Optimization of intermittent CO_2 supply and flocculation recovery for the cultivation of a haptophyte *Isochrysis galbana*

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CONTENTS

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ABSTRACT

The requirement for high fatty acids and fucoxanthin content in *Isochrysis galbana* is rising year by year, therefor, the low-cost cultivation and harvesting in large-scale has become an indispensable issue. The culture of microalgae is affected by a number of parameters, including the amount of solar irradiation, the availability of nutrients, the agitation, and the condition of the CO_2 supply. Continuous CO_2 supply for accounts for a significant portion of microalgae cultivation's total energy consumption. While the intermittently CO_2 supply has the possibility to reduce the aeration cost, it may induce carbon deprivation and inhibit microalgae growth. Microalgal harvesting also a challenge due to its small cell size. There is a method of flocculation that reduces the cost of harvesting algae by simply adding flocculant and adjusting the pH.

Firstly, Chapter II examined the effects of CO_2 concentration and frequency on the productivity of the marine microalgae *I. galbana*. Different CO_2 concentrations and frequencies (0.04 -10% CO_2 and continuous, 1/9 minutes of supply) were applied to semi-continuous *I. galbana* cultivation. In Chapter III, the effects of various pH values (between 8 and 10) and PO_4 concentrations (between 0 and 10 mM) on the sedimentation efficiency of microalgal were evaluated. As a result, under air supply conditions, biomass productivity was achived 0.17 g L^{-1} d^{-1} with continuous supply(control). The 5% continuous condition reached highest biomass productivity (0.35 g L^{-1} d^{-1}). In continuous of CO_2 supply, the productivity was only 0.11 g L^{-}

^{1} d^{-1} with its concentration of 10%, indicating that a higher DIC concentration also would inhibit the growth of microalgae. On the other hand, the condition with CO_2 concentration of 5% and 10% showed 0.3 and 0.22 g L^{-1} d^{-1} of productivity in intermittent CO_2 supply of 1/9min/min, respectively. These productivities exceeded those of the control group. In addition, under supply condition of air, a higher fucoxanthin concentration of 0.61 mg/g-DW was attained than 5% and 10% with continuous supply (0.18 and 0.11 mg/g-DW), respectively. However, the fucoxanthin content reached 0.56 mg/g-DW under 5% continuous supply with 1/9 min/min agitation frequency. This result show that continuous supply of high CO_2 concentration inhibited the accumulation of fucoxanthin, but intermittent supply was advantageous to fucoxanthin accumulation. On the other hand, Fast and efficient sedimentation efficiency developed (within 30 min) at a high PO_4 concentration (5,10 mM) at high pH (10), the sedimentation rate was achieved 40%.

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CHAPTER 1 General Introduction

1.1. Production process of microalgae

Microalgae are promising photosynthesis-producing microorganisms that are used to make food for humans, animals, and biofuel. There are several steps for the cultivation of microalgae to obtain dry microalgal biomass, which requires to be harvested and extracted before their usage as commercial products. Firstly, certain open or closed systems are used to grow microalgae (Suganya et al., 2016). Secondly, the microalgae will be harvested and subsequently dried (Salim et al., 2013). Thus, additional downstream processing, such as cell disruption and extraction, is required to create certain microalgae-derived compounds (Fig 1.1).

Microalgae are natural biofactories that have garnered increasing attention for their sustainable and versatile applications. They offer long-term solutions in areas such as food or feed production, generation of biochemical or bioenergy, and mitigation the change of global climate (Ratnapuram et al., 2018; Vu et al., 2018) The biomass of microalgae serves as a natural food for significant aquaculture organisms like fish, mollusk, and shrimp, and (Selvarajan et al., 2015). Moreover, microalgae are a rich source of valuable bioactive compounds, including proteins, lipids, carotenoids, carbohydrates and vitamins, which have numerous commercial applications. Carotenoids, for instance, exhibit cancer-preventive and anti-tumor properties (Suganya et al., 2016). According to their structural components, carotenes, which are made of hydrogen and carbon (such asα-carotene, β-carotene, and lycopene), and xanthophylls, which are made of hydrogen, carbon, and oxygen (such as zeaxanthin, β-cryptoxanthin, astaxanthin, lutein, fucoxanthin), are the two main groups of carotenoids. (Jaswir et al., 2011).

Fucoxanthin, a type of carotenoids, possesses a unique structure characterized by an unusual allenic bond. Once absorbed into the human body, fucoxanthin is metabolized into fucoxanthinol, amarouciaxanthin A, and halocynthiaxanthin (Sangeetha et al., 2010). Notably, fucoxanthin exhibits higher potency than astaxanthin and β-carotene in terms of anti-obesity activity and the induction of apoptosis in human leukemia (Kim and Pangestuti, 2011; Peng et al., 2011; D'Orazio et al., 2012). Despite its valuable biological activities, the utilization of fucoxanthin has been limited due to challenges in extracting it efficiently from sea sources and difficulties in synthesis of chemical (Kanazawa et al., 2008; Kajikawa et al., 2012). While some studies have focused on extracting fucoxanthin from brown macroalgae (Kanazawa et al., 2008; Kim and Pangestuti, 2011). The low concentrations of fucoxanthin in these macroalgae, coupled with their traditional food status in Southeast Asia and some European countries, render commercial production unfeasible (Kim et al., 2012). Finding alternate sources of fucoxanthin is therefore important.

1.2. A marine microalga *Isochrysis galbana*

Isochrysis galbana, belonging to the phylum Haptophyta, is primarily found as a unicellular flagellate microorganism. It possesses two long isoquant flagella emerging from a gullet-like structure. Due to its favorable nutritional qualities, such as high carotenoid and polyunsaturated fatty acid content, *I. galbana* is extensively used in aquaculture, particularly as a feed for early-stage growth of mollusk larvae, fish, and crustaceans (Vandamme et al., 2013). Moreover, microalgae with elevated levels of fatty acids, including eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), as well as carotenoids like fucoxanthin, hold promise for applications on the food, cosmetic, and industries of pharmaceutical (Vandamme et al., 2013). Fucoxanthin, a

commercially significant carotenoid, is extracted from marine brown algae and diatoms and is valued at approximately 30,000 US dollars per kilogram (Su et al., 2019). While previous extraction methods primarily focused on large algae, recent studies have highlighted the advantages of cultivating microalgae, which are simpler and more easily grown organisms with better culture conditions and growth rates (Vandamme et al., 2013). Therefore, there is a necessary for technological advancements to increase the production of valuable compounds from microalgae to improve the growing social and economic need (Vandamme et al., 2013). Table I-1 summarize microalgae producing fucoxanthin and their contents, with *I. galbana* exhibiting relatively high levels of fucoxanthin (Kim et al., 2012), which is one of the reasons for selecting *I. galbana* in this PhD study. Additionally, *I. galbana* has a faster growth rate and shorter cultivation period, which can contribute to cost savings, making it highly suitable for commercial applications.

Regarding the current development status of *I. galbana*, there have been numerous studies indicating the influence of cultivation conditions on algal growth. For instance, research has shown that *I. galbana* tend to thrive under high nitrogen (N) conditions, with a productivity increase of 16 times when the concentration is raised from 0 to 144 mg/L (Zarrinmehr et al., 2020). Additionally, studies have analyzed the impact of light intensity on *I. galbana* growth, suggesting that a light intensity of 300 μmol/m^2/s is favorable for microalgal growth (Guo et al. 2016) but unfavorable for fucoxanthin accumulation (Li et al., 2019). Currently, various methods have been able to achieve their desired outcomes; however, the high cost associated with cultivation is an issue. This problem is not exclusive to *I. galbana* cultivation but represents a bottleneck faced by all microalgae cultivation processes.

1.3. Cost of microalgae cultivation and harvesting

The previous study reported an estimated cost of 596 cts. € $kg^{-1}DW^{-1}$ for microalgal biomass production in flat panels (Norsker et al., 2011). In this production process, the electricity power costs of aeration alone accounted for 40% of the total cost (Fig. I-2(a)). It is crucial to explore avenues to reduce the expenses associated with aeration. Also, there is a need to emphasize on the cost of harvesting. Infect, the cost of harvesting is related to the type and scale of the microalgae cultivation, and the equipment used for harvesting. Depending on the type and scale of cultivation, the harvest cost accounts for about 1-28% of the total cost (Molina Grima et al., 2003; Norsker et al., 2011). Table I-2 presents the harvesting cost percentages for microalgae production is dependent on the type and scale of reactor. The calculation in this table not only includes the power consumption but also the cost of the equipment. For instance, in the case of the flat-panel reactor, cultivating microalgae on a 100-hectare area resulted in a 2% harvesting cost. However, the use of tubular photobioreactor, despite a smaller cultivation scale, led to increase in the harvesting costs to 28%. Similarly, cultivating microalgae in raceway ponds, with the same reactor size as the flat-panel reactor, resulted in a 6.5-fold increase in harvesting costs (13%) due to the lower cell density of microalgae in the raceway pond compared with flat panel reactor, tubular reactor and so on. For small and medium-sized microalgae cultivation, if the harvest cost can be reduced from 28%, the total cost of microalgae culture will be greatly reduced. Therefore, this PhD study focuses on reducing power consumption during microalgae cultivation and harvesting through intermittent CO_2 supply and flocculation, respectively.

1.4. Microalgal cultivation

Despite the advantages of growing the microalgae *I. galbana*, there are numerous issues with its development. For instance, the culture of microalgae is relatively expensive due to the low biomass yield and low fucoxanthin content. To increase microalgae output, numerous tools and growing techniques have been improved over time. Although microalgae are simple to cultivate in the lab with strict controls, it is still challenging to achieve their high output in outdoor large-scale production.

Microalgal cultivation are influenced by various factors, including light, availability of nutrient, agitation, and conditions of supply CO_2. CO_2 continuous supply of aeration accounts for a significant portion of the total energy cost in microalgal cultivation (Norsker et al., 2011). There is a need to reduce the cost of this, otherwise it becomes a problem. The supply of carbon dioxide has roughly two functions, first, is to provide the carbon source needed for algae growth, and second, is agitation process of the microalgae, so that the microalgae can evenly receive the light irradiation. The concentration of carbon dioxide controls the supply of carbon sources. Studies on the impact of CO_2 supply concentrations on microalgal development have been conducted. A previous study (Vandamme et al., 2013) showed that intermittent CO_2 supply can achieve the similar result as continuous supply of CO_2. The growth rate of Chlorella vulgaris cultivation at various CO_2 concentrations is displayed by Liu (Liu et al., 2017). The biomass productivity of *Chlorella vulgaris* grown in 10% CO_2 conditions enhanced. However, under conditions of 20% CO_2 supply, biomass decreased. That may imply that a constant supply of high CO_2 levels may be the cause of the microalgae's growth inhibition. However, the optimum amount varies depending on the type of microalga; it is unknown what the optimum concentration is for *I. galbana*. Although the

regulation of CO_2 concentration can improve the growth of microalgae, it cannot solve the bottleneck problem, that is, the high culture cost. The problem of high culture costs may be ameliorated by intermittent high CO_2 supply. Thus, the aim of this research is to find a balance between intermittent gas supply and CO_2 concentration in order to cultivate high-density microalgae and accumulate more pigments, while minimizing the cost of culture.

1.5. Microalgal harvesting

After cultivation, the microalgae need to be harvested. One of the key steps in the processing of microalgae is microalgae harvesting. As a result of the high capital expense and energy requirement, several studies have estimated that it accounts for 1% to 28% of the total production cost (Uduman et al., 2010; Norsker et al., 2011; Barros et al., 2015). This huge difference is due to the scale of microalgae production, which means the larger the scale, the smaller the cost of harvesting. For small and medium-scale cultivation, microalgae harvest costs account for a considerable part. To facilitate subsequent processing, such as the extraction of bioactive compounds, all harvesting procedures aim to remove as much culture medium as possible from the microalgal biomass.. Biomass has been harvested using a variety of techniques, incorporating filtering, centrifuging, flotation and flocculation,. (Singh and Patidar, 2018). In some cases, a combination of two or more approaches is used to boost harvesting effectiveness. So, a low-cost, low-energy pre-concentration technique is needed. Microalgal flocculation and sedimentation can be an effective pre-concentration method because of its simplicity and fastness. Combinations of cation addition and pH adjustment is an effective method. Although, there have been some previous studies on this method, the results were not consistent probably because most studies only measured the change of algal density in the supernatant. It is necessary to optimize the

analysis methods for algal flocculation and sedimentation. Optimizing the analysis methods can enhance the efficiency and accuracy of microalgal flocculation and sedimentation, facilitating a greater comprehension and command of the procedure.

1.6. Objective

This PhD. study aimed to optimize the cultivation conditions and sedimentation harvesting method for reducing the cost of the power consumption in the cultivation of *I. galbana* by performing the following objectives:

1) To investigate the effects of semi-continuous cultivation of *I. galbana* under different CO_2 concentrations and supply frequencies (Chapter II).
2) To depict the impact of pH and cationic substances on the flocculation and sedimentation of marine microalgae *I. galbana* (Chapter III)

After achieving a balance between intermittent gas supply and CO_2 concentration in order to cultivate high-density *I. gralbana* and accumulate more fucoxanthin (Chapter II) and an optimum flocculation and sedimentation condition for harvesting microalgae (Chapter III), this PhD study aimed the practicability of using a photobioreactor (column reactor) in terms of electricity consumption and cost-effectiveness as a part of the general discussion (Chapter IV).

Figures

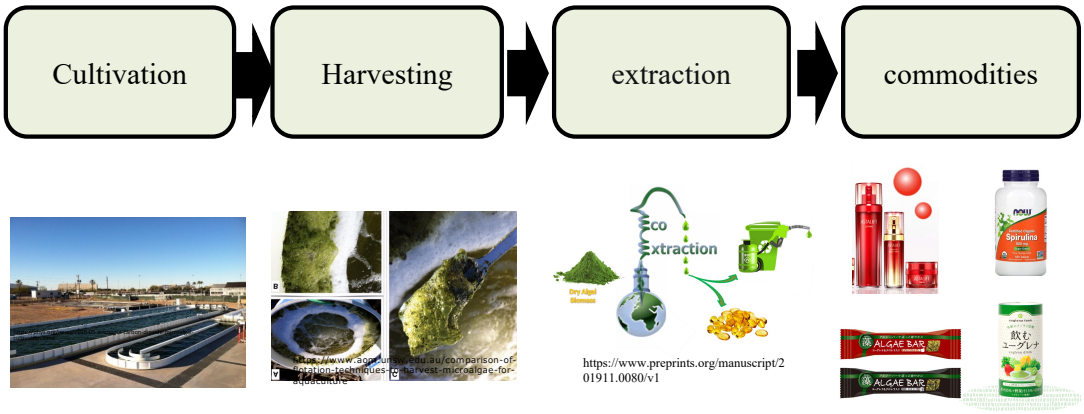


Fig. I-1. Process for microalgal biomass production

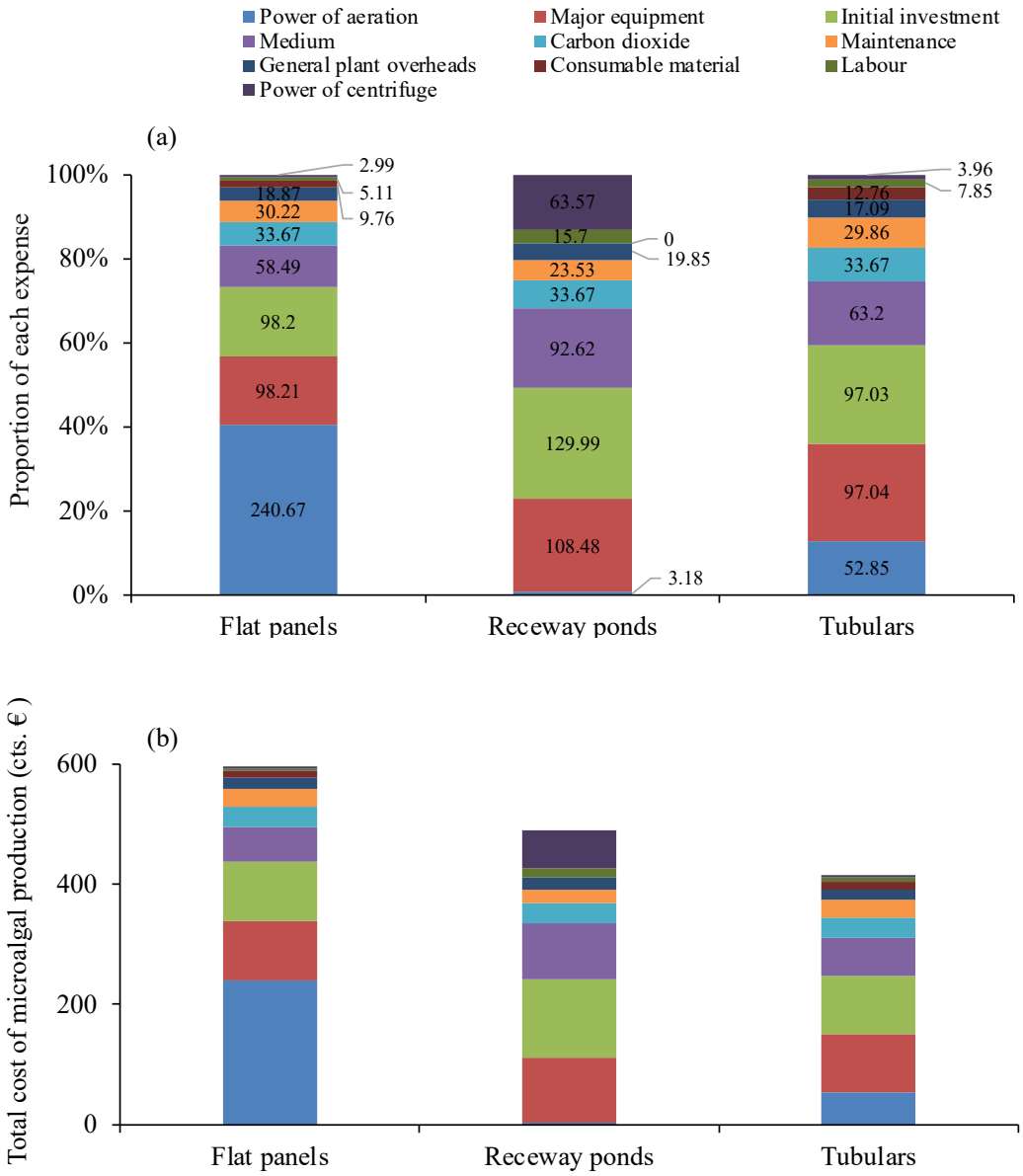


Fig. I-2. (a)The cost of each expense (cts. €) and its proportion for flat panel, raceway ponds and tubular reactor. (b) The total cost of microalgal cultivation until harvesting in flat panel, raceway ponds and tubular reactor.

Tables

Table I-1: The contents of fucoxanthin produced by various microalgae

	Microalgae Species	Fucoxanthin content (mg/g)	Ref.
Microalgae	*Chrysotila carterae*	1.04	Ishika et al. (2017)
	Navicula sp.	1.49	Ishika et al. (2017)
	Amphora sp.	1.21	Ishika et al. (2017)
	Chaetoceros muelleri	2.92	Ishika et al. (2017)
	Chaetoceros calcitrans	2.08	Foo et al. (2015)
	Chaetoceros calcitrans	5.25	Foo et al. (2015)
	Chaetoceros gracilis	2.24	Kim et al. (2012)
	Cylindrotheca closterium	5.23	Pasquet et al. (2011)
	Isochrysis aff. galbana	18.23	Kim et al. (2012)
	Isochrysis galbana	6.04	Kim et al. (2012)
	Isochrysis galbana	0.14	Gómez-Loredo et al. (2015)
	Pheodactylum tricornutum	1.87	Ishika et al. (2017)
	Phaeodactylum tricornutum	15.42–16.51	Kim et al. (2012)
	Phaeodactylum tricornutum	8.55	Kim et al. (2012)
	Phaeodactylum tricornutum	0.2	Gómez-Loredo et al. (2016)
	Phaeodactylum tricornutum	5.5	Hualian et al. (2016)
	Nitzschia sp.	4.92	Kim et al. (2012)
	Odontella aurita	18.47	Xia et al. (2013)

Table I-2. Harvesting cost in different type and size of reactor

Reactor type	Reactor size	Percent of the total cost(%)	Ref.
Flat panel reactors	100ha	2	Norsker et al., 2011
Tubular photobioreactor	0.8 m^3	28	Grima et al., 2003
Raceway ponds	100ha	13	Norsker et al., 2011

CHAPTER 2 Optimization of CO_2 supply frequency and concentration in high cell density culture of *Isochrysis galbana using* bubble column photobioreactor

2.1. Introduction

In general, microalgae rearing systems may be divided into two types: open ponds and photobioreactors. Each method has advantages and disadvantages.

One of the oldest and most essential methods for the development of microalgae on a big scale is known as open pond cultivation. Due to their low constructing, maintenance, and operating costs, open ponds are utilized frequently in industry. Additionally, open pond systems are scalable, need little energy, and are simple to operate and maintain. The benefit of the open pond approach is that it is the least expensive form of production. Microalgae produced in natural water have a low cell concentration despite a large culture area, requiring a highly effective harvesting procedure (Sun et al., 2016). In addition, concerns such as precipitation discharge, which modifies the growth conditions of algae, such as pH and salinity, which results in overflow and increased water contamination, possible to have a significant effect on microalgae production in open pond. Another concern that arises with open pond culture systems is the likelihood of protozoa and bacterium contamination, which causes the products to be poisonous and useless. The current available solution is to cultivate microalgae capable of living in severe alkaline or saline conditions, as only a few pollutants can flourish in these conditions. (Stark M, 2012). Besides, because the system is open, it is more difficult to manage some growth of parameters such as temperature , pH and light intensity, which may impact the microalgae growth rate (Stark M, 2012).

The photobioreactor, a reactor system used for cultivating the phototrophs such as algae in a closed reactor that does not permit direct material exchange between the culture medium and the external environment. The photobioreactor can circumvent numerous limitations that are frequently encountered in the design of open pond cultures. First, the bioreactor is smaller in size than an open pond, enabling greater efficient land utilization. Second, the method provides a confined and high regulated development environment for the cultivation, allowing it to growth the microalgae culture of a single strain that is free of contamination(Posten, 2009). In addition, the carefully regulated growth conditions can translate into increased nutritional and efficiency of metabolic, causing an increase in biomass production for every unit of substrate. However, the practical application of photobioreactors is hindered by their limited scalability, which makes their use in large-scale production uneconomical (Gupta et al., 2015). Moreover, photobioreactors with highly controlled growth conditions always incur high capital and operating expenses. This study aims to reduce the cultivation cost of photobioreactors. In this study, bubble column photobioreactor was used, and intermittently gas supply were used to reduce the aeration power consumption of microalgae cultivation.

As factors influencing the proliferation of microalgae, light intensity, temperature, nutrients, and aeration play a significant role. Among them, the cost of aeration represents a substantial proportion. A previous study estimated the electricity consumption at 448 kWh/day when using photobioreactors for microalgae cultivation (Acién et al., 2012). And 21% (96 kWh/day) of this energy consumption was attributed to cultivation aeration. Consequently, there is a pressing need for a cost-efficient method to cultivate microalgae. One possible approach to reduce cultivation costs is by adjusting the frequency of CO_2 supply, employing intermittent CO_2 supply. However,

while intermittent CO_2 supply has the potential to lower aeration costs, if the supply frequency is insufficient, it might also result in carbon starvation and cell sedimentation, creating a critical concentration of CO_2. Therefore, maintaining an appropriate CO_2 concentration is equally important when implementing intermittent gas supply. A previous study demonstrated that intermittent CO_2 supply (1 minute every 9 minutes (1min/9min)) can reached the similar growth rate as continuous supply (Yago personal communication). On the other hand, *Isochrysis galbana* is also good spacies for producing fucoxanthin. Allelic carotenoid fucoxanthin has demonstrated significant nutraceutical bioactivity, exerting antioxidant, anti-cancer, anti-obesity, anti-angiogenic, anti-photoaging, and anti-metastatic properties (Miyashita et al., 2011; D'Orazio et al., 2012). The fucoxanthin composition in microalgae is also influenced by factors such as pH, CO_2 concentration, light intensity, cell density, nutrient concentration, aeration, and the physiological state of the culture. Hence, intermittent supply could potentially have a positive impact not only on biomass production but also on carotenoid accumulation.

This study investigated the influence of CO_2 concentration and intermittent CO_2 supply (1min/9min) on the productivity of the marine microalgae *I. galbana. I. galbana* was cultivated semi-continuously at various carbon dioxide concentrations and frequency, and production of biomass and accumulation of fucoxanthin were measured.

2.2. Materials and Methods

2.2.1. Microalgae strain and culture conditions

Isochrysis galbana UPMC-A009, a marine microalga, was acquired in Malaysia. With the aid of 10 mL mono-specific seed culture, the population of these genera was increased in test tubes. The growth medium was Conway medium, which

contained KNO_3 at a concentration of 100 mg/L, Na_3PO_4 at 20 mg/L $Na_2H_2EDTA \cdot 2H_2O$ at 45 mg/L, H_3BO_3 at 33.6 mg/L, $FeCl \cdot 6H_2O$ at 1.3 mg/L, $MnCl_2 \cdot 4H_2O$ at 0.36 mg/L, $ZnCl_2$ at 2.1 mg/L, $CoCl_2 \cdot 6H_2O$ at 2 mg/L $(NH_4)6Mo7O_{24} \cdot 4H_2O$ 0.9 mg/L, $CuSO_4 \cdot 5H_2O$ at 2 mg/L Thiamin HCl at 200 µg/L Cyanocobalamin at 10 µg/L and Na2SiO3·9H2O at 30 mg/L. The medium was autoclaved at 121 °C for 20 min. Microalgae was cultivated with 300 µmol photons m^{-2} s^{-1} of light intensity, and a 12 hours light and 12 hours dark cycle at 25 °C. The pH was 8.0 ± 0.3 at the start. Aeration of the culture occurred at 0.2 L. min^{-1}.

2.2.2. Column reactor Setup

Three components made up the culture system: a column reactor with a mechanism for delivering culture medium, a LED light and a thermoregulator with a water reservoir (Fig. II-1). The column reactor had a 52 cm height and the diameter of 6.0 cm constructed of transparent glass. This reactor had a 1.2 L for working volume and a light-receiving area of 0.021 m^2. The reactor was lined with LED lighting. Using a quantum sensor (QSPL-2101, Bio Spherical Instruments, United States), the light intensity was verified at the reactor's surface. A flow meter was used to regulate the amount of carbon dioxide present, and air was constantly injected into the reactor at 0.2 L min^{-1}. A thermosregulator with a water bath was used to regulate the temperature. To dissipate heat, and fan was mounted on the near of the light. Before the light period, a sample was taken once a day, and medium was added until 1.2 L. Sterilized conditions were used for all procedures.

2.2.3. The conditions of CO_2 concentration and supply frequency

In order to understand the microalgal growth characteristics, the impacts of carbon dioxide condition and frequency of supply on microalgal growth was studied. The

cultivation of *I. galbana* was conducted under four conditions of carbon dioxide concentration (air, 2, 5, and 10%) and two conditions of supply frequency (continuous and supply CO_2 for 1 minute every 9 minutes (1/9min)). Gas was added through to 0.2 μm filter at the flow rate of 0.2 L min^{-1}, with the temperature kept at 25 ± 1◦C. 1.2 L of fresh medium were provided, and 0.25 d^{-1}. of dilution rates were used. In all dilution rate treatments, a intensity of light was 300 μmol m^{-2} s^{-1}. In every experiment, the light was kept constant at 12 hours light and 12 hours dark. The control test was carried out with a steady supply of air.

2.2.4. Measurement of growth

Using a total organic carbon analyzer (TOC-L CPH/CPN, Shimadzu, Japan), a pH meter (D-51, Horiba, Japan), and a UV-visible spectrophotometer (UV-2450, Shimadzu, Japan), the dissolved inorganic carbon (DIC) concentration, pH, and optical density at 750 nm (OD750) of the culture were determined. A standard curve of OD_{750} vs DW that was created before the experiment was conducted and used for determining the dry weight (DW). OD_{750} and DW values were determined for each of the five concentrations. (≤ 1 OD_{750}), at which the stock culture was diluted. As a result, with $p < 0.01$ and $R^2 = 0.9989$., a correlation between OD_{750} and DW was verified. The following this conversion formula was created from the acquired standard linear line, and it was used to calculate DW. (Eqn 1):

$$\text{DW concentration} = 1.2749 \times OD_{750} - 0.046 \quad . \quad (1)$$

Using Equation (2), biomass productivity (g/L/d in the stationary experimental phase was calculated using the DW of 3 days. Eqn (2):

$$\text{Biomass productivity} = 0.25 \times S_{DW} \quad , \quad (2)$$

where 0.25 is the daily dilution rate, and S_{DW} (g/L) is the DW (g/L) for the stationary

phase.

On the first and last days of the experiment, the fucoxanthin content was determined.

2.2.5. Pigment

The microalgal cells were purified using a GF/F filter, repeatedly washed with distilled water, and then extracted in 5 ml N,N-dimethylformamide (DMF) for 24 h at 20°C for the pigment compositions (Furuya et al., 1998). Prior to analysis, 1 ml of the extracted material was filtered using a 0.22-μm PTFE filter (Millex-FG, Merck, Japan). Using an LCMS system (ACQUITY UPLC H-Class, Waters, USA), the carotenoids in the samples were determined and identified. Solvent A (100% acetonitrile), Solvent B (100% 2-propanol), and Solvent C (10% methanol) made up the mobile phase. Solvent A was 50%, Solvent B was 40%, and Solvent C was 10% at the beginning of the measurement. After that, there was a linear gradient to Solvent A being 2%, Solvent B being 40%, and Solvent C being 58%. The mixture was changed to solvent A 50% after 12 minutes. The Danish Hydraulic Institute, Hrsholm, Denmark, sold the chemical standards for astaxanthin, canthaxanthin, lutein, β-carotene, zeaxanthin, and violaxanthin, which were then purchased and analyzed using a reverse phase column (ACQUITY UPLC BEH C18, 1.7μm; 2.1 × 50 mm, Waters, USA) By contrasting the absorption and mass spectra with the reference values, the sample chromatographic peaks were located, and the peak regions were then integrated to determine their quantitation.

2.2.6. Lipid measurement

The sulfo-phospho-vanillin technique was used to test lipids. Initial preparation of the phosphovanillin reagent involved stirring constantly while 0.6 g of vanillin was

dissolved in 10 ml of pure ethanol and 90 ml of deionized water. The combination was then mixed with 400 ml of strong phosphoric acid, and the reagent was then kept in the dark until it was needed. For maximum activity, the reagent was freshly produced just before each experimental run. The GF/F filter-filtered cells were next treated with 2 ml of concentrated sulfuric acid, heated for 10 minutes at 100°C, and then chilled for 5 minutes in an ice bath. After centrifuging the microalgal cells at 4000 rpm for five minutes, 1 ml of the supernatant was poured into a glass vial. The bottle was then filled with 2.5 ml of sulfo-phospho-vanillin, which was then incubated for 15 minutes at 37°C. The samples were quantified by comparing them to a calibration curve using commercial canola oil (200 mg) in 100 ml chloroform as a standard. The absorbance was read using a spectrophotometer (HACH) at 530 nm.

2.3. Results and discussion

2.3.1. Biomass productivity

The effects of CO_2 concentration and supply frequency on the growth of *I. galbana* microalgae were investigated. The temporal changes in pH, DIC, and DW is shown in Fig. II-2. The control condition is a constant supply of air. Similar outcomes to the control were obtained with a concentration 2% CO_2 supply (DW was 0.7 g L^{-1}) (Fig. II-2c). Under concentration of 5% CO_2 condition, the maximal concentration of biomass (1.32 g L^{-1}) was attained. The settings with 5% growth exhibited the strongest growth, which was consistent with the growth of *I. zhangjiangensis.* (Li et al., 2019). However, the 5% CO_2 intermittent supply conditions, however, similarly displayed nearly identical high dry weight and growth rates to the continuous supply in 5%. Regardless of how frequently it was supplied, the CO_2 supply of 5% boosted biomass content of *I. galbana.* Under conditions of 0 and 2% CO_2 for 1 min per 9 min conditions,

the highest dry weight was only 0.29 and 0.26 g L^{-1}, respectively. These settings demonstrated slower rates of growth than the other conditions. The quick decrease in optical density was observed at 10% CO_2 continuous supply settings, indicating that excessively high continuous of CO_2 supply concentrations may inhibit microalgal growth. This is different from the results obtained for the microalgae of *C. vulgaris* mentioned in the introduction. The optimal culture condition of C. *vulgaris* is a CO_2 supply concentration of 10%, and the growth of microalgae is inhibited when the supply concentration is increased to 20%. This is because the two types of microalgae are different, and the minimum pH value they can withstand is also different. The freshwater microalgae C. *vulgaris* can withstand a lower pH value, so it can survive under a higher concentration of CO_2. Under the condition of 10% and 1-min/9-min , development was slow at first during culture, but it picked up around day 7. The ultimate (highest) DW was greater than control, indicating that the cells had become used to the culture conditions. Under circumstances of air continuous and air for one minute every nine minutes throughout the culture phase, pH climbed to roughly 9 after three days, but under other conditions, it declined to approximately six days (Fig. II-2a). Similar to this, DIC level decreased under circumstances of continuous air and air for one minute every nine minutes, although an increase to around 35 mg C L^{-1} was seen under other conditions. The high CO_2 concentration supply was to blame for the pH drop and rise in DIC (Fig. II-2b). Previous research has demonstrated that excessive CO_2 input will be acidify the medium and make lower pH, preventing the development of microalgae. (Dineshkumar and Sen, 2020). Additionally, it has been found that inadequate DIC slows development. (Qiu et al., 2017). Therefore, the absence of DIC under conditions and intermittent air supply with 2% CO_2, as well as the pH reduction under conditions of continuous 10% may have contributed to the decline in growth.

However, the 10% intermittent CO_2 supply showed a recovery tendency in the growth rate, and the pH fell below 6.3. This finding implies that in long-term cultures, large quantities of intermittent CO_2 delivery may only have a little impact on algae development. Due to the semi-continuous nature of this experiment, I carefully compared with each condition during to the stationary phase.

On the stationary phase, the biomass production and pH are shown in Fig. II-3. Low biomass productivity was seen in the air and 2% circumstances, whereas the maximum biomass productivity was seen in the 5% settings. The experimental settings can be split into 2 categories: lower pH circumstances of about 6 (5% and 10%) and higher pH circumstances of about 10 (air and 2%); the biomass concentration of productivity was 0.12 to 0.33 and 0.07 to 0.18 g $L^{-1}d^{-1}$, respectively. In the pH range of 5 to 9, Kaplan et al. (Kaplan et al., 1986) shown that there has no discernible difference in the microalgae growth, while other studies have demonstrated that a lower pH (6-7) was favorable for the growth of the microalga *Isochrysis sp.* (Brown et al., 1993); these results agree with those of the present investigation. However, DIC concentration was lower at air and 2% CO_2 concentrations than at 5% and 10%, indicating that the DIC concentration may be the limiting factor for growth (Fig. II-4a). The growth of microalgae between 5% and 10% also need be considered. The carbon supply might be in the form of – CO_2, HCO_3, or CO_3 – depending on the pH (Fig. II-10). Even though DIC's overall composition is the same, the amounts of CO_2 vary depending on the pH (Fig. II-11). However, no prior research has revealed which shape is more beneficial for *I. galbana* development. Figure II-9 displays Masao's(Masao, 2000a). measurements of the ratio of total DIC's carbonic acid (CO_2, HCO_3^-, and CO_3^{2-}) concentration at various pH levels. It suggests that the HCO_3^- content in seawater is very high between pH 7 and 8. (Masao, 2000b). The pH values at 5% and 10% in the

current investigation, using the supply of continuous scenario as one example, achieved 6.7 and 6.1, respectively. HCO_3 made up around 88% and 62% of the mixture, respectively (Fig Ⅱ-9). The HCO_3 level was much lower in 10% circumstances than in 5% conditions. Additionally, biomass productivity increased with increasing HCO_3 concentrations (Fig Ⅱ-10(a)). Fig Ⅱ-11 shoed the relationship between biomass productivity and HCO_3 and CO_2 content. From the result, it can infer that an HCO_3 carbon source might promote *I. galbana* development (Fig II-11(a)), and there is no relationship between CO_2 and biomass productivity (Fig II-11(b)). In earlier investigations of the productivity of biomass of microalgal utilizing *I. galbana*, continuous aerated culture only produced 0.048 d^{-1} of specific growth rate. (Sánchez et al., 2013). By adding 1 g L^{-1} of carbonate to the culture, Danesh (Danesh et al., 2019) were able to produce 0.38 g L^{-1} of biomass production and 0.22 d^{-1} of specific growth. *I. galbana* was grown under 5% continuous conditions in other research (Picardo et al., 2013), and the results showed high biomass output and specific growth of 0.51 g L^{-1} and 0.34, respectively. This investigation demonstrated that *I. galbana* can still produce at high rates under intermittent CO_2 delivery since biomass yields of up to 0.56 g L^{-1} and specific growth of 0.36 were attained.

2.3.2. Pigment and Lipid production

The concentration of fucoxanthin generation with photobioreactors is influenced by several significant parameters, including nutrition, light, temperature, salt level, aeration, pH, and cell density (Boderskov et al., 2016). By carefully monitoring and changing these parameters, the photobioreactor's fucoxanthin production may be raised to its maximum potential. This research examined the impact of pH and aerated of carbon dioxide concentration on formation of pigment. The findings of the pigment's

fucoxanthin content are shown in Fig. II-4b, c. As can be observed, under continuous settings, fucoxanthin content decreases as supply concentration of CO_2 rises. However, with intermittent supply, it is evident that the level of fucoxanthin accumulation peaked at 5% CO_2 supply. However, a constant carbon dioxide supply provides the cells with sufficient and uniform illumination, which would not result in a significant fucoxanthin accumulation. Previous research has indicated that low light is better for fucoxanthin accumulation (Gómez-Loredo et al., 2016); This showed that, under the appropriate CO_2 conditions, intermittent delivery might encourage fucoxanthin buildup. Researchers looked at how three different carbon dioxide concentrations (0%, 1%, and 2%) affected the growth and production fucoxanthin in *P. tricornutum.* (McClure et al., 2018). The biomass content of this microalga decreased as the CO_2 concentration increased. For instance, cultures with 2% CO_2 showed lower biomass concentrations than cultures without CO_2, with the biomass concentration reducing by 62.22%. Biomass was produced at a rate of 0.45 g/L without the presence of CO_2. The medium's pH fell to 6.9 with the addition of 2% CO_2 due to the CO_2 acidification. In contrast, carbon dioxide concentrations of 1 and 2% resulted in larger fucoxanthin accumulations than those of air, suggesting that either sufficient amounts of CO_2 may be required to create fucoxanthin or that this microalga may have been stressed by high CO_2concentrations. In another study, the growth cells of *I. zhangjiangensi*s and the production of the fucoxanthin were examined in response to the addition of three different CO_2 concentrations (0% (air), 2%, and 5%) (Li et al., 2019). In contrast to 0% supplementation, the concentration of biomass and fucoxanthin concentration of this microalgae were both enhanced by 2 and 5%. The highest amounts of biomass (1.34 g/L) and fucoxanthin (2.32% DW) were reached at a CO_2 concentration of 5%. The difference in growth efficiency between these two research might be explained by

microalgae's resistance to rising pH levels with the addition of CO_2. *I. zhangjiangensis*, a microalga, may tolerate higher pH levels and can flourish in situations with CO_2 concentrations as low as 5%. Therefore, more investigation is required to determine if microalgae require CO_2 to create fucoxanthin or whether the stress brought on by high CO_2 concentrations increases fucoxanthin content.

Additionally, studies have shown that light plays a crucial role in microalgae's capacity to synthesize carotenoids. *I. galbana* and *P. tricornutum* both had increased fucoxanthin concentrations at low light intensities, with increases of 1.2 and 10.9 times, respectively, as well as improvements in total fucoxanthin production, with increases of 2.2 and 16.4 times, respectively. Low irradiances appear to be able to boost the carotenoid content by five times while also increasing pigment concentration, which has previously been linked to pigment involvement in light harvesting (Alabi et al., 2002). Additionally, it has been hypothesized that cells exposed to and accustomed to sub-saturating light for an extended period of time may produce more carotenoids (Mulders et al., 2014). According to research, as the number of photosystems increases in low light, so does the cellular concentration of the primary pigments (Mulders et al., 2014). For instance, a recent study discovered that *I. galbana* had higher levels of proteins, carbohydrates, lipids, and carotenoids when it was cultured in an aerated 5 L bubble column under low light conditions (27 μmol photons m^{-2} s^{-1}) (Guermazi et al., 2014). The carotenoid production in microalgae can also be influenced by the growth media. *Isochrysis sp.* was cultivated to its highest cell density in both F/2 and Conway media in a prior study. Under the identical circumstances, the Conway medium's cell density concentration was 32.4% greater than the F/2 medium (Lananan et al., 2013).

Due to the potential benefits that the carotenoid may have on human health as well as the fact that microalgae farming is more economically feasible than macroalgae

cultures, the production of fucoxanthin from microalgae is quite intriguing. This study discovered that an intermittent supply of CO_2 was favorable for the accumulation of fucoxanthin at the right CO_2 concentration (5%), but a continuous supply did not support the formation of fucoxanthin. For the purpose of establishing the optimal fucoxanthin production parameters, factors such as temperature, light intensity, and harvesting time should be carefully investigated in addition to the impacts of CO_2 concentration and supply frequency.

The control (Air continuous) showed the best fatty acid content in the findings for fatty acids, and there were no significant changes in fatty acids between circumstances. This suggests that CO_2 frequency and concentration have essentially little impact on the buildup of fatty acids.

2.3.3. Compare with other study

Table II-2 lists the earlier investigations on the productivity of microalgal biomass employing *I. galbana*. The species of microalgae, culture medium, CO_2 concentration, supply frequency, and particular growth are listed clockwise from the left. In the first investigation, constant aeration was utilized to cultivate microalgae, but only 0.048/d of specific growth was attained. The biomass output and specific growth in the second trial, which employed 1g L^{-1} carbonate to cultivate microalgae, were 0.38g L^{-1} and 0.22 d^{-1}. In the third study, they grew the microalgae at 5% continuous conditions and got higher yields of biomass (0.51g L^{-1}) and specific growth (0.34). The biomass yielded in my investigation reached up to 0.56g L^{-1}, and the specific growth was 0.36. In this work, it has been demonstrated that intermittent CO_2 delivery may still produce the best results for microalgal growth.

2.4. Conclusion

It was discovered that the best biomass productivity occurred under 5% CO_2 continuous supply circumstances. Due to the lower concentrations of DIC, biomass production was decreased when CO_2 was in low levels (air and 2%). Microalgae cannot thrive in environments with a high continuous CO_2 supply (10%) because the pH drops, causing the HCO_3 level to decrease. At a CO_2 concentration of 5%, however, supply of intermittent—which permits lower consumption of power—showed nearly the similar microalgae growth rate as supply of continuous. Additionally, *I. galbana* accumulated fucoxanthin due to irregular feeding. It is obvious that more research is required to determine the impact of additional elements including temperature, light intensity, and light hue on the fucoxanthin levels of *I. galbana.*

Figures

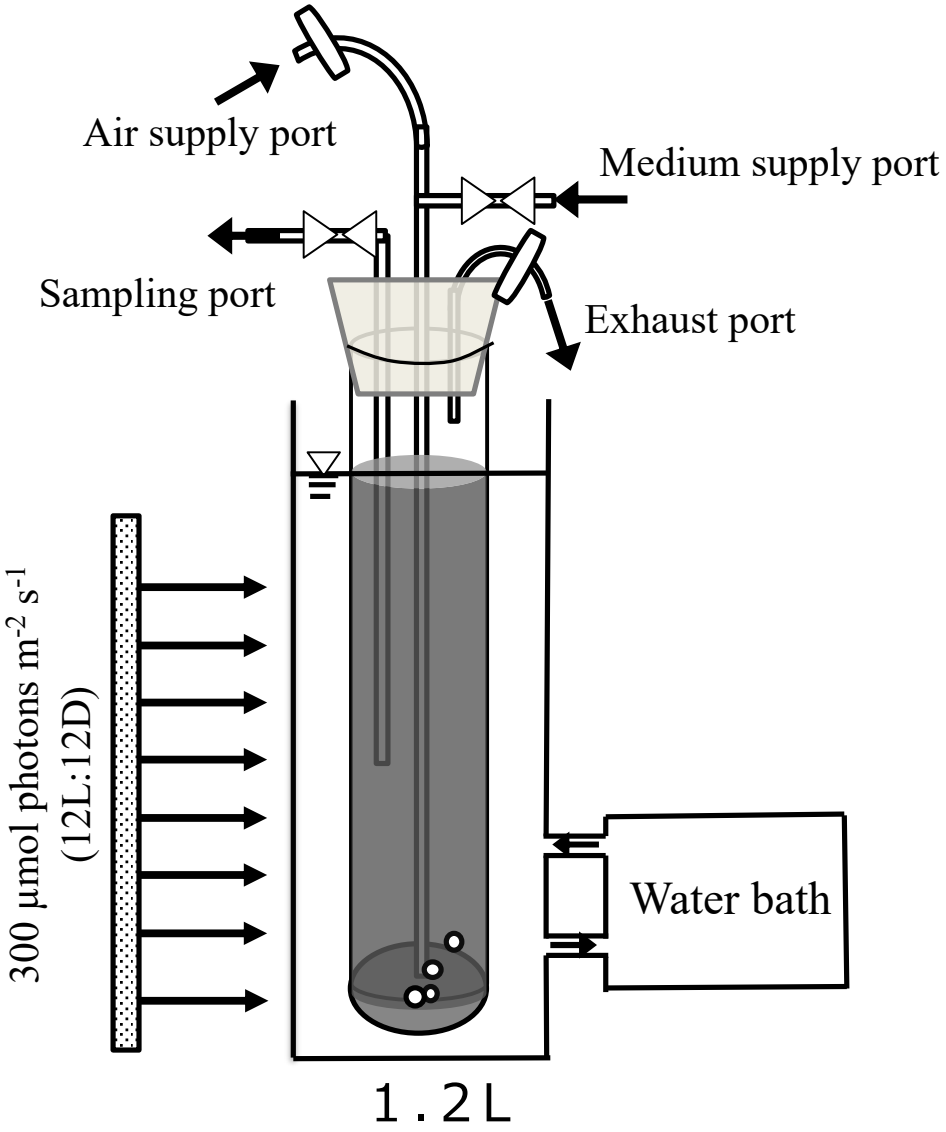


Fig. II-1. Schematic diagram of the column photobioreactor: inner diameter of 6.0 cm, working volume of 1.2 L. Light was provided to the surface of the water bath at 12L:12D. Filtered air (0.2 µm) was added with air, 2, 5, 10% CO2 from the bottom of the column reactor at a flow rate of 0.2 L min−1.

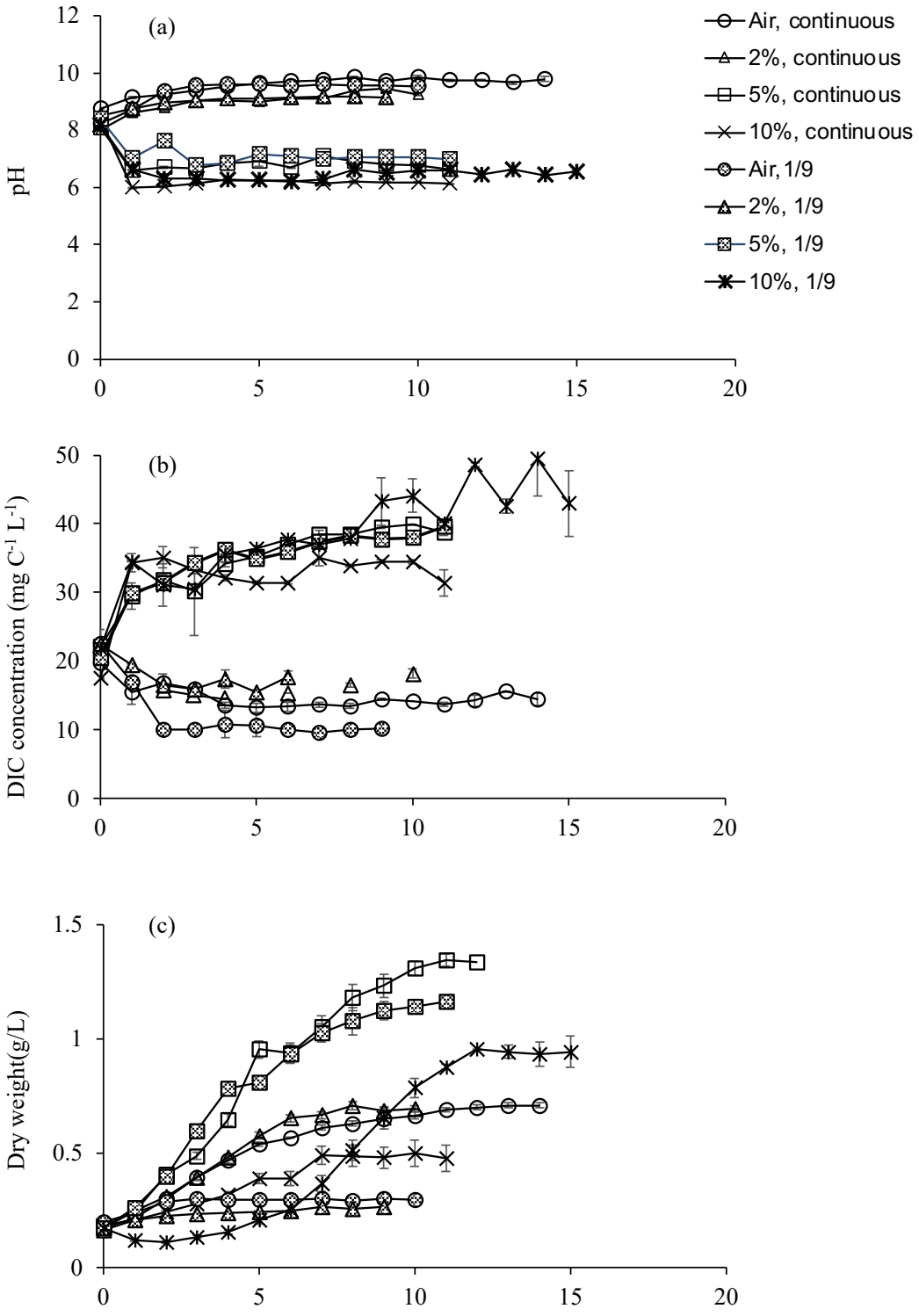


Fig. II-2. (a) pH, (b) dissolved inorganic carbon concentration and (c) dry weight (g/L) of each CO_2 supply concentration (air, 2%, 5%, 10%) and frequency (continuous, 1/9 min) condition.

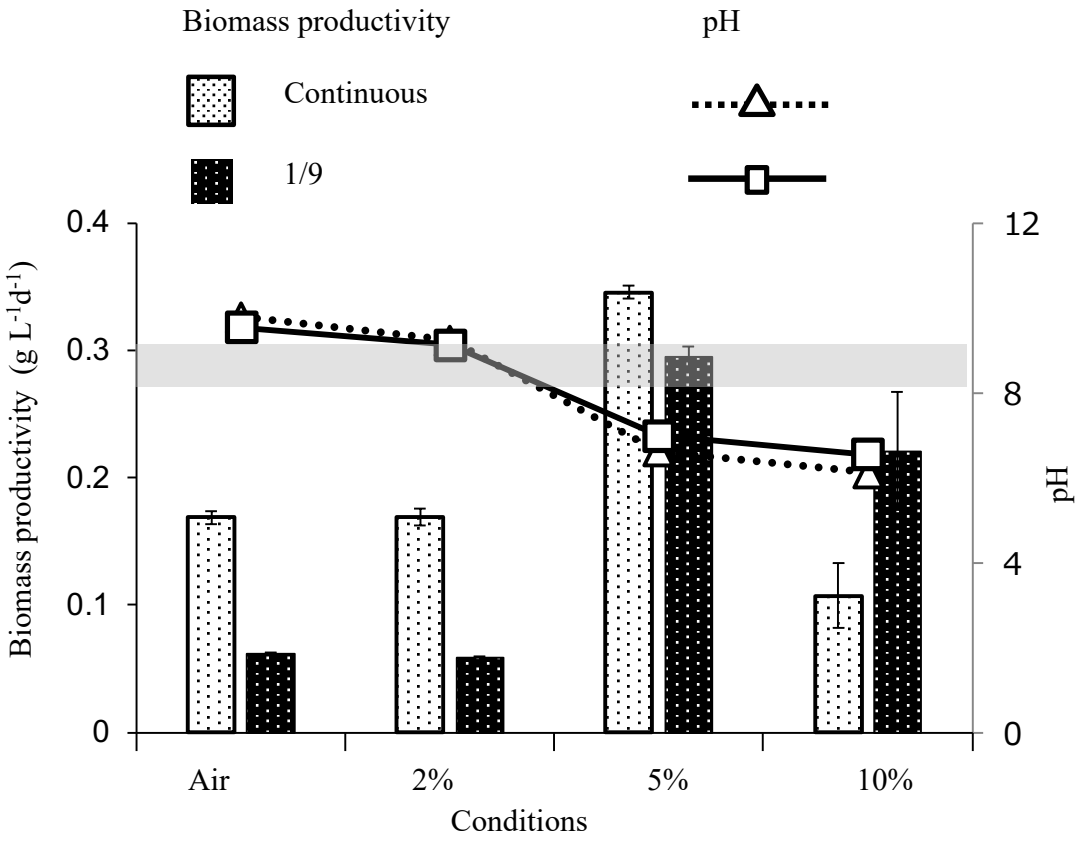


Fig. II-3. Biomass productivity, pH in stationary phase of each CO_2 supply concentration (air, 2%, 5%, 10%) and frequency (continuous, 1/9 min) condition. every condition. Shaded part of the optimum pH conditions for culture this *Isochysis galbana.*

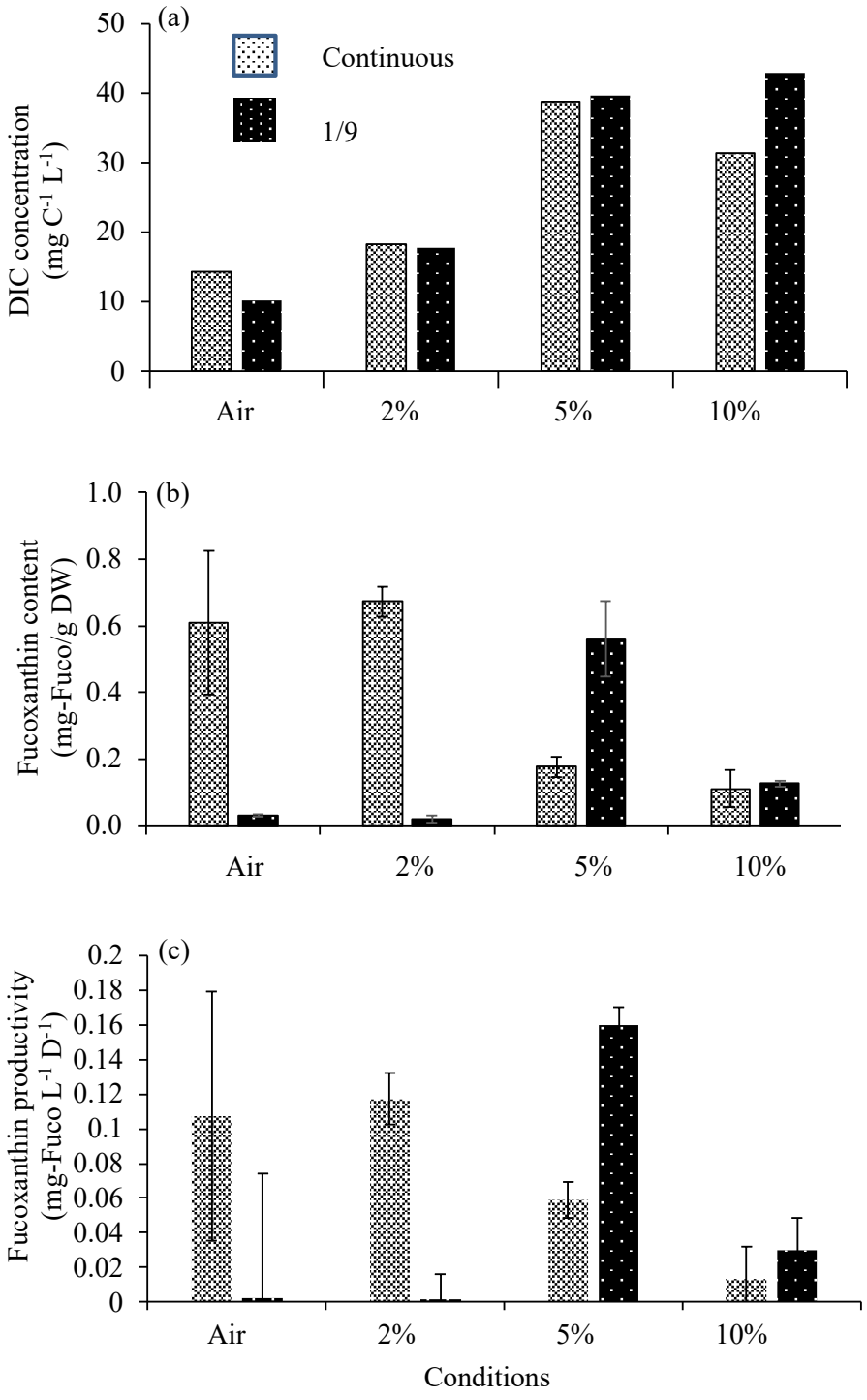


Fig. Ⅱ-4. (a) DIC concentration in stationary phase of each CO_2 supply concentration (air, 2%, 5%, 10%) and frequency (continuous, 1/9 min) condition. every condition. (b) Fucoxanthin content of each CO_2 supply concentration (air, 2%, 5%, 10%) and frequency (continuous, 1/9 min) condition. (c) Fucoxanthin productivity of each CO_2 supply concentration

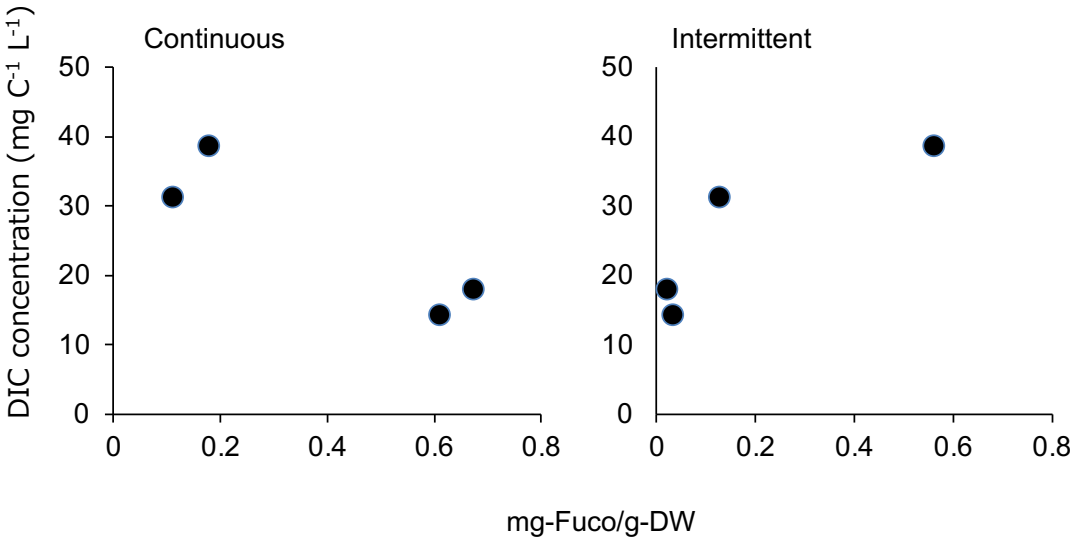


Fig. II-5. Relationship between DIC concentration and fucoxanthin content of continuous and intermittent conditions.

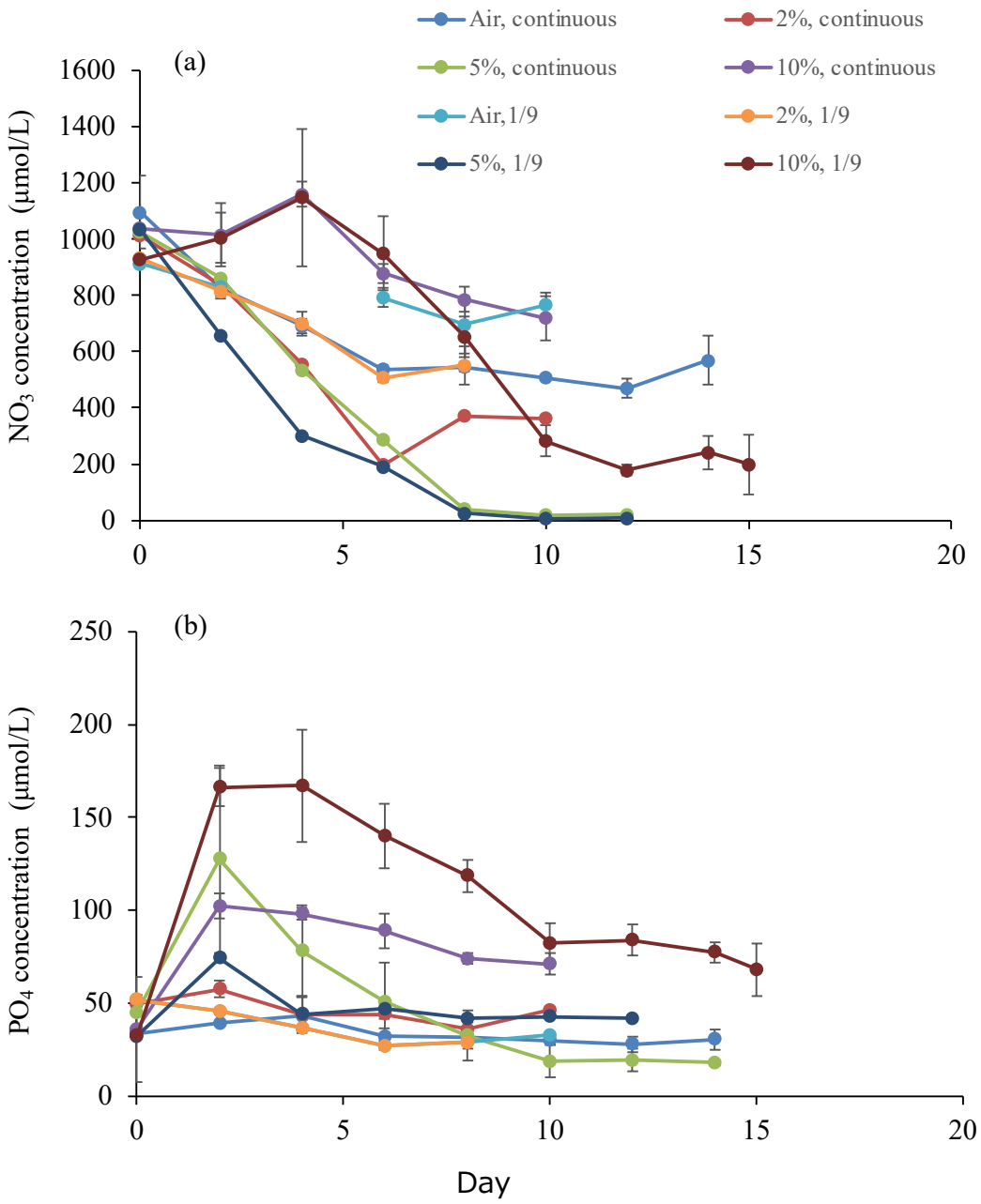


Fig. II-6. The concentration of NO_3 anf PO_4.contant.

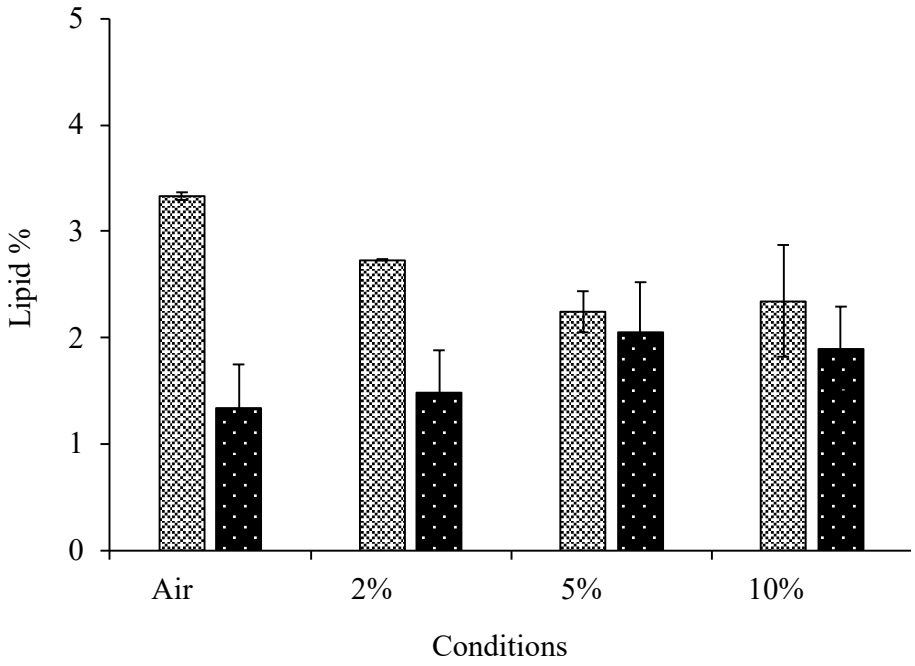


Fig. II-7. Fatty acid content of each CO_2 supply concentration (air, 2%, 5%, 10%) and frequency (continuous, 1/9 min) condition.

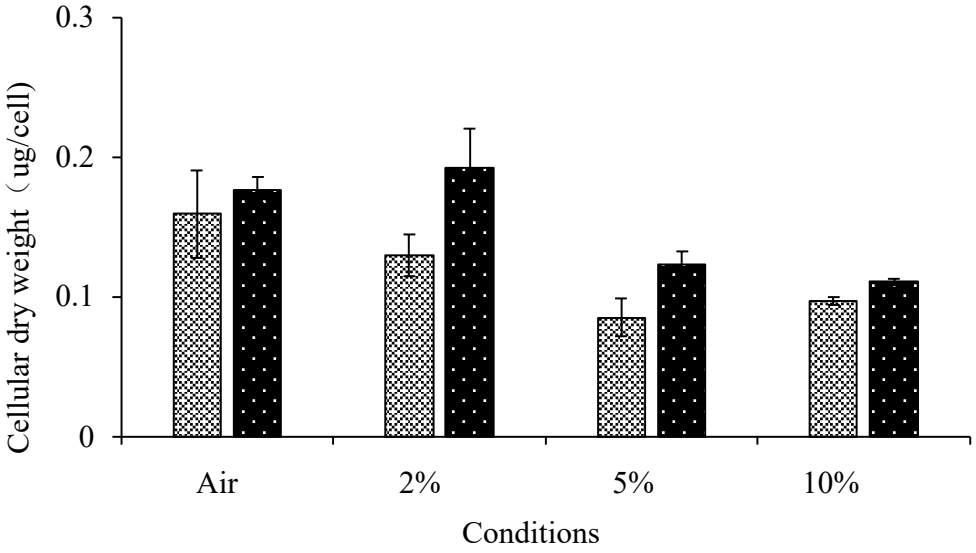


Fig. II-8. Cellular dry weight of each CO_2 supply concentration (air, 2%, 5%, 10%) and frequency (continuous, 1/9 min) condition.

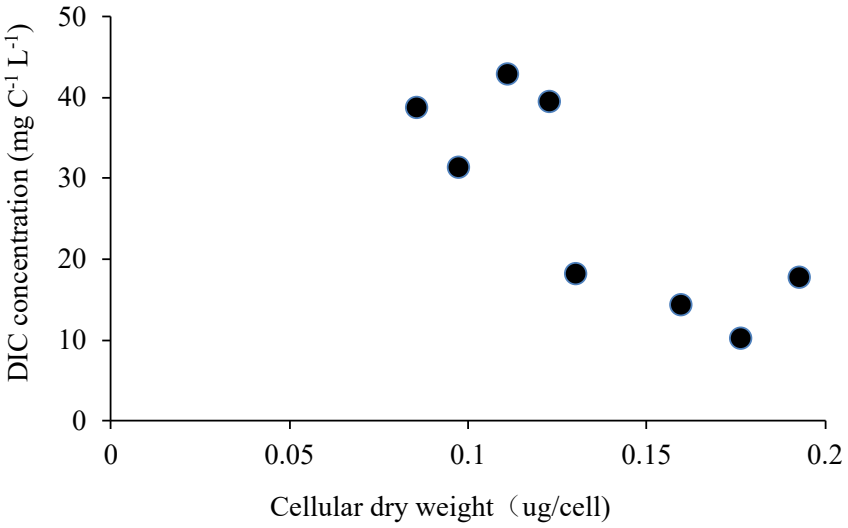


Fig. II-9. Relationship between DIC concentration and cellular dry weight in continuous and intermittent conditions.

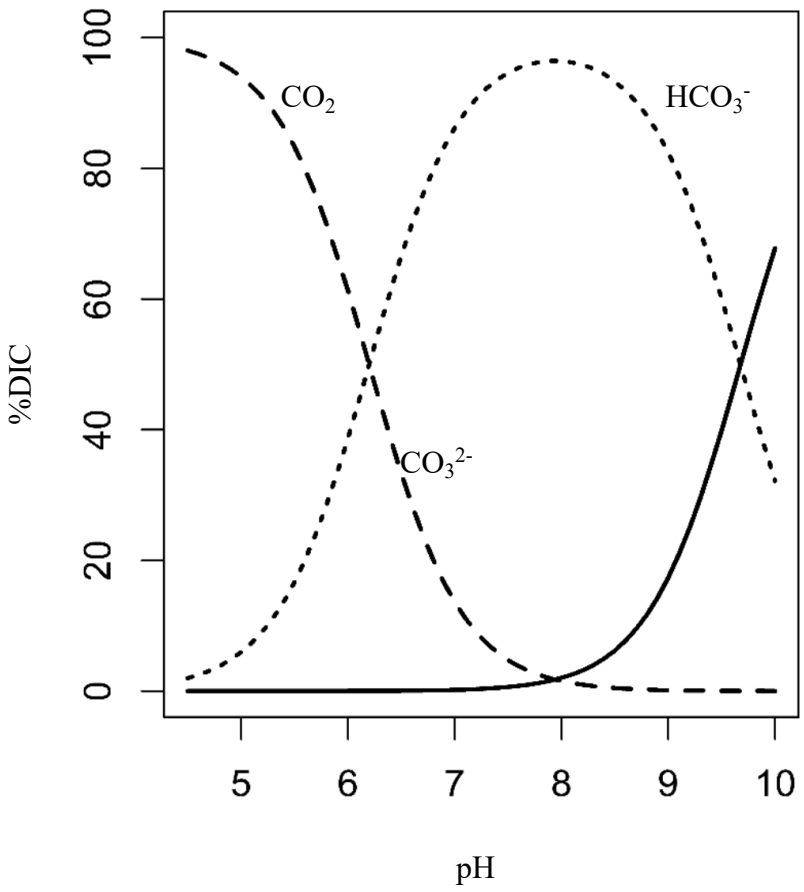


Fig II-10:Relative proportion of CO_2, HCO^{3-} and CO_3^{2-} of total dissolved inorganic carbon (DIC) under a range of pH values.

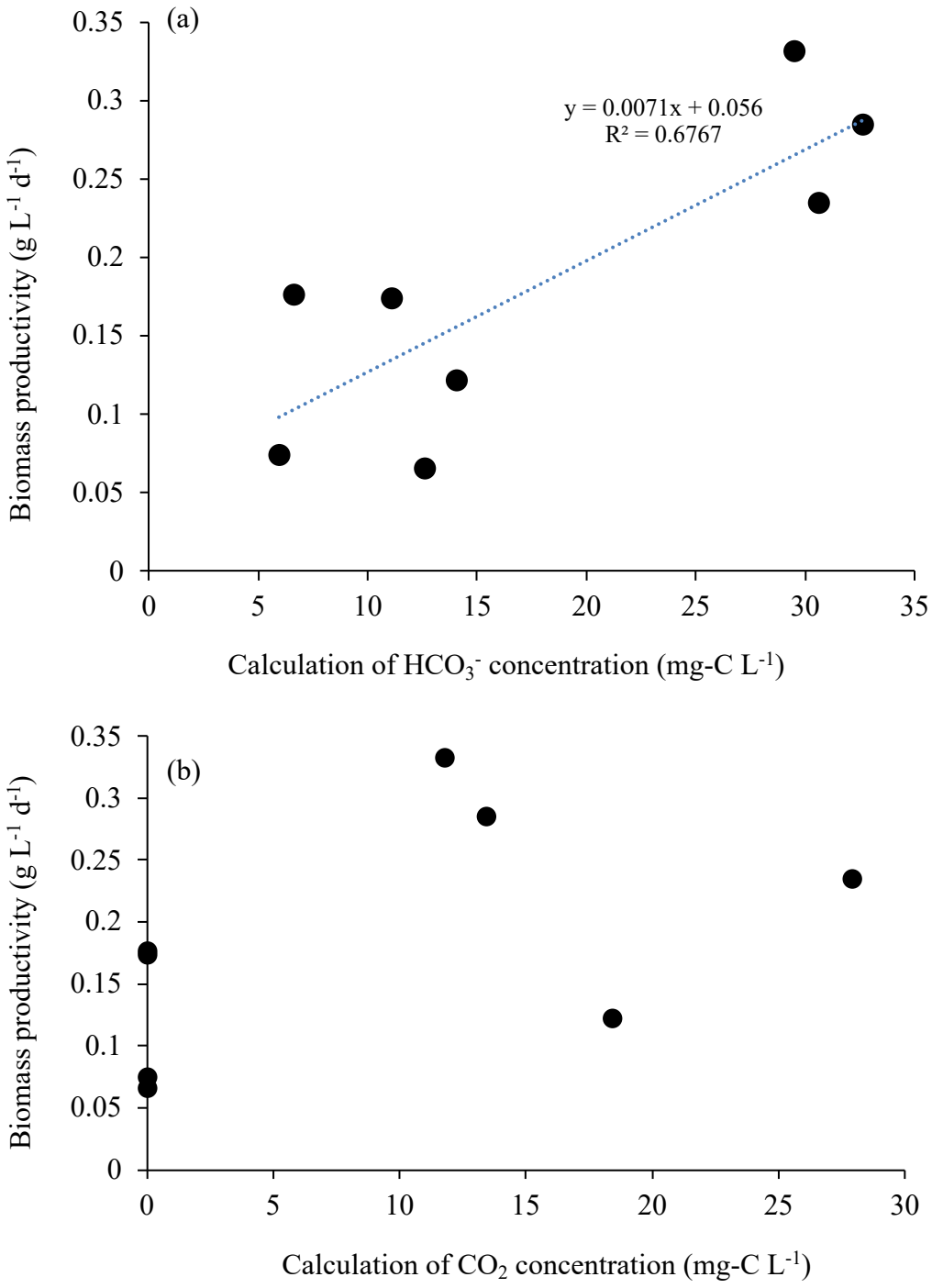


Fig II-11. Relationship between biomass productivity and HCO_3^- (a) and CO_2 (b).

Tables

Table II-1: The conditions of CO_2 concentration and supply frequency

	Air	CO_2 2%	CO_2 5%	CO_2 10%
Continue (control)	○	○	○	○
1/9 (min/min)	○	○	○	○

Table II-2: Previous studies of microalgal specific growth using *Isochrysis galbana*

Microalgae	Medium	CO_2 concentration	Supply frequency	Specific growth (d^{-1})	Reference
Isochrysis galbana (T-ISO)	F/2	Air	continuous	0.048	Sánchez et al., 2013
Isochrysis galbana ABC007	Conway	carbonate (1 g L^{-1})	-	0.22	Danesh et al., 2019
Isochrysis galbana	F/2	5%	continuous	0.34	Picardo et al., 2013
Isochrysis galbana UPMC-A009	Conway	5%	1/9 min	0.36	This study

CHAPTER 3 Harvesting of freshwater microalgae *Chlorella vulgaris* and seawater microalgae *Isochrysis galbana* by flocculation and sedimentation method

3.1. Introduction

Microalgae are potential materials to produce biofuel, human and animal food. However, most microalgae species' cells for such applications are small with two to twenty μm, and to harvest them can be challenging (Guo et al., 2013; Kumar et al., 2022). Centrifugation and filtration are two best ways to harvest microalgae, which has high energy consumption and costs much operating and labor. Hence, it requires an energy-efficient and economical preliminary concentration method.

Flocculation can be an effective means of preliminary concentrating microalgae before centrifugation and filtration, because a flocculant is able to sediment microalgae immediately (de Godos et al., 2011; Li et al., 2021). After adopting a chemical coagulant instead of a biological coagulant, microalgae biomass concentration that is more stable, effective and cheaper is maintained (Vandamme et al., 2013). Chemical flocculants induce flocculants by altering the electrical charge of microalgae cells by modifying the pH of solution and ions. The simplest chemical technique involves adding an acid to a microalgae solution; generally, the negatively charged surfaces of microalgae cells exclude each other. These surfaces can be neutralized by mingling the solution with an acid for carboxylate ions on the surfaces accept protons. The neutralizing process flocculates the microalgae (Spilling et al., 2011). Alternately, cations such as calcium (Ca^{2+}), alum, magnesium (Mg^{2+}), or ferrous ions can be addition to the culture with microalgae in order to neutralize the cell charges of surface and cause the cells to aggregate. (Banerjee et al., 2012; Smith and Davis, 2012; Blockx et al., 2019; Li et al., 2021). Ca^{2+} and Mg^{2+} are favorable cations for microalgae harvest.

They are prevalent in various media and are less hazardous than ferrous ions and alum. Nurdogan and Oswald (Nurdogan and Oswald, 1995) found that scant microalgae flocculation reacted in water in low magnesium and calcium. However, the lime added into it greatly improved the flocculation and sedimentation of microalgae biomass, the efficiency of which rose from 45% to over 90%. Young (Young et al., 2021) also found that the solution's turbidity fall from one hundred to nearly zero by increasing the dosage of magnesium from 10 to 80mg/L. On the whole, microalgae flocculation with a cation coagulant, is effective at high pH (Hable et al., 2019). Flocculation with a high pH is suggested for magnesium and/or calcium salts (e.g., calcium phosphate) can lead to precipitation (Shelef and Sukenik, 1984). Calcium phosphate's precipitation can flocculate cells with neutralized charges and thus, result in quick coagulation (Terhi Suopajärvi, 2015). Previous studies show that flocculation with calcium phosphate produce shapeless and big floccule (Sukenik et al., 1985), leading to much sediment. However, few previous researches focus on its thickness, even though optimizing sediment thickness could act a crucial role in harvesting algae.

Most previous research assessed the sedimentation efficiency of microalgae only by detecting the changes in concentration of microalgae at supernatant. However, both the thickness and speed of sediment cannot be assessed from the changes in microalgae concentration at the supernatant. The sediment thickness has a direct impact on the cost of microalgae recovery. The lower the sediment volume becomes; lesser energy is needed for the following process of centrifugation. Hence, modifying the methods to measure sedimentation is necessary. Further, to precisely observe the effect of sedimentation imposed on microalgae, this research explored the sedimentation variances by measuring chlorophyll *a*.

Few studies have reported the flocculation ability of seawater microalgae *Isochrysis*

galbana. And since *I. galbana* is a marine microalgae, the structure of compounds in seawater is complex, so it is difficult to determine the flocculation characteristics. In this chapter, freshwater microalgae were first used to study the effect and principle of flocculation, and then the method was tested for seawater microalgae *I. galbana.*

Therefore, with a modified method to measure chlorophyll *a*, this chapter assesses the impacts of Ca^{2+} concentration and the pH imposed on the microalgae sedimentation as well as sediment density. First, tests at different Ca^{2+} concentrations and pH indexes are conducted, and the clarity of the surface supernatant is measured for a preliminary screening of the experimental conditions (Experiment 1). The sedimentation dynamics is analyzed in detail, with experiment in cylinders and measurements of microalgae concentration changes at five heights (Experiment 2), illustrated in Fig. III-1. Lastly, the above results were used to verify its influence on the flocculation and sedimentation of Marine microalgae *I. galbana* (Experiment 3).

3.2. Materials and Methods

3.2.1. Experiment 1: Effects of the pH and Ca^{2+} concentration on the sedimentation efficiency

3.2.1.1. Algal strain and culture medium

The Japanese National Institute of Environmental Studies provided the green microalga *Chlorella vulgaris* Beijerinck var. *vulgaris* NIES-227. Mineral salt medium (MSM) was used as the growth medium and contained the following ingredients: KH_2PO_4: 1.25 g/L , KNO3: 1.25 g/L, $MgSO_4 \cdot 7H_2O$: 1 g/L, H_3BO_3: 0.1142 g/L, $CaCl_2$: 0.0835 g/L, $7H_2O$: 0.0882 g/L, $FeSO_4 \cdot 7H_2O$: 0.0498 g/L, MoO_3: 0.0071 g/L, $ZnSO_4 \cdot MnCl_2 \cdot 4H_2O$: 0.0144 g/L, $Co(NO_3)_2 \cdot 6H_2O$: 0.0049 g/L. $CuSO_4 \cdot 5H_2O$: 0.0157 g/L, , and ethylenediaminetetraacetic acid at 0.5 g/L (Sorokin and Krauss, 1958). The

medium underwent a 20-minute autoclave at 121 °C. At a temperature of 25 °C, microalgae were grown under conditions of 130 µmol photons m^{-2} $s^{-1}$and a 12 h:12 h light: dark cycle. The pH was 7.0 ± 0.1 at the start.

3.2.1.2. pH and Ca^{2+} concentrations

To understand microalgae flocculation, calcium and pH impacts on sedimentation efficiency were examined. The test was carried out use for MSM medium that was devoid of both calcium and magnesium (Ca and Mg-free MSM). Aliquots the MSM were mixed with $CaCl_2$ to create test medium with Ca^{2+} concentrations of zero (control), 0.5, 3, and 5 mM (labeled Ca0, Ca0.5, Ca3, and Ca5, respectively). Experiments were conducted at pH 3, 7, 9, and 11. Adding 1 N HCl or 6 N NaOH to adjust the pH. Each of the 16 conditions under which experiments were conducted was replicated three times. The control measurement (in pH7-Ca0) was conducted at pH 7 in the absence of Ca^{2+}. In each measurement, sedimentation efficiency was measured.

3.2.1.3. Sedimentation efficiency

Using the procedures outlined here, the sedimentation efficiency was measured. A microalgae culture was diluted with Ca and Mg-free MSM medium to an OD750 of 0.8±0.01 after centrifuging at 8000g for 10 minutes at 25 °C. A portable DR/2400 spectrophotometer was used to determine the OD750. (HACH, USA). Fifty milliliters (mL) of the liquid was taken out and put into a 60 mL test tube, where it was stirred for a full minute. After waiting 30 minutes, a sample of the suspension was taken from just 1 centimeter below of the surface and placed in a 6 milliliter tube. The concentration of Chlorophyll *a* (Chl. *a*) was then calculated for the aliquot. Chl. a was extracted by adding the 5 milliliter aliquot of N,N-dimethylformamide to the 0.5 milliliter sample

and freezing the mixture at -20 °C for 24 hours. The 10AU (Turner Design) fluorometer was then used to measure the Chl. a concentration. Check out the formula for converting Chl. *a* concentration to OD_{750} at 750 nm(OD_{750}) below.

Chl. *a* concentration= $11.65 \times OD_{750} - 0.4504$ (1)

Using an equation, the sedimentation efficacy was calculated.

Sedimentation efficiency (%)= $\left(1 - \frac{B}{A}\right) \times 100$ (2)

where A is the concentration of Chl. *a* at time 0 and B is the concentration of Chl. *a* in the supernatant after 30 minutes.

3.2.2. Experiment 2: detailed sedimentation dynamics

After evaluating the sedimentation efficiencies determined in Experiment 1, seven experimental conditions were chosen, and each condition was tested in triplicate. First, the initial OD_{750} was altered to 0.8±0.01 by centrifuging the pre-cultured microalgae suspension, followed by dilution with Ca and Mg-free MSM. Then, 150 milliliter culture medium of the microalgae with optical density of 0.8±0.01 were added to every 300 milliliter cylinders. The suspension volume in every cylinder were then altered to 300 milliliters by adding Ca and Mg-free MSM. The pH was modified by the addition of 2NHCl or 2NNaOH, and the concentration of Ca^{2+} was modified by the addition of $CaCl_2$. Less than 2 mL of the liquid was added to accomplish the adjustments. The mixture was then transferred to a 500 mL Erlenmeyer flask and agitated for 10 minutes at 100 revolutions per minute. The sedimentation dynamics of microalgae were evaluated after transferring a 250 mL aliquot to a modified cylinder (see Fig. III-1). The sedimentation rate was determined by measuring Chl. *a* concentration at various heights (1, 6, 8, 12, 5, 18, 2, and 23.2 cm) and times (0, 1, 3, 5, 10, 20, and 30 minutes) from

the cylinder's base. The side of the plastic mixing cylinder was drilled, and the resultant void was filled with a rubber closure. The sample was performed with the injection needle, and the author marked the silicone stopper to ensure the needle was always inserted in the same location. As depicted in Fig. III-1, the sediment thickness, or the height of the boundary layer between the sediment and supernatant, was measured at each sampling interval. Each test's sediment was examined with a DMIL LED light microscope (Leica Microsystems, Germany).

The percentage of microalgae cells that were recovered was determined by taking the total chlorophyll a concentration and dividing it by the chlorophyll a concentration in the supernatant. This calculation was done based on the assumption that the chlorophyll a concentration in the supernatant was the same at each height. The formula that was used to determine the amount of microalgae cells recovered was:

$$\text{Algal cell recovery (\%)} = \left(1 - \frac{\Sigma(x_i \times V_i)}{x_0 \times V_{total}}\right) \times 100 \quad (3)$$

where x_i and V_i are the Chl. *a* concentration and volume in the supernatant layer *i*, respectively, x_0 is the initial Chl. *a* concentration, and V_{total} is the total volume of the microalgae suspension in the cylinder. The supernatant was defined as the liquid above the boundary layer. The supernatant volume was assumed to be the volume of the layer above and including the boundary layer. The concentrating effect achieved in each test was assessed by calculating the harvesting volume reduction using the equation

$$\text{Harvesting volume reduction (\%)} = \left(1 - \frac{V_s}{V_{total}}\right) \times 100 \quad (4)$$

where V_s represents the supernatant volume and V_{total} represents the total culture volume.

Using Surfer 11 (Golden Software, USA) and the Kriging method to interpolate Chl. a data, the sedimentation dynamics were visualized by plotting contour maps using

the Kriging method to interpolate Chl. *a* data. Each contour map was used to visualize the boundary layer under the assumption that the Chl. a concentration immediately above the boundary layer was identical to the Chl. a concentration immediately above the boundary layer in the supernatant layer. A mass balance of the Chl. an in the entire cylinder was used to estimate the Chl. *a* concentration at the bottom of the cylinder (xbottom). For instance, xbottom was calculated using the equation if the boundary layer was above the first layer (1.0 cm from the bottom).

$$x_0 \times h_{total} = x_{supernatant} \times (h_{total} - h_{boundary}) + \frac{(x_{boundary}+x_1)\times(h_{boundary}-h_1)}{2} + \frac{(x_1+x_{bottom})\times h_1}{2} \quad (5)$$

where h is the height (cm) and x is the Chl. *a* concentration (mg L^{-1}) for the layer of interest. where h is the layer's height in centimeters and x is the Chl. a concentration in milligrams per liter.

3.2.3. Experiment 3: Harvesting of seawater microalgae Isochrysis galbana by flocculation and sedimentation method

The high cost of harvesting microalgae is a significant obstacle for the microalgae industry, with harvesting costs accounting for approximately 20% of the total cost; therefore, an efficient pre-concentration method is required. Previous research examined the flocculation and sedimentation characteristics of freshwater *Chlorella vulgaris* (Hable et al., 2019). Microalgae can be flocculated and settled under conditions of high pH and cationic concentration. At high pH, calcium ion can react with phosphoric acid (PO_4) to form large flocs, thereby achieving flocculation and sedimentation. In contrast to microalgal flocculation in freshwater, seawater contains more calcium and less phosphorus. The purpose of this experiment was to examine the

flocculation effect of pH and phosphoric acid concentration on the marine microalgae *Isochrysis galbana.*

3.2.3.1. Algal strain and culture medium

University Putra Malaysia obtained the marine microalga *I. galbana* UPMC-A009 sample. Pre-cultivation was carried out in a one liter Conway medium with continuous airflow. This medium contained KNO_3 at a concentration of 100 mg/L, Na_3PO_4 at 20 mg/L $Na_2H_2EDTA \cdot 2H_2O$ at 45 mg/L, H_3BO_3 at 33.6 mg/L, $FeCl \cdot 6H_2O$ at 1.3 mg/L, $MnCl_2 \cdot 4H_2O$ at 0.36 mg/L, $ZnCl_2$ at 2.1 mg/L, $CoCl_2 \cdot 6H_2O$ at 2 mg/L $(NH_4)6Mo7O_{24} \cdot 4H_2O$ 0.9 mg/L, $CuSO_4 \cdot 5H_2O$ at 2 mg/L Thiamin HCl at 200 µg/L Cyanocobalamin at 10 µg/L and Na2SiO3·9H2O at 30 mg/L (Ershad-Langroudi et al., 2010). The pH of the medium was 8.0 ± 0.3 The medium and all culture instruments were autoclaved for 20 minutes at 121 degrees Celsius. The pre-cultivation conditions included 100 µmol photons m^{-2} s^{-1} of light intensity, 12 hours light and 12 hours dark cycle, and a temperature of 25°C.

3.2.3.2. Effects of the pH and PO_4^+ concentration on the sedimentation efficiency

To acquire a comprehension of the flocculation characteristics of the microalgae, the effects of the PO_4 concentration and pH on sedimentation efficiency were investigated. The experiment was conducted using Conway medium devoid of PO_4 (Conway medium devoid of PO_4). The Conway medium was diluted with Na_2HPO_4 to produce test solutions with PO4 concentrations of 0, 5, and 10 mM. Experiments were conducted at pH 8, 9, and 10. Adjusting the pH by introducing 2 N NaOH. Each of the nine conditions under which experiments were conducted was replicated three times. The control was carried out at a pH of 8 and in the absence of

PO4. In each measurement, sedimentation efficiency was measured.

3.2.3.3. Sedimentation efficiency

The measurement method of sedimentation efficiency explained here. The culture medium of microalgae was centrifuged at 1000rpm for 10 minutes at 25 °C and then dilution with no PO_4 Conway medium to achieve an OD at 750 nm (OD_{750}) of 0.2 ± 0.02.

The pH was adjusted by the addition of NaOH, and the concentration of PO_4 was adjusted by the addition of Na_2HPO_4. After transferring a 250 milliliter aliquot to the modified cylinder, the sedimentation dynamics of microalgae were calculated. The sedimentation rate was measured by concentration of Chl. *a* at various heights (1.0, 6.8, 12.5, 18.2, and 23.2 cm) and durations (0, 1, 3, 5, 10, 20, and 30 minutes) from the bottom of the cylinder. The plastic mixing cylinder's side was drilled, and the gap left behind was filled with a rubber cover. The sampling was performed with an injection needle, and the author marked the silicone stopper to ensure the needle was always inserted in the same location. Using an equation, the sedimentation efficacy was calculated.

$$\text{Sedimentation efficiency (\%)} = \left(1 - \frac{B}{A}\right) \times 100$$

where A is the concentration of Chl. *a* at time 0 and B is the concentration of Chl. *a* in the supernatant after 10, 30 and 60 min..

3.3. Results and discussion

3.3.1. Experiment 1: effects of the pH and Ca^{2+} concentration on the sedimentation efficiency

After 30 minutes of sedimentation, the control (Ca0-pH7) had a sedimentation efficiency of just 3.9%, as shown in Fig. III-2. At pH 3, but without $Ca^{2+,}$ the sedimentation efficiency was 51%. Since pH 3 is where microalgae reach their isoelectric point (Crist et al., 1981), the floc surfaces would have been somewhat negatively charged, causing the particles to coagulate since the cells would not have been strongly attracted to one another. According to Fig. III-2, the addition of Ca2+ decreased sedimentation efficiency at a pH of 3, most likely because it altered the balance of positive and negative charges.

At pH 7, adding 5 mM Ca^{2+} resulted in a high sedimentation efficiency of 93%, however when the Ca^{2+} concentration was lower, the sedimentation efficiency was 6.9%, as shown in Fig. III-2. However, significant sedimentation efficiencies were reported at Ca^{2+} values of 3 and 5 mM at pH 9 and 11, as shown in Fig. III-1. Even at a Ca^{2+} concentration of 3 mM, the elevated pH exacerbated the effect of Ca^{2+} on sedimentation. Ca^{2+} most likely improved sedimentation efficiency by causing calcium phosphate precipitate flocculation. At high pH levels, calcium phosphate precipitates and microalgae flocculate, as found by Sukenik (Sukenik and Shelef, 1984) and Wu (Wu et al., 2020). They reasoned that the flocculation was caused by the calcium phosphate's positive surface charge canceling out the negative surface charge of the microalgae cells.

Despite the high sedimentation efficiency at high pH values (9, 11) even at a relatively low Ca^{2+} concentration (3 mM), care must be taken to prevent the pH from reaching a level high enough to kill the microalgae cells. Vandamme (Vandamme et al.,

2012) and Knuckey (Knuckey et al., 2006a) discovered that the majority of microalgae cells remained intact following sedimentation at pH 12 and pH 9, respectively. Blanchemain (Blanchemain et al., 1994) discovered, however, that microalgae lysis happened one hour after sedimentation at a pH of nine. These results indicated that the duration of the treatment must be selected to prevent cell deterioration while obtaining a high harvesting efficiency. Therefore, it was necessary to evaluate the sedimentation dynamics to determine the optimal time to harvesting cells under every setting of conditions.

3.3.2. Experiment 2: effects of the pH and Ca^{2+} concentration on sedimentation dynamics

In most of the preceding researches (Knuckey et al., 2006b; Zheng et al., 2012), and Experiment 1, in the supernatant, the cell density (a few millimeters under the suspension surface) was measured to assess sedimentation efficiency. However, the surface clarity does not always ensure harvest efficiency and the reason is that the sediment may not be accumulated efficiently despite a clear surface. Information about both sediment thickness and surface clarity over time is need for precise estimation. Hence, Experiment 2 was designed to measure the Chl. *a* distribution dynamics at five heights (0, 6.8, 12.5, 18.2, and 23.2 cm from the bottom) of a 250 mL cylinder 0, 1, 3, 5, 10, 20, and 30 minutes after the experiment begins. Select the eligible conditions when all assessments in Experiment1 finish. The selected conditions, which had sedimentation efficiencies >50% (see Fig. III-2), taken as the control (pH7-Ca0).

Fig. III-3a shows the Chl. a concentration of the control group (pH7-Ca0) has scant changes, which indicates that no sedimentation happens here. As for the pH3-Ca0 test, under the same conditions in Experiment 1, the sedimentation efficiency rises to 51% ,

but no obvious boundary layer was detected. Fig. III-3b demonstrates that the sedimentation under the acidic conditions only clarified the suspension's surface. It is possible that part of the sedimentation happened under these conditions, because according to the Schulze–Hardy rule (Terhi Suopajärvi, 2015), by putting the microalgae to the isoelectric point with added H^+, only some weak coagulation was formed. Probably, the floc was too small to effectively sediment and no obvious change takes place both before and after flocculation with pH 4 in metal ion content in the medium, which means no inducement of microalgae flocculation(Fan et al., 2017).

Fig. III-3c–3g shows that, with Ca^{2+} concentrations as well as higher pH values, much more sedimentation can take place. The treatments mainly include fast sedimentation (pH 9–11, 5 mM Ca^{2+}; Fig. III-3f–3g), slow sedimentation (pH 9–-11, 3 mM Ca^{2+}; Fig. III-3c–3d), as well as the pH7-Ca5 treatment (Fig. III-3e). Table III-1 shows that 72%–77% after 10 min was fit for the microalgae cell recovery for the fast sedimentation treatments, while for the slow ones, the microalgae cell recovery became 33%–53%. However, after 30 minutes, the sediment is over two times of thicker for the fast sedimentation treatments (1.62–2.06 cm) than the slow sedimentation ones (0.68–0.79 cm). The thicker sediment needed a bigger harvesting capacity. Therefore, the volume decrements were lower for the fast sedimentation treatments (91%–93%), compared with the slow sedimentation ones (97%). With a high pH, flocs (Beuckels et al., 2013; Fan et al., 2017; Branyikova et al., 2018; Wu et al., 2020) was produced from reactions among phosphate, Ca^{2+} and carbonates. Generally speaking, Calcium phosphate has a positive surface charge (Sukenik and Shelef, 1984), which permits microalgae cells, which is charged negatively, to stick to the its surfaces (Formosa-Dague et al., 2018). Adhesion of microalgae makes huge flocs to form and quick sedimentation happen. Translucent "hazy" flocs were observed to form by a microscope

(Fig. III-4b and 4c). The varying thickness and speeds of sedimentation for the fast and slow sedimentation treatments possibly result from the various dosages of added Ca^{2+}. The flocs turned to be fewer and smaller. Besides, the amount of non-flocculated microalgae cells became obviously lower in the fast sedimentation treatments (displayed in Fig. III-4b), in comparison with the slow ones (Fig. III-4c). The non-flocculating cells as well as small flocs could have result in inefficient and slow sedimentation in the slow sedimentation treatments. But, Table III-1shows the slow sedimentation ones had a tendency of thicker sediment, which indicates that the cells were concentrated effectively. The reason might be the small floc size, which permitting accumulation of dense sediment.

Regarding the pH7-Ca5 treatment, that the huge capacity decrease (96% in just ten minutes) and the fast microalgae cell recovery with 69%, indicates the effectiveness of the pH7-Ca5 treatment. In addition, in this treatment, the sediment layer became more condensed and thinner than that of in the high pH treatments and calcium concentration. The sedimentation rate was high despite no formation of a “hazy floc” in the this treatment. This microalgae cells were neutralized by Ca^{2+} instead of sticking to calcium precipitate to produce large particles. Fig. III-4a displays dense aggregations of microalgae cells with no identification of a hazy precipitate of the pH7-Ca5 product by a micrograph. Probably, Ca^{2+} neutralized the microalgae cells’ surface negative charges, which may weaken the zeta potential and increase flocculation. Previous studies show that the microalgae’s negative charges can be penetrated by a cationic flocculant and therefore make the particles tightly stick to each other, indicting a compact sediment(John Bratby, 2016).

Both pH11-Ca5 and pH9-Ca5 treatments presented the quickest sedimentation as well as microalgae cell recoveries, but the pH7-Ca5 treatment also showed the highest

volume decrease and a similar recovery. In this case, the three conditions were adopted in the following experiment on calcium recovery.

3.3.3. Experiment 3: Effects of the pH and PO_4 concentration on the sedimentation efficiency of seawater microalgae Isochrysis grlbana

In the marine microalgae *I. galbana*, the sedimentation efficiency only achieved 3.2% under control condition (No PO_4-pH 8) (Fig. III-5). The sedimentation efficiency increased under the condition of increasing pH, the highest can reached 10.7% (No PO_4-pH 10). The sedimentation efficiency achieved 40% under high PO_4 concentration (10 mM) and pH 10, that was higher than control condition (No PO_4-pH 8) (Fig. III-5). The results under each condition were not significantly different. In addition, under the conditions of high pH and high PO_4 concentration, the difference in the results of the triple test is relatively large. This is because the sedimentation layer and the supernatant layer are critically close to 1cm, and sampling is particularly difficult, so the results are relatively large. This is also the defect of this experiment. In future research, sampling at more points can be used to obtain more accurate results. It can be known from the above results that the sedimentation efficiency in seawater is lower than in fresh water. This may be due to the smaller size of *I.galbana* than freshwater microalgal *C. vulgaris*. In contrast, at elevated pH, Ca^{2+} and phosphate and carbonate reactions produce flocs (Nurdogan and Oswald, 1995). Calcium phosphate typically has a positive surface charge, allowing microalgae cells with a negative charge to adhere to its surfaces. Since seawater contains more anions, a higher pH may be necessary to induce microalgal flocculation and sedimentation.

3.3.3.1. Comparison of flocculation and sedimentation between freshwater and Marine microalgae

The highest flocculation efficiency of freshwater microalgae *Chlorella vulgaris* reached more than 91% under the conditions of high pH (9， 10) and Ca (3, 5mM) concentration, but under the same conditions, seawater and *I. galbana* can only reached 40% sedimentation efficiency. This may be due to the smaller cell size of *I. galbana*, which did not produce larger flocs even if flocculation occurred, that way flocculation efficiency was lower. In addition, the content of negative ions in seawater is much higher than that in fresh water, which may be the main reason for the gap in sedimentation efficiency. This means that a higher pH may be required to induce flocculation, additional experiments were performed in this theses. The experimental conditions were the same as before (PO_4 condition was 10mM), except that the pH was increased from 10 to 11, however, the sedimentation efficiency (30%) did not increase significantly within 60 minutes, but the supernatant showed an abnormal state (Fig. III-6). This indicates that the pH can increase the clarity of the supernatant, but the area of the sediment layer is larger, which directly affects the cost of subsequent centrifugation. On the other hand, regarding the chemical reagent (2N NaON) used for pH adjustment, when the pH is adjusted to 10, the required NaON capacity is 1.2mL, but it does require 41ml to adjust to pH11, it is not feasible in actual operation. It is needed to find other ways to improve the flocculation efficiency in the future.

On the other hand, the microalgae used in this experiment were cultured under air continuous supply conditions, not the microalgae cultured under the optimal conditions (5% intermittent) obtained in the experiments in the chapter 2. The authors concluded that culture conditions had no effect on flocculation and sedimentation. As shown in the figure Ⅱ-6, when the culture reaches the steady state, the nitrogen concentration and phosphorus concentration contained in the culture solution under

condition air continuous and 5% intermittent were basically the same, so the basic conditions for their flocculation are the same. Therefore in this experiment I can assume that the microalgae cultured condition (Air continuous, 5% intermittent) will not affect the final effect of flocculation and sedimentation.

3.4. Conclusion

A method improvement permitted the microalgae cell concentrations at different heights in a system to be determined at different intervals during the experiments, as well as the sedimentation characteristics to be thoroughly analyzed. According to the results, a high Ca^{2+} and PO_4 concentration prompted rapid sedimentation. A high pH (9 or 10) caused rapid sedimentation, but the sediment thickness (harvest volume) was lower than at higher pH values, most likely due to dense coagulation resulting from ionic charge neutralization. On the basis of the presented results, it is possible to optimize the flocculation of microalgae and accomplish rapid and effective recovery of microalgae.

Figures

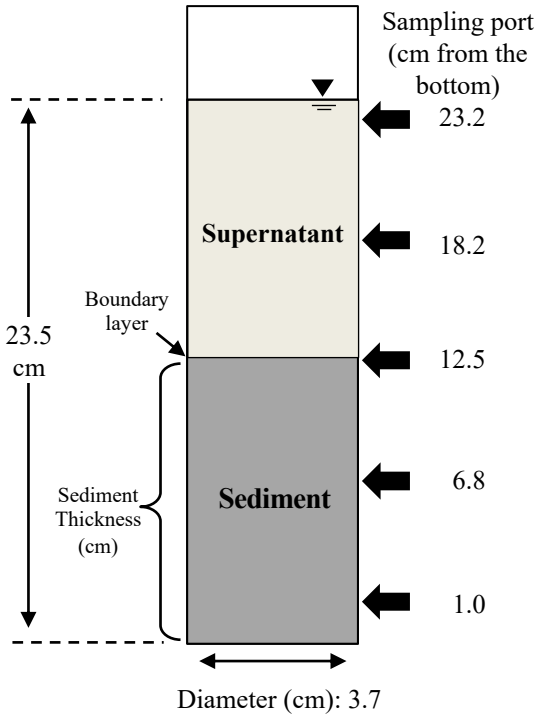


Fig. III-1. Side view schematic of the cylinder used in the study. Light gray is the supernatant, dark gray is the sediment.

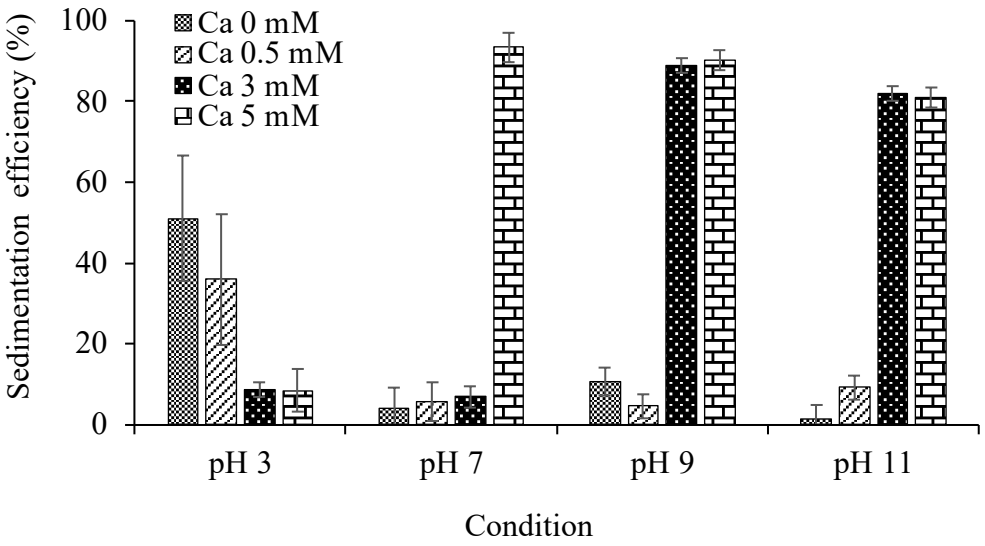


Fig. III-2. Microalgae sedimentation efficiencies at pH 3, 7, 9, and 11 and calcium concentration (Ca) of 1–5 mM (the mean values are shown, and the error bars are the standard deviations; n = 3).

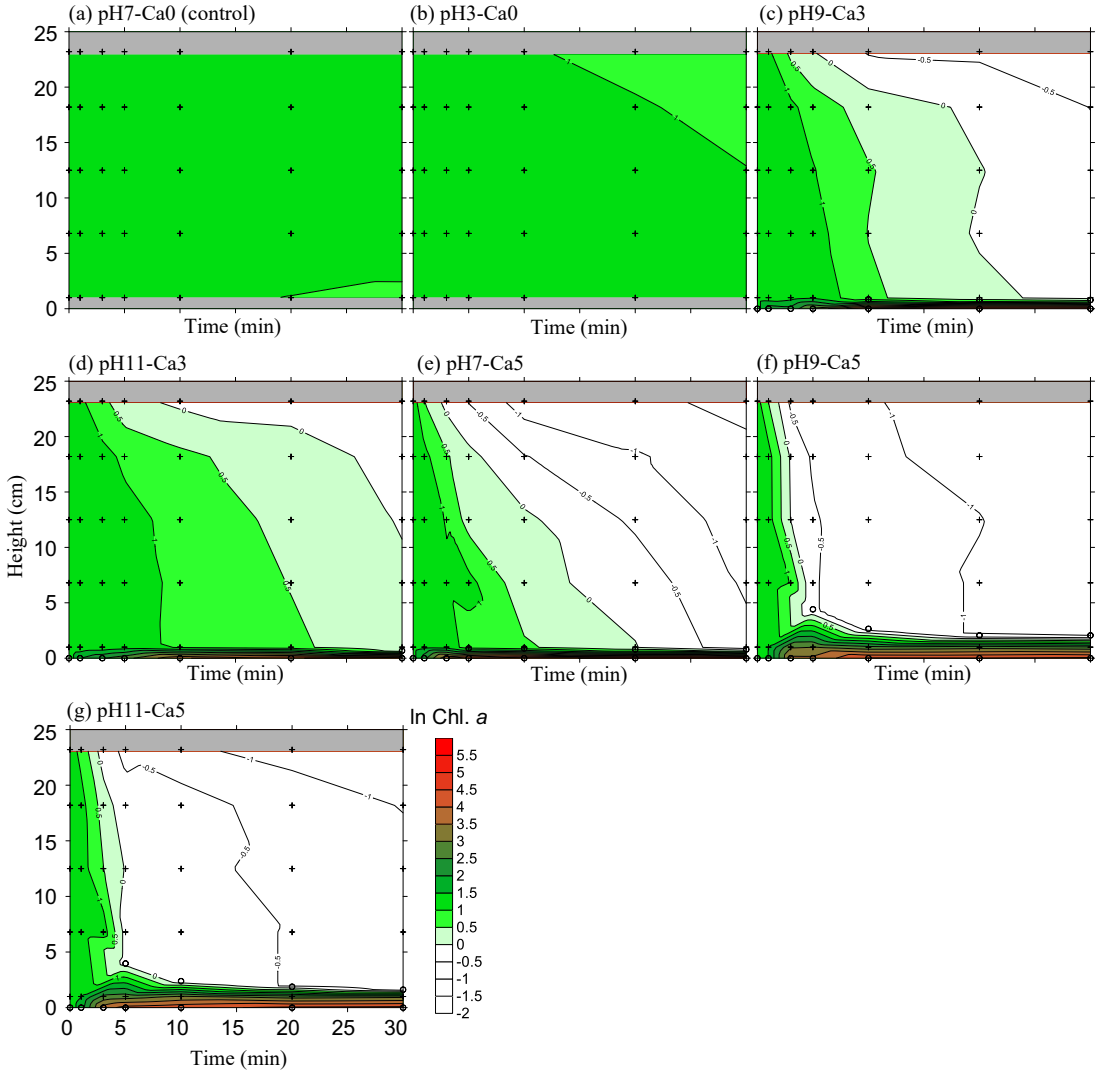


Fig. III-3. Spatiotemporal variations in the chlorophyll *a* concentration (Chl. *a)*. Measured and assumed values are indicated as crosses (+) and circles (○), respectively. Seven sedimentation dynamic series are shown as the ln Chl. *a* concentrations found when systems with various pH and calcium concentrations (Ca, in mM) were used.

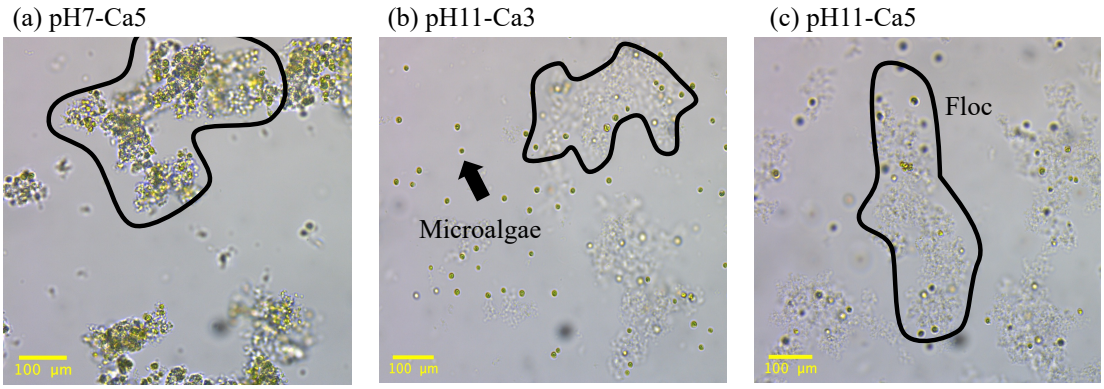


Fig. III-4. Micrographs of the sediment produced at pH 7 and 11 and calcium concentrations (Ca) of 3 and 5 mM. The black areas are flocs, and the arrows indicate microalgae that have not formed flocs.

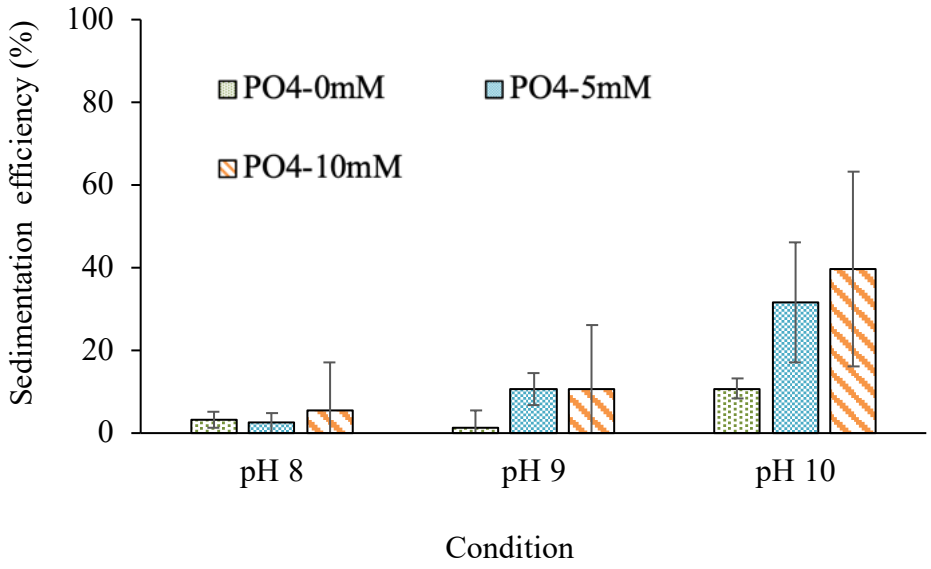
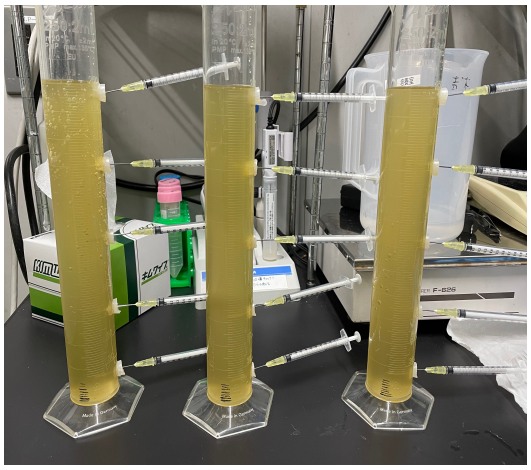
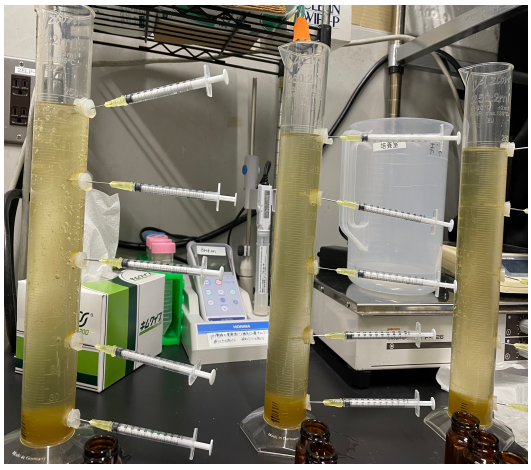


Fig. III-5. Microalgae sedimentation efficiencies of *I. galbana* at pH 8, 9, and 10 and PO_4 concentration (PO_4) of 0, 5, 10 mM (the mean values are shown, and the error bars are the standard deviations; n = 3).

(a) pH-10, PO4-10mM

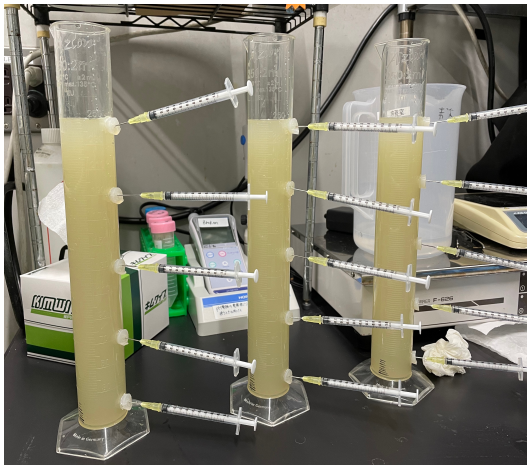


0min

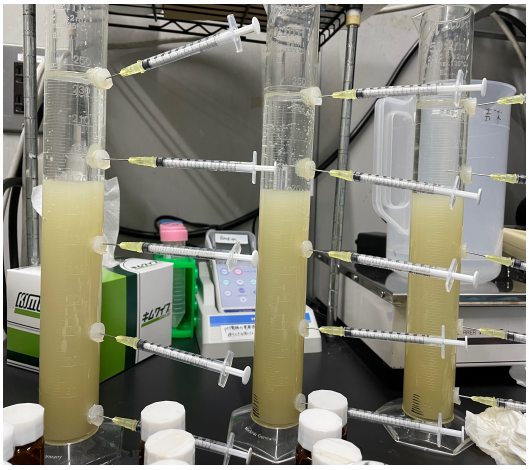


After 60min

(b) pH-11, PO_4-10 mM



0min



After 60min

Fig. III-6. Microalgae *I. galbana* sedimentation figures at pH 10 (a) and 11 (b) with PO_4 concentration 10 mM.

Tables

Table III-1. Recoveries of algae cells from below the boundary layer after sedimentation for 10 and 30 min. Ca = calcium concentration in mM.

Condition	Algae cell recovery (%)	Sediment thickness (cm)	Volume reduction (%)
10 min sedimentation			
pH 9-Ca 3	53	0.85	96
pH 11-Ca 3	33	0.62	97
pH 7-Ca 5	69	0.88	96
pH 9-Ca 5	77	2.65	89
pH 11-Ca 5	72	2.38	90
30 min sedimentation			
pH 9-Ca 3	76	0.79	97
pH 11-Ca 3	68	0.68	97
pH 7-Ca 5	88	0.79	97
pH 9-Ca 5	83	2.06	91
pH 11-Ca 5	81	1.62	93

Table III-2. Amount of acid solution(1N HCl) added (mL/L) to reach the target pH.

	Acidification to pH 5	**Acidification to pH 3**	**Acidification to pH 1.5**
pH7-Ca5	10	17	30
pH9-Ca5	20	40	60
pH11-Ca5	26	45	80

CHAPTER 4 General Discussion

4.1. Effect of biomass concentration and fucoxanthin accumulation

In this PhD study, the effects of CO_2 concentration (Air, 2%, 5%, 10%) and supply frequency on the proliferation of *Isochrysis galbana* microalgae were investigated. The condition of continuous supply of air was the control. The condition of continuous 2% CO_2 supply showed identical results to the control (DW was 0.7 g L^{-1}). Under conditions of continuous supply of 5% CO_2, the maximal biomass concentration (1.32 g L^{-1}) was attained. However, the 1-min/9-min intermittent CO_2 supply conditions exhibited nearly the same high growth rates and DW (1.32 g L^{-1}) as the 5% continuous CO_2 supply. Regardless of supply frequency, the supply of 5% CO_2 increased the biomass concentration of *I. galbana.* In comparison with previous study, this PhD study achieved a high biomass concentration. Table IV-1 shows the previous studies of microalgal biomass productivity using *I. galbana*, CO_2 concentration, and supply frequency. The first study used 1g L^{-1} carbonate to culture microalgae, biomass concentration achieved was only 0.38 g L^{-1}. Second study used aeration to culture microalgae, biomass yield achieved was 0.6g L^{-1}. The third and fourth research they used 5% continuous condition to grow the microalgae and high biomass yield achieved was 1.2g L^{-1} and 1.35, respectively. This proves that the concentration of CO_2 supply directly affects the growth of microalgae. In this PhD study, the biomass yield achieved was up to 1.34g L^{-1}, and specific growth was 0.36 in 5 % intermittent CO_2 supply condition (1/9 min), however, the result was similar than previous study. It is proved that intermittent CO_2 supply can still achieve the higher effects for microalgal culture in this study. On the other hand, carbon dioxide concentration serves a crucial function in microalgal growth and accumulation of fucoxanthin. Previous study examined the supplementation of 3 different CO_2 concentrations (air, 2 and 5%) on the microalgal

growth and accumulation fucoxanthin of *Isochrysis zhangjiangensis* (Li et al., 2019). Both 2% and 5% supplementation boosted the fucoxanthin content of this microalga as compared to 0% supplementation. The fucoxanthin content was increased from 18.4 to 22.6 mg/g-DW at air and 5% carbon dioxide concentration. However, distinct results were found in this PhD study. In continuous conditions, the higher the CO_2 supply concentration, the lower the fucoxanthin concentration. The fucoxanthin content was decreased from 0.6 to 0.1 mg/g-DW at Air and 10% CO_2 supply. The difference between these two investigations may be explained by the tolerance of microalgae to an acidic pH after the addition of carbon dioxide. *I. zhangjiangensis* might be able to survive in more acidic environments. Therefore, it could thrive well and accumulate fucoxanthin in the presence of 5% CO_2. Therefore, additional research is required to confirm the microalgal demand for CO_2 in fucoxanthin production.

According to the above described studies, it can also be seen that the fucoxanthin concentration in this study is not as good as that in other studies, and further research is still needed on how to increase the fucoxanthin content. Many factors, including temperature, light, nutrition, salinity, cell numbers, aeration, and pH, influence the rate of fucoxanthin production in photobioreactors. (Boderskov et al., 2016). Among them, light intensity is considered to be an important condition affecting the accumulation of fucoxanthin in microalgae. Table IV-2 showed fucoxanthin (fx) production in different light density condition. It can be known from the table that the increase of light intensity leads to the decrease of fucoxanthin content. A previous study revealed that a fluctuating light intensity of 10 to 100 μmol/m^2/s induced the highest fucoxanthin content in microalgae, ranging from 0.52 to 4.28 % dry weight (DW). Increased light intensity decreased the fucoxanthin content. This recurrent occurrence may be explained by the fact that fucoxanthin is a light-harvesting pigment and the

increased fucoxanthin compensates for the diminished light intensity (Lananan et al., 2013). As shown in Table IV-2, the content of fucoxanthin in this study was low, which may be attributed to the excessive irradiation intensity of light. But high light intensity is good for algae growth. Therefore, in the following research, the conditions of algae culture and fucoxanthin content should be optimized step by step. For example, higher light intensity is used in the algal culture stage, and when the algal concentration reaches a certain concentration, the light intensity can be reduced to promote the increase of fucoxanthin content.

4.2. Acid treatment to recover calcium in freshwater microalgae Chlorella vulgaris

Chapter 3 showed that an microalgae suspension with added calcium could achieve clear supernatant and fast sedimentation. However, the sediment with high calcium content could influence the harvested microalgae quality in a negative way. In fact, Fig. IV-2a illustrated that the flocculation as well as sedimentation of ~80% of the calcium added to the suspension. Also, it harvested with the microalgae biomass and a 5 mM calcium concentration. For instance, the Ca^{2+} concentration in the supernatant after sedimenting, turned to be only 0.037 g L^{-1}, in the pH11-Ca5 treatment, despite the 0.20 g L^{-1} initial Ca^{2+} concentration. It revealed that 1 g microalgae adhered to 0.12 g calcium. Consequently, to elevate the biomass quality, it became fundamental for calcium recovery from the harvested biomass. Also, the process of recovery would recycle the recovered calcium in the following steps of harvesting biomass to cut down costs.

Assess the impact of acidification on Ca^{2+} removal via altering the pH values of the aliquots of harvested biomass to 5, 3, and 1.5. Acidification to pH 5 removed little calcium. As for the efficiency of calcium recovery for the pH7-Ca5, pH9-Ca5, and

pH11-Ca5 treatments, they were 23%, 33%, and 60%, illustrated in Fig. IV-2b. Fig. IV-2b displayed that acidification to pH 3 elevated the efficiency of calcium recovery, by 46%, 70%, and 82% for the pH7-Ca5, pH9-Ca5, and pH11-Ca5 treatments. Yet, acidification to pH 1.5 did not elevate much efficiencies. The minimal variance ranging from pH 3 to 1.5 showed pH 3 was qualified for nearly the optimal recovery of calcium from microalgae biomass collected by flocculation which includes calcium. It becomes favorable according to the needed amount of acid. Table III-2. shows that the needed acid rises exponentially as the pH falls. For instance, changing the pH11-Ca5 sample to pH 5, 3, and 1.5 needed 26, 45, and 80 mL of acid per milliliter, respectively. Two times needed acid to alter the sample to pH 3 was necessary for changing it to pH 1.5. The pH with in-depth optimization could reduce the dosage of needed acid.

Under varying conditions of flocculation, compare the calcium recoveries of the samples. Noticeably, among all the samples, the efficiency of calcium recovery for the pH7-Ca5 was lowest. At pH 9 and 11, microalgae adsorption to calcium carbonate or phosphate was the possible major flocculation mechanism. With ionic bonds, the solid flocculants can dissolve easily in acid. Nevertheless, within the pH7-Ca5 system, flocculation most likely happened by means of communications between microalgae cells and ions. Ca^{2+} electrostatic bonding to the microalgae cells may make it relatively tough to detach Ca^{2+} via ion exchange. If taking the calcium recovery and sedimentation rate into consideration, and Ca^{2+} concentration 5mM system and high pH (9~11) was a better choice for flocculating and sedimenting *Chlorella vulgaris.* The results of this study may also be applicable to the flocculation of other microalgae, such as marine microalgae *I. galbana*. Also, growing microalgae usually adopts carbon dioxide as one of the sources, for it accelerates growth and stabilizes the culture medium's pH value, which, however, rises once the carbon dioxide supply is shut off. To a large extent, the

above method can cut down pH regulation cost, which is worthwhile to try concerning application for microalgae flocculation in the future.

4.3. Cost of electricity

4.3.1. Reduction in the cultivation and harvesting cost

As shown in general introduction (Fig. I-2(a)), the cost of agitation (aeration) with microalgal cultivation accounts for 40%, 13%, 1% of the total cost (240, 53, 3 cts. Euro per gram) in flat panels, tubular reactors and raceway ponds respectively (Fig. I-2(b)). In this study, it was concluded that intermittent (1min/9min) supply of 5% CO_2 gas can achieve almost the same results as continuous supply. However, the results of this study may only apply to flat panels and similar reactors. The tubular reactors might not be suitable for this intermittent supply of CO_2 because the reactor consists of no vents and the supplied CO_2 cannot escape. Another important factor to consider for tubular reactors is agitation. If intermittent agitation is used, it may lead to uneven nutrient circulation and precipitation of microalgal cells.

As a result, under different air supply conditions, the biomass and fucoxanthin productivity reached 0.17 g L^{-1} and 0.10 mg/g-DW with continuous supply (control). However, the 5% intermittent (1min/9min) condition showed highest biomass concentration and fucoxanthin content (0.30 g L^{-1} and 0.16 mg/g-DW, respectively). Thus, regarding the biomass and fucoxanthin content of *I. galbana* even intermittent supply once every 9 min produced results comparable to those obtained using continuous supply. According to the previous knowledge, using intermittent supply can reduce the energy and cost of aeration. In this PhD study, the energy consumption of continuous and intermittent (1min/9min) supply was calculated. Aeration supply energy (E aeration) requirement was calculated based on aeration's energy consumption in one cubic meter (kWh/m^3).

$$E\ aeration = (W \times 24h \times Af) \times 10^{-3}/1000$$

where W is Watt (2.8), Af is aeration frequency (continuous=1, 1min/9min=1/9).

In terms of energy required for supply, the intermittent supply mode reduced the supply energy consumption as compared to the continuous supply mode. Intermittent power supply mode and continuous power supply mode were 7.5 and 67.2 kWh/m^3, respectively. The energy consumption of culture one cubic meter from intermittent supply was more than 89% lower than that of continuous supply. And the biomass productivity per electricity of continuous and intermittent supply was 5.1 and 38.8 g kWh^{-1}, respectively, the biomass productivity of intermittent supply increased by 7.6 times. According to the results obtained in this study, the condition of continuous supply of 5% CO_2 showed excellent growth rate and maximum concentration. Simultaneously ventilating 5% CO_2 for one minute within nine minutes (intermittent supply) gave almost the same results as continuous supply. This means that using the results of this study it is possible to achieve a 80% reduction in aeration costs, from 240 to 48 cts. Euro per gram of microalga produced. This will greatly reduce the cost of microalgae cultivation in the future.

About the calculation of the cost of microalgae harvesting. The formula below calculated the volume required for centrifugation when producing 1kg-DW of microalgae.

$$\text{No flocculation (L)} = DW \times 1000$$

$$\text{Flocculation (L)} = DW \times 1000 \times 40\% \times \frac{1}{23}$$

Where DW is Dry weight (g/L), 40% is the best sedimentation efficiency (%) from study 3, $\frac{1}{23}$ is the volume of centrifuged cells after flocculation.

Without flocculation, centrifugation of 746 L culture is required. The volume required for centrifugation was 81 L when 1 kg of microalgae was produced using the

flocculation sedimentation method. It decreased 9.2 times from the condition without flocculation.

4.3.2. Reduction in the cost of reused medium

As shown in the figure (Fig. I-2), using the results of the first chapter, the semi-continuous culture method is used to cultivate algae, and then the cultivated algae are recovered by flocculation and sedimentation (Chapter 2). Finally, in order to save costs and reduce the environmental pollution, the supernatant after precipitation must be recycled. Under flocculation condition of pH10 and PO_4 10mM, the contents of NO_3 and PO_4 in the supernatant after flocculation and sedimentation were 0.99 and 0.89 mM, respectively. However, the contents of NO_3 and PO_4 in the Conway medium originally before the microalgal cultivation were 0.99 and 0.81 mM, respectively. The NO_3 and PO_4 content in the supernatant after flocculation was similar as that of the original. Therefore, it can be concluded that there is no need to add NO_3 and PO_4 when the supernatant is reused, which will greatly reduce the cost of the culture medium for the microalgal cultivation.

4.3.3. About CO_2 cost

In this study, the optimal culture condition of microalgae was intermittent (1/9min) supply of 5% CO_2. The cost calculated in this chapter does not include CO_2, because in the future it will consider using CO_2 released from the exhaust of the thermal power plants, iron factories, automobiles, etc. to cultivate microalgae, which can greatly reduce the cost of purchasing CO_2 in microalgal cultivation, thus can reduce the impact on the environment. In this study, CO_2 concentrations in thermal power plants, coal-fired power plants, ironworks, and automobile exhaust were investigated. The results

show that the CO_2 concentrations in the exhaust gas from gas thermal power plants, coal thermal power plants and iron factories are 10%, 14% and 25%, respectively, and the CO_2 concentrations in automobile exhaust vary with different models, for example, the exhaust gas of a 1500cc car is 9% to 11%. Based on the above results, it can be proved that the concentration of CO_2 contained in the exhaust gas of power plants and automobiles is sufficient to cultivate microalgae, and this method can be used to cultivate microalgae in the future.

4.4. Future studies

It was discovered that conditions of CO_2 supply at 5% reached the maximum biomass productivity. Although, due to low DIC concentrations, biomass productivity was reduced when CO_2 levels were low (air and 2%). However, intermittent supply, which permits reduced power consumption at a CO_2 concentration of 5%, exhibited a growth rate nearly similar to that of continuous supply. In addition, intermittent supply promoted the accumulation of fucoxanthin in *I. galbana*. On the other hand, the flocculation and sedimentation characteristics of freshwater *Chlorella vulgaris* and *I. galbana* were explained in this PhD study. It was demonstrated that high pH and high cationic conditions can induce flocculation and sedimentation of microalgae. In the future research, it should be aimed to raise the pH without adding chemicals for cost saving and environmental reasons (Fig. IV-3). The rationale being that the microalgal culture would absorb CO_2 from the culture solution, causing the pH increase. Thus, the flocculation experiment was designed to stop the supply of CO_2 at the end of the microalgal culture, by increasing the pH in the medium, and subsequently adding cationic substances to flocculate the microalgae. However, how the increase in the pH can be maintained, remains to be understood.

Furthermore, the optimal condition between biomass productivity and fucoxanthin biosynthesis frequently results in the highest fucoxanthin yield under cultivation conditions. As the responses of fucoxanthin synthesis to temperature, pH, and salinity have been discussed infrequently, additional research is required to strengthen this field. In addition, integrated investigations of the factors affecting the production of biomass and fucoxanthin are required. However, the biomass and fucoxanthin production-related information, such as molecular and metabolic studies, is still understudied. Future research should concentrate on the molecular and metabolic processes of fucoxanthin under these conditions, the upscaling of fucoxanthin production, and the economic and environmental assessments of fucoxanthin production (technoeconomic and life cycle assessment studies) in order to establish sustainable commercial fucoxanthin production.

Figures

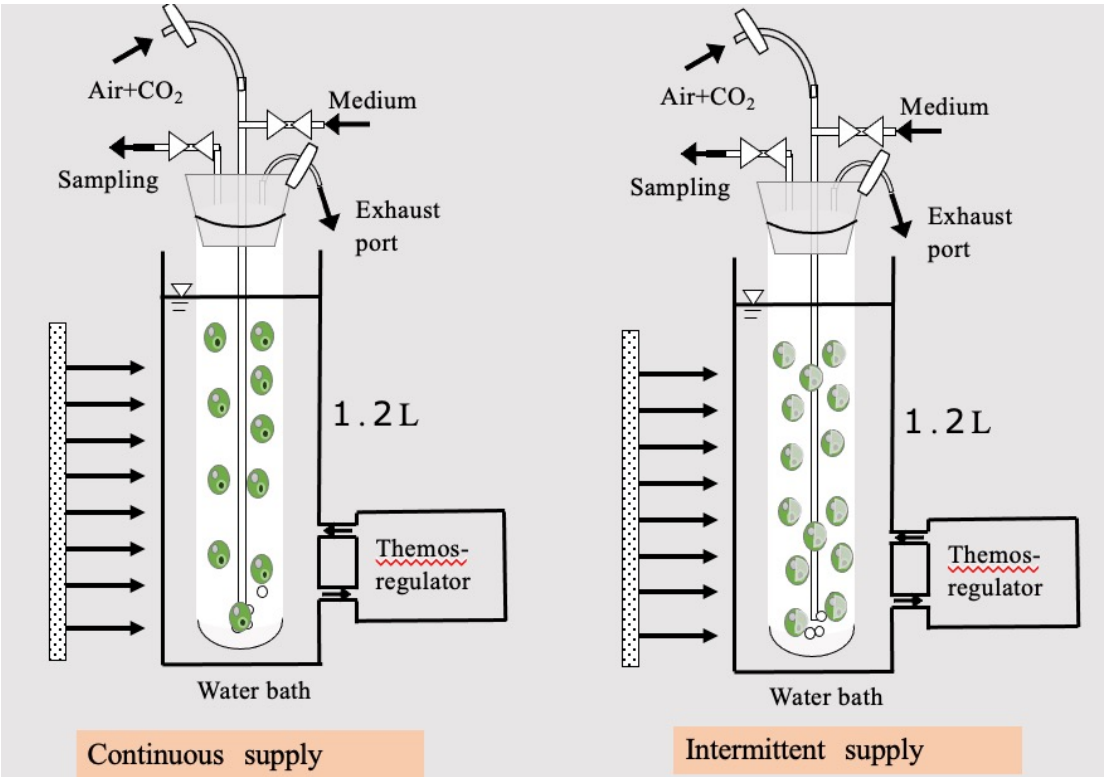
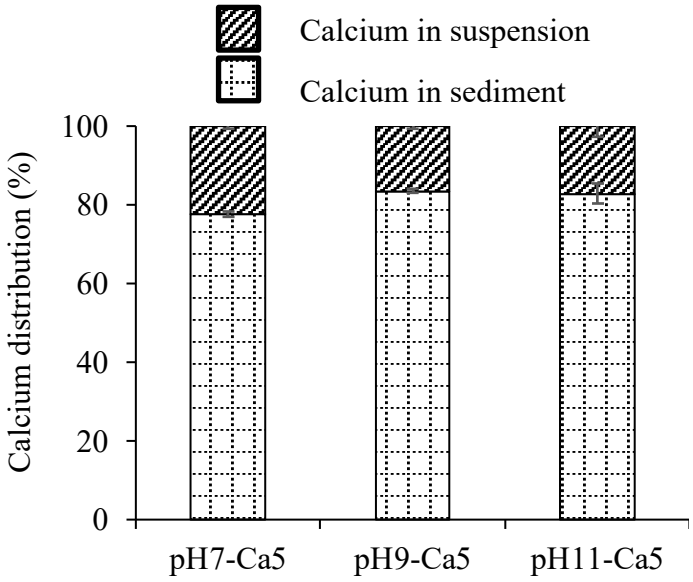


Fig. IV-1. Light absorption effect in continuous and intermittent supply conditions.

(a) Calcium distribution after flocculation



(b) Calcium recovery from sediment after acid treatment

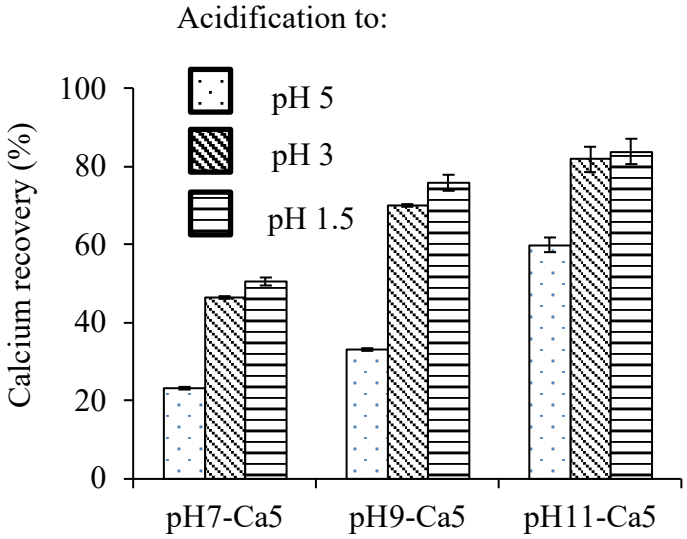


Fig. IV-2. (a) Calcium distribution after flocculation and (b) calcium recovery from sediment after acid treatment (at pH 5, 3, and 1.5).

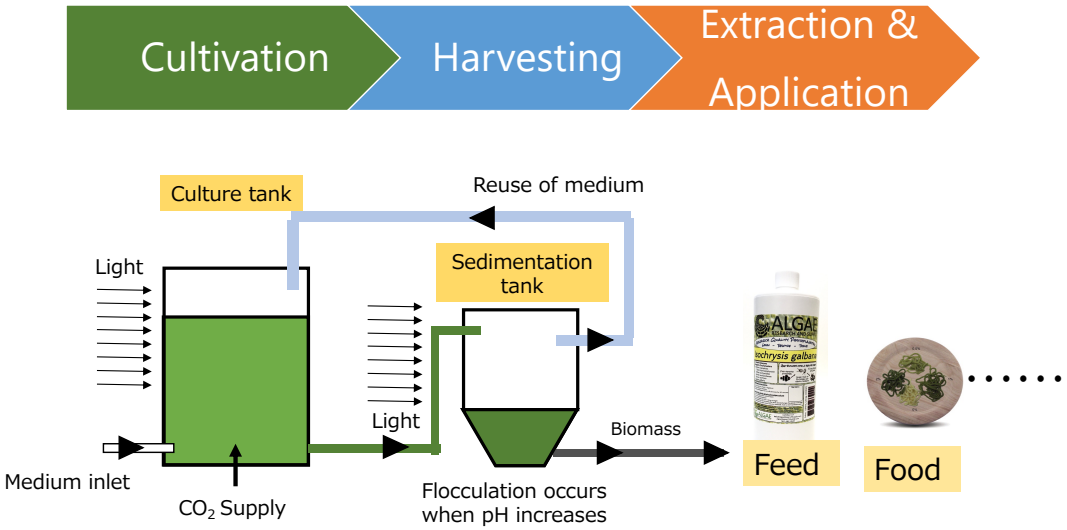


Fig. IV-3. (a)Selection of optimal cultivation and flocculation condition for culture and harvesting of the fucoxanthin-rich marine microalga *Isochrysis galbana* use this system. The microalgae will be growth in culture tank, and recovery in sedimentation tank. The supernatant solution will be reused to microalgae culture. .

Tables

Table IV-1. Previous studies of microalgal concentration(Bc) using *Isochrysis galbana*

Microalgae	Carbon dioxide	Supply frequency	Bc (g L^{-1})	Reference
Isochrysis galbana ABC007	carbonate (1 g L^{-1})	-	0.38	Danesh et al., 2019
Isochrysis sp. ISO-FJ	Air	continuous	0.6	Li et al., 2023
Isochrysis galbana	5%	continuous	1.2	Picardo et al., 2013
Isochrysis zhangjiangensis	5%	continuous	1.35	Li et al., 2023
Isochrysis galbana	5%	continuous, 1/9 min	1.32	This study

Table IV-2. Fucoxanthin (fx) production in in different light density

Microalgae	Light density (μmol/m2/s)	Initial microalgae concentration	Fx Content (mg/g-DW)	Reference
Isochrysis sp. CCMP1324	30	0.3 g/L	14.1	Sun et al., 2019
	120		10	
Isochrysis zhangjiangensis	40	-	22.6	Li et al., 2019
	300		6.84	
Tisochrysis lutea	50	OD_{750}:0.4	5	Gao et al., 2020
	500		1.5	
Isochrysis galbana	300	0.15 g/L (OD_{750}:0.1)	0.56	This study

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