microalgae Beads

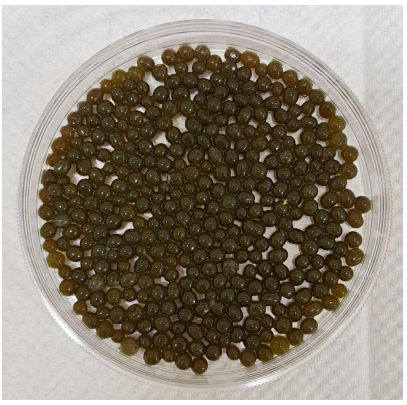
The conditions of the alginate-encapsulated beads were compared before and after treatment after 15 days of cultivation. Based on **Table 3** below, it can be seen that the beads cultivated in BG-11 media (**Table 3.1b**) and 20% food waste (FW 20%) treatment (**Table 3.2b**) mostly retained their dark green color after treatment. On the contrary, the FW 40% FW (**Table 3.3b**), FW 60% (**Table 3.4b**), FW 80% (**Table 3.5b**), and FW 100% (**Table 3.6b**) beads had color alteration into yellowish-brown color. It was also observed that the color of the FW 100% alginate beads was more yellowish than that of the other brown beads (**Table 3.6b**).

Table 3 Bead appearance comparison before and after food waste treatment (Plate view)

Treatment	Bead Appearance Before Treatment	Bead Appearance After Treatment
Control (BG-11)	 (1a)	 (1b)
FW 20%	 (2a)	 (2b)
FW 40%	 (3a)	 (3b)

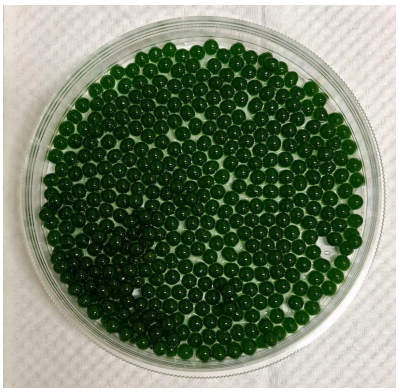
FW 60%

(4a)



(4b)

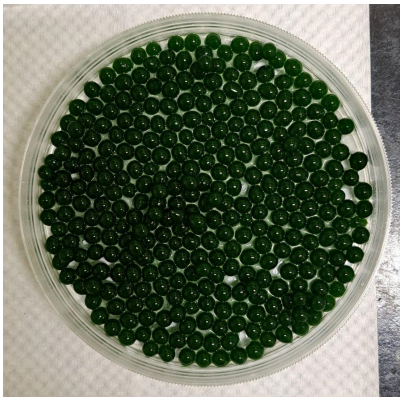
FW 80%



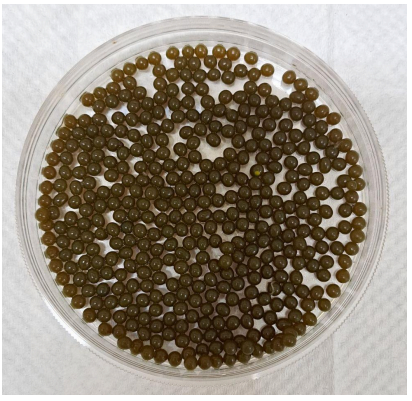
(5a)

(5b)

FW 100%



(6a)



(6b)

Microalgae are known to contain pigments, including chlorophylls (green color), carotenoids (orange to yellow color), and phycobiliproteins (red to blue color). *Chlorella vulgaris* mostly contains chlorophyll a, which is responsible for its dark green color. However, when microalgae are exposed to stress conditions or nutrient imbalance, carotenoid is produced more as an antioxidant while chlorophyll is degraded. This condition shifts its color from dark green to yellowish (Dharma et al., 2017; Oo et al., 2017; Khairunnisa et al., 2024).

Cultivation in food waste media can expose the microalgae to a stressful environment. Although food waste contains a relatively abundant amount of macronutrients and micronutrients, the composition and ratios of these nutrients highly depend on the food materials used to make the food waste media, which may not be suitable for optimal microalgal growth. For instance, beef contains protein associated with ammonium content (Chang & Zhang, 2017). A research by Zheng et al. (2019) which elevates ammonia content from 220 mg/l to 110 mg/l demonstrated a decrease in cell viability of *Chlorella vulgaris* from 89 ± 2% to 61 ± 4%.

To balance nutrient composition in food waste, it is recommended that a formulation for the food waste media be developed by determining the suitable nutrient composition for microalgae growth and conducting nutrient profiling. Another recommendation is adding sufficient supplementary nutrients if the nutrients in the food waste media do not suffice. Another stress factor is high COD levels in food waste media as reported and explained in section 4.1.1.2. This explains why beads cultivated in higher food waste concentrations have a more yellowish color, as food concentration directly correlates with COD levels. Hence, higher food waste concentration or organic compound content is correlated to alterations of microalgae biomass color (Third alternative hypothesis or H_1 accepted).

4.1.2.2. Bead Stability of Alginate-microalgae Beads

Aside from their color, the size of the alginate-microalgae beads was addressed. **Figure 6** below illustrates the side-by-side comparison between the beads before and after food waste treatment. The diameter of ten beads from each treatment is measured, and the average value is displayed in the figure. Although the beads remained in smoothly edged spherical shapes, their average diameter became smaller. The bead size lowered more significantly in 20% FW, 40% FW, and 60% FW compared with other treatments, with an average diameter decrease between before and after treatment of 0.909 mm, 0.904 mm, and 0.9016, respectively. Meanwhile, the beads cultivated in 80% FW and 100% FW had an average diameter decrease of 0.601 and 0.608, whilst the control treatment beads had the lowest average diameter decrease of 0.463.

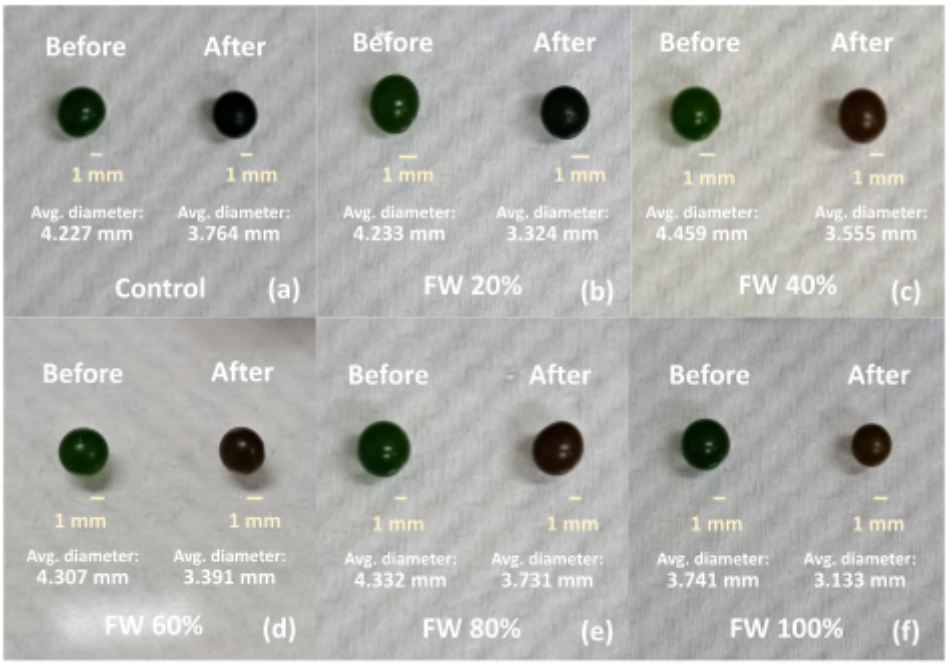


Figure 7 Bead size comparison before and after food waste treatment (Side-by-side view), with 1 mm of scaling. [Top left to right: Control (a), FW 20% (b), and FW 40% (c); Bottom left to right: FW 60% (d), FW 80% (e), and FW 100% (f)]

The obtained diameter values of the measured beads were then inputted for bead stability analysis with the bead stability values displayed in **Figure 8** below (Calculation in **Appendix C**). As can be seen, the bead stability for FW 20%, FW 40%, and FW 60% were 30%, 40%, and 40% respectively meaning that more than 50% of the measured beads had ≥ 20% diameter size reduction from average bead diameter before food waste treatment. Besides size reduction, the texture of the beads became more mushy after treatment. It is deduced that the size reduction and texture change of the beads were caused by the alginate lyses enzymes produced by microalgae which leads to alginate degradation. Fungi and bacteria existing in food waste media also produce alginate lyses, accelerating the degradation process (Zhu & Yin, 2015; Dharani et al., 2020). Therefore, it is speculated that fungi and bacteria contamination caused more significant bead stability reduction in FW 20%, FW 40%, and FW 60% due to their higher presence, indicated by their more conspicuous appearance as precipitates in the food waste media (**Figure 4b**).

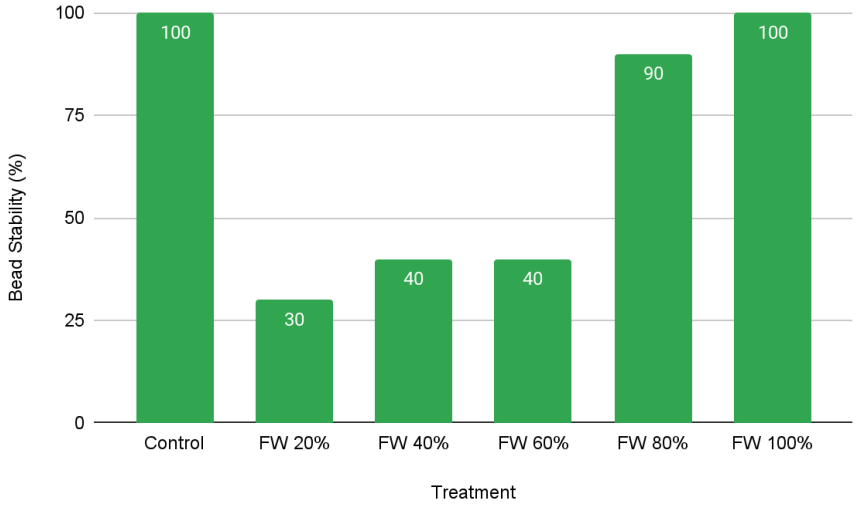


Figure 8 Bead stability percentages of alginate-microalgae beads

On the other hand, the beads treated in food waste concentrations of 80% and 100% had higher bead stability of 90% and 100% respectively. This indicates that the cultivated beads had less degradation due to containing fewer microbes. Another confirmation is that the microalgae-alginate beads cultivated in BG-11 media as a control treatment had 100% bead stability, as it is assumed the inorganic media did not contain fungi before the treatment. Hence, food waste media cultivation affects bead stability depending on fungi presence within the media (H_1 accepted).

4.2. Nutrient Composition of Microalgae Biomass

In this analysis, the dried biomass was isolated from the sodium alginate after food waste treatment and analyzed for its lipid and carbohydrate composition. This analysis also included a nutrient content comparison between treated biomass and biomass that was not exposed to food waste treatment. The calculated composition values are shown in **Table 4** below (**Appendix D**). It is observed that the untreated microalgae biomass had the highest lipid and carbohydrate content of 214.83 mg/g and 355.819 mg/g compared to the treated ones. This is aligned with the results of research done by Pleissner et al. (2017) that analyze the lipid, and carbohydrate content of *Chlorella pyrenoidosa* cultivated inside food waste hydrolysate. It is reported that the nutrient content of the biomass from batch culture tends to decrease after 3 days of cultivation. Similar to the experiment, this research also incorporates batch culture where there is no continuous supply of nutrients in the media. Therefore, the nutrient content within the biomass eventually dwindles as it is metabolized for microalgal cell growth (Yang & Sha, 2019).

Another observation is the lipid content of the *C. vulgaris* cultivated in 100% food waste has the lowest lipid composition of 114.17 mg/g compared to other treatments. This finding is similar to the obtained results in research by Chew et al. (2018) which showcases the lowest lipid content of 85.8 mg/g in *C vulgaris* cultivated with 100% food waste compost medium (FW 100%). Another research by Zeng et al. (2018) which increased food waste hydrolysate content reported a decrease in lipid content from 44.75% to 16.9%. The aforementioned experiment also mentioned that sugar presence within the organic food waste media inhibits the expression and activity of photosynthetic enzymes, leading to the biomass and lipid content decrease. This research found the reduction of carbohydrate composition in biomass cultivated in organic media (FW 100%) of 247.522 mg/ml, in comparison to inorganic media (BG-11 media) with carbohydrate content of 285.776 mg/ml. Additionally, Ramandani et al. (2024) reported a lower carbohydrate content of 248.24 mg/g in *C. vulgaris* FSP-E cultivated in 100% food waste media compared to 322.71 mg/g carbohydrate composition in BG-11 cultivation. Hence, cultivation in food waste media affects microalgae nutrientl composition and the fifth alternative hypothesis (H_1) is accepted.

Table 4 Nutrient (lipid and carbohydrate) composition within dry microalgae biomass after food waste treatment

Treatment	Lipids (mg/g)	Carbohydrates (mg/g)
Control (BG-11)	154.67	285.776
FW 20%	158.5	225.431
FW 40%	148	280.388
FW 60%	186.17	239.978
FW 80%	135.67	261.530
FW 100%	114.17	247.522
Untreated	214.83	355.819

4.3. Performance of Microalgal Biomass as Biofertilizer

Aside from biochemical composition analysis, the isolated *C. vulgaris* biomass was implemented into lettuce (*Lactuca sativa*) plants as biofertilizers. To assist its role in lettuce growth, several parameters

including stem length, leaf number, leaf length, and leaf width were measured 14 days after sowing (Measurement results displayed in **Appendix E**). **Figure 8** below illustrates the stem length increment from observation day 0 until day 24. As could be seen, the graph plot representing all treatments increases between observation day 0 until day 6, becomes relatively stationary between observation day 6 and day 14, and displays another increment between observation day 14 and day 24.

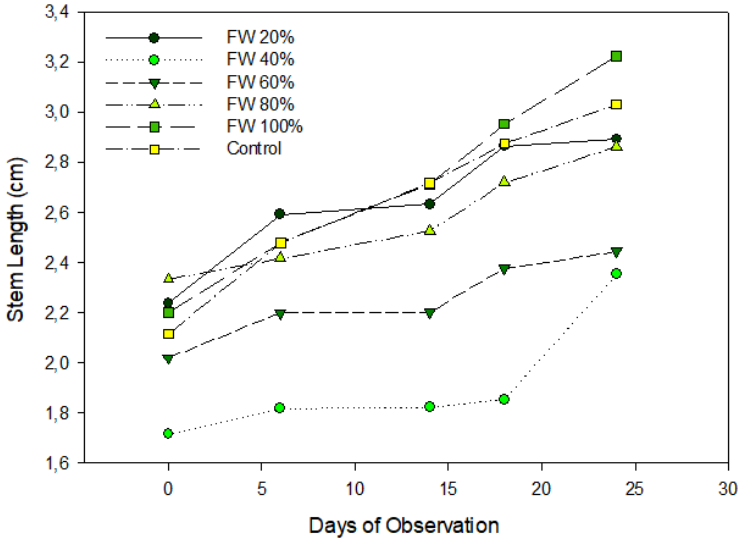


Figure 9 Stem length increment (in cm) for lettuce treated with biomass from FW 20%, FW 40%, FW 60%, FW 80%, and FW 100%, along with no biomass treatment (control) within 24 days of plant observation

Additionally, **Figure 10** below illustrates the leaf number increment from observation day 0 until day 24. Only the number of true leaves is being considered in this analysis as the cotyledon leaves were excluded. The lettuce plants treated with biomass from FW 20%, FW 60%, and FW 80% had the earliest leaf number average increment from observation day 0. Meanwhile, the plot of FW 40% and control (distilled water) treatment escalated from day 6 onwards. FW 100% treatment experienced the latest leaf number increment (from day 14 onwards). Overall, FW 20% had the highest leaf number average of 3.5 after 24 days of observation, followed by FW 40% and FW 80% with leaf number average of 2.5, while FW 100% treatment had the lowest leaf number average of 1.5.

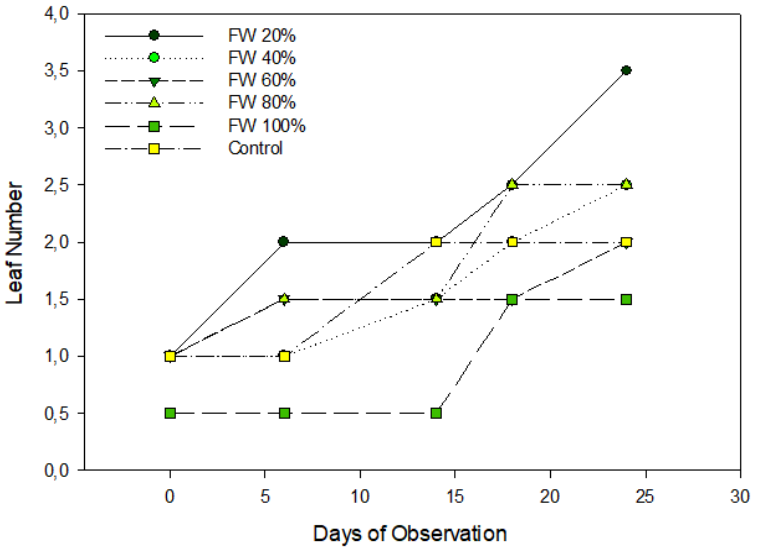


Figure 10 Leaf number increment for lettuce treated with biomass from FW 20%, FW 40%, FW 60%, FW 80%, and FW 100%, along with no biomass treatment (control) within 24 days of plant observation

The lettuce leaf length and leaf width were analyzed on observation day 18 and day 24 when the true leaves had developed, with the measurement results shown in **Table 5** below. Overall, the FW 20% treatment had the most leaf length and leaf width increments of 0.6 cm and 0.515 cm respectively. In contrast, the control treatment had the least leaf length and leaf width increments of 0.003 cm and 0.01925 cm respectively.

Table 5 Lettuce leaf length and leaf width (in cm) in observation day 18 and day 24

Treatment	Leaf length day 18 (cm)	Leaf length day 24 (cm)	Leaf width day 18 (cm)	Leaf width day 24 (cm)
Control (No biomass)	1.311	1.314	0.801	0.82
FW 20%	1.536	2.136	0.918	1.433
FW 40%	1.249	1.341	0.817	0.905
FW 60%	1.027	1.066	0.575	0.6
FW 80%	1.197	1.244	0.74	0.789
FW 100%	1.001	1.069	0.602	0.673

The paired t-test analysis was conducted toward the stem length, leaf length, and leaf width to determine the significance of the biofertilizer treatment. The treatment is determined to be significant when p-value ≤ 0.05. Based on the p-value shown in **Table 6** below, only FW 20% was

determined to be significant towards the leaf width with the p-value being 0.002. Despite that, the treatment was proven to be ineffective as the rest of the p-values > 0.05, concluding that the sixth null hypothesis (H_0) is accepted.

Table 6 p-value from paired t-test results of plants implemented with biofertilizers

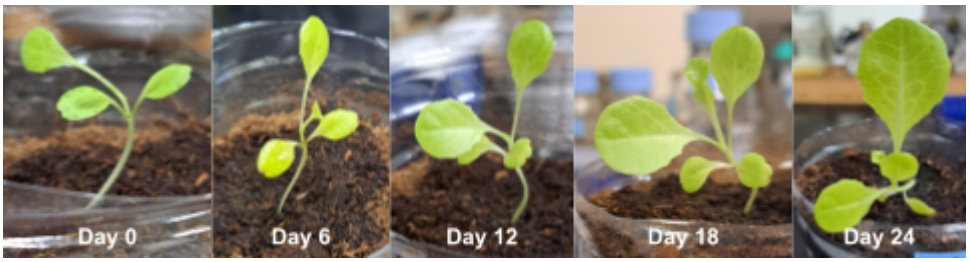
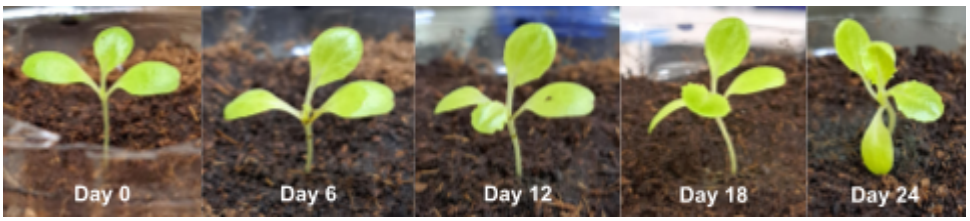
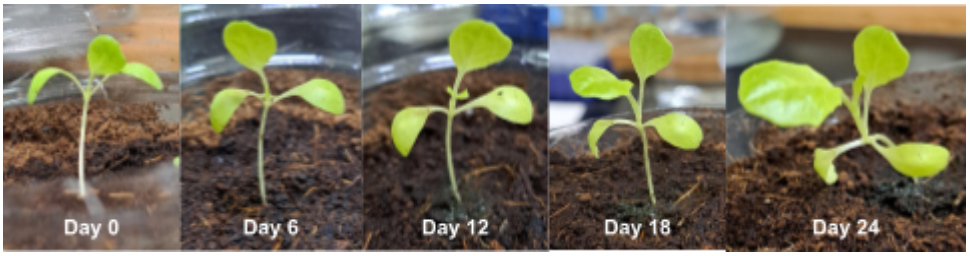
Treatment	Stem length	Leaf length	Leaf width
Control (No biomass)	0.095	0.134	0.127
FW 20%	0.22	0.059	0.002*
FW 40%	0.431	0.180	0.113
FW 60%	0.103	0.198	0.1
FW 80%	0.548	0.226	0.09
FW 100%	0.371	0.165	0.175

*p-value ≤ 0.05

However, it needs to be considered that there are factors that lie in the ineffectiveness of the treatment. First, the processing and planting method of the lettuce seeds. A study by Wichaphian et al. (2024) sterilized the lettuce seeds by soaking them in 1.2% (v/v) NaClO solution, followed by washing them using sterilized deionized water three times. To induce disease resistance and promote growth, the seeds were soaked in concentrated S*treptomyces thermocarboxydus* S3 spores before planting in the cultivation tray. Furthermore, the peat moss was used as potting soil in difference to cocopeat in the experiment, and tap water irrigation was implemented for 10 days to grow the seedlings. A higher concentration of biomass also needs to be used as the research used 0.5 g of de-oiled *Chlorella* biomass in 10 ml of water. Additionally, other plant parameters need to be assessed including the substance inside the leaves because the research reported increased yields of chlorophyll a, chlorophyll b, total chlorophyll, and carotenoids despite the reduction of shoot length, leaf number, and root length.

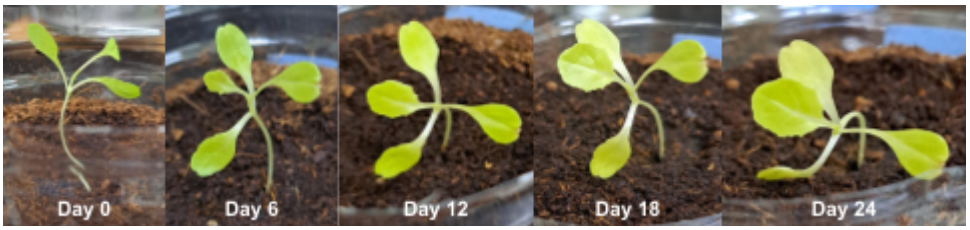
The development of the lettuce plant during 24 days of observation is illustrated in **Table 7** below. Initially, the seedlings had three leaves (two cotyledon leaves and one true leaf). Within six to twelve days of observation, another true leaf had emerged. Some plants managed to grow more than one true leaf on observation day 12 and day 18. Overall, it is seen that plant 2 of FW 20% achieved the highest development on observation day 24 despite the ineffectiveness of the treatment.

Table 7 Lettuce development as depicted in a six-day interval within 24 days of observation (day 0, day 6, day 12, day 18, and day 24)

Treatment	Plant Observation
Control (Plant 1)	
Control (Plant 2)	
FW 20% (Plant 1)	
FW 20% (Plant 2)	
FW 40% (Plant 1)	
FW 40% (Plant 2)	

FW 60%
(Plant 1)

FW 60%
(Plant 2)

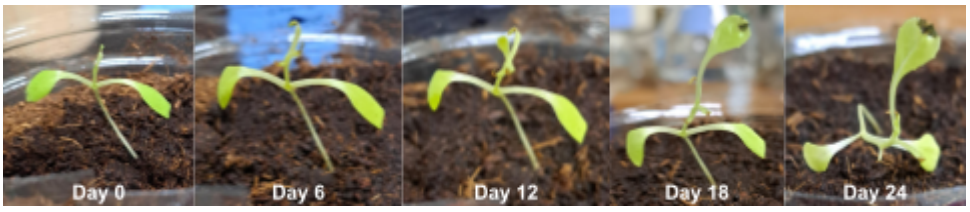


FW 80%
(Plant 1)

FW 80%
(Plant 2)

FW 100%
(Plant 1)

FW 100%
(Plant 2)



V. SELF REFLECTION

From this internship experience, I learned various hard skills and soft skills related to:

1. Food waste pre-treatment (Food waste collection, mechanical disruption, refinement, acid pre-treatment, and heat pre-treatment)
2. Microalgae encapsulation with sodium alginate
3. Food waste treatment (Cultivating alginate-microalgae beads for food waste media treatment)
4. Processing the separated microalgae biomass to make biofertilizer solution
5. Biofertilizer implementation to lettuce
6. Lettuce observation and measurement
7. Statistical analysis (Paired t-test)
8. Soft skills (Communication, time management, team-work, etc.)

These skills are also supported by my participation in i3L theoretical and laboratory courses, specifically the courses within my Sustainable Biotechnology specialization. They are also beneficial especially if I decide to focus on research work related to microalgae biotechnology, waste management, and bioprocessing after graduation.

During my internship time, I found myself to be quite detail-oriented, hard-working, persistent, communicative, able to communicate my findings, and willing to give my best based on my abilities. However, I also identify that I am often clumsy and careless during lab work, need work in controlling my emotions, need work in handling stress, occasionally lack of consideration towards others, and quite lack of time management skills. From these weaknesses, I learn to be more careful and double check everything after lab work, not using my emotion for thinking, more caring towards others, and improving my time management skills. Although I realize I still have many flaws related to research work, I hope that my presence and experience in Algae Bioseparation Research can still contribute to developing the research group.

VI. CONCLUSION

Microalgae are known to be capable of treating wastewater, including food waste, due to their ability to reduce food waste's environmental impact. It has also been discovered that encapsulation can facilitate microalgae performance during food waste cultivation. Therefore, this research proposes using alginate-encapsulated *Chlorella vulgaris* for food waste treatment. Based on the findings, it was observed that the treated food waste media had appearance changes to lighter color shades. The alginate-microalgae beads also performed COD removal, meaning organic material was reduced within the food waste to a certain extent. In this research, it is found that microalgae can perform COD removal of > 90% until FW 60%. However, the main challenge in food waste cultivation is associated with the presence of microorganisms (i.e., fungi and bacteria) that can impact the viability of the microalgae as they affect the growth conditions. The altered growth conditions can be reflected by the bead conditions after food waste treatment, in which the bead color changes from dark green to yellowish-brown due to the stress growth environment and decrease of bead stability depending on fungi presence. Therefore, it is suggested that fungi growth should be minimized to prevent such contamination. Further research for other parameters such as nitrogen, phosphate, and ammonium removal is also needed to discover more about the role of alginate-microalgae beads in food waste treatment. Additionally, it is also found that food waste treatment affects the carbohydrate and lipid composition of microalgae biomass. Furthermore, the biomass implementation as biofertilizer towards lettuce plants is deemed insignificant mainly due to improper planting techniques. For future experiments, it is suggested to consider different planting media (i.e., peat moss instead of cocopeat) and provide irrigation to grow the lettuce seedlings. Implementing higher biomass concentration as a biofertilizer solution is another way to ensure more significant biofertilizer treatment.

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APPENDICES

Appendix A. BG-11 Media Preparation

The BG-11 media serves as a main nutrient source during biomass cultivation of *Chlorella vulgaris* FSP-E. To prepare 1 liter of BG-11, 1.5 g of $NaNO_3$, 0.03 g of K_2HPO_4, 0.075 g of $MgSO_4.7H_2O$, 0.006 g citric acid, 10 ml of stock 1 solution, 10 ml of stock 2 solution, 10 ml of stock 3 solution, and 1 ml of stock 4 solution, as listed detailedly in **Table A1** below. After all the required chemicals were transferred into a DURAN® bottle, distilled water was added until the total volume reached 1 liter. The media was finally autoclaved at 121°C for 20 minutes for sterilization.

Table A1 Chemical composition of BG-11 media

Chemical Name	Composition
Main Chemicals	
$NaNO_3$	1.5 g/l
K_2HPO_4	0.03 g/l
$MgSO_4.7H_2O$	0.075 g/l
Citric Acid	0.006 g/l
Stock 1 (10 mg/l)	
Na_2CO_3	2 g/l
Stock 2 (10 mg/l)	
$CaCl_2.2H_2O$	3.6 g/l
Stock 3 (10 mg/l)	
Ferric ammonium citrate	0.6 g/l
EDTA	0.1 g/l
Stock 4 (10 mg/l)	
H_3BO_4	2.86 g/l
$MnCl_2.4H_2O$	1.81 g/l
$ZnSO_4.7H_2O$	0.222 g/l
$Na_2MoO_4.2H_2O$	0.3 g/l

$CuSO_4.5H_2O$	0.07 g/l
$Co(NO_3)_2.6H_2O$	0.04 g/l

Appendix B. COD Values of Food Waste Media

Table B1 The COD value of food waste media before treatment (Day 0). The measurements were performed in three technical replicates, with the actual COD value being calculated based on the average COD value and dilution factor of each food waste sample

Food Waste Concentration (%)	Dilution factor	Measured COD (mg/l)			Average COD (mg/l)	Actual COD (mg/l) = Average x Dilution
		1	2	3		
20	40	433.9	435	435	434.63	17385.33
40	40	861.8	866.1	871.5	866.47	34658.67
60	60	908.4	918.2	913.8	913.47	54808
80	80	918.2	919.2	921.4	919.6	73568
100	100	832.5	826	829.3	829.27	82926.67

Table B2 The COD value of food waste media after treatment (Day 15). The measurements were performed in three technical replicates, with the actual COD value being calculated based on the average COD value and dilution factor of each food waste sample

Food Waste Concentration (%)	Dilution factor	Measured COD (mg/l)			Average COD (mg/l)	Actual COD (mg/l) = Average x Dilution
		1	2	3		
20	10	167.5	170.8	171	169.77	1697.67
40	20	108.4	111.7	109.5	109.87	2197.33
60	20	113.9	119.5	118.4	117.27	2345.33
80	20	720.9	719.8	722	720.9	14418
100	25	862.9	861.8	859.6	861.43	21535.83

Table B3 COD removal value of food waste media based on the COD value difference between before and after treatment

Food Waste Concentration (%)	COD Removal (%)
20	90.2
40	93.7
60	95.7
80	80.4
100	74.03

Appendix C. Alginate-Microalgae Bead Diameter and Bead Stability

Table C1 Bead diameter before food waste treatment (Day 0). Ten beads from each treatment were measured for their diameters. 80% of the average diameter was calculated to determine which beads measured after the treatment were stable

Treatment	Measured Bead Diameter (mm)										Average diameter (mm)	80% Average (mm)
	1	2	3	4	5	6	7	8	9	10		
20% Food Waste	4.8	4.39	4.27	4.12	4.35	3.79	4.23	4.2	4.1	4.08	4.233	3.39
40% Food Waste	4.41	4.71	4.59	4.43	4.44	4.35	4.35	4.76	4.45	4.1	4.459	3.57
60% Food Waste	4.2	4.12	4.49	4.22	4.64	4.64	4.06	4.06	4.53	4.11	4.307	3.45
80% Food Waste	4.47	4.47	4.43	4.43	4.43	4.24	3.98	4.13	4.37	4.37	4.332	3.47
100% Food Waste	3.94	3.93	3.7	3.7	3.68	3.68	3.66	3.82	3.65	3.65	3.741	2.99
Control (BG-11)	4.29	4.15	4.15	4.15	4.73	4.27	4.26	4.26	4.02	3.99	4.227	3.38

Table C2 Bead diameter after food waste treatment (Day 15). Ten beads from each treatment were measured for their diameters. The values highlighted in yellow are classified as unstable beads as they are lower compared to 80% of average bead diameter before treatment

Treatment	Measured Bead Diameter (mm)										Number of stable beads
	1	2	3	4	5	6	7	8	9	10	
20% Food Waste	3.17	3.45	3.42	3.16	3.31	3.37	3.34	3.25	3.36	3.41	3
40% Food Waste	3.87	3.88	3.99	3.25	3.1	3.36	3.54	3.33	3.47	3.76	4
60% Food Waste	2.97	3.25	3.27	3.04	3.04	3.13	3.69	3.93	3.79	3.8	4
80% Food Waste	3.86	4.09	3.77	3.72	3.77	3.51	3.77	3.4	3.57	3.85	9
100% Food Waste	3.46	3.13	3.17	3.17	3.07	3.11	3.09	3.18	2.78	3.17	10
Control (BG-11)	3.97	3.51	3.59	3.8	3.9	3.74	3.65	3.83	3.85	3.8	10

Table C3 Bead stability value of food waste media based on the percentage of stable beads out of all ten measured beads

Food Waste Concentration (%)	Bead Stability (%)
20	30
40	40
60	0
80	90
100	100
Control (BG-11)	100

Appendix D. Biochemical Composition Analysis

Table D1 Lipid composition of microalgae biomass after food waste treatment. The lipid composition value was calculated by dividing the lipid amount by the mass of the dry biomass sample (0.02 g)

Treatment	Lipid Amount (g)	Lipid Amount (mg)	Lipid Composition (mg/g)
20% Food Waste	0.00317	3.17	158.5
40% Food Waste	0.00296	2.96	148
60% Food Waste	0.00372	3.723	186.17
80% Food Waste	0.00271	2.713	135.67
100% Food Waste	0.00228	2.283	114.17
Control (BG-11)	0.00309	3.093	154.67
Untreated	0.004297	4.297	214.83

Table D2 Carbohydrate absorbance value for carbohydrate composition standard curve. The absorbance values were measured in three technical replicates

Concentration (mg/g)	Absorbance			
	1	2	3	Average
2	0.351	0.352	0.352	0.3517
4	0.826	0.827	0.827	0.827
6	1.237	1.238	1.238	1.238
8	1.823	1.824	1.824	1.8237
10	2.173	2.173	2.173	2.173

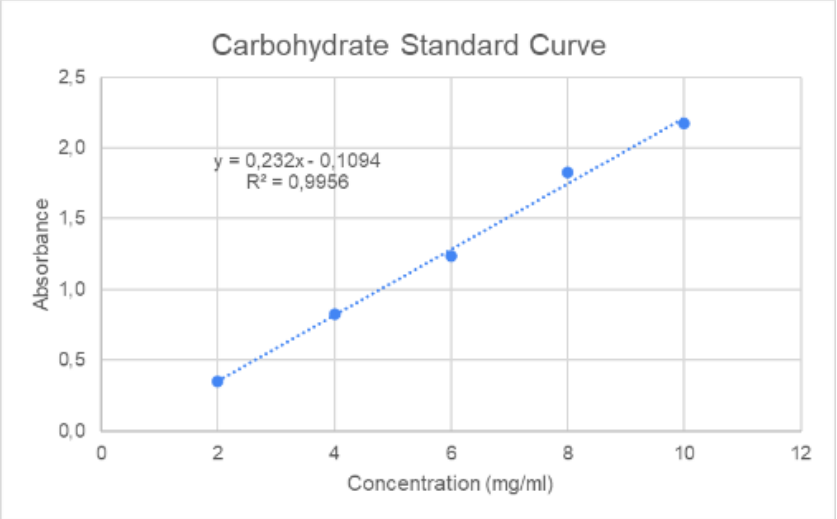

Figure D1 Carbohydrate standard curve for carbohydrate concentration (in mg/ml) determination

Table D3 Carbohydrate composition of microalgae biomass samples after food waste treatment. The absorbance values were measured in three technical replicates. The carbohydrate concentration is determined based on the standard curve, while te carbohydrate amount is calculated by multiplying the concentration value by the sample volume (5 ml). Lastly, the carbohydrate composition value is determined by dividing the carbohydrate amount value by the mass of the dry biomass sample (0.04 g)

Treatment	Absorbance				Carbohydrate Concentration (mg/ml)	Carbohydrate Amount (mg)	Carbohydrate Composition (mg/g)
	1	2	3	Avg			
20% Food Waste	0.309	0.309	0.309	0.309	1.803	9.017	225.431
40% Food Waste	0.411	0.411	0.411	0.411	2.243	11.216	280.388
60% Food Waste	0.336	0.336	0.336	0.336	1.920	9.599	239.978
80% Food Waste	0.376	0.376	0.376	0.376	2.092	10.461	261.530
100% Food Waste	0.35	0.35	0.35	0.35	1.980	9.901	247.522
Control (BG-11)	0.421	0.421	0.421	0.421	2.286	11.431	285.776
Untreated	0.551	0.551	0.551	0.551	2.847	14.233	355.819

Appendix E. Lettuce Parameter Assessment

Table E1 Stem length (in cm) of lettuce treated with biomass from FW 20%, FW 40%, FW 60%, FW 80%, and FW 100%, along with the control group (no biomass given). The measurements were performed in biological duplicates (two lettuce plant)

Date	Observation Day	FW 20%			FW 40%			FW 60%		
		1	2	Avg	1	2	Avg	1	2	Avg
18/11/24	0	2.433	2.043	2.238	1.687	1.741	1.714	1.631	2.409	2.020
24/11/24	6	2.84	2.343	2.5915	1.799	1.836	1.8175	1.898	2.501	2.200
2/12/24	14	2.845	2.423	2.634	1.803	1.84	1.8215	1.898	2.506	2.202
6/12/24	18	2.846	2.882	2.864	1.809	1.901	1.855	1.985	2.768	2.377
12/12/24	24	2.852	2.933	2.8925	1.812	2.892	2.352	1.986	2.902	2.444

Date	Observation Day	FW 80%			FW 100%			Control		
		1	2	Avg	1	2	Avg	1	2	Avg
18/11/24	0	2.104	2.562	2.333	1.933	2.466	2.1995	2.165	2.065	2.115
24/11/24	6	2.039	2.795	2.417	2.187	2.774	2.4805	2.378	2.579	2.4785
2/12/24	14	2.039	3.015	2.527	2.191	3.237	2.7140	2.552	2.886	2.719
6/12/24	18	2.041	3.397	2.719	2.207	3.701	2.9540	2.85	2.9	2.875
12/12/24	24	2.312	3.413	2.8625	2.283	4.163	3.2230	2.943	3.119	3.031

Table E2 Leaf number of lettuce treated with biomass from FW 20%, FW 40%, FW 60%, FW 80%, and FW 100%, along with the control group (no biomass given). The measurements were performed in biological duplicates (two lettuce plants)

Date	Observation Day	FW 20%			FW 40%			FW 60%		
		1	2	Avg	1	2	Avg	1	2	Avg
18/11/24	0	1	1	1	1	1	1	1	1	1
24/11/24	6	2	2	2	1	1	1	1	2	1.5
2/12/24	14	2	2	2	2	1	1.5	1	2	1.5
6/12/24	18	2	3	2.5	2	2	2	1	2	1.5
12/12/24	24	3	4	3.5	3	2	2.5	2	2	2

Date	Observation Day	FW 80%			FW 100%			Control		
		1	2	Avg	1	2	Avg	1	2	Avg
18/11/24	0	1	1	1	1	0	0.5	1	1	1
24/11/24	6	1	2	1.5	1	0	0.5	1	1	1
2/12/24	14	1	2	1.5	1	0	0.5	2	2	2
6/12/24	18	2	3	2.5	2	1	1.5	2	2	2
12/12/24	24	2	3	2.5	2	1	1.5	2	2	2

Table E3 Leaf length (in cm) of lettuce treated with biomass from FW 20%, FW 40%, FW 60%, FW 80%, and FW 100%, along with the control group (no biomass given). One to two true leaves were measured from each plant

Date	Observation Day	FW 20%					FW 40%				
		1-1	1-2	2-1	2-2	Avg	1-1	1-2	2-1	2-2	Avg
6/12/24	18	1.216	1.166	2.082	1.681	1.536	1.152	1.152	1.524	1.167	1.24875
12/12/24	24	1.626	1.42	3.257	2.242	2.136	1.163	1.185	1.769	1.247	1.341

Date	Observation Day	FW 60%					FW 80%				
		1-1	1-2	2-1	2-2	Avg	1-1	1-2	2-1	2-2	Avg
6/12/24	18	0.91	-	1.058	1.114	1.027	1.436	0.709	1.687	0.955	1.1968
12/12/24	24	0.946	-	1.063	1.19	1.066	1.445	0.848	1.714	0.968	1.24375

Date	Observation Day	FW 100%					Control				
		1-1	1-2	2-1	2-2	Avg	1-1	1-2	2-1	2-2	Avg
6/12/24	18	1.342	0.717	0.944	-	1.001	1.221	0.994	1.675	1.355	1.31125
12/12/24	24	1.366	0.846	0.994	-	1.069	1.222	0.999	1.675	1.361	1.31425

Table E4 Leaf width (in cm) of lettuce treated with biomass from FW 20%, FW 40%, FW 60%, FW 80%, and FW 100%, along with the control group (no biomass given). One to two true leaves were measured from each plant

Date	Observation Day	FW 20%					FW 40%				
		1-1	1-2	2-1	2-2	Avg	1-1	1-2	2-1	2-2	Avg
6/12/24	18	0.807	0.494	1.43	0.941	0.918	0.725	0.749	0.954	0.841	0.81725
12/12/24	24	1.215	0.98	2.076	1.46	1.433	0.733	0.873	1.134	0.879	0.90475

Date	Observation Day	FW 60%					FW 80%				
		1-1	1-2	2-1	2-2	Avg	1-1	1-2	2-1	2-2	Avg
6/12/24	18	0.622	-	0.857	0.716	0.575	0.909	0.562	1.01	0.477	0.7395
12/12/24	24	0.639	-	0.861	0.733	0.6	0.928	0.67	1.052	0.506	0.789

Date	Observation Day	FW 100%					Control				
		1-1	1-2	2-1	2-2	Avg	1-1	1-2	2-1	2-2	Avg
6/12/24	18	0.827	0.466	0.514	-	0.602	0.725	0.596	0.941	0.942	0.801
12/12/24	24	0.834	0.591	0.595	-	0.673	0.735	0.615	0.944	0.987	0.82025