SELECTION AND OPTIMIZATION OF BIOMASS PRODUCTION FROM MICROALGAL CONSORTIUM USING BIOGAS EFFLUENT WASTEWATER FOR POTENTIAL BIODIESEL GENERATION

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การคัดเลือกและหาสภาวะที่เหมาะสมในการผลิตชีวมวลจากกลุ่มประชากรสาหร่ายจากน้ำเสียที่ออกจากระบบก๊าซชีวภาพเพื่อการใช้อย่างมีศักยภาพในการผลิตไบโอดีเซล

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คำสำคัญ : สาหร่ายขนาดเล็ก, การบำบัดน้ำเสีย, การใช้เชื้อร่วมกัน, แอคติโนไมซิส, เชื้อรา, ความสามารถในการเก็บเกี่ยว, ศักยภาพในการผลิตเชื้อเพลิงชีวภาพ

การบำบัดน้ำเสียอย่างยั่งยืนด้วยศักยภาพของการผลิตพลังงานชีวภาพ ทำได้โดยการบำบัดน้ำเสียแบบควบคู่กับการผลิตชีวมวลเพื่อวัตถุประสงค์ด้านพลังงานชีวภาพ ดังนั้นจึงต้องมีการบำบัดอย่างเหมาะสมเพื่อหลีกเลี่ยงผลกระทบที่ไม่พึงประสงค์จากการปล่อยน้ำเสีย ในบรรดาเทคนิคในปัจจุบันระบบการบำบัดด้วยสาหร่ายกำลังได้รับความสนใจมากขึ้น อย่างไรก็ตาม การประยุกต์ใช้เทคนิคนี้ยังไม่ถูกนำมาใช้ในเชิงอุตสาหกรรม อันเนื่องมาจากข้อจำกัดด้านการเพิ่มผลผลิตของสาหร่ายขนาดเล็ก และเทคนิคการเก็บเกี่ยวเซลล์สาหร่าย วัตถุประสงค์หลักของการวิจัยนี้คือการคัดเลือกจุลินทรีย์ที่เป็นประโยชน์ร่วมกับสาหร่ายขนาดเล็ก เพื่อเพิ่มประสิทธิภาพการผลิตชีวมวลจากกลุ่มสาหร่ายขนาดเล็ก และลดปัญหากระบวนการเก็บเกี่ยวเซลล์ของสาหร่ายขนาดเล็ก เพื่อบำบัดน้ำเสียและนำผลิตชีวมวลของสาหร่ายไปใช้เพิ่มประสิทธิภาพในการผลิตไบโอดีเซลจากน้ำเสียของการผลิตก๊าซชีวภาพจากมันสำปะหลัง ในการศึกษาครั้งนี้ได้แบ่งจุลินทรีย์ออกเป็น 2 กลุ่มโดยถูกแยกและคัดเลือกเพื่อนำมาเพาะเลี้ยงร่วมกัน โดยจุลินทรีย์กลุ่มแรก ได้แก่จุลินทรีย์ที่เป็นสาหร่ายขนาดเล็กได้แก่ *Chlorella sorokiniana* และแบคทีเรียในสกุล *Streptomyces thermocarboxydus* BMI 10 กลุ่มจุลินทรีย์ที่สองได้แก่ กลุ่มสาหร่ายขนาดเล็กในสกุล *Chlorella vulgaris* สายพันธุ์ TISTR 8580 และเชื้อราในสกุล *Aspergillus niger* สายพันธุ์ F5 โดยทำการทดสอบปฏิกิริยาซินโทรฟิกโดยทำการเพาะเลี้ยง *S. thermocarboxydus* BMI 10 ร่วมกับ *C. sorokiniana* P21 ผลการศึกษาพบว่า กรดอินโดล-3-อะซิติก (IAA) ที่ผลิตจาก *S. thermocarboxydus* BMI 10 ส่งผลต่อการเจริญเติบโตของสาหร่าย P21 ในน้ำเสียที่ผ่านการฆ่าเชื้อ ในขณะที่อีกระบบที่มีการปลูกเชื้อสายพันธุ์ F5 ที่มีสมบัติในการย่อยสลายฟอสเฟตลงไปในระบบกำจัดน้ำเสียจากมันสำปะหลัง หลังจากที่ค่าการกำจัดฟอสฟอรัสรวมมีค่าคงที่ ร่วมกับสาหร่ายขนาดเล็ก สายพันธุ์ 8580 พบว่าสามารถเพิ่มประสิทธิภาพในการกำจัดฟอสฟอรัส แต่สามารถเก็บเกี่ยวเซลล์ของสาหร่ายสายพันธุ์ 8580 ได้น้อย อย่างไรก็ตาม ปริมาณไนโตรเจนทั้งหมดมีปริมาณลดลงเมื่อใช้การกำจัดทั้งสองวิธี เมื่อทำการเพาะเลี้ยงสาหร่ายขนาดเล็กสายพันธุ์ P21 ร่วมกับ *Streptomyces* สายพันธุ์ BMI 10 พบว่า สามารถส่งเสริมมวลชีวภาพได้ดีกว่า (2.11 กรัมต่อลิตร), ประสิทธิภาพในการกำจัดปริมาณไนโตรเจนทั้งหมด ปริมาณฟอสฟอรัสทั้งหมด และค่า COD คิดเป็น 76.76, 86.56 และ 72.94 % ตามลำดับ ซึ่งการ

เพาะเลี้ยงในระบบนี้ให้ผลดีกว่าเมื่อเทียบกับการเพาะเลี้ยงร่วมระหว่างสาหร่ายขนาดเล็กสายพันธุ์ 8580 กับเชื้อราสายพันธุ์ F5 นอกจากนี้พบว่า การเพาะเลี้ยงร่วมระหว่างสาหร่ายขนาดเล็กสายพันธุ์ P21 ร่วมกับ *Streptomyces* สายพันธุ์ BMI 10 มีประสิทธิภาพในการบำบัดได้ดีกว่าการใช้เซลล์สายพันธุ์ใดสายพันธุ์หนึ่ง อย่างไรก็ตามผลผลิตของการเพาะเลี้ยงเชื้อร่วมกันในน้ำเสียนั้นต่ำกว่าในสภาพปลอดเชื้อ การวิเคราะห์ปริมาณ และองค์ประกอบของกรดไขมันจากสิ่งมีชีวิตต่อหน่วยพื้นที่ที่มีการเพาะเลี้ยงเชื้อร่วมนี้พบว่า เป็นที่น่าพอใจสำหรับการผลิตไบโอดีเซล (กรดไขมันอิ่มตัว 54.11-61.52% ที่มีระดับความไม่อิ่มตัวที่ 0.59-0.82) โดยภาพรวมแล้ว ผลการวิจัยแสดงให้เห็นว่าการเพาะเลี้ยงเชื้อร่วมกันของสาหร่ายและแบคทีเรีย สามารถเพิ่มประสิทธิภาพแบบองค์รวมที่ควบคู่กับการบำบัดน้ำเสียและการผลิตไบโอดีเซล โดยสามารถลดพลังงานในการผลิตไบโอดีเซลได้ถึง 35% ต่อหน่วยการผลิตไบโอดีเซลทั้งหมด อย่างไรก็ดีประสิทธิภาพในการกำจัดสารอาหารยังไม่สามารถทำได้เต็มที่ ซึ่งอาจส่งผลกระทบต่อสิ่งแวดล้อม โดยเฉพาะการเกิดปรากฏการณ์ Eutrophication ดังนั้น การศึกษาความเป็นไปได้ในอนาคต จำเป็นต้องมีการขยายระดับการทดสอบที่ใหญ่ขึ้น เพื่อให้มีสภาพใกล้เคียงกับระบบการกำจัดจริง

สาขาวิชาวิศวกรรมสิ่งแวดล้อม
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ลายมือชื่อนักศึกษา .....
ลายมือชื่ออาจารย์ที่ปรึกษา ..... Nittaya Boonian

MOHAMAD PADRI : SELECTION AND OPTIMIZATION OF BIOMASS PRODUCTION FROM MICROALGAL CONSORTIUM USING BIOGAS EFFLUENT WASTEWATER FOR POTENTIAL BIODIESEL GENERATION. THESIS ADVISOR : ASST. PROF. NITTAYA BOONTIAN, Ph.D., 222 PP.

Keyword : MICROALGAE, WASTEWATER TREATMENT, CO-CULTURE, ACTINOMYCEYES, FUNGI, HARVESTABILITY, BIODIESEL POTENCY

Coupling bioenergy in the biodiesel form and wastewater treatment system has been emerging nowadays to address an increase of energy demand and environmental protection action. Combination microalgae culture in the wastewater with the beneficial microbial would address the problem of algal production and harvestability process. The primary objective of this research is to select and optimize biomass production from microalgal consortia using cassava biogas effluent wastewater to treat the wastewater and produce algal biomass for potential utilization in biodiesel production. Two different co-cultures were developed based on the isolation and screening processes. The first one was alga-actinomycete co-culture that consisted of *Chlorella sorokiniana* strain P21 and *Streptomyces thermocarboxydus* strain BMI 10 were potential for alga-actinomycete co-culture. The second co-culture was *Chlorella vulgaris* strain TISTR 8580 and *Aspergillus niger* strain F5 for alga-fungus co-culture. The syntrophic interaction of *S. thermocarboxydus* BMI 10 with *C. sorokiniana* P21 was also observed. The Indole-3-acetic acid (IAA) mechanism of growth-promoting affected the growth of alga P21 in sterilized wastewater. Additional fungus F5 pellets with the phosphate-solubilizing activity after the total phosphorus (TP) removal became stationary in cassava wastewater treatment using alga 8580 significantly increased TP and chemical oxygen demand (COD) removals. Another approach by co-culturing the fungus F5 and alga 8580 also increased the removal efficiencies but less effective in trapping algal cells during the cultivation. However, total nitrogen (TN) removal decreased with both methods of application. On the other hand, co-culture of alga P21 and actinomycete BMI 10 showed the better total biomass production (2.11 g L^{-1}), TN, TP, and COD removals as much as 76.76, 86.56, and 72.94 %, respectively than co-culture of alga 8580 and fungus F5. Alga P21 and actinomycete 8580 co-culture showed a higher result than the single culture of alga P21 in the actual wastewater utilization. Nevertheless, the productivity of the co-culture in the real wastewater was lower than

in the sterilized condition. Analysis of the amount and composition of fatty acids from this co-culture biomass revealed that it was quite satisfactory for biodiesel production (54.11-61.52% saturated fatty acids with a 0.59-0.82 degree of unsaturation). Overall, the results showed the co-culture of the alga and bacterium is a holistic enhancement that couples wastewater treatment with biodiesel production with a total 35 % reduction of energy demand per unit biodiesel production. Nevertheless, the significant removal of nutrients was still not a complete removal and might still carry eutrophication potency to the environments. Further, upscaling process into a bench-scale may be required before the application of co-culture in the real situation.

School of Environmental Engineering
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Student's Signature .....
Advisor's Signature ..... Nittaya Boonnorm

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LIST OF ABBREVIATIONS

CBEW	=	Cassava Biogas Effluent Wastewater
WW	=	Wastewater
N	=	Nitrogen concentration
P	=	Phosphorus concentration
BOD	=	biochemical oxygen demand
COD	=	chemical oxygen demand
TKN=	=	total Keldjah nitrogen
NH_4	=	ammonium nitrogen
NO_3	=	nitrate nitrogen
NO_2	=	nitrite nitrogen
TP	=	total phosphorous
PO_4	=	phosphate
A_{xxx}	=	Absorbance at the wavelenght xxx
IAA	=	Indole-3-Acetic Acid
PKV	=	Pikovskaya
PDA	=	potato dextrose agar
SEM	=	Scanning Electron Microscope
Chl *a*	=	Chlorophyll *a*
B_T	=	Total biomass
B_A	=	Algal biomass
B_M	=	Microbial biomass
X	=	Biomass
X_m	=	Maximum biomass
X_0	=	Initial Biomass
t	=	time
μ	=	growth rate
S	=	Substrate concentration
S_{na}	=	non-assimilated substrates
S_0	=	initial concentration of substrate
Y_0	=	initial microalgae coefficient
HRT	=	Hydraulic rentention time
Q	=	Flowrate

LIST OF ABBREVIATIONS (Continued)

V	=	Reactor working volume
P	=	Productivity
FA	=	Fatty acid
FAME	=	Fatty acid methyl ester
DU	=	Degree of saturation
N	=	Number of carbon-carbon bond
Mf	=	Mass fraction of saturated fatty acids
υi	=	kinematic viscosity
SG	=	specific gravity
CP	=	cloud point
CN	=	cetane number
IV	=	iodine value
HHV	=	higher heating value
EPS	=	Extracellular polymeric substance
BLAST	=	Basic Local Alignment Search Tool
-S	=	Sterilized
-U	=	Unsterilized
OD	=	Optical density
PUFA	=	Poly-unsaturated fatty acid
TUFA	=	Tri-unsaturated fatty acid
DUFA	=	Di-unsaturated fatty acid
MUFA	=	Mono-unsaturated fatty acid
SFA	=	Saturated fatty acid.

CHAPTER 1
INTRODUCTION

1.1 Background

Globalization and rapid industrial growth worldwide cause an increase in energy supply in which the energy demand is increasing simultaneously with the growth of population (Overland, 2016). Nowadays, energy sources are clustered into three domains: fossil fuels, renewable energy, and nuclear sources (Forsberg, 2009). For fossil fuels, prime sources are coal, oil, and natural gasses. Limited sources and detrimental effects of fossil fuels treat the sustainability of the environment. Another source of energy is the nuclear source. The production can generate high-energy power instantly, yet the risk and development of this source still require more consideration. Therefore, the renewable energy source is critical to fill the gaps between the demands of environmentally sustainable fuels and the feasible generated fuel.

Biofuel from biomass seems to have more advantages than other renewable energy sources (Li et al., 2008). Among biofuel sources from biomass plants such as coconut, palm, soybean, sugarcane are well known for their potency to produce biofuel through several physicochemical processes. Nevertheless, mentioned crops are also food crops, and food security can be threatened due to the conversion. Thus, extensive agriculture does not solve the problem since it could damage existing carbon sequester sites. However, the application of agroforestry can tackle a small portion of this issue currently (Aertsens et al., 2013). Animal feedstock, as other bioenergy sources, performs promising results in several lab-scale studies. However, this technology's industrialization must have a high supply of raw materials. In addition, animal and cooking oil cannot be utilized as substrate sources. Thus, there is a need for renewable energy to produce high raw materials without affecting the current supply of food or other vital industries.

Biofuel from algae recently obtained massive attention since it can grow without utilizing large space and has a very high biomass production (Mata et al., 2010; Pierobon et al., 2018). The growth of algae is fast and produces a high biomass yield. Another critical issue is the energy from algae does not compete with the food supply, as previously mentioned to be drawback factors for crop plants (Chen et al., 2011). Algal biofuel has thus fulfilled the requirement of renewable energy development without any significant conflict with other demands, including food security concerns.

Additionally, biodiesel, as the primary fuel generated from algae, is possibly integrated into the current transportation and industrial system since it has a remarkable similarity to the current fuel that fits with the installed infrastructures (Forsberg, 2009).

Water as a resource has a limited amount of supply, and thus it makes the usage of water has many priorities. Water supply for food, agriculture, and industry have taken early places to its concern due to its importance to supporting human life. Accordingly, it also puts microalgae cultivation into highlight since it requires water as the primary medium. The supply for this demand is under concern after water depletion since the water footprint of this technology is relatively high (Guieysse et al., 2013). Consequently, water utilization as the medium of cultivation shall be limited and separated from the primary usage as the water supply.

Limitation of water availability can be overcome by coupling the wastewater treatment with algae cultivation since algae have the potency to reduce the organic matter in wastewater. The wastewater availability is considered sustainable. This coupling is expected to reduce nutrient contaminants in the wastewater since the nutrients and other pollutants are assimilated into algae cells as proper growth substrates of microalgae. Comparing this coupling system with physical and chemical wastewater treatments, potential biomass product is the added value. Most conventional approaches such as water filtration do not have this potential valuable by-product (Kumar et al., 2015b).

Several advantages of the application of algae cultivation using wastewater as the medium have been summarized by Mata et al. (2010) as follows:

1. The application of microalgae can reduce the CO_2 from industrial flue gases by algae bio-fixation. Thus, it can reduce carbon emissions from industrial activity.

2. The presence of NH_4^+, NO_3^-, PO_4^{3-}, and other forms of nitrogen and phosphorus are severe problems while the algae need these forms for synthesizing the biomass.

3. The by-product of biomass after oil extraction can still be processed for ethanol, methane, livestock feed, used as organic fertilizer since it has a high N:P ratio. Direct combustion is also possible for obtaining the extra value of this substance.

4. The Unnecessity of clean water usage with no specific requirements for fertile land and soil is the other advantage since most of the raw materials of biofuel are yielded from cropping plants that need to deal with food security issues.

5. The ability of algae that can generate more economical products than oil, such as natural dyes, sugars, pigments, antioxidants, and high-value bioactive compounds, is worth consideration for optimum utilization of this coupling.

6. The possibility of compounds produced in this system is potential for other applications such as biotechnology in cosmetics, pharmaceuticals, nutrition and food additives, aquaculture, as well as pollution prevention

Although some nutrients can be vital for algae growth, the excess of nutrients in the water body can lead to counter-productive growth of algae and inefficient removal. This situation needs another treatment to ensure that the effluent contains fewer nutrients before flowing to the water body. Standing in contrast, lack of nutrients can mean an additional cost of the treatment for additional nutrients (Xu et al., 2015). Currently, studies have been conducted to cultivate the algae using many wastewater sources, and the results showed promising improvement in this system for industrialization. However, the amount of biomass with the derivatives and the growth rate have been reported insufficient. The lipid content of microalgae cultivated in wastewater has been found in a wide range of percentages. Microalgae species cultivated from the wastewater vary from many genera and families.

Several attempts have been conducted to increase the productivity of microalgae. Creating a consortium by isolating indigenous microalgae species emerges as the promising option for adaptable cultivation with many possible changes and functions (Subashchandrabose et al., 2011). The effect of combining two different organisms to produce high yield biomass was reported in many studies (Kumsiri et al., 2018; Qi et al., 2018; Thøgersen et al., 2018). Some applied commercial and genetically-engineered strains (Ghosh et al., 2016). Indigenous strains showed a higher increase of biomass production than commercial microalgae strains and dominant algae strains (*Chlorella, Chlorococcum, Ankistrodesmus, Chlamydomonas,* etc.) in monoculture collection for the dairy farm wastewater treatment (Hena et al., 2015). Nevertheless, a study of microalgae cultivation in wastewater showed a higher production of the commercial strain of *Desmodesmus communis* than the indigenous strain using urban wastewater as the growth medium (Samorì et al., 2013). However, optimum applications between monoculture and consortium are different between each wastewater.

High productivity of biomass can lead to increased production of biodiesel. As 18-52% of microalgae biomass consists of lipid (Liu et al., 2011), it is essential to enhance the biomass and its lipid percentage (Xin et al., 2010). Excess lipid that increases in the cells can only be achieved primarily under stress conditions. It often leads to low productivity. Thus, it is difficult to enhance lipid productivity simultaneously with the increase of total biomass in the culture. *C. vulgaris* has been reported to increase productivity in phosphorus starvation but with the sufficient amount of other nutrients (Mujtaba et al., 2012). Yet, the state of phosphorus in low

concentration is rarely found because it is difficult to remove this nutrient without any reduction of COD and other constituents. Based on this condition, the early mentioned nutrient removal can be significantly reduced through the nutrient limitation mechanism. Consequently, wastewater treatment objectives cannot be achieved. However, the most promising way to increase productivity is by generating as much biomass as possible from microalgae culture.

Several factors also limit the density of microalgae. Light (for autotroph and mixotroph), CO_2 supply, and nutrient content and its availability determine the maximum possible concentration of algal cell per working volume in a photobioreactor (Pegallapati & Nirmalakhandan, 2011) and open pond (Kumar et al., 2015a). It is possible to extend this limitation in the photobioreactor because almost all the factors mentioned can be controlled and adjusted to optimum growth. In contrast, density is limited to a narrow extent in the open pond system. The open pond and closed photobioreactor systems are considered the two main options with the additional system features in several current studies such as the air-lift tubular system (Converti et al., 2006), bubbling column (Wu & Merchuk, 2002), and high depth photobioreactor (Sawant et al., 2018).

Biomass production rate is defined as the amount of biomass produced by the total substrate available per time. The increase of production rate can be maintained to be positive in the cost balance if the biomass production can increase and the time for producing the biomass reduces. Nowadays, the algae cultivation have a capital cost of 8-15 USD kg dry ash^{-1}, and it needs to be reduced to about 0.25 USD for competing with fossil fuel (James & Boriah, 2010). Compared with increasing the optimum biomass of microalgae that is possible to yield, time reduction and total biomass yield are the most affordable enhancement for this idea to be industrialized.

Several mechanisms have been tried on the microalgae for producing biomass in a short period. Coexisting growth of microalgae and growth-promoting microbes has been proven to increase the production rate to a specific extent (Fuentes et al., 2016). Co-culture of microalgae and other organisms has been documented widely as the emerging solution for increasing the feasibility of biodiesel generated from algae by nutrient exchange and additional production of hormones (Yao et al., 2019). Microalgae excrete growth regulators, vitamins, and nitrogen compounds (Fuentes et al., 2016). On the other side, the growth-promoting microorganisms utilize excreted organic carbon and photosynthesis oxygen produced from the photosynthetic microalgae. This synergistic interaction can potentially lead to a high yield of biomass production with an increase of removal efficiency.

A combination of microalgae *Chlorella sorokiniana* and a mixed bacterial culture from an activated sludge process have been tested to remove 75% of COD, 99% of NH_4^+, and 86% of PO_4^{3-} contents in swine wastewater (González et al., 2008). Several potential strains have also been tested in the monoculture system. The results showed a high density of microalgae cells could be achieved in shorter with higher efficiency of nutrient removal period than the single culture. Actinomycete *Nocardia* sp. with microalgae *Tetradesmus obliquus* (Kumsiri et al., 2018), bacterium *Bradyrhizobium japonicum* with microalgae *Chlamydomonas reinhardtii* (Wu et al., 2012), and fungus *Aspergillus fumigatus* with microalgae *Thraustochytrid* sp. (Wrede et al., 2014) were among the co-culture that showed the positive results of co-culture. The actinomycete doubled biomass productivity in the chicken manure medium by producing Indole-3-acetic acid that enhances the algae productivity. Another type of co-cultivation of the microalgae and yeast cultivation reported that yeast *Rhodotorula glutinis* and microalgae *Scenedesmus obliquus* showed a mutualistic interaction of gas exchange and trace elements for one another and it resulted a higher biomass concentration up to two-fold (Yen et al., 2015). Nonetheless, the condition of microalgae in the reactor lacks stabilization since the pure and culture are very susceptible to slight change and contamination.

Microalgae co-cultivation is not limited to the single culture of bacteria or yeast to obtain an optimum increase of biomass productivity. Freshwater microalgae *Chlorella* sp. has been reported to be cultivated with activated sludge from sewage treatment plans (Leong et al., 2018). With the ration 4:3 of activated sludge and microalgae, 130 mg L^{-1} of microalga biomass has been reached. Furthermore, Qi et al. (2018) studied the pure culture of microalgae *Chlorella sorokiniana* with the three most dominant bacteria from fermentation wastewater after treatment. They resulted in a one-fifth increase of the wastewater produced from pure culture cultivation. Nevertheless, the same study reported that co-cultivation only resulted in 42% of flocculation efficiency.

Although high biomass production rate can be achieved, harvestability and post-harvest processes of this biomass, including dewatering (Uduman et al., 2010), lipid extraction (Pragya et al., 2013), and transesterification of the fatty acid, still need improvement to optimize the industrialization of this technology (Zhang et al., 2016). Current effective techniques to harvest the microalgae biomass rely on high-energy demand technologies. The previous methods of harvesting mainly include simple screening, filtration, and flotation (Show & Lee, 2014). On the other hand, lipid extraction depends on how the biomass can be thickened and dried effectively from the previous step (Mubarak et al., 2015). Eventually, transesterification, as the most

well-known method to synthesize fatty acid methyl ester (FAME), is designed and conducted based on the product from initial steps (Amin, 2009). Among these processes, harvesting possesses an essential role that interferes with the industrialization of biofuel from microalgae (Mathimani & Mallick, 2018). It costs 30-50 % of the total production cost of algal-based biofuel (Uduman et al., 2010).

Coagulation-flocculation is the most employed method to harvest algae biomass via sedimentation and filtration since the algae have slow sedimentation because of negative cell surface and small particle size (Gerardo et al., 2015). Additionally, these processes were also employed to increase the efficiency and reduce the operational cost of other methods such as centrifugation and floatation. The coagulation process is started by adding chemicals (coagulants) to reduce or neutralize the negative surface charge on algal cells (Chatsungnoen & Chisti, 2019). Some organic and biological flocculants are employed to increase the flocculation efficiency, such as chitosan and tannin (Bracharz et al., 2018; Kirnev et al., 2018) or biological agents as flocculants such as bacterial polymers (Jimoh et al., 2019). The latter flocculants group are preferable for a sustainable reason. It also lowers the total cost compared with the former method (Bracharz et al., 2018).

Available coagulants and flocculants in the market for the water treatment systems are mostly inorganic coagulants such as $FeCl_3$, Fe_2SO_4, polyaluminium chloride (PAC), and cationic polymers such as polyacrylamides (PAM), chitosan, cationic starch, inorganic metal salts, and many other chemical flocculants. However, it is also important to note that there is no specific flocculant suitable for all types of microalgae (Milledge & Heaven, 2013). These coagulants are identified as the reducing agents for algae since they reduce the algal dispersion (Pieterse & Cloot, 1997) and some of them produce by-products at the end of the process (Zdybel et al., 2019). Interestingly, several studies have been conducted to obtain and develop microbes that potentially be bio-coagulant for replacing the synthetic coagulants using actinomyces (Li et al., 2017), fungi (Wrede et al., 2014), and bacteria (Lee et al., 2013). Since the bio-coagulants are commonly free from any by-products, the objective of nutrient removal can be achieved without unfavorable compounds in the final products.

Interestingly, actinomycetes, a group of Gram-positive bacteria, and fungi, have these potencies to address these two drawbacks. The bacteria have the ability to increase productivity by enhancing the growth using growth-promoting activity (Barka et al., 2016; Doumbou et al., 2001; Jog et al., 2014; Khamna et al., 2008; Khamna et al., 2010) and increase the harvestability as it possesses mycelial forms to attach the algal cells (Li et al., 2017; Sivasankar et al., 2020). Similarly, several fungi strains also

include both advantages as the natural traits (Kumar et al., 2021). It is noteworthy that these advantages have never been studied in a holistic investigation before. Previous studies were focused on one of these advantages rather than combining these two advantages. This study was conducted to demonstrate the possibility of the co-culture of indigenous microalga in the cassava effluent wastewater and filamentous microorganism isolated from similar wastewater.

1.2 Objectives of the Study

a. To develop the microalgae-symbiont consortia with synergistic cooperation in cassava biogas effluent wastewater (CBEW).

b. To examine the most promising consortium for treating raw wastewater and evaluate its application feasibility in CBEW.

c. To evaluate biomass generation, nutrient evolution, harvestability, oil production, and biodiesel quality from the selected consortium cultivated in CBEW.

1.3 Scope of the Study

To address the bottleneck of microalgae cultivation and wastewater treatment coupling system, this study is designed to construct the co-cultivation of microalgae with indigenous synergistic microorganisms and advance the coagulation-flocculation process to ease the sedimentation and harvesting phase. Consequently, adjustments in wastewater content, including pretreatment of the wastewater, were excluded in the focus of this study.

The scope of this study covers the characteristics of wastewater that imply the growth rate of algae and determining water constituents that need to be removed, the combination of two different microorganisms (algae and other co-culture microorganisms) for wastewater treatment, and emerging the potency of synergistic organisms to be the bioflocculant to enhance the harvestability simultaneously were the novelty of the study. Lipid content of the biomass and several kinetic parameters were also calculated to display the improvement and differentiate the result between the combination of algae and the other organisms. However, this study did not profoundly examine the native microbial community and its shift after additional co-culture.

1.4 Significance of The Study

One intended outcome of the study is to reduce the gap between production requirements of algae in terms of biomass yield and to ease the harvesting process through the formation of flocs where production costs can be reduced. In other words, this study mainly offers the method of algal cultivation by co-culturing the algae with co-culture microorganisms to enhance the biomass productivity and removal efficiency of wastewater contaminants with high harvestability. Significant distinctive improvement from the previous investigations was also found in this study. The comprehensive development in the raw wastewater based on the mechanisms in the sterilized condition was the first to demonstrate by this study.

This study also makes other several contributions as follows:

a. Understanding microalgae-symbiont advantages and challenges using the co-culture approach in the cassava biogas effluent wastewater.

b. The effect of this co-culture on the end-product of the biomass for biofuel purposes.

c. Alternative nutrient removal for CBEW to fulfill the wastewater discharged standards

1.5 Contribution of the Study

Several contributions for the knowledge and practical uses were generated based on the results and findings of this study. The wastewater-based cultivation of microalgae with the co-culture studies has demonstrated several key findings. This study contributed to the additional knowledge of co-culturing for the growth and harvestability of the microalgae. This knowledge may be further applied in other algae with potential end products with different substrates. Additionally, this co-culture also shared a new perspective on the mechanisms of algae and the symbiont. Furthermore, since one of the main focuses of this research is on the wastewater treatment system, it offers some contribution to today's problem of wastewater treatment, especially regarding the removal of nutrients such as N and P. An alternative way to treat wastewater using algae-based treatment system and future improvement on biofuel production using algae biomass based on the increasing total yield potency from the algal biomass were also elaborated based on this study.

1.6 Overview of the Research Stages

The study was started by isolating indigenous microalgae from the wastewater effluent of the biogas plant. The isolates were then screened for their ability to grow under mixotrophic conditions. Only highly mixotroph strains were further be used. Morphological characterization, physiological activity in terms of carbon sources utilization, and molecular identification were conducted for standard identification and characterization processes. Commercial strains and natural communities of microalgae were also prepared to obtain the most optimum algal inoculants for wastewater treatment. All the inoculants were tested in terms of wastewater treatments and biomass production.

Similarly, fungi and actinomycetes isolation was also conducted. Strains with growth-promoting activities were co-cultured with the most optimum inoculants of microalgae that previously tested. The compatibility between the algae and symbiont was then tested to obtain the most suitable strain based on several parameters, namely nutrient removal efficiencies and biomass generation. An experiment in the flask scale was employed here to obtain the details of this co-culture. The harvestability of the constructed consortium was also tested. Self-sedimentation was performed to demonstrate the ability of biomass recovery from the system.

After alga and growth-promoting microbe were constructed in the previous step, the application in wastewater was measured to observe their ability to treat raw wastewater. Lastly, lipid content from the total biomass generated was assessed. The lipid produced eventually characterized to obtain fatty acid profile. Biodiesel properties were then described based on the fatty acid profile to compare the quality of the obtained biodiesel.

CHAPTER 2
LITERATURE REVIEWS

2.1 Microalgae

The microalgae term refers to the photosynthetic microorganisms that are identified to be able to grow and develop in unusual and scarce resource conditions due to their traits (Mata et al., 2010). Studies have reported that microalgae has been found in wide range of environments, from the freezing ecosystem, complete darkness annually or continuous light exposure and UV (Lyon & Mock, 2014). The traits of growing in numerous conditions are supported by the vast number of species appearing in almost all environments. Microalgae can thus be classified by the environment where they are available.

Some green microalgae are also known to possess high percentage of lipid in their biomass. This oil and other compounds in these biomasses are potential to be use as affordable source of valuable bioproducts. Since these microalgae usually occur in the freshwater, some studies have tried to obtain green microalgae near by the wastewater or other media to further cultivate those algae. It is also important to note that the advantages of green microalgae are not limited only for generating bioenergy. Some other advantages of algae biomass utilization include supply of the food materials (Ötle et al., 2001) and cosmetics (Hagino & Saito, 2004). In these latter applications, quality of the medium plays an important role and thus the utilization of wastewater often avoided due to the high-standard quality.

Among the utilization of algal biomass for the bioenergy, biofuel especially biodiesel is the most emerging development for algal based energy nowadays. Biomass generation that targeting biodiesel production requires lipid content quantity and quality. Biodiesel production requires certain fatty acid contents for transesterification process in order to produce fatty acid methyl ester (FAME) product (Alcantara et al., 2000). This product is classified as the algal biodiesel content that can be directly consumed by diesel engines. FAME production can be simplified as alcohol displacement from ester group by another alcohol or usually called alcoholysis (Fukuda et al., 2001). The oil percentage thus plays a key role to precisely apply algae species for biodiesel generation (Chi et al., 2019).

Table 2.1 Biomass yield with fatty acid percentage of several green microalgae.

Microalgae	Biomass ($g\ L^{-1}$)	Lipid content (%)	Sources
Chlorella sp.	1.7	13.7	(Wang et al., 2010)
Chlorella sp.	1.1	11	(Chi et al., 2019)
C. protothecoides	24.01	34	(Mu et al., 2015)
C. pyrenoidosa	0.73	59	(Tu et al., 2016)
C. sorokiniana	11	38	(León-Vaz et al., 2019)
C. minutissima	0.97	37	(Chandra et al., 2019)
Spirulina maxima	0.8	7.30	(Oliveira et al., 1999)
S. platensis	0.6	7.24	
S. platensis	0.8	13.70	(Lu et al., 2019)
Spirulina sp.	.3	60.13	(Andrade et al., 2019)
Scenedesmus acutus	0.9	30.4	(Alva et al., 2013)
S. dimorphus	2.5	24.7	(Ruangsomboon et al., 2013)
S. abundans	1.1	44	(Mandotra et al., 2014)
Desmodesmus spp.	-	58	(Pan et al., 2011)
Desmodesmus sp.	0.73	12.9	(Rugnini et al., 2018)
D. communis	1.23	19.0	(Pezzolesi et al., 2019)
Chlamydomonas sp.	4.15	19.4	(Tan et al., 2019)
C. reinhardtii	-	50	(La Russa et al., 2012)
C. reinhardtii	0.73	18.8	(Ahmad et al., 2015)

Thousands of algal strains and inoculants have been reported for versatile purposes and one of them is to generate qualified biomass in large scale. Tailoring the microalgal utilization with the proper medium is vital to start the initial process of biomass production, which is screening process. To generate biofuel from algae, several important notes shall be considered. Maximum density, rate of growth and nutrient utilization, and wide range environmental niches are among them (Bux, 2013). Further analysis of lipid content is also important for generating high amount of end product. Several genera were reported to render high lipid product with considerable density level, namely *Chlorella*, *Spirulina*, and *Scenedesmus* (Table 2.1).

2.1.1 Chlorella

Genus *Chlorella* is often found freshwater ecosystems as this group can moderately tolerate the occurring pollutants (Bellinger & Sigee, 2015). Apart from the versatility, this group still manages to produce up to 59 % lipid content in considerable amount of biomass concentration (Table 2.1). High tolerance trait of this genera is often considered to use this group for inoculants, and one of them is the salinity tolerance (Guccione et al., 2014). Another supporting fact of this trait is the well-known physiology and widely development strains and inoculants.

Chlorella is often found in almost all trophic condition, namely autotroph and heterotroph as well as mixotrophic states. These characteristics are important for the organic matter removal in the wastewater. Mu et al. (2015) reported 24.01 g L^{-1} production of biomass by utilizing sugarcane bagasse hydrolysate with the mixotrophic *C. protothecoides*. Further, with 82.02 % removal of nutrients, this study also proved that this genus renders potency to be cultivated in open pond system, the system where the stability and high result of removal are essentially required. In the high initial concentration, the unassimilated nutrients can occur above the discharge standard. This situation often leads to demands of further treatments where removal cost can be even higher. *C. sorokiniana* was also reported to be applied in the mixotrophic cultivation using with the specific growth rate of 0.052 h^{-1} and total biomass of 11 g L^{-1} (León-Vaz et al., 2019). Similar with the commercial strains that previously developed and adjusted to be applied in the new condition, naturally occur strains or often called native strains of Chlorella are also cultivated for generating biomass. Numerous advanced approaches were also developed for optimization. One of the approach is by N^{+} beam implantation technique on *C. pyrenoidosa* for oil purpose (Tu et al., 2016). this approach resulted an increase of the lipid content from this native strain up to 32.4 %. The approach was also proven to be employed for biodiesel production. Thus, the remarkable reports and developments of this genus has emerged the possibility of this strain for nutrient removal and wastewater treatment system.

2.1.2 Spirulina

Spirulina is a well-known algal genus for its potency as pharmaceutical and cosmetics materials, livestock feeds and food products (Sharma & Sharma, 2017; Zhang et al., 2019). The purposes of food product and livestock feeds require standard of end product with high quality of substrates. The artificial medium with controlled condition is often applied to this genus. Only a few strains from this genus that aimed for nonfood purpose. Bioethanol generation process from *Spirulina* biomass as

substrate was previously reported to obtain promising result (Markou et al., 2013). Biodiesel was also produced through transesterification process using the strains from this genus (Mohamadzadeh Shirazi et al., 2017). High biomass productivity is among the consideration for the application of this alga.

Carbohydrates, lipids, and protein are the main biomass composition of this alga. Most of the applications of this alga focus on the protein as the highest valued components from the algal biomass. Often the utilization of this protein harvesting resulted unutilized lipid and carbohydrates. Parallel utilization of protein, lipid, and carbohydrates, on the other hand, is still feasible (Borowitzka, 1999). It is possible to have biomass utilization of *S. plantesis* simultaneusly with the protein extraction. using proper technique, this combination has been performed previously in protein extraction up to 60.7 % using pH adjustment and biofuel generation of 8 % (Parimi et al., 2015). The residual biomass was reported to possibly recovery to produce up to 8.9 % biofuel from the initial biomass by chloroform-methanol extraction method (Sumprasit et al., 2017). In line with that number, Prates et al. (2018) also reported 12.7% lipid content from the total biomass yielded from *Spirulina* sp. by different technique.

Protein and lipid contents shall possess a high portion with low carbohydrates percentage in the algal biomass in order to address the food production purpose and alternative biodiesel source. Prospective Protein : carbohydrate:lipid ratio (0.47:0.13:0.32) of the biomass was reported to be achieved under modification in nitrogen availability and CO_2 supply (Morais et al., 2018) while another also achieved 46–63 % of protein content (Lupatini et al., 2017). The high amount of lipid did not guarantee high amount of biodiesel product. Transesterification of algal fatty acids only produced 19.8 % weight of biodiesel from the total biomass production of 4.86 g L^{-1} (Mostafa & El-Gendy, 2017). This result depicted the importance of utilized method to result optimum production. Another important value of *Spirulina* is the high amount of phycocyanin content in its biomass (Lupatini et al., 2017). It is also worth noting that this kind of proteins requires to be extracted from *Spirulina* sp using high standard methods (Mourelle et al., 2017). Thus, for application in the wastewater this genus is also potential with minor adjustment.

2.1.3 Scenedesmus

Scenedesmus genus is one of the common genera to apply in the wastewater because it possesses the survival ability in low-nutrient environments. This genus is often found to be dominant in the eutrophic and hypertropic environments (Bellinger & Sigee, 2015). Numerous laboratory and pilot scale projects for coupling wastewater treatments and biodiesel production have used these strains to

demonstrate the possibility of the coupling (Mandal & Mallick, 2011; Xin et al., 2010) . Studies have also demonstrated the durability of this genus in different characteristics of wastewater for bioenergy purpose based on the genus traits to store high amount of lipid and carbohydrates (Choi et al., 2019).

Remarkable nutrient intake is the key for the vast possible applications of this genus, although the optimizations and adjustments are still required for better removal activity. The condition of nutrient initial concentration often creates some obstacles to achieve high removal efficiency. Immobilized microalga study showed that the removal of ammonium by *S. obliquus*. in wastewater was optimum at the initial concentration of 50 mg L^{-1} rather than 70 and 30 mg L^{-1} (Liu et al., 2019), indicating that this genus requires proper conditioning before actual application.

Scenedesmus as the main organisms in the biological wastewater treatment system commonly showed high removal activity. *S. acutus* that cultured in the wastewater produced higher biomass yield than that in the enrich or artificial medium (Alva et al., 2013). As much as 98.5% of total TN and TP with 96.6 % of ammonium removal were reported to achieve by culturing this microalga (Liu et al., 2019; Xin et al., 2010). With the 97% of P removal and 90% of N removal in direct application in domestic wastewater, this genus showed an incredible potency of wastewater treatment (Zhang et al., 2014).

With the long-time development that aimed for the wastewater treatment purpose and considerable amount of lipid in the biomass make *Scenedesmus* the alga that suits for coupling wastewater treatment and biofuel generation. Suitable separation and feasible father processes still need to be improved. In some studies, odor was also reported to cause undesired effluent (Bellinger & Sigee, 2015). Thus, these whole adjustment and issues are still needed to be solved in this algal application.

2.2 Algae-microbes Interaction

Interactions between algae and other microorganisms are commonly grouped into mutualistic and parasitic interactions. Mutualistic interaction between microalgae and other microbes occurs naturally while bacteria or fungi are also sometimes considered as contaminant in algal cultures (Cooper & Smith, 2015). There are several modifications of the interactions between algae and the other microbes in the liquid media. Nonetheless, most of them rely on mutualistic and antagonistic interactions.

The mutualistic mechanisms of algae and other microbes usually occur in one the these three types of enhancements, namely fixation of nitrogen, exchange of the

nutrients, signal transduction and transfer of the gene (Kouzuma & Watanabe, 2015). As nitrogen fixation whereas the bacteria would provide nitrogen for algae as their hosts (Cooper & Smith, 2015). In addition to nitrogen fixation, bacteria are often found to protect the algae from toxic compounds such as heavy metals (Park et al., 2008) as it is knwon that some bacteria can reduce the toxicity of the heavy metals by several mechanisms such as precipitation, adsorption, or transformation (Goecke et al., 2010). The nutrient exchange between algae and bacteria is also counted to be advantageous for these organisms (Kouzuma & Watanabe, 2015). This interaction can be found as the production and intake of macro- and micronutrients such as vitamins by the organisms (Cooper & Smith, 2015). Bacteria provide algae with vitamin B12 and in turn algae would provide exudates and oxygen which essentially important for the bacteria (Fuentes et al., 2016). Cell debris of the algae is also utilized for heterotrophic bacteria (Kouzuma & Watanabe, 2015).

Bacteria are able to modify the forms of nutrients, providing carbon dioxide for the algae as well as rendering essential minerals and growth promoting compounds (Kazamia et al., 2012). The growth factors in the forms of growth promoting components are found mostly to work as phytohormones and biostimulators for the algal cell to elongate and divided (Cole, 1982). Current study also reported that bacterial community in the biofilm formation plays a role on the algal spore germination with further colonization the substrates by the algae. Bacterial strains that forming a biofilm isolated from marine surfaces was utilized by *Ulva* spp. settlement (Patel et al., 2003; Tait et al., 2005)

Apart from the former interaction among the algae and bacteria, antagonistic interaction is also commonly found in both organisms. It has been reported that glucosidases, chitinases, cellulases and other enzymes produced by the bacteria disrupted the algal cells and it leads to the cell lysis (Goecke et al., 2013). This situation provided the algal cytoplasm which is very favorable for the bacteria to utilize. Another form of antagonism is the competiton of substrate utilization where the limited substrate may result lower maximum algal or bacterial amount (Fuentes et al., 2016). Several microalgae were also reported to effectively reduce the bacteria colonization by the inhibiting mechanisms by this competition. Conversely, bacterial biofilm was discovered to negatively affect the algae by penetrating the algal cells (Goecke et al., 2013).

The relationship between algae and the bacteria is not limited only for the mutualism and antagonism. Syntrophic and commensalism between algae and bacteria were also documented previously (Wang et al., 2015; Watanabe et al., 2005). Syntrophic bacteria are the bacteria that only can grow utilizing the excreted

compound or exudates from the microalgae, whereas the commensalism can be interpreted as the relationship between two microorganisms does not involve any detrimental or positive effect in at least one of the organisms. Here, combination of the abiotic factors such as availability of the nutrients and metabolism pathways of both algae and bacteria play important roles to determine the interactions between both organisms.

2.2.1 Microbes influence the biomass of microalgae cultivation

Other microbes that live together with the algae can increase biomass yield during the production process. The enhancement was based on the mediator molecules (i.e. vitamins) that affect the microalgae and bacteria interaction (Fuentes et al., 2016). Previous study reported *Alteromonas* sp. and *Muricausa* sp. enhanced *Dunaliella* sp. biomass yield (Le Chevanton et al., 2013). In this study, nitrogen assimilation was also increased, indicating nitrogen availability for microalgae was affected by the occurrence of related bacteria (Cui et al., 2020; Le Chevanton et al., 2013). *Bacillus licheniformis* was also found to enhance green alga *C. vulgaris* for treating wastewater (Liang et al., 2013). In line with that, Cho et al. (2015) also stated that several bacteria with growth promoting ability in the phycosphere affected the total biomass and lipid productivity of the alga. The other studies found that *Rhizobium* sp. possible to use as inoculum for mass cultures of *Botryococcus braunii*, where it could enhanced 50% of *B. braunii* biomass (Rivas et al., 2010).

2.2.2 Nutrient Removal and Wastewater Treatment

Besides the above explanation, the symbiotic microalgal-microbial process could also be applied for wastewater treatment. Liang et al. (2013) revealed that the system that containing *C. vulgaris* and *Bacillus licheniformis* performed higher removal of NH_4^+ and total P in wastewater treatment. The highest removal was also achieved by algae-bacteria consortium. The consortium removed 97% NH_4^+, P, and DOC of 97, 98, and 26 %, respectively in unsterilized wastewater. In addition the bacteria also play a role on the organic matter dynamic in the wastewater (He et al., 2013). The other studies also observed *C. vulgaris* for bioremediation of municipal wastewater and production of biodiesel simultaneously. The result showed using untreated wastewater the growth of *C. vulgaris* was higher than using sterilized water. This result suggesting that bacteria was one of the wastewater components that could promote the growth of *C. vulgaris* (Ryu et al., 2014).

Another study also utilized the microbes influence on algal biomass production. The alga and bacterium combination were demonstrated through co-immobilized process. *C. vulgaris* and *Pseudomonas putida* was combined to simultaneous remove NH_4^+, P, and OC in wastewater using single-stage reactor (Shen

et al., 2017). Using the same substrate, the non-immobilized and single cultures showed lower growth compared with the combination of the culture. The occurrence of the alga in the immobilized technology was also aimed to add the aeration for the heterotroph bacteria (Praveen & Loh, 2015). Additionally, antibacterial compound from the microalga can also act as the screening system for the advantageous bacteria to reduce the growth of the unnecessary bacteria that can reduce the effectiveness of the culture (Praveen & Loh, 2015).

2.3 Wastewater Characteristic and the Importance to Algal Growth

Wastewater utilization to be the algal substrate for biomass generation is gaining more attention nowadays. It is caused by the fact that undesired wastewater constituent are often the important nutrients for the algal growth. Some contaminants in the wastewater, however, also act as the inhibitory factors for the algal growth. Nitrogen and phosphorous that contained in the wastewater can be found in wide range of concentrations and forms. Differently, chemical oxygen demand (COD) can only be found in the low or moderate concentrations (Hamza et al., 2019). These mentioned contaminants are classified as the wastewater content that may be utilized for algal growth. Nevertheless, some other contaminants such as metals specifically heavy metals, can be harmful for the algae and considered as toxic substances. Here, the utilization of wastewater contaminants by the algae also needs to carefully elaborate the operational factors namely light intensity and availability of carbon dioxide (CO_2) (Figure 2.1).

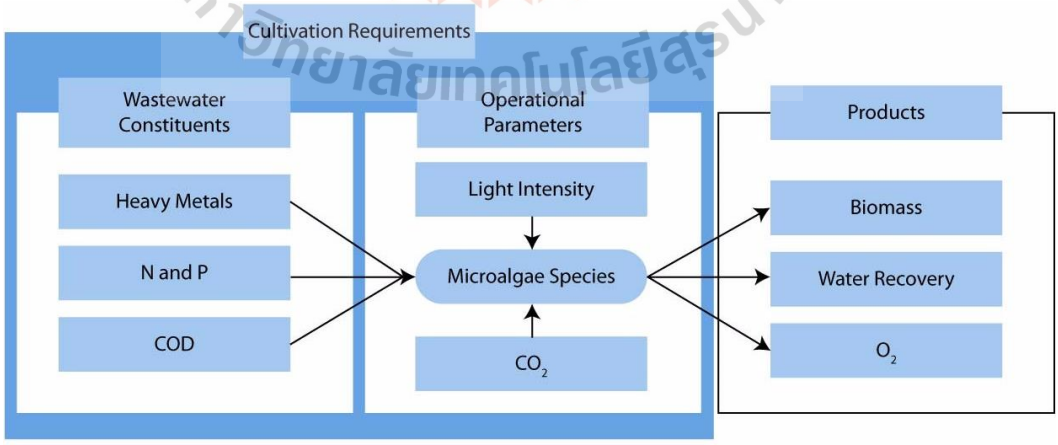

Figure 2.1 Wastewater constituents that affect the algal growth with the operational factors for biomass production and water recovery process.

2.3.1 Chemical Oxygen Demand

Chemical oxygen demand (COD) consists not only easily degradable substrate containing the organic carbon chains but also the hardly degradable substances. Few studies examined the algal based treatment and found that the algal-based treatment often removed COD for only less than half of influent concentration (Huang et al., 2015). Aside from the hardly degradable materials counted in COD parameter, the fact that the alga are naturally autotrophic organisms leads to consume carbon dioxide in higher portion than the organic carbon source that available in the medium (Barsanti & Gualtieri, 2014). On the other hand, organic matters in the medium at the moderate or low concentration was found to provide suitable condition to the algae to produce higher algal biomass than that in the pure autotroph condition (Huang et al., 2015).

Table 2.2 Removal of chemical oxygen demand with the biomass production in several wastewaters by several microalgae strains.

Microalgae	Wastewater	COD (mg L^{-1})	removal (%)	Biomass (g L^{-1})	Sources
Scenedesmus sp.	Vinasse	27,100	36.2	4	(de Mattos & Bastos, 2016)
Chlorella vulgaris	Municipal wastewater	61.5	41	5.1[1]	(Lee et al., 2016)
Selenastrum gracile			53	4.3[1]	
Scenedesmus quadricauda			63	0.5[1]	
Indigenous algal population			45	1[1]	
Scenedesmus obliquus	Piggery wastewater	3200	65.06	1.68	(Xu et al., 2015)
		2200	73.41	1.84	
		1600	75.29	2.18	
		1200	72.29	1.58	
		800	63.02	1.20	
		400	61.58	0.87	
C. vulgaris	municipal wastewater	490	96	2.20	(Mujtaba et al., 2018)

Table 2.2 Removal of chemical oxygen demand with the biomass production in several wastewaters by several microalgae strains. (Continued)

Microalgae	Wastewater	COD ($mg\ L^{-1}$)	removal (%)	Biomass ($g\ L^{-1}$)	Sources
C. vulgaris	Aquaculture wastewater	8.5	-	0.07	(Gao et al., 2016)
S. obliquus			-	0.06	
Chlorella kessleri	wastewater from the WWTP	70	-	2.70	(Caporgno et al., 2015)
Chlorella vulgaris	centrate from the WWTP			2.91	
Microspora willeana and minor portion	Dairy manure	149	>99	4.98[2]	(Mulbry & Wilkie, 2001)
		79.7	>99	5.00[2]	
		128	>99	4.99[2]	
Chlorella sp.	Polluted water	1,200	51	-	(Safonova et al., 2004)

[1] mg L^{-1} of chlorophyll a content. [2] g day^{-1} biomass generated from 1 m^3 pond.

Xu et al. (2015) reported the actual COD concentration in wastewater were often not the optimum concentration for the algae to reach their optimum concentration. The absence or minor COD, reciprocally, also Adversely affects the alga, resulting the final concentration of the algal to be half of the highest concentration in the adjusted COD level. High production of algae biomass was relatively found in the less than 1000 mg L^{-1} COD content (Table 2.2) which proves that the inhibition factor of COD is significant for algae biomass.

2.3.2 Nutrients

Levels of nitrogen and phosphorous in the wastewater that discharged into the environments are often potential to cause the eutrophication in the natural water body. The proper treatment for these constituents is thus essential to be properly treated. Phycoremediation for these nutrients has been developed for these recent years (Wang et al., 2014a). Algal application in this specific purpose is also supported by the potency of energy crisis mitigation. Occurrence and structure of the nutrients here play a crucial role as the initial concentration somehow affects the total biomass that generated after the treatments (Table 2.3).

Nitrogen is known to be an important building-blocks of amino acids and proteins, chlorophylls, energy storage molecules, and genetic substances in cells where all the metabolism activities involves these molecules (Sniffen et al., 2018). In the natural biomes, algae are found to have significant effect to convert inorganic substances to be organic substances (Liu et al., 2019). Availability of the nitrogen in the substrate essentially affects growth of the algae as this element is an irreplicable substance algal anabolism process. A report of nitrogen concentration level showed that 9.61×10^{-4} M of nitrogen in waterbody rendered the highest biomass that potentially produced (Fried et al., 2003). Apart from the concentration, availability of nitrogen in several molecules also determines the algal growth. *D. tertiolecta* was revealed to nitrate rather than ammonia as the preferable nitrogen form (Chen et al., 2011b). It is also essential to note that nitrogen and phosphorous may possess different limitation for the occurrence and related effects to the algae. Scarcity of nitrogen in growth medium was found to still suitable for the algae to survive and grow even in the much slower rate (Rhee, 1978). Differently, P showed no dramatical decrease on algal differentiation and developments (Chen et al., 2011b).

Phosphate, as it is mentioned earlier, is also a crucial part in algal metabolism because it is needed to synthesize numerous macromolecules, namely sugars, lipids, proteins, and nucleic acid. Furthermore, inorganic phosphate is often irreplaceable for those mentioned macromolecules. Martínez et al. (1999) showed that $H_2PO_4^-$ and HPO_4^{2-} are the most suitable P available for the algae to intake. It was also revealed that these forms are essential nutrients for phosphorylation pathway in order to build the organic compound. Cell molecules that incorporated with the energy generation namely, ATP and ADP require phosphate group in their molecules. Dissimilation of these groups results the energy production for the cells. The process of oxidation of these molecules the photosynthesis activity, on the other hand, releases the phosphate through plasma membrane. These activities result the flow of intake and release of the phosphate are very complex. Nonetheless, even though the posphate can be released and incorporated only for a very low level compared with the nitrogen, algal can still adsorb and release phosphate in organic and inorganic forms. Few algal strains, however, are reported to assimilate organic phosphate in the form of esters groups rather than the inorganic forms for building the biomass (Kuenzler, 1965).

Nitrogen occurrence (often expressed as total nitrogen, TN) can be found in vast range. Extremely high concentration of TN potentially decreases the yield of alga and insufficient TN level may result the lower productivity (Wang et al., 2014a). The susceptibility of the changes of the nutrient concentration thus becomes one of

the important factors for the algae. Algae with high consumption rate be beneficial for the wastewater treatment purpose. *Desmodesmus* sp. was reported to be able to generate 4 mg L^{-1} yield after the cultivation in wastewater containing TN up to 1,420 mg L^{-1} (de Mattos & Bastos, 2016). *Chlorella* sp. generated 34.6 mg L^{-1} chlorophyll, depicting high biomass in the high phosphate flowrate of 392 g DW $m^{-2}day^{-1}$ (Min et al., 2011). As consequences, Nutrient removals of those studies resulted only 70 %, indicating the low removal efficiency over the high concentration of nutrient inflow.

States of trophic that often called the carbon source preferences of the algae are also important for the holistic removal activity. Mixotrophic condition was reported numerous times to have higher final biomass and efficient removal of the nutrient (Choi et al., 2019; Liu et al., 2019; Mandal & Mallick, 2009). Here, trophic conditions possess higher difficulties for the wastewater treatment since it has to act as heterotroph and autotroph at the same time. Uneven level of one of the nutrients (carbon, nitrogen, and phosphate) can affect the removal activity of the whole process of removal and biomass yield (Figure 2.2). Among the problem that faced by the unbalance level of wastewater nutrients for sufficient removal. The carbon source was often found in the lower concentration that the requirements and thus the additional carbon source may be needed to sufficiently remove the other nutrients. This additional carbon source can add considerable cost into the system. Here, proper construction of system of mixotroph is needed to avoid the fall on the feasibility study. Mixotrophic with the flexible concentrations can answer this problem. With the great niche in the removal and biomass production, especially in the pilot and industrial scale, it is expected that the removal may work simultaneously by combining autotroph and heterotroph activities of the constructed biological agents, i.e., microalgae inoculants.

Table 2.3 Nitrogen and phosphorus effects in the generation of the algal biomass cultivated in the several wastewaters.

Algae Species	Wastewater	Total N (mg L^{-1})	Total P (mg L^{-1})	Biomass (g L^{-1})	Sources
Desmodesmuss sp.	Vinasse	1,420	2.61	4	(de Mattos & Bastos, 2016)

Table 2.3 Nitrogen and phosphorus effects in the generation of the algal biomass cultivated in the several wastewaters. (Continued)

Algae Species	Wastewater	Total N (mg L^{-1})	Total P (mg L^{-1})	Biomass (g L^{-1})	Sources
C. vulgaris	Municipal wastewater	32.2	3.90	5.1[1]	(Lee et al., 2016)
Selenastrum gracile				4.3[1]	
S. quadricauda				0.5[1]	
Indigenous algal population				1[1]	
S. obliquus	Piggery wastewater	120.69	129.22	241.67	(Xu et al., 2015)
C. vulgaris	Municipal wastewater	50	10	2.2	(Mujtaba et al., 2018)
C. vulgaris	Aquaculture wastewater	6.81	0.42	0.07	(Gao et al., 2016)
S. obliquus				0.06	
C. kessleri	WWTP effluent	140	5.76	2.70	(Caporgno et al., 2015)
C. vulgaris				2.91	
Auxenochlorella protothecoides	Concentrated municipal wastewater	134	212	1.16	(Zhou et al., 2012c)
Algal consortium	Domestic wastewater	50.0	50.0	0.43	(Park & Craggs, 2011)
Lygnbya sp. and *Spirogyra* sp.	River water	1.29	0.23	16.3[2]	(Mulbry et al., 2010)
		1.03	0.14	3.6[2]	
		1.05	0.11	3.8[2]	
Chlorella sp.	Centrate effluents	275	392	34.6[2]	(Min et al., 2011)
Chlorella sp.	digested manure	200	2.5	6.83[2]	(Chen et al., 2012)

[1] indicates the unit chlorophyll a content in mg L^{-1} of. [2] indicates unit of g DW $m^{-2}day^{-1}$

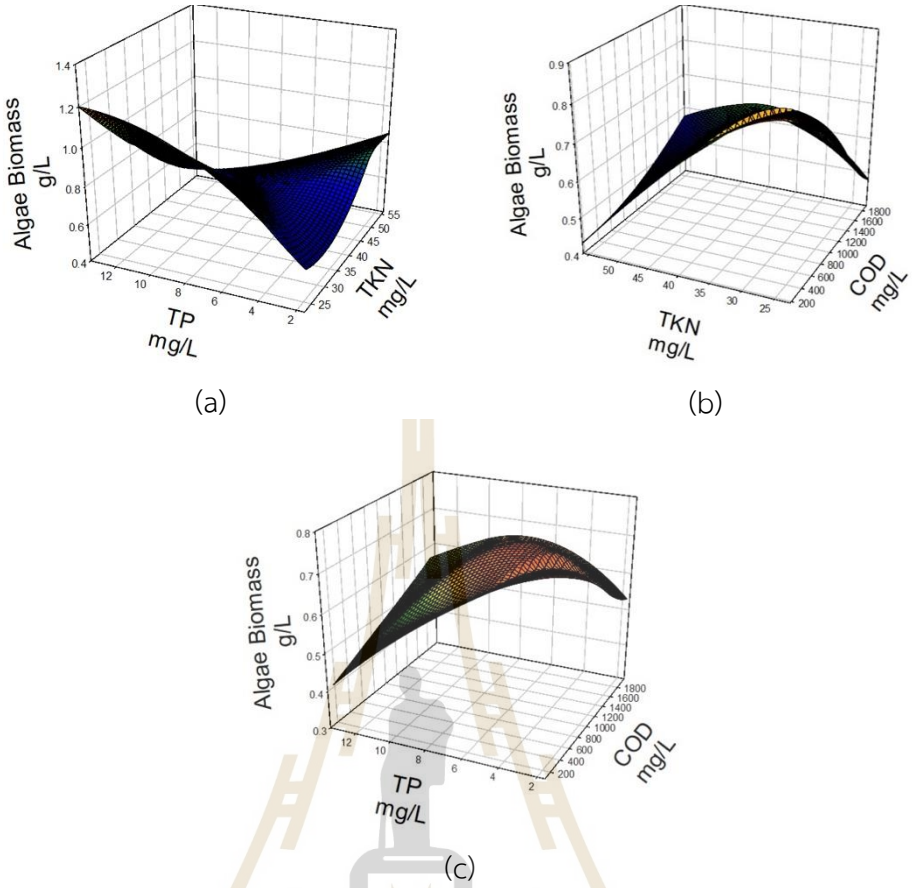

Figure 2.2 Relationship between three main nutrients for algae growth.
(**a**) relationship of TP and TKN combination for algae biomass;
(**b**) relationship of TKN and COD combination for algae biomass;
(**c**) relationship of TP and COD combination for algae biomass.
Data were generated analyzed from Table 2.3.

Combination of both COD and nutrients are important to support algae growth. However, it is rare to obtain all the parameters contained in the single wastewater source. Municipal wastewater usually contains moderate concentration of three parameters (Bux, 2013) where it needs less COD and high amount of phosphorous and nitrogen (Figure 2.2). On the other hand, decrease of COD detected in the less nutrients. Thus, proper selection of algae strain from wild type is among the important factors to generate optimum biomass (Hopkins et al., 2019). Another prospect is to obtain the algae with modified engineering.

2.3.3 Metals

Metal constituents in the micro concentration are basically essential for the algal metabolism. The metals such as copper, iron, zinc and manganese are identified as important enzymes' cofactors in the chloroplast and mitochondria (Hanikenne et al., 2005). Although the metals are important for these specific purposes the excess amount can cause detrimental effects for the algae. The occurrence of these elements in the medium can be the source of low productivity or even decrease total biomass for the microalgae. On the other hand, there are numerous transporter protein binding in the algal membrane which facilitate the adsorption of such elements. Adsorbed elements will be further accumulated in the cells or converted into less toxic and harmful conditions. Among the mechanisms of intoxicating activities microalgae, oxidation state change to less reactive elements may take place as the most useful mechanism (Monteiro et al., 2012) (Figure 2.3).

Metal and more importantly the heavy metals removals in the wastewater mostly employ the immobilized microalgae technology. Only several other treatments use the alga in the free-living cells (Kumar et al., 2015b). Copper, zinc, lead, cadmium, and manganese are often studied in the bench and laboratory scales for the algal based treatments system (Table 2.4). Several microalgae, namely *Cladophora glomerata*, *Oedogonium westii*, *Vaucheria debaryana* and *Zygnema insigne* were once studied and found to accumulate number of metals in industrial wastewater such as cadmium, chromium, lead and nickel with removal of 80.3, 63.3, 92.1, and 93.0 % by *C. glomerata*, *O. westii*, *V. debaryana* and *Z. insigne* (Khan et al., 2017). Coper removal of 85% was found employing batch system of algal beads forming from sodium alginate using *Spirulina* sp. biomass (Prathima et al., 2017). These abilities of removal depend on the structure and form of the heavy metals (Shamshad et al., 2014). Among the studied metals, it has been stated that chromium was found to be pertinent for algal-based removal and accumulation (Ahmad et al., 2017; Jin et al., 2017). Electrical conductivity reductions of this accumulant causes this pertinence and it is affected by the BOD, COD, TDS, and nitrate of the wastewater. The removal also strongly related with the saturation of oxygen in the medium.

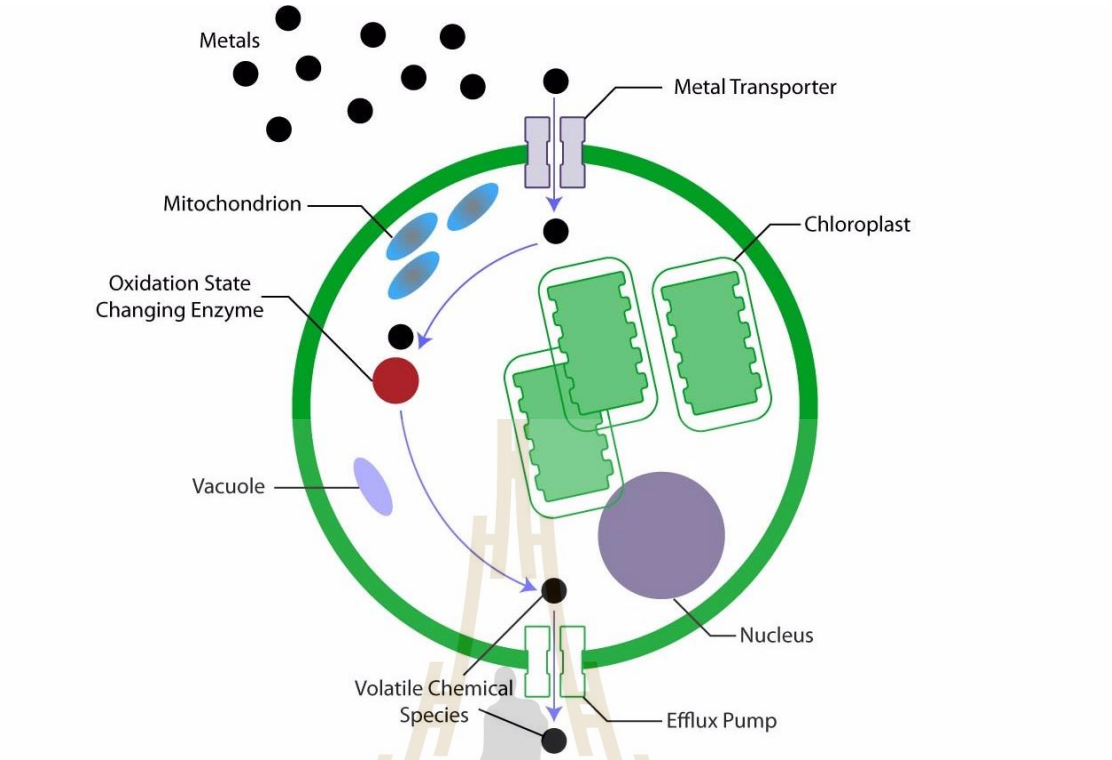

Figure 2.3 Fate of metals in the algae cell.

Additional effects of the metals including heavy metals resulted the additive effects for *Chlorella sp.* whereas other combination of algae and their treated metals can be found in the synergistic interaction (Mo et al., 2017). Zinc and copper decreased the cell abundance of *Chlorella* sp. with the former one eliminated the alga growth after 5-day incubation and the latter decreased the pigment of the alga (Kondzior & Butarewicz, 2018). Thus, it is noteworthy that the removal mechanisms can only work under proper contamination level and with the very limited number of algal strains.

Table 2.4 Accumulation of metals and heavy metals by microalgae.

Strains	Heavy metals	Accumulation in the biomass (mg g^{-1})	Occurrence in the initial stage (ppm)	removal (%)	Sources
Chlorella spp.	Cu	15×10^{-3}	79	-	(Krishnamurti et al., 2015)
	Zn	1×10^{-3}	23	-	
	Pb	2×10^{-3}	10	-	

Table 2.4 Accumulation of metals and heavy metals by microalgae. (Continued)

Strains	Heavy metals	Accumulation in the biomass (mg g^{-1})	Occurrence in the initial stage (ppm)	removal (%)	Sources
C. minutissima	Zn	9.17	2[1]	62.05	(Yang et al., 2015)
	Mn	4.04	2[1]	83.68	
	Cd	4.27	2[1]	74.34	
	Cu	1.40	2[1]	83.60	
Alginate-*Spirulina*[2]	Pb(II)	12.9	100	-	(Kőnig-Péter et al., 2016)
	Cd(II)	4.5	100	-	
	Cu(II)	4.1	100	-	
Chitosan-*Spirulina*[2]	Pb(II)	4.8	100	-	
	Cd(II)	3.6	100	-	
	Cu(II)	2.7	100	-	
Arthrospira	Ce	18.1	80–800	-	(Sadovsky et al., 2016)
		38.2		-	
S. platensis	Pb	-	100	91	(Al-Homaidan et al., 2016)

[1] concentration in mM. [2] immobilized state of algae.

Heavy metal removal by the algae is affected by the affinity and specific surface area of the algal culture. These factors can further diverse the mechanisms in every algal that previously described. The morphological occurrence, cell wall constituents and structure as well as the composition of cell membrane may play key factors for each algal ability (He & Chen, 2014). To sum up, before the mechanisms of metal valency reduction or other specific mechanisms in the cytoplasm, binding cites, and other transporter proteins play key aspects in the early stage.

2.3.4 Other abiotic factors

2.3.4.1 Light

Light utilization by microalgae is one of the most important factors to generate biomass yield as intensity, duration, and portion are able to activate or deactivate certain metabolism pathways by which yield of biomass is critically determined (Chang et al., 2011). Bux (2013) explained that photosynthesis of the algae was affected mostly by the light intensity. Activity of the photosynthesis is in line with the degree of the intensity. After the saturation level of light intensity was exceeded,

number of photons in photosystem II of the algal chloroplast will not increase and the rate of photosynthesis will be similar with no additional enhancement. Light intensity will be adversely affecting the photosystem by rendering damage to the photosynthetic apparatus.

The intensity of the light is also related to the light penetration which also affect algal cells. Previously, it has been reported that *C. sorokiniana* cultivated under high density showed lower gross productivity than those with less density (Holdmann et al., 2018). It was related to the amount of the light that can penetrate the suspension of the alga. Extremely dense suspension can reflect the light and avoid complete penetration to provide sufficient light intensity for the photosynthesis process. *C. vulgaris* was reported to be exposed with various light intensity and showed the favorable intensity of 360 μmol m^{-2} s^{-1} of photon for algal biomass generation as the lower or higher intensities resulted lower biomass (Sun et al., 2018). On the other hand, Mondal et al. (2017) found that the light intensity that exceeded 150 μmol m^{-2} s^{-1} tended to inhibit algal growth whilst 80 μmol m^{-2} s^{-1} was reported to be sufficient to give *C. vulgaris* condition for the highest yield production. Low photon availability often activated the mixotrophic culture. It created the switch of the trophic condition from the complete autotroph to be facultative autotroph. Although, heterotrophic condition may result higher biomass than the autotroph or mixotroph (Kim et al., 2013), for the mixotrophic cultures, low light intensity could activate the heterotroph trait of the alga in the maximum portion of metabolisms. Additionally, different application technology may require different light intensity. Huang et al. (2016) revealed that the immobilized algae, the light intensity of 120 μmol m^{-2} s^{-1} was proper to produce optimum algal biomass concentration.

Here, the optimum intensity also depends on the cultivated strains. *C. protothecoides* was reported to preferably growth under the 420 μmol photons m^{-2} s^{-1} while *A. platensis* requires slightly moderate light intensity of 100 μmol m^{-2} s^{-1} (da Silva et al., 2016). Thus, technology for the cultivation or algal application in the medium with the wastewater and the strains that selected for that application are important to determine the total light intensity for optimum growth. Eventually this parameter was important to determine operational and capital costs for industrializing algal biomass utilization from wastewater.

2.3.4.2 Carbon Dioxide

Carbon dioxide (CO_2) level that soluble in the water is strongly related to the total yield that may be obtained in the cultivation condition, since it is an irreplaceable molecules that demanded during the photosynthesis and most of the

green algae are naturally photoautotrophic with slight mixotroph traits potency when it faces the abundant organic compound or scarce of light (Singh & Singh, 2014). It is also important to note that the carbon sequestration of microalgae is ten times higher than that the ability of terrestrial plant (Wang et al., 2011). This fact supports the algal cultivation for not only the purpose of removal nutrient in the wastewater but also mitigate the CO_2 emission in the atmosphere. Microalgae thus are projected to simultaneously remove the nutrient removal, generate biomass, but also act as the sequester agent for mitigate the CO_2 emission (Aslam et al., 2019).

System conformation of the cultivation may determine the availability of the CO_2 concentration in the solutions. Through diffusion process CO_2 is provided in the soluble phase, by which the alga can assimilate it. Gas dispersion and air bubble size thus contribute significantly to CO_2 dilution factors. The gas that infused to the medium has two critical factors which are type of diffuser and gas flow rate. Several diffusers, namely sintered stone, porous curtain, perforated ring, and porous wood diffusers were employed to provide sufficient gas including CO_2 and resulted different *Spirulina* biomass concentration with similar available CO_2 concentration of 0.05 vvm (Moraes et al., 2016). Interestingly, in the same study it was also found that the higher concentration of CO_2 that injected in the form of gas potentially lowered the uptake efficiency.

Flue gas has been correlated to this advantages of algal cultivation since some of the industries that produced wastewater aimed for algal based treatment also emitted the CO_2 during their operations. Among the studies, an examination on *Spirulina* sp. achieved 24% adsorption of the CO_2 in the flue gas using autotrophic condition (Costa et al., 2015). In the same study, an increase for up to 35% of the growth rate has been achieved as the implication of CO_2 supply. However, the low carbon that assimilated during the process was still an important issue to be addressed. The total carbon fixation was only 7.5 % from the total carbon emitted to the culture.

Wang et al. (2019) was reported to sparge 1 L min^{-1} (vvm) of CO_2 as the flue gas into *Spirulina* sp. culture in the batch tank cultivation. The content of CO_2 has been reduced for 75 % from the initial concentration. The remaining 25 % may still be doubtful to be accepted in the environmental threshold. Nevertheless, the vast removal of CO_2 indicates the possibility of this concept to widely apply as the main method to remove CO_2. With the similar CO_2 concentration, Uggetti et al. (2018) could achieve 66-100 % additional biomass yield and increase removal of COD and NH_4^+-N. The increase of CO_2 thus became advantage for the treatment and biomass

generation. It also proved that the level of CO_2 tolerance may not be exceeded. Bicarbonate ion in the suspension can be a very critical parameter here. The excess CO_2 generate high bicarbonate ion that available in the suspension and thus pH will be very acidic for the algae to growth (Wang et al., 2011). Increase of bicarbonate ion concentration, on the other hand, was confirmed to be not related with the potential biomass yield (Mokashi et al., 2016).

The additional carbon dioxide gas was also once combined with the heavy metal remediation. The removal of CO_2 and heavy metals was achieved simultaneously with emphasizing of 30 % metal removal (Aslam et al., 2019). Here, numerous advantages of the factors determining the removal rate and biomass production have been described. Heavy metal removal and carbon sequestration may add additional values to the coupling wastewater treatment with biomass generation. Nevertheless, the flue gas as the source of CO_2 is still need sequence of experiments before it is ready for real scale application.

2.4 Constrain Between High Biomass Productivity and Removal Efficiency

Numerous algal strains were reported to cultivate in various substrates aiming biomass, removal of nutrients and other specific purposes. Wastewater, nowadays, has been gaining more attention as the substrate for this cultivation. Daily manure, municipal, chicken, piggery and brewery wastewaters are among the tested substrate from the waste sources (Zheng et al., 2018, Wang et al., 2010, Alva et al., 2013, Kumsiri et al., 2018). Wastewater utilization was economically advantageous than most of the synthetic media for the algal cultivation. As the main objectives of the cultivation apart from the recovery of water and removal of nutrients, lipid content and other biomass constituents are critically important to be enhanced in the limited media culture. Numerous attempts to enhance this yield based on the substrate modification have been demonstrated such as, varying the salinity (Ruangsomboon et al., 2013), nutrient starvation (Ruangsomboon et al., 2013; Yeesang & Cheirsilp, 2011) and the addition of metal as a trace elements in the medium (Ruangsomboon, 2012; Ruangsomboon et al., 2013; Rugnini et al., 2018; Yeesang & Cheirsilp, 2011). As previously mentioned, the light also needs to properly penetrate medium including the wastewater with the low intensity (Yeesang & Cheirsilp, 2011). Thus, combination of proper nutrient content, conditioning of the media, and the availability of the light are important to scrutinize in this coupling concept.

Several attempts to couple this removal and biomass generation have been demonstrated with various results. It is previously discussed that Spirulina is not a

common genus to be cultivated for this particular purpose. However, an examination of this alga resulted total biomass of 1.47 g L^{-1}after intensive cultivation in the cow effluent (Çelekli et al., 2016). Apart from their dense biomass at the end of cultivation, removal efficiency of more than 90% has been achieved of this unusual alga in TP and COD levels in high salt condition (Zhou et al., 2017). TN and TP levels were also reported to remove for 92.58 and 94.13%, respectively from their initial concentration with the algal biomass generated as much as 262.50 mg L^{-1} in the similar condition with the former study (Zhai et al., 2017). However, some issues are still needed to solve. For the small size algae like Spirulina, separation process would be considerably more difficult than the larger or aggregating algae. Considering the advantages offered by this genus, such as high biomass accumulation, the application for animal feeding may still feasible for several wastewater sources as the content of important feeds is completed in this algal biomass (Zhang et al., 2019).

It is also important to carefully consider the percentage of removal that the algal cultivation achieved. Fails in the removal can cause additional process that requires even higher cost than the primary or secondary treatments. A study has previously reported ammonia, TN, TP, and COD removals were relatively high in therms of the percentage yet the effluent of the system was still not sufficient for direct discharge into the reservoir (Wang et al., 2010). Available solutions for this problem may come from the adjusting the initial concentration of the wastewater. High dilution factor can reduce the initial amount of the nutrient and the cultivated algae can effectively remove the nutrient in the solutions. ratio of organic and inorganic carbon may play important roles to provide the suitable condition for trophic conditions that preferable for the algae, as this may result significant amount of biomass change and eventually affect the removal of the essential nutrients (Roostaei et al., 2018).

Another current development for this coupling is by installing the cultivation reactor next to the primary treatment. As it was known that primary treatment aims to remove the suspended solids and relatively large molecules whereas the secondary treatments that often installed are biological based treatment. By installing these treatments in sequence has been tested in the bench and laboratory scale. Anaerobic digestion effluent was directly treated in algal cultivation with promising nutrient removal and biomass generation (Wang et al., 2010). Nonetheless, it is also essential to consider the anaerobic digestion may not remove the COD sufficiently and as a consequence, inflow for the algal cultivation may contain high COD. This situation can create the reduction of biomass as previously described in COD parts.

Algal based treatment also depends on the occurrences of the micro contamination of heavy metals or highly toxic elements for animals and humans. It

has been discussed prior that these components may still be needed for enhancing the biomass production. However, the improper amount of these elements in the influent can only cause banned for the application in animal feeding. Thus, to utilize the algal biomass that generated in this system, influent adjustment and strict control must be conducted to aim this application of harvested biomass. The study of food additives wastewater was reported to have remarkable result in *Spirulina* sp. cultivation by producing biomass with 25% protein and 50% lipid portions (Jiang et al., 2015). Interestingly, the application of the food industry wastewater in this study can provide sufficient nutrient and at the same time it is also claimed to be free from the metal contaminants.

Substrate utilization and the strains application may play an important role in the successful process of cultivation. Nonetheless, period and intensity of light exposure are still important apart from the described factor that prior scrutinized. Reactors have been designed to cope the demand of sufficient supply of aeration and light. Some of the reactors employ additional light with continuous while the others focus on the periodic exposure. Conversely, open pond cultivations which rely on the natural sunlight exposure are susceptible for insufficient sunlight in some period of the year. For the tropical area, period of the sunlight exposure is commonly stable and with slight reduction during the rainfall seasons. Nonetheless, in subtropical area this period may change during the spring/fall as well as the midsummer. This change can create detrimental effect in the total production and removal efficiency. It is thus important to consider geographical factor such as sunlight in thew sunlight feeding system, capital and operational cost. Nonetheless, the acclimatization process of algae is able to address the light factors drawback. The ability for modulation of light harvesting capacity in order to optimizing light suggests the production of algae can be managed in low light conditions (Bux, 2013). However, growth rate and productivity of algae are strictly related to light factor and alteration, or limitation of light intensity affects the algae yield.

Apart from water treatment to remove undesired substances point of view, the production of high biomass yielded from the system shall be focused. Taking *Chlorella* sp. as the most cultivated microalgae for biodiesel, the percentage of the lipid content was relatively wide which around 13.2-60 % of the total biomass (Table 2.1). The utilization of this alga should be considered. This range of percentage is necessary to be noticed for further decision of algae cultivation. It is important to note that wide range of this percentage can create gap in the production system and capital cost calculation for the system to be built. To each high biomass of this alga, it is also necessary to create the condition based on the wastewater characteristics. Optimum

biomass concentration up to 11 g L^{-1} is still possible to reach by using wastewater alongside with effective removal of undesired substances (León-Vaz et al., 2019). To obtain such concentration, proper water recovery, sufficient but not excess concentration of N and P solely for building biomass and other metabolism activities, mixotrophic condition for algae to digest organic carbon, and heavy metals defense mechanisms are expected to address both importance (Figure 2.4).

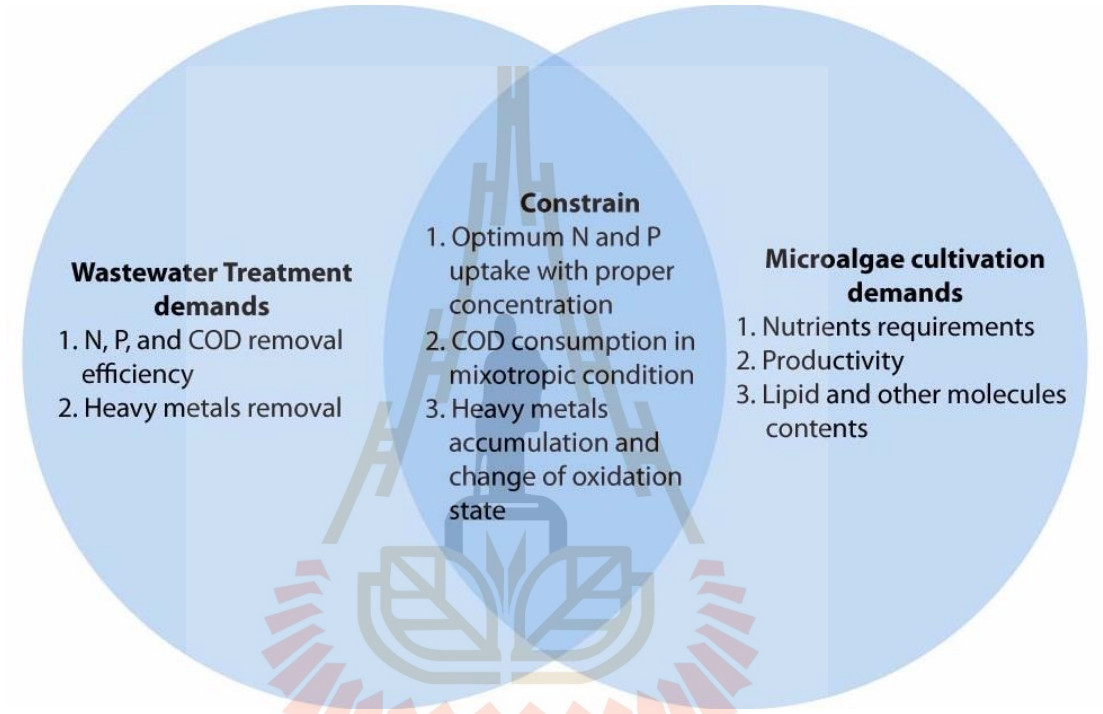

Figure 2.4 Constrain between wastewater treatment demands and microalgae cultivation demands.

2.5 Cultivation and harvesting methods for generating algae biomass

2.5.1 Cultivation Condition

Cultivation condition plays an important role in the production of biomass and the state of the cell. Here the production of biomass and related studies are summarized to obtain several cultivation condition descriptions based on previous studies.

2.5.1.1 Phototrophic Condition

Most of microalgae are photoautotrophic, they can produce energy by using renewable and inexpensive resources as sunlight, inorganic salts, water

and CO_2 (Caprio et al., 2016). Phototrophic microalgae consuming carbon dioxide to produce oxygen (Hajar et al., 2017). Besides, microalgae can store high amounts of neutral lipids (bio-oil), carbohydrate, carotenoids (such as lutein, astaxanthin, β-carotene), proteins and other molecules (Caprio et al., 2016).

Some of microalgae species showed a higher growth rate under phototrophic cultivation such as *Chlorella* sp. and *Scenedesmus* sp (Visca et al., 2017). In addition, microalgae which cultivated by phototrophic could consume 9-35% of Mn, S, Fe, N, Mg and less than 5 % of P, Mo, Co, B, Zn, and Ca (Bohutskyi et al., 2014).

2.5.1.2 Heterotrophic condition

Heterotrophic cultivation is a term used when microalgae use organic carbon for both energy and carbon source (Chojnacka & Marquez-Rocha, 2004). The advantage of heterotrophic algae culture are fast growth, high production rate, and convenient harvesting. In addition, heterotrophic mode of cultivation of microalgae had shown better *biomass* and metabolites productivities than the phototrophic mode. Morales reported that heterotrophic conditions can enhance the biomass concentration of microalgae by as much as 25-fold compared with phototrophic conditions. Nutrient supplementation strategy improves the biomass growth as well as lipid, carbohydrate and protein productivities (Guldhe et al., 2017).

A series of heterotrophic microalgae species were successfully used in industry-scale polyunsaturated fatty acids production. Heterotrophic culture can be used as a better process to produce seed cells for the alga *Chlorella sorokiniana* in large scale open systems, since it had much higher productivity but similar performance compared with its phototrophic counterpart (Zheng et al., 2012). Recently, heterotrophic microalgae culture to produce biodiesel was reported and showed its promise, however, a high cost of organic carbon is one of limiting factors for this process (Chi et al., 2011).

Heterotrophic cultivation on aquaculture wastewater, organic waste as well as municipal wastewater could act as a nutrient substrate for cultivation of microalgae (Zheng et al., 2012). Moreover, microalgal biomass generated using aqua culture wastewater shown high lipid, carbohydrate and protein yields which can be used for biofuels and feed application. This biorefinery concept forms the basis for sustainable and economic integration of aquaculture and microalgae industry (Guldhe et al., 2017).

2.5.1.3 Mixotrophic Cultivation

Mixotrophic growth was preferable for some microalgae. Mixotrophy means growth whereby organic carbon is simultaneously assimilated with

light and CO_2. Much work has been done on the mixotrophic growth of the green algae *Chlamydomonas reinhardtii, Chlorella, and Haematococcus pluvialis* (Liu et al., 2009). The energy yields of mixotrophic cultures were 4–6 times higher than those from photoautotrophic cultures. One of the reason of a better microalgae growth because it combine the advantages of autotrophic and heterotrophic and overcome the disadvantage (Zheng et al., 2012).

Chlorella is one of the species of microalgae which was reported that could obtained higher lipid production under mixotrophic cultivation(Yeh & Chang, 2012). The other species is *Isochrysis galbana* could grow successfully in mixotrophic culture than in heterotrophy. The optimal glycerol concentration to support the mixotrophic growth of *I. galbana* was 50 mmol glycerol (Alkhamis & Qin, 2013).

2.5.1.4 Photoheterotrophic Cultivation

Organisms that use light for energy but cannot use CO_2 as their carbon source called photoheterotrophs. As the result, they use organic compounds from the environment as carbon source which are carbohydrates and fatty acids. *Chlorella vulgaris* ESP-31 which was grown under photoheterotrophic cultivation with glucose as carbon source showed a highest biomass and lipid content 3.5 g L^{-1} and 26 % respectively than other carbon sources (Yeh et al., 2012).

Ettlia texensis has also been reported to grow photoheterotrophically with glucose and yeast extract. In the optimized medium, the highest biomass productivity and total lipid content achieved were 0.97 g L^{-1} d and 26% of dry weight basis, respectively (Isleten-Hosoglu et al., 2013). Moreover, two green marine microalgae *Tetraselmis gracilis* and *Platymonas convolutae* could reach and elevated levels of biomass, lipids, and fatty acids as well as polyunsaturated fatty acids (PUFAs) under photoheterotrophic growth condition (Selvakumar & Umadevi, 2014).

2.5.2 Cultivation Process of the Algae

Microalgae cultivation involves many methods to obtain provide suitable environment for algae to grow in order to produce high biomass. The biomass can be important as the main objective apart from the recovery of water or other specific purposes where biomass can be a second option or only be the by product. For optimum production, microalgae should be well cultivate using proper production reactors. Some reactors have more than one supply of nutrient and controlled system whereas the other are supported by natural supply of light and stirrer. Followings are the system usually used to cultivate algae.

Microalgae have several cultivation condition regarding the source of energy and carbon. Both are important factors for synthesizing the biomass and other metabolism activities in the system. Phototrophic, heterotrophic, mixotrophic, and

photoheterotrophic are the conditions of algae to grow based on algae carbon and energy source (Table 2.5) (Chen et al., 2011a). The cultivation condition is important to understand for better development of the cultivation reactors. Cultivation of algae using wastewater needs this consideration since the source of energy and carbon shall be provided in optimum condition with consideration of cost production.

Wastewater constituents are ranging from algae from the nutrients such as nitrogen and phosphorus to organic content in terms of chemical oxygen demand (COD) and Biological oxygen demand (BOD). These are the parameters which indicating the state of the wastewater (Dionisi, 2017). Coupling the cultivation of algae with the wastewater treatment system must concern on the removal of these constituents in the waters. Among the cultivation conditions, mixotrophic condition is able to accommodate both importance (algae cultivation and wastewater treatment) (Ghosh et al., 2016).

Table 2.5 Microalgae cultivation conditions based on energy and carbon sources.

Condition	Energy source	Carbon source	Possible species
Phototrophic	Light	CO_2	Green and Blue green algae
Heterotrophic	Organic	Organic	Diatoms, Dinoflagellate
Mixotrophic	Light and organic	CO_2 and organic	Green algae, Blue Green algae
Photoheterotrophic	Light	Organic	Blue green algae

2.5.2.1 Open Ponds

Open pond system refers to a pond in open area with direct sunlight harvesting as the main source of light using natural or additional agitation system where the algae are mixed together with the medium. Open pond systems usually use phototrophic condition since microalgae can harvest energy from the sunlight directly and yield inorganic carbon source in the form of carbon dioxide (Costa & de Morais, 2014) (Bhatia, 2014). The most occupied model of open pond systems is raceway pond. Using paddles in the ponds, water is agitated and rotated in one direction though the rotation of the pond. The purpose of this mechanism is to keep water and algae mixed and move along the racetrack for prevalent exposure to light and ambient air. Thus, the mixing where algae shall be agitated is important to keep

the algae in exposure of all requirements of growth. Vertical mixing plays an important role in this system to bring the algae upward for obtaining proper light and expose to the ambient air or injected CO_2 in opposite direction (Kumar et al., 2015a).

Table 2.6 Several lab and pilot scale design of algae cultivation in the open pond system.

Strains	Biomass Yield (Total Yield/ Productivity)	Design	Sources
Botryococcus braunii	1.8 ± 0.13 g L^{-1}	Concrete raceway pond (Length 6.1 m; Width 1.52 m; Height 0.3 m) volume 2000 L.	(Ashokkumar & Rengasamy, 2012)
Dunaliella salina	0.342 g $L^{-1}.d^{-1}$	1 m^2 surface area outdoor fibreglass paddle wheel-driven raceway ponds at 11–21 cm depth (normal depth = 16 cm)	(Moheimani & Borowitzka, 2006)
Pleurochrysis carterae	0.220 g L^{-1} d^{-1}		
Botryococcus braunii	1.8 g L^{-1}	Length 1.13 m; Width 0.6 m; Depth 0.3 m) with capacity 80 L	(Ranga Rao et al., 2012)
Tetraselmis sp.	36 ± 2 mg L^{-1} d^{-1}	1-m^2 paddle wheel-driven open raceway ponds 20-cm depth with additional CO_2	(Raes et al., 2014)

Open pond systems are also affiliated with mass culture system in some of the industries. The economical capital cost is among the main reasons to adapt this system for mass cultivation of algae. Since the large scale culture of open pond is larger nearly 10,000 m^2 compared to closed PBR which only up to 100 m^2 (Klein-Marcuschamer et al., 2013). The advantages of the open pond are also coupled with other factors that need to be considered. Some of the important factors to be considered in the open pond are location, pond depth, power consumption, mixing rate, CO_2 delivery into raceway pond, volumetric mass transfer coefficient, light, salinity, oxygen accumulation, and contamination (Kumar et al., 2015a). Some of the factors are very dependent on nature condition namely light intensity, contaminant,

and salinity while volumetric mass transfer, mixing rate, and CO_2 are completely controlled under the operational system. The natural depending factors thus become a challenging factors of open pond system.

2.5.2.2 Photobioreactor

The system of photobioreactor is a closed system where all the components are closed and isolated from the environments. However, some of the system of photobioreactor still export Thus, most of the operational parameters are controlled with specific agitation with or without additional light apart from the sun. with the advantages of isolated system, contamination risk can be negligible. Several models of this system have been developed and applied in the lab scale system to run experimental study about algae (Table 2.7).

2.5.2.3 Comparison of Open Pond and Photobioreactor.

In open pond system the production of biomass is lesser than that in the photobioreactor. Comparing outdoor open raceway pond and a helical tubular photobioreactor, Raes et al. (2014) found that the production of biomass has been found higher in the PBR 5.5 times than in the open pond system. Nevertheless, using illuminated area productivities, open raceway pond was found to have three times higher productivity compared to the PBR. The contamination risk of open pond is relatively high than that in the PBR.

Table 2.7 Photobioreactor used in the algae cultivation.

Strains	Biomass Productivity	Design	Sources
Nannochloropsis sp.	12 g L^{-1} d^{-1}	A flat plate with 10 cm light-path, vertical reactor made of 10-mm glass plates	(Cheng-Wu et al., 2001)
Chlorella zofingiensis	8.4 mg L^{-1} d^{-1}	Flat plate photobioreactor with dimension of length 90 cm, thickness 17 cm, and height 40 cm with working volume 60 L	(Feng et al., 2011)
Scenedesmus acutus	9.0 ± 0.6 g m^{-2} d^{-1}	Panels photobioreactor with width 1.17 m, height 1.17 m and thickness 3.8 cm with 60 L working volume.	(Eustance et al., 2016)
Tetraselmis sp.	67 ± 5 mg L^{-1} d^{-1}	Helical tubular photobioreactor, 60-m in diameter of clear tubing with working volume 40 L	(Raes et al., 2014)

Water loss in the system of PBR is considered almost none where in the open pond reduction of water by evaporation is considered very high. System of closed PBR and open pond are similar in terms of water demand and water footprint. Although different amount of water is required, considered the water that needs to be supplied both still occupy high amount of water in production (Guieysse et al., 2013). With closed system, weather dependence can be reduced in the PBR while in the open pond small change in the weather can cause failure in the system while in the closed system some of the systems are free from weather dependence.

Table 2.8 Comparison between Open Pond reactor and PBR system

Parameters	Open Pond	PBR
Location	affects the temperature, medium cost, transportation, rainfall precipitation	Temperature still plays important role in outdoor cultivation.
Dimension	Important dimension scale to ease the light penetration, agitation, and mixing with regard in the power consumption	Affected by mixing and agitation process, cylinder complete circulation.
Power consumption	Mainly to operate the paddles for circulation and agitation	For paddling, aeration, light generation and materials pumping.
Mixing rate	Crucial for even exposure to the ambient air and light	Shear mixing in some case can cause damage in the cells, however bubbling and paddle are preferably.
CO_2 delivery and intake	Important but not essential for cultivation since diffusive CO_2 is supplementary for enhance the production.	Injection of CO_2 is usually occupied in this system to provide carbon source to the system.
Light	Yielded from sunlight, thus the shallow pond is favourable for optimum light penetration	Moderate, some models occupy sunlight where the others use bulb or illumination from other sources

Table 2.8 Comparison between Open Pond reactor and PBR system (Continued)

Parameters	Open Pond	PBR
Salinity	Fluctuate due to high evaporation	Mostly controlled since the evaporation is negligible.
Oxygen accumulation	Optional to remove since low density of algae produces less oxygen that inhibitory in excess level	Highly accumulated since closed system produces and accumulate more O_2 in higher rate.
Contamination	Highly risk for contamination since the airflow can bring the contaminants as well as the predatory such as protozoa and other organisms.	Very small and often negligible. Closed system cause material exchange is only from the gasses injection and feed water or medium.

Since all the nutrients intake and other supporting condition are adjusted to fit the optimum condition of algae in PBR, period of production is shorter than in the open pond system. It relates with the turbulence in the system. PBR is able to maintain high turbulence in the system thus the mass transfer from the medium into the algae cells is relatively fast (Grobbelaar, 2008). Efficiency of water treatment is relatively high in the open pond rather than that in the closed system such as PBR.

Scale of bioreactors is mainly the consideration of using open pond rather than the PBR. Limitation in scaling up is the main factor. Increasing the size of PBR tube or cylinder is not similar with the increasing the dimension of the pond since too large tube of PBR can cause lower penetration of the algae into the deeper layer of PBR (Camacho et al., 2011).

2.5.3 Alga Harvesting Methods

Large biomass of algae from several cultivation methods should be harvested for further process of biofuel production. Harvesting process is the process of separating algae from its growth substrates. This process includes dewatering, removing water from the algae in order to confirm that the algae biomass is in condition of containing proper water content to enter further process. Failure in the dewatering and or thickening can cause excess water entering oil step, which will increase demands in the next steps. This step, thus, is playing a critical role to maintain the oil produced by the algae in the ratio that still acceptable for extraction process and eventually biofuel production.

Generally, methods of algae harvesting are divided into several conditions (Figure 2.5). Of the microalgae, mechanical methods are usually employed while for in the methods of macroalgae harvesting, strenuous methods are mostly used to collect the algae. Compared with macroalgae with physical activities of harvesting, microalgae in which the production rate can be incredibly faster than that of microalgae, needs chains of process to reduce the content of substrate from the algae aggregate to obtain less containing water microalgae slime or cake.

It is important to note that although algae can be growing in the high rate and more productive than other biomass production in land such as maize and cane, dealing with the harvest and post-harvest of this particular crop can be the most difficult obstacle to conquer in order for industrial application. Following are several possible methods that have been developed to harvest the microalgae.

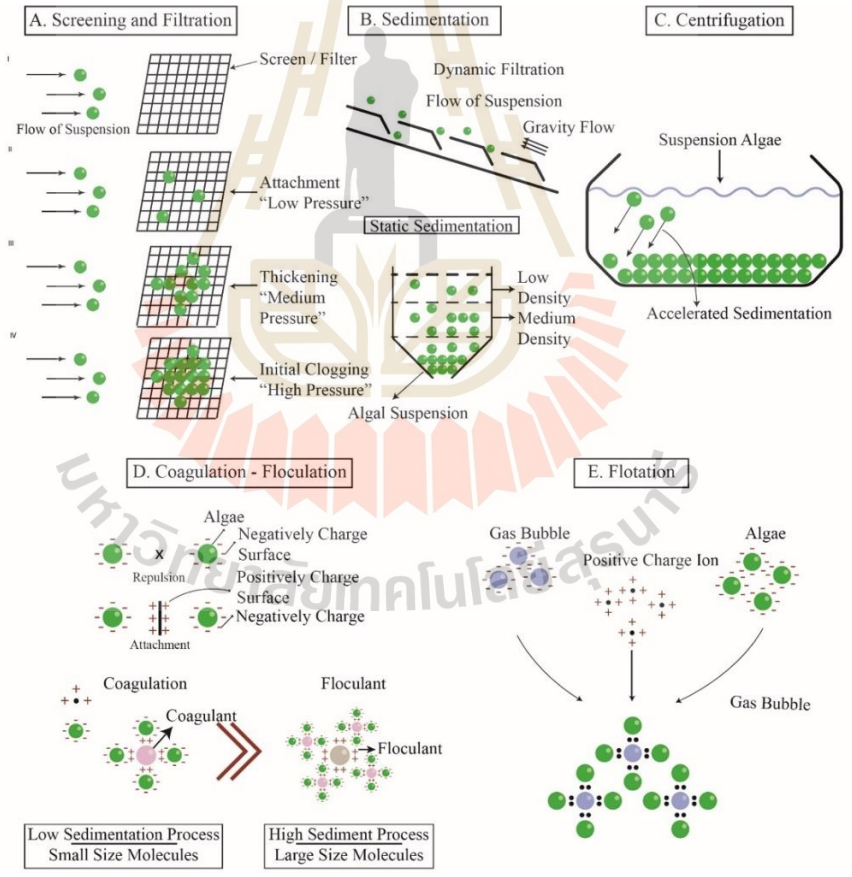

Figure 2.5 Several principal methods of harvesting microalgae. (A) screening and filtration. (B) dynamic and static sedimentations. (C) Centrifugation. (D) Coagulation-flocculation. (E) Flotation.

Table 2.9 Combination of traditional harvesting methods

Methods	Screening-filtration	Sedimentation	Coagulation-flocculation	Flotation	Centrifugation
Screening-filtration		-	✓	-	✓
Sedimentation			✓	-	✓
Coagulation-flocculation				✓	✓
Flotation					✓
Centrifugation					

2.5.3.1 Screening and Filtration

The terms filtration in some case can be put together with screening. Although both of the techniques use the similar concepts, the application of each has specific different. In the screening process, the algae are introduced to the screen with specific aperture or sieve size. Thus, the screening effectiveness really depends on the size of screen opening or sieve and the algae biomass. On the other hand, filtration is done with a force of flow to bring algae meet the filter medium using certain pump. The algae suspension is trapped and further it becomes denser due to an increase of biomass cells with the decrease of medium concentration.

Several innovations and modification regarding these two mechanisms were done. The combination particle of filter medium was examined to find the high deposited cells with optimum fluid flow. Some of the filter mediums combine sieve and charge of the filter medium elements. This combination results the increase of trapped suspended solid into the surface of filter medium. As the suspended solid contains opposite charge from the element, the filter medium will attract more suspended solid toward the elements and the algae deposition is form faster than that filter without any charge. The Magnetic particles with Flame-derived silica-coated had shown a high separation efficiencies >95% under 5 min filtration process (Cerff et al., 2012). It is important to note that since this combination involve charge, a slight change in pH is a crucial factor for separation efficiencies. While Magnetic separation has great potential for efficiently harvesting the algae, big gap of work including the mechanism of adsorption and particle recovery still need to be examined.

Table 2.10 Technology of Screening and Filtration

Algae	Method	Efficiency	Description	Ref
Chlamydomonas reinhardtii and *Chlorella vulgaris*	High gradient magnetic filtration	> 95%	By diluted the magnetic separation molecules into liquid for adsorption into algae before applying magnetic plate.	(Cerff et al., 2012)
Botryococcus braunii	Filtration	≈ 100%	Using usable material for further application such as coal for filtration process. Filter media and algae suspensions is already usable.	(Rettenmaier, 2013)
Scenedesmus quadricauda	Membrane filtration	98%	Ultrafiltration membrane module with air backwash mechanism	(Zhang et al., 2010)
Filamentous algae	Screening	-	With filamentous algae, raking is appropriate for collecting filamentous algae with microalgae suspended in between the filament	(Grayburn et al., 2013)
Nannochloropsis gaditana and *Phaeodactylum tricornutum*	Membrane filtration	50*	Microfiltration in the wastewater for collecting the algae	(Nurra et al., 2014)

*Permeabilitiy in L h^{-1} m^{-2} bar^{-1}

Nannochloropsis gaditana and *Phaeodactylum tricornutum* had been tested to try the effectiveness of dynamic filtration. Although the result was not convincing since there is a dropping value from ultrafiltration to microfiltration because of fouling reduction, this result is still better than conventional filtration (Nurra et al., 2014).

Microalgae is also considered to be suspended solid that needs to be removed, technology for removing suspended solid from water is also available for this circumstance. Membrane filtration is fitted well to the purpose of generating clean water and obtain the materials apart from the water. Forward osmosis system is simply an opposite version of reverse osmosis that world-widely used for treating the water. Final concentration of microalgae reported by (Kim et al., 2015) using this techniques was 1.45 g L^{-1} in which lower than the value of other method from the same study.

Interestingly, if the concentrate contains sufficient number of microalgae with short period of filtration, this method can be viable for microalgae harvesting since it requires low energy input with easy maintenance compared with centrifugation method and coagulation process.

2.5.3.2 Sedimentation

This process depends on the force of gravity to attract the suspension into the bottom of fluid. To occupy the gravity, microalgae shall be in high gravity potential condition. However, to increase settling velocity additional flocculation needs to be added. Eventually, harvesting from the bottom of the medium can be conducted by collecting the sludge in the bottom of the sedimentation place. Sedimentation experiments have been done to highly hydrophobic algal suspension of *Synechocystis salina* co-cultured with other growth promoting microorganisms (Gonçalves et al., 2015) and the results showed that all microalgal suspensions presented low microalgal recovery efficiencies. Nonetheless, even in the low recovery efficiencies the complete process still uses low energy less cost compare with other methods. However, a negative linear relationship between microalgal removal percentage and free energy of hydrophobic interaction was obtained.

Combination of Sedimentation with other method such as membrane filtration can lead to considerable less operation costs with a noticeable total rejection and high final concentrations. The combination eventually creates a cheaper cost than centrifugation. It has been teste by (Hapońska et al., 2018) that the final concentration of *Dunaliella tertiolecta* after this two combination methods was 184.58 g L^{-1} with 81.5% of water content in the sludge. This concentrations was high enough to reduce the total cost of further steps before cell disruption.

2.5.3.3 Coagulation-Flocculation

The two previous methods of harvesting really depend on the size of the particles. Both filtration and sedimentation are employed by the principle of size of the suspended solid (in sedimentation, size linearly related to gravity force). the flocculation and coagulation are the sequences of harvesting method and in most of harvesting methods nowadays these two steps are included. The coagulation is the process where the suspended solid of microalgae becomes attached in to the coagulant due to the charge in the surface. Aggregate of the microalgae and coagulant are still too small to settle down. As the aggregate is getting bigger, the floc is formed. The flow which is heavy and big enough can be easily stuck on the sieve in filtration process or settling in the bottom of sedimentation ponds. Thus, this phase is supercritical to determine the capital cost of harvesting process. However, this method

also relies on the state of cation and anion (pH) concentration to provide condition suitable for attachment (Wu et al., 2012).

Table 2.11 Several Technology of Sedimentation

Algae	Method	Efficiency (%)	Description	Sources
C. sorokiniana	Sedimentation-filtration	≈100	Sedimentation using pH adjustment in the reactor was followed by the vibrating filtration.	(Hapońska et al., 2018)
C. vulgaris	inclined sedimentation	80*	Inclining settler applied in the flow of the microalgae cultivation in continuous flow.	(Smith & Davis, 2013)
S. dimorphus	inclined sedimentation	80	Using incline sedimentation to settle the algae in continuous feed of water	(Wang et al., 2014b)
C. vulgaris		55		
C. vulgaris	inclined sedimentation	65	Enhanced incline sedimentation using vibration	(Smith et al., 2013)
Nannochloropsis oculata	sedimentation	70	Ultrasonic wave by aucostophoresis in the inclined settling for low density algae	(Hincapié Gómez & Marchese, 2015)

*fold greater than conventional method

Since the suspended solid is assumed to have negative charge surface, positive charge coagulant needs to be added for coagulation process. Cationic coagulant such as aluminum sulfate ($Al_2(SO_4)_3$) and cationic starches are often used to flocculate cells of *Chlorella spp.*, *Scenedesmus* spp., *Chlamydomonas reinhardtii*, *Schizochytrium limacinum*. Cationic starch was found to primarily recoomended due to its ability to flocculate cells 4–28 times greater than $Al_2(SO_4)_3$. These starches have

high degree of substitution and they have been proven to provide an efficient and effective method to harvest the algae. Again, the pH is a crucial when it employs different charge mechanism (Gerde et al., 2014). Another organic flocculant usually used for microlage is chitosan. Several studies used *Tetraselmis sp.* (Kwon et al., 2014) and *Chlorella sp.* (Mohd Yunos et al., 2017) to investigate pH changes and optimal flocculants concentrations for this coagulant and found that the optimal concentrations chitosan is ranged from 0.3 to 0.4 mg/L with neutral pH. These result showed that chitosan did not change the pH in flocculation process and demonstrated a feasible application to the water treatment system.

Apart from the organic and inorganic flocculants, bio-flocculants are getting more attention recently due to its environmental factors. It is considerably free from by-product and effectiveness. The bio-coagulant agents are from fungi and bacteria group. *Aspergillus sp.* has reported to have roughly 100 % conversion of algae in the free-suspended state into pellet form. As the algae getting denser and bigger in the flocculation, the size of pellet can reach 2–5 mm by which the filtration can be easily conducted using simple filter with relatively big pores (Zhou et al., 2012a).

Several methods try to combine the organic and inorganic agents for better performances. Yahi et al. (1994) suggested to combine sodium hydroxide and calcium hydroxide into the flocculant total with granular sand or inert resin can enhance solids abatement to be more than 95% with excellent settleability and good mechanical resistance. Moreover, Loganathan et al. (2018) has designed the combination of coagulant-flocculant substances. using alum as coagulant and chitosan as flocculent, microalgae removal 98 % with 5 minutes reduction of process time. This result can be important and a groundbreaking system to split the coagulation and flocculation into different agents. the differentiation can be important to ease the minimum requirement of each step and optimize the usage based on usage of each.

It is important to note that pH is important in this method, yet Wu et al. (2012) who also conducted the experiement on medium pH value which proven to enhance the highest flocculation efficiency of up to 90% in *Chlorella vulgaris*, *Scenedesmus* sp., *Chlorococcum* sp. (freashwater microalgae) *Nannochloropsis oculata* and *Phaeodactylum tricornutum* (marine microalgae) found that although the efficiency of removal was high, variation of the efficiency was tremendously affected by the metabolite synthesized from the algae.

Table 2.12 Coagulant in the Coagulation and Flocculation Method

Coagulant	Algae	Efficiency (%)	concentration	Description	Ref
chitosan	*Scenedesmus sp.* and *S. obliquus*	>90	20 mg L^{-1}	Based on the cost NHO_3 was the most effective	(Wu et al., 2015)
polyacrylamide			200 mg L^{-1}		
$Al_2(SO_4)_3$			80 mg L^{-1}		
NaOH			1200 mg L^{-1}		
HNO_3			600 mg L^{-1}		
ferric chloride ($FeCl_3$)	*Chlamydomonas reinhardtii*	>90	10 mM	Different strains of alga showed different flocculation efficiency	(Fan et al., 2017)
calcium chloride ($CaCl_2$)			30 mM		
magnesium chloride ($MgCl_2$)			5 mM		
$Al_2(SO_4)_3$	*Chlorella* sp.	93.8	200 mg L^{-1}	-	(Nayak et al., 2019)
$FeCl_3$		97.3	300 mg L^{-1}		
$Fe_2(SO_4)_3$		98.8	350 mg L^{-1}		
chitosan		99.6	10 mg L^{-1}		
$Al_2(SO_4)_3$	*D. salina*	>80	1 mM	$FeCl_3$ was considerably the most promising.	(Pirwitz et al., 2015)
$AlCl_3$		>80	1 mM		
$FeCl_3$		>80	1 mM		
$FeSO_4$		<50	1 mM		
$Fe_2(SO_4)_3$		>80	1 mM		

2.5.3.4 Floatation

The method of flotation is suggested for the algae with low settling velocity. As the flotation is formation process of algae layer in the upper medium. This method is the opposite side of sedimentation. The mechanism is by contacting the algae with air bubble introduced from the bottom of the tank in column for air dispersion. As the algae reaches the surface, the layer of algae is formed thus the skimming method of floating algae can be done. Thus, it important to control the amount of air bubbled in the pond as well as the urgency of additional light flocculants for better performance.

The application of the flocculent is needed to capture the bubble in high effectiveness. The combination of those two methods in separate way tends to be redundant and cost consuming. Thus, combination of flocculent in sequence with the air bubble can be a solution. (Kwon et al., 2014) displayed a method

of pressurizing the microlage in the floc form with high pressure (2 atm) to trap the micro-air bubble inside the floc before floating it int he normal ambient pressure. Paradoxically, (Rashid et al., 2013) finding that high flocculant concentration can reduce floatation phenomenon but enhance sedimentation while they were trying to sediment the floc. Thus, it is interesting to find out whether these two methods can be combined or put in a sequence later.

Other technology tries to combine dispersed air flotation with foam fractionation to not only harvesting but also optimizing the process of water reduction. Floatation process in *Chlorella sp*. using polystyrene latex beads showed a promising results (Coward et al., 2013). Yet, the factors that need to be controlled and pinpointed namely, states of cationic ions, surfactant concentrations, the height of the column are still considered as a complex system. Apart from that, microalgae foam residence time is also necessay to be contemplated for effectiveness. Deeply deliberating, this method has a sufficient result for being adapted with high control of the factors.

Table 2.13 Flotation method for Algae Cultivation

Method	Algae	Efficiency (%)	Description	Sources
Electro-coagulation–flotation	*Microcystis aeruginosa*	>90	Using electrode material, the floc forming was triggered and later floated in the medium.	(Gao et al., 2010)
Iron pretreatment floatation	*M. aeruginosa*	99	Application of Fe- increase the flocculation efficiency and later affecting the floatation harvesting	(Qi et al., 2018)
Air flotation	*C. vulgaris, M. homosphaera,* and *M. aeruginosa*	>90	By adding cationic polyelectrolyte poly into air bubble system, for algal organic matter to enhance the floatation.	(Hanumanth Rao et al., 2018)
Floatation	*C. vulgaris*	80.53 89.23 (TAB), (saponin)	Additional of tea saponin and hexadecyltrimethyl ammonium bromide (TAB) for sulfactant to enhance algae floatation.	(Shen et al., 2018)
Surfactant aided foam flotation	*C. vulgaris*	96.3	sodium dodecyl sulphate was used as the sulfactant to increase the floatation activity of the culture	(Umar et al., 2018)

2.5.3.5 Centrifugation

The method of centrifugation is based on the principal of sedimentation yet the process of particle to settle on the bottom is accelerated by centrifugal force, which is much higher than gravity force. This method is well known to separate the phase of solid and liquid in the medium with high-energy demand. Despite its practical use and quality of the harvesting, this method still needs pre-harvesting method such as flocculation for efficient process and maximum quality of tick slurry of the algae.

Some of the distinguise characters of centrifugations are the low flow rates and high energy consumptions (Sim et al., 1988). To subsidize the high energy consumption, at least one of the drawbacks needs to be reduced. It can be either the flowrate or low energy consumption. The former one can be achieved by designing the proper apparatus with high efficiency process while the latter one focused on the quality and quantitiy of the harvested slurry.

Several studies put the effort to overcome the problem. For designing improvement, *Tetraselmis suecica* has been chosen to test the hydrocyclones centrifuge (Shakeel Syed et al., 2017). By modifying the common model of hydrocyclone with smaller cut-size for high-throughput particle/cell sorting, final microalgal biomass concentration was achieved to increase 7.13 times than the conventional centrifuge. This particular model might be advantageous in the near future with proper scaling up.

The Centrifucation of *Nannochloris* sp. has been tested for the high flow rates (above 1 L min^{-1}) and showed lower capture efficeincies (<90%) (Dassey & Theegala, 2013). To comprimize the low effluent quality, high dense effluent of microalga culture was proposed to balance the energy consumption. This increase of influent flow also proposed that in low algae harvested (roughly 25 %) with the the flowrate up to 18 L/min, the total energy consumption was reduced by 82 %. Although the effluent qauntitiy only contain one fourth of the totm biomass available in the influent, this model is still worthy try when it comes to the energy balance.

Reflecting to the disc stack centrifuge system, another future task for the development of centrifugation method is to optimize the centrifuge as a separator for liquid and lipids contents by achieving cell destruction. When further steps are eased by the centrifugation by increasing the quality or partly merging the function of down process such as the oil separation and algal biomass separation in a single operation, the energy required of the harvesting by centrifugation can be neglected. It is due to the contribution for reducing the total energy demand by

eliminating the energy requirement in the process operations of cell fracture and lipid extraction (Milledge & Heaven, 2011).

2.6 Application of Algal-Microbial Co-culture for wastewater treatment and biomass generation

Biomass generation, wastewater treatment, and separation process are among the main focuses of the algal based treatment. Additional microbes into the system of cultivation or harvesting are widely employed throughout the systems to enhance the efficiency. Based on those objectives, combinations the algae and other microbials to achieve the goals are divided into four types (Figure 2.6). The first type is where co-cultures act merely to enhance the algal growth. The second type is where the co-cultures act as the biological agent that also treats the wastewater by consuming the nutrients. The third type is where the microbes act as harvesting aids by producing extracellular polymeric substance or by entrapping the algal cell on the hyphae. The fourth type is where the microbes act both as the algal growth promoting agent and for the harvesting aids.

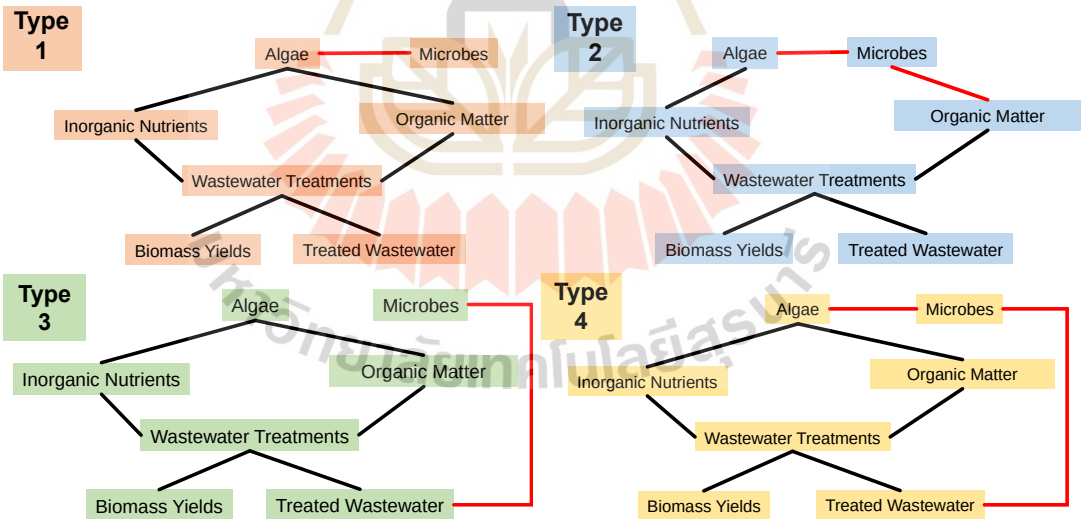

Figure 2.6 Four types of microbes' application in algal-based wastewater treatments.

The first type of co-culture is that the algae was the main agent to reduce the wastewater content by consuming it or through other mechanisms that change the condition of the water and eventually the contaminants are conversed to other states. Several previous attempts were made based on this concept. Kumsiri et al. (2021) has

combined the Actinomycete *Piscicocus intestinalis* WA3 to enhance the growth of *Tetradesmus obliquus* AARL G022. Here the application was based on the removal by the algae where most of the function of the *P. intestinalis* was to produce phytohormone and other growth promoting molecules so that the algae could growth in higher growth rate than before. It is also important to note that some of the interaction in this type may be syntrophic, where the exudate from the algal metabolisms. Thus, the microbial that available for this type can come from the natural symbiont or normal flora as it previously described by Wang et al. (2015).

Microbes can also directly remove the wastewater through the direct intake. Occurrence of the additional microbes can enhance the growth of the algae but also the nutrient removal may be more important in this type. Several studies have demonstrated this type. Guo and Tong (2014) stated that this type of promoting organisms can grow independently in the same substrate with the algae without any occurrence of the algae. Further, as the microbes can utilize the substrate from the raw medium, the growth will be enhanced in the co-culture of the algae. Thus, in this report, the main objective was still to enhance the algal growth for utilizing the medium. Differently, Nguyen et al. (2020) employed activated sludge (AS) to grow with microalgae. The results showed that the growth with 3:1 ratio of alga:AS was the most optimum inoculum for nutrient removal. However, the algae concentration in this particular study was not significantly enhanced. Nevertheless, removal of COD, TP, and TN were dramatically enhanced. Here, it can be concluded that the main objective of enhancement can be the wastewater removal or the biomass production.

Type three of this co-culture is mostly related to the trait of microbe's morphology and physiology. Some of the microbes in this type are in the hyphae form, while others are in single or colony forms without any hyphae or mycelium formation. There are two main mechanisms in this type. The first one is by entrapping the algal cells on the surface of EPS produced by the bacteria while the second one is by entrapping the cells between the hyphae of the microbes (Leng et al., 2021). For the former mechanism, the attachment varies under different condition of organic carbon sources and mixing rate applied to the system (Lee et al., 2009). Mostly, carbohydrate composition in EPS plays important role to bridge the cells and neutralize the charge on the surface (Choi et al., 2020). However, the EPS can come from microalgae after bacteria induction (Vu et al., 2019), single strain bacterium (Li et al., 2017b), or the bacterial consortium in the form of activated sludge (Choi et al., 2020). For entrapment of the algal cell through mycelium, high biological activity, fast sedimentation speed, and easy solid–liquid separation are the most utilized trait from the filamentous microbes (Li et al., 2020). Condition such as pH and calcium concentration determine

the efficiency of this harvesting (Li et al., 2017a). The application of cultured algae was previously conducted using *Aspergillus oryzae* pellets to entrap *Chlorella vulgaris* UMN235 cells (Zhou et al., 2013).

Type four of the co-culture involves combination of harvestability enhancement and biological enhancement of the removal. Here, combination of both advantages was often obtained through multilayer screening and characterizations. The harvestability of algal cells, increasing of the lipid composition with advantageous compositions, and removal through several growth promoting activities and independent removal process (Muradov et al., 2015). The combination was previously reported in fungus *Aspergillus fumigatus* and alga *C. protothecoides* (Muradov et al., 2015), *A. fumigatus* with *Thraustochytrid sp.* (Wrede et al., 2014), and *Aspergillus sp.* UMN F01 and UMN F02 with *C. vulgaris* UMN235 (Zhou et al., 2012b). Similarly, application of this type was also demonstrated by Lakshmikandan et al. (2021) who combined the actinomycete *Streptomyces rosealbus* with alga *C. vulgaris* to enahnce the growth and harvestability of the co-culture.

2.7 Conceptual Framework

Based on the introduction and the review of literature sections, conceptual framework has been designed (Figure 2.7). This study focuses on the development of the algal based treatment to treat cassava biogas effluent wastewater. By screening several potential inoculants in the sterilized condition and combining the most promising algal inoculants with the selected strains of actinomycetes and fungi, the co-culture was constructed. The values of the wastewater nutrient removal, self-sedimentation of the alga, and the biomass production were normalized to choose the suitable co-culture. Further, the selected co-culture was tested in the raw wastewater in batch and semi-continuous systems. The oil production and other factors were calculated to construct the life cycle assessment for the co-culture. However, the recirculation and microbial community were excluded from this study. The system that generates the wastewater was also excluded from this study while the starting point of this study was emphasized on the wastewater generated from the cassava starch industry.

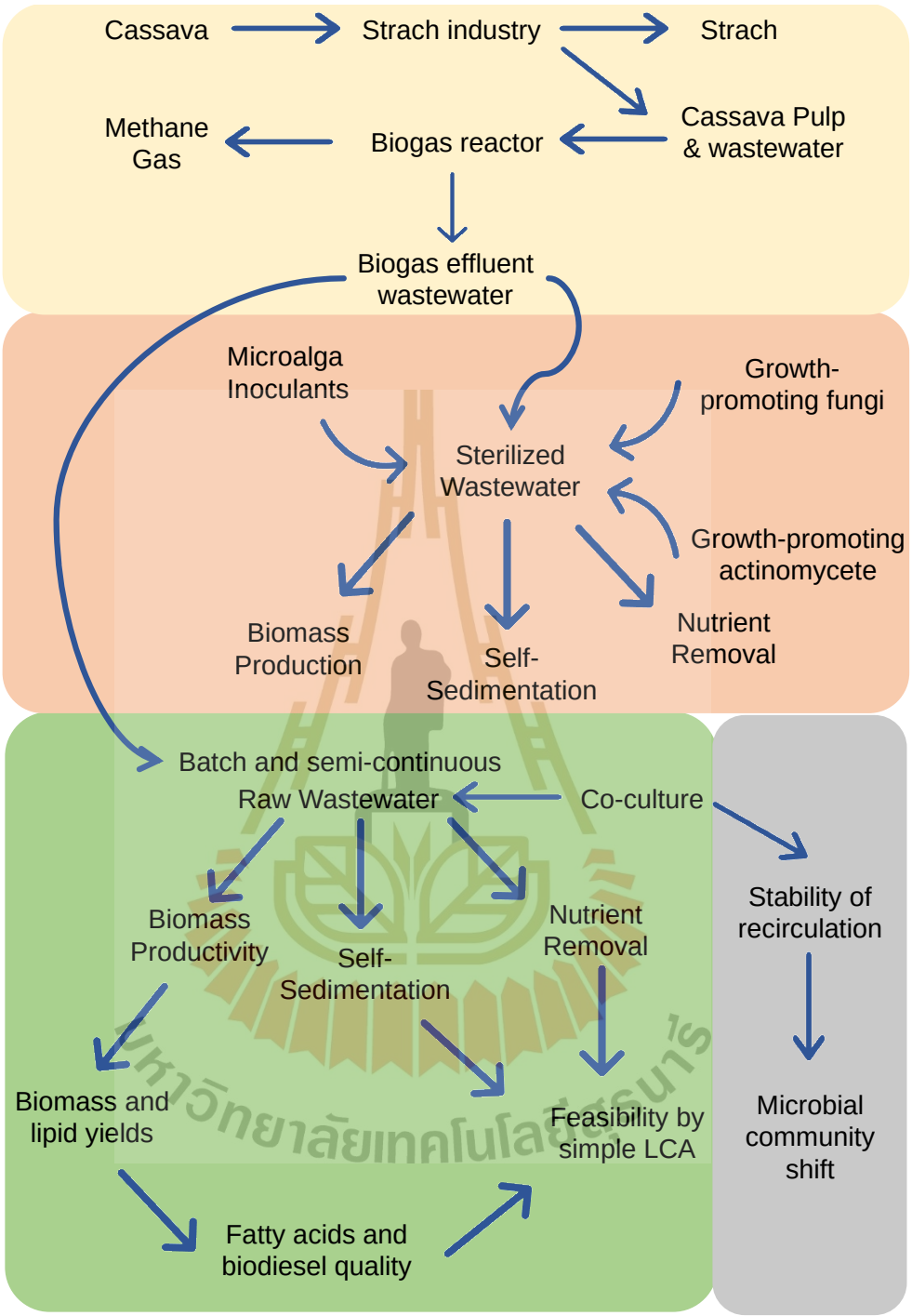

Figure 2.7 Conceptual Framework of the study.

CHAPTER 3
RESEARCH METHOD

3.1 Algae Isolation and Construction of Consortia

3.1.1 Water Sample Collection

Cassava biogas effluent wastewater (CBEW) from Cassava Starch Company Ltd. was collected to obtain indigenous algae strains and communities for further experiments. The sources were varied among several sampling sites. Generally, the sampling sites were classified from the sources. Cassava starch industry (Korat Inc, Nakhon Ratchasima, Thailand) was the source of cassava wastewater (Figure 3.1). This biogas wastewater is chosen because the cassava industry is among the potential emerging technologies of renewable energy in Thailand (Prasertsan & Sajjakulnukit, 2006) but the wastewater generation from this activity creates some burdening factors to environments since it emits unpleasant odor and potentially causes eutrophication due to its nutrient contents. The generated wastewater contains relatively low C:N ratio which suitable for algae to grow in several conditions.

Table 3.1 Wastewater Sampling Sites for Algae Isolation

Wastewater Sampling Source	N	E
Diluted pond 1 of biogas effluent wastewater	14°53'52"	102°03'57"
Diluted Pond 2 of biogas effluent wastewater	14°53'52"	102°03'54"
Diluted Pond 3 of biogas effluent wastewater	14°53'52"	102°03'51"
Diluted Pond 4 of biogas effluent wastewater	14°53'54"	102°04'51"
Conditioning Pond before biogas reactor influent	14°53'53"	102°04'00"
Wastewater effluent from biogas process	14°53'53"	102°03'58"

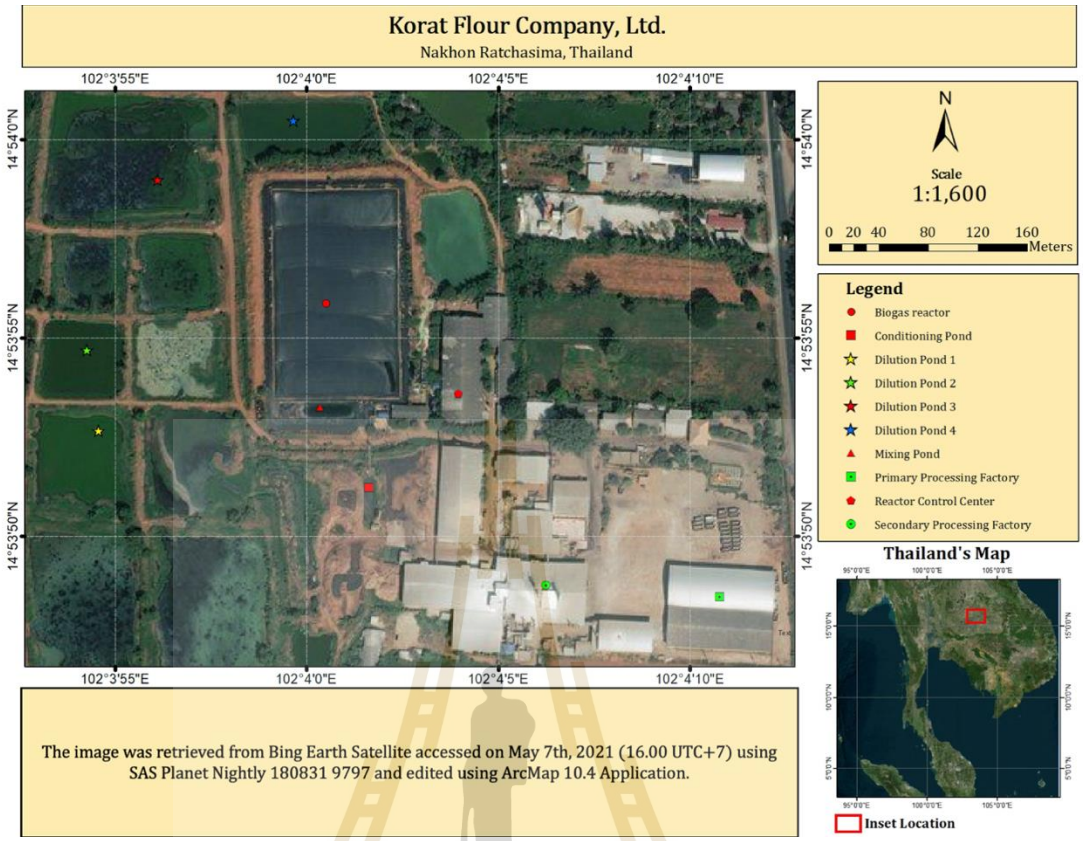

Figure 3.1 Map of the cassava starch industry site.

Cassava industry area was the area where the cassava starch produced. It has many ponds to supports the system of water circulation in cassava factory. Around the factory, wastewater and recirculated water can be found. In this study, the sampling sources of algae isolation process are chosen based on the appearance of the water body in several ponds. After observation, four dilution ponds, conditioning pond before biogas reactor influent and biogas effluent are chosen. The ponds are commonly used to sediment the organic matter and other suspended and dissolved solid in the wastewater after biogas production. Thus, water samples from pond 4 of this system was used as the dilution of wastewater effluent from biogas. The influent and effluent of biogas reactor were collected as well.

3.1.2 Wastewater source and its characterization

Cassava biogas effluent wastewater (CBEW) was used throughout this study. Briefly, the wastewater was generated from the biogas reactor. The reactor was supplied with the acidified cassava pulp from the waste of starch flour production. To understand characteristics of the wastewater, several standard measurements including physico-chemical measurement were conducted based on the standard methods (APHA, 2005). Mainly, parameters were as follows; alkalinity, conductivity, biochemical oxygen demand (BOD), chemical oxygen demand (COD), alkalinity, total Keldjah nitrogen (TKN), ammonium nitrogen (NH_4), nitrate nitrogen (NO_3), nitrite nitrogen (NO_2), total phosphorous (TP), and phosphate (PO_4). Furthermore, pH, dissolved oxygen (DO), and salinity were also determined using a YSI 556 MPS Multiprobe System (Xylem, Ohio).

3.1.3 Isolation of microalgae

The isolation of microalgae was based on the method by Tale et al. (2014). Briefly, basal medium of BG 11 agar was made before spread agar plate was conducted. One Liter BG 11 contained 10 mL Stock A (Na_2Mg EDTA 0.1 g L^{-1}, Ferric ammonium citrate 0.6 g L^{-1}, Citric acid·$1H_2O$ 0.6 g L^{-1}, $CaCl_2$ ·$2H_2O$ 3.6 g L^{-1}), 10 mL Stock B ($MgSO_4$·$7H_2O$ 7.5 g L^{-1}), 10 mL Stock C (K_2HPO_4·$3H_2O$ 4.0 g L^{-1}), 1 mL Stock D (H_3BO_3 2.86 g L^{-1}, $MnCl_2$·$4H_2O$ 1.81 g L^{-1}, $ZnSO_4$·$7H_2O$ 0.222 g L^{-1}, $CuSO_4$·$5H_2O$ 0.079 g L^{-1}, $COCl_2$·$6H_2O$ 0.050 g L^{-1}, $NaMoO_4$·$2H_2O$ 0.391 g L^{-1}), 0.02 g Na_2CO_3, and 1.5 g $NaNO_3$. pH of the medium was adjusted to be 7.5-8. To isolate single microalga strains from the field water samples, standard plating method was used to separate algal populations. The field samples were first diluted to aid in the isolation process. Sterilized petri dishes containing approximately 20 mL of agarized medium were used to plate these diluted samples. One hundred µL of the diluted sample was transferred to a media plate and spread evenly across the surface. The algae were allowed to grow for about 14 days under continuous light exposure by a white bubble lamp (5000 lux). Single algal colonies were picked and restreaked to new BG 11 agar medium and placed back under the light exposure for purification. This streaking method was repeated until isolation into unialgal cultures was achieved.

The number of colonies that transferred from each dilution plate onto other nutrient media plates depended on the amount of contamination and the identification of the colonies present based on the colony morphology and the microscopic cellular morphology of each isolate. Following the isolation of individual microalgae colonies, each strain was initially labeled based on the sampling location. Isolated algae were maintained as stock cultures and stored on a cool, low light shelf. These stock cultures were maintained by re-plating each onto new nutrient media at

least once a month, or more frequently depending on the nature of each isolated strain. Wastewater characteristics and removal efficiency of the algae were conducted further to understand the dynamic of algae in the wastewaters.

3.1.4 Screening of potential algal strains

Microalgae strains were collected and further tested to utilize organic carbon as a sole carbon source. The ability to utilize organic carbon source under no light indicates the algal ability to remove organic carbon from the wastewater in the mode of mixotrophic. Screening of mixotrophic microalgae was conducted using several sources of carbon. Glucose, sucrose, fructose, mannitol, and galactose were used as carbon sources. The strains were tested in BG 11 medium with addition of 0.5% of each of the carbon sources. BG 11 medium with no additional organic carbon source was inoculated as a control. The algae were grown for 14 days in 250 mL of BG 11 until reaching their stationary growth phase. Then, the cultures were diluted to achieve an optical absorbance at a 680 nm wavelength (A_{680}) of 0.5 (Fazal et al., 2021; Salgueiro et al., 2016). Next, 100 µL of each dilution was inoculated to individual wells of a sterile twenty-four well microplate with 1 mL working volumes to culture the strains with the absence of the light. Shaking was the only agitation for the alga at 200 rpm (Supplementary Figure 1). After seven days of incubation A_{680} of the culture was measured. These results were used to determine the most suitable microalgae for further experiments.

3.1.5 Isolation and screening of actinomycetes

Several samples from several ponds, wastewater effluent and influent, soil in the pond, and dried sludge around the CBEW sites were collected for bacterial isolation. Bacteria were isolated using a spread plate method. After diluting 1 g of soil sample or 1 mL of water and sludge samples, serial dilutions from 10^{-3} to 10^{-6} were performed before spreading 100 µl of the diluents onto a Starch Casein Agar medium (10 g L^{-1} starch, 0.3 g L^{-1} casein, 2 g L^{-1} KNO_3, 2 g L^{-1} NaCl, 2 g L^{-1} K_2HPO_4, 0.05 g L^{-1} $MgSO_4.7H_2O$, 0.02 g L^{-1} $CaCO_3$, and 0.01 g L^{-1} $FeSO_4 \cdot 7H_2O$) (Peng et al., 2020). The plates were then incubated at 28 °C for 1–2 weeks. The hard-to-pick colonies as one of the actinomycetes characteristics were restreaked and purified using the same medium. Purified strains were stored in a 25% glycerol suspension at −80 °C.

Potential isolates were further screened from their growth regulator compound production by determining the production of Indole-3-Acetic Acid (IAA) using Salkowski reagent test. All strains will be inoculated into 10 mL International Streptomyces Project (ISP) II medium (4 g L^{-1} yeast extract, 10 g L^{-1} malt extract, and 4 g L^{-1} dextrose) supplemented with 0.2 % L-tryptophan in the sterilized conical tube 50

mL and shaken for 7 days at 150 rpm at room temperature. Supernatant of the culture was extracted by centrifugation at 500 rpm for 10 min. A colorimetric technique using Salkowski reagent (48:1:1 of 35% $HClO_4$, 0.5 M $FeCl_3$, and DI water) was employed to detect the ability of IAA occurrence in the culture (Lebrazi et al., 2020) (Supplementary Figure 2).

The ability of the isolates to solubilize phosphate was also tested using $Ca_3(PO_4)_2$ solubilization on Pikovskaya medium (10 g glucose, 5 g $Ca_3(PO_4)_2$, 0.5 g $(NH_4)_2SO_4$, 0.2 g NaCl; 0.1 g $MgSO_4 \cdot 7H_2O$, 0.2 g KCl, 0.5 g yeast extract, 0.002 g $MnSO_4 \cdot H_2O$, and 0.002 g $FeSO_4 \cdot 7H_2O$). Actinomycete discs (5 mm) of 7-day old culture on ISP II medium were placed on PVK agar plates supplemented with 0.5% tricalcium phosphate as the sole phosphorus source. The plates were incubated at 30 °C for 7 days. Clear zones around the colony were indicated the capacity to solubilize phosphate (Supplementary Figure 3).

3.1.6 Isolation and screening of fungi

Isolation of fungi from the similar source was also conducted. Similar with the actinomycetes, fungi also could entrap the algal cells through the mycelium pellet with several growth promoting activity that reported previously (Li et al., 2020; Li et al., 2017). Isolation of fungi was conducted by diluting wastewater and sediment samples from the sampling sites by factors of 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6}. The low dilution factors were used as the fungi may occur in the less density than the actinomycete. Potato dextrose agar (PDA) (Himedia, India) was prepared as an isolation medium. Around 20 mL of sterilized PDA medium was poured into individual plates. Approximately 200 µL of each dilution were spread onto the PDA medium using a sterile glass bar. After 2-5 days, single hyphae of fungi on the PDA was thus reinoculated onto new plates until pure isolates were obtained (Doilom et al., 2020). Growth-promoting activity was tested using similar assays with the actinomycetes (Section 3.1.6). Pikovskaya agar was used for phosphate solubilizing activity test with spores as an inoculum. However, for the IAA test, potato dextrose broth (PDB) was used instead of ISP II. Positive strains were further tested for its ability to enhance the growth of microalgae.

3.1.7 Identification of isolated strains

The single isolates were proceeded into identification process. Microalgae were initially separated based on morphological examination of the colonies. Similar colonies were thus observed under Light microscopy (Primo Star, Zeiss). Characteristics such as shape of colony, cell, and other morphological information were used to determine the strains classification. This general classification

method was only used to distinguish isolates on the most basic level. Identification of these isolates to the genus level was based on the morphology of the individual cells.

Molecular identification was conducted based on previous studies (Fawley & Fawley, 2004; Jagielski et al., 2017). The 18s rRNA region of algal and fungal strains was amplified using the primers, NS1: 5′-GTAGTCATATGCTTGTCTC-3′ and NS8: 5′-TCCGCAGGTTCACCTACGGA-3′ (Ding et al., 2020). Meanwhile, the 16s rRNA region of actinomycetes was amplified using the primers, fD1: 5′- AGAGTTTGATCCTGGCTCAG -3′ and rP2: 5′-ACGGCTACCTTGTTACGACTT -3′ (Dione et al., 2018). After PCR reactions were performed for the strains, the sequence of the gene was analyzed using the NCBI BLAST tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi). A phylogenetic tree was constructed using MEGA version X (MEGA, USA) after multiple alignments of data using the Muscle Tool. Evolutionary distances and clustering were constructed using a neighbor-joining method and evaluated using bootstrap values based on 1000 replications.

3.1.8 Differentiation of alga and other microbial biomass

Microalga was not the only organisms that can contribute to the turbidity of the water in the co-culture system. There was discrepancy and possibility of the flocs and sedimentation made by the combination of actinomycete or fungus mycelia that could significantly reduce the absorbance. The growth the algal biomass was then measured based on the chlorophyll contents. Before the cultivation, standard curve for the function of chlorophyll content to the algal biomass was also made. Later, the biomass of alga was expressed as dry weight per volume based on its chlorophyll content. To measure the chlorophyll *a* content (Chl *a*), method of Halfhide et al. (2014) with minor modification was employed. Briefly, as much as 10 mL of algal suspension was extracted by centrifugation at 8000 rpm for 10 mins. After a centrifugation step, supernatant was discarded, and 15 mL of ethanol was added to the pellet. After 10 sec of vortexing, the mixture was sonicated for 5 mins to disrupt the cells. Another centrifugation step was conducted before taken the solution to measure the absorbance using spectrophotometer based on equation (1) (Cuaresma Franco et al., 2012),

$$\text{Chl a} = (16.72 \times A_{665} - 9.16 \times A_{665}) \times DF \quad (1)$$

where *Chl a* is Chlorophyll *a* concentration (mg L^{-1}), A_{xxx} is absorbance of the solution at wavelength xxx nm, and DF is dilution factor.

Similar suspension of axenic alga was also gravimetrically measured. The biomass was determined gravimetrically by taking a 10 mL cell suspension and passed

it through a pre-weighed 4.7 cm GF/C Whatman filter. The paper was then washed with acidified water and dried at 60 ºC for 48 hr. The paper then was weighted after the drying process. Biomass of the alga and Chl *a* values were used to construct the standard curve (Supplementary Figure 4). This gravimetric method was also applied in the co-culture to obtain the total biomass. The microbial biomass was then differentiated with the algal biomass using Equation (2).

$$B_T = B_A + B_m \quad (2)$$

where B_T is the total biomass from direct gravimetric measurement, B_A is the algal biomass after conversion of Chl *a* concentration to be biomass, and B_m is the microbial biomass. All the units are expressed in mg L^{-1}.

3.2 Co-cultures construction and comparison

3.2.1 Screening of inoculum for algae treatments

Studies have been conducted to compare the feasibility of single strains, commercial strains, and natural blooms to treat wastewater (Mennaa et al., 2015). Here, Similar comparison was also made from the commercial strains, natural algae blooms, and the isolated strains. The result of this particular experiment was used to determine the feasibility of algal inoculum for wastewater treatment. Commercial strains purchased from Thailand Institute of Scientific and Technological Research Culture Collection (Bangkok, Thailand), namely *Chlorella* sp., TISTR 8411, *Coelastrum* sp. TISTR 8508, *Scenedesmus obliquus* TISTR 8522, *Chlorella vulgaris* TISTR 8580, and *Chloroccocum* sp. TISTR 8583.

Four communities of algae were also obtained from the sites of samplings, namely Dilution Pond 1,2,3 and 4 (P1, P2, P3, and P4) (Figure 3.1). To obtain inocula from this type of samples, the greenish waters in three different points of each pond was taken. The third type was native algal bloom. A native algal bloom culture was obtained by exposing the CBEW wastewater under sufficient light to grow the indigenous algae. Briefly, A 50 mL of unsterilized wastewater was agitated (150 rpm) under 2000 lux light in 250 mL capped flask. Algal suspensions of all cultures were extracted by centrifugation and the solids washed three times with DI water to ensure that they had no extraneous nutrient content prior to cultivation. All the cultures were treated by antibiotics (10 mg L^{-1} tetracycline, 10 mg L^{-1} kanamycin, and 10 mg L^{-1} ampicillin) for 3 days in 50 mL sterilized CBEW (Han et al., 2016) to remove excess

bacterial contamination. Confirmation of axenic community was conducted by spreading 10 µL of suspension into LB agar medium. After antibiotic treatments, all the inoculums were washed and adjusted to an optical absorbance of A_{680} = 0.75 in CBEW. Composition of green microalgae was obtained by microscopic observation under a light microscope and their morphological appearance were used to determine the genus of the microalgae in order to obtain the characterization of algal community (Barsanti & Gualtieri, 2005).

Mixotroph isolates that prior isolated which can utilize all the carbon sources were chosen to test in the sterilized condition, alongside the commercial strains, natural algal bloom in the CBEW, and the algal communities that had been treated with antibiotics. Sterilized wastewater has slightly lower nutrient concentrations after being autoclaved at 120° C and one bar for 15 mins. Here, the focus was to observe the growth of algae by measuring absorbance 680 nm (A_{680}) daily.

All the inoculates were prepared at the same time and cultivated in parallel. The flasks were shaken on an orbital shaker at 200 rpm for seven days under a 2000 lux light. The suspension was daily measured by taking and replacing 10 mL of the solution and the algal biomass was measured. At the end of the cultivation period, nutrient removal was also measured. To comprehensively consider all the parameters that have been tested, normalization of the important parameters was calculated to obtain the most feasible inoculants. Equation (3) was used to normalize all the parameters obtained for all inoculants of the algae in the sterilized wastewater.

$$\text{N-Value} = \frac{V_{actual}}{V_{max}} \qquad (3)$$

where, N-value = normalization value, V_{max} is the highest value in mentioned parameter, and V_{actual} is value obtained from the selected treatment. The inoculants with the highest scores were then further tested for actual CBEW treatments and their possibility to grow in the co-culture formation.

3.2.2 Construction of alga-bacterium consortium

Consortium of selected alga and bacterium was constructed by co-culturing the potential microalga. Algal inoculum with the highest score of performance in the sterilized CBEW was cultured in 100 mL of sterilized BG 11 medium until its growth reached the exponential phase (A_{680}=0.75). A synergistic test was conducted in a 250 mL capped flask. Ninety mL of BG 11 medium was added into the flask, followed

by a 5 mL microalga suspension. All of the positive IAA-producing and phosphate-solubilizing actinomyces strains were individually added to algal cultures after the actinomycete cultivation in 5 mL ISP II medium for 3 days with initial concentration of 10^5 spores mL^{-1} (Kumsiri et al., 2018). The flasks were shaken on an orbital shaker at 200 rpm for seven days under a 2000 lux light. After 7-day period of cultivation, the suspension was sampled and measured as prior described.

3.2.3 Construction of alga-fungus consortium

Similar with the previous test of actinomycete, the isolated fungi were also tested in terms of the growth promoting activity to the algae. The screening process was based on the ability to produce IAA and the phosphate solubilizing activity. However, the determination of the applied fungal strain was based on its ability to enhance the growth and harvestability of the algae. It is also important to note that the risk of antagonistic interaction between the algae and fungi is rarely found compared with the actinomycetes (Zheng et al., 2013). Thus, the approach of the screening was slightly different from the actinomycete-based co-culture.

In order to screen the applied strain, a sequence of experiments was conducted. The experiment was emphasizing the development and applicability of the fungal pellets as the main trait of the fungus that advantageous for algal cultivation and harvesting (Leng et al., 2021). The pellet formation ability was observed in several concentration of sugar supplementation (0.5, 1, and 1.5 %). Dilution of 0, 0.9, 0.8, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2, and 0.1 of the wastewaters with the optimum glucose concentration were also conducted to examine the pellet formation capacity. Lastly, the adsorption capacity of the range of glucose supplementation was observed to display the mechanisms of harvestability aid of the fungi to the algal cells. To prepare these tests, the fungal isolates were previously grown on the PDA medium. After spores were occurred in the sufficient amounts (5-7 days), harvesting was conducted and diluted to DI water. As much as 1 ml of 10^7 spores L^{-1} suspension was grown in the mentioned media with various carbon supplementation and wastewater dilution (Wrede et al., 2014). The pellets that formed in the BG 11 supplementation were also used to adsorb the alga suspension. The adsorption capacity of the fungi was also measured by analyzing the SEM images to describe the mechanisms of the harvestability enhancement by the fungi as it was previously described in the Pei et al. (2021)

3.2.4 Comparison of algal inoculum with the co-culture

Axenic algae with the highest removal and growth activity were combined with selected strains of fungus and actinomycete with the highest promoting

activities. Several methods of co-cultures and single organism treatments were tested here, depending on the characteristics of the co-inoculants (Leng et al., 2021). Similar treatments were used for actinomycete and fungus co-inoculants. Sterilized wastewater was used in this experiment to observe the mechanisms between microalgae and co-culture. Concentrations of nutrients and key parameters such as DO and pH were measured daily. Biomass of the algae and the co-culture were measured daily. Harvestability of the co-cultures was also measured using previously described method. Removal efficiency (*R*) was determined using equation (4):

$$R = \frac{(S_0 - S_x)}{S_n} \times 100\% \quad (4)$$

where S_0 is the initial concentration of a particular nutrient (mg L^{-1}) and S_x is the unassimilated concentration of that nutrient (mg L^{-1}). Similar formula was also used for the harvestability measurements.

The growth rate and removal rate of nutrients were the main parameters examined in these experiments. Based on the Verhulst kinetics model (Mennaa et al., 2015), algal growth can be expressed as Equation (5):

$$\frac{\delta X(t)}{\delta t} = \mu X(t)\left[1 - \frac{X(t)}{X_m}\right] \quad (5)$$

where *X* (mg SS L^{-1}) is the concentration of a specific component at time *t* (days), μ is the specific growth rate (day^{-1}), X_m was the maximum concentration of biomass in the culture (mg SS L^{-1}). By integrating Eq. (5), a formula for *X* used throughout the experimental period is expressed as Eq. (6):

$$X = \frac{X_0 X_m e^{-\mu t}}{X_m - X_0 + X_0 e^{-\mu t}} \quad (6)$$

where X_0 is the initial biomass concentration in the reactor (mg SS L^{-1}). Productivity is an important parameter to consider in the technology for cultivating microalgae, as it shows the capacity of a reactor to produce biomass under specific operating conditions

and defined as the biomass produced per reactor volume and per unit time. Equation 7 was used to calculate productivity of the alga in the sterilized culture.

$$\text{Productivity} = \frac{\mu(0.9 \cdot X_m - 1.1 \cdot X_0)}{\text{Ln}\left(\frac{9\left(X_m - 1.1 \cdot X_0\right)}{1.1 \cdot X_0}\right)} \quad (7)$$

Equation (8) was used to model the nutrient uptake. Substrate availability was related to its removal rate as previously proposed and used to model substrate concentration as a function of *t* time.

$$S = \frac{\left(\frac{X_0}{Y_0} + S_0\right)(S_0 - S_{na}) - S_{na}\left(S_0 - \left(\frac{X_0}{Y_0} + S_0\right)\right)e^{\mu t}}{(S_0 - S_{na}) - \left(S_0 - \left(\frac{X_0}{Y_0} + S_0\right)\right)e^{\mu t}} \quad (8)$$

S is the substrate concentration in mg SS L^{-1} at (*t*) time in units of days, and Y_0 is the initial microalgae coefficient. Y_0 represents the yield coefficient (SS mg N^{-1} or mg P^{-1} at day 0). Thus, $1/Y_0$ is the nutrient content of the biomass used for inoculation (mg N or P mg SS^{-1}). S_{na} and S_0 are the non-assimilated substrates and initial concentration of substrate (mg L^{-1}), respectively. Thus, the algal growth rate and nutrient removal were calculated based on these formulas. Consumption rate of the nutrients was calculated based on the Equation 9.

$$CR_{max} = \left(\frac{-ds}{dt}\right) \approx \left(\frac{-\Delta S}{\Delta t}\right)_{max} = \left(\frac{S_t - S_{t+\Delta t}}{\Delta t}\right)_{max} \quad (9)$$

Where CR_{max} is the maximum daily consumption rate of total substrates (mg L^{-1} d^{-1}), and *S* is substrate concentration of the substrates at a specific time *t* (mg L^{-1}).

Another important parameter to choose the suitable co-culture through the screening process was the harvestability test. The degree of self-sedimentation of the co-cultures was compared using the simple harvestability assessment. The experiments were conducted in capped 50 mL conical tubes. Briefly, 40 mL of the culture samples were added into the tubes. The tubes were shaken at maximum speed for 1.5 min. After that, all the tubes were shaken at 50 rpm for 20 min to allow flocculation. Another 20 min of sedimentation time was given for the flocs to settle as sediment before measuring their optical density at the wavelength 680 nm (OD_{680}) values. Sampling of the clarified water was done at three water levels. These levels were the 20-, 30-, and 40-ml depths of the liquid level, to obtain homogeneity of the water samples. The biomass removal efficiency was calculated using Equation (10):

$$\text{Removal Efficiency (\%)} = \left(1 - \frac{A_{680 \text{ in final condition}}}{A_{680 \text{ in initial condition}} } \right) \times 100\% \qquad (10)$$

Jar test has been known for simple coagulation test. However, in this study the coagulation-flocculation test was conducted in the smaller scale. To ensure that there was no bias in the data, the comparison between actual jar test and the tube test was demonstrated. Validation has been conducted using iron coagulant ($FeCl_3$) into the suspension of alga as previously described by Mennaa et al. (2015). Here, the result showed that there was no significant difference between the actual scale of jar test and tube test. Moreover, the results of the tube test from different depths also showed a similar removal, indicating the feasibility of the method (Supplementary Figure 5 and 6).

3.2.5 Microscopic observation of algal and co-culture interaction

After cultivation and self-sedimentation process in the previous section, three random sediments were chosen and prepared for observation under compound microscope or scanning electron microscopy (SEM). One of these two observations were chosen for each co-culture based on its characteristics. Light microscopy (Primo Star, Zeiss) was employed to observe the sediment or pellets in the filamentous actinomycete. Meanwhile, as the fungi contain more compact pellets and rarely released the filament as suspended solids, the pellet that contains the algae was observed. A freeze-drying method was chosen for sample preparation to observe the binding sites of algal cell walls to fungal filaments in the pellets since the freeze drying method can directly immobilize and stabilize the algal-fungal pellet specimens (Read, 1991). The preparation of sample using freeze drying was based on (Zhang et al., 2018).

Briefly, three pellets were prepared in a freeze dryer (Alpha 2-4 LSC, Christ) using a 0.011 mbar pressure with a shelf temperature of -20° C for 5 hrs. After drying, the pellets were sputter coated with gold (NeoCoater MP-19020NCTR) for 2 mins. Observations were done using an Auriga SEM (Zeiss) with magnifications ranging from 2500-10000x.

3.3 Application of co-culture in the raw wastewater

3.3.1 Batch kinetics of the co-culture

Application of raw CBEW treatment were conducted in the closed photobioreactor. It was aimed to examine the kinetics of the chosen co-culture. A simple reactor, made from the PET bottles. A 5 L working volume was used in all reactors with a 6 L total volume (Figure 3.2).

The reactors were equipped with an air stone for continuous aeration (2 L min^{-1}). Continuous light exposure was provided using a white LED 1950 lm lamp (Hi-Tek, Thailand) with an incident light intensity of 81 µmol m^{-2} s^{-1} measured using a Digital Lux Meter (Nicety LX 801). As much as 500 mL of inoculum of each culture was inoculated into 4.5 mL raw CBEW in the reactor.

Selected co-culture based on the scores in the sterilized wastewater experiments was applied in this reactor. The preparation was similar with the previous sterilized experiments, including the initial concentration of inoculants and the condition of the wastewater. Cultivation was conducted until the cultures achieved a stationary growth phase. Nitrogen, phosphate, and COD were monitored daily.

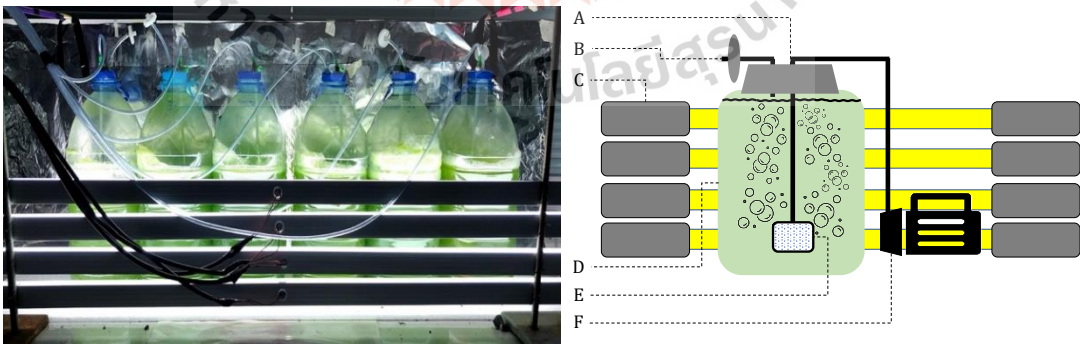

Figure 3.2 Reactor used in this study. A. Cap, B. Gas outlet with filter, C. Lamp, D. Tank, E. Bubble stone, and F. Pump.

3.3.2 Semi-continuous kinetics of the reactors

To further examine the stability of the co-culture, after reaching the stationary phase, co-culture was further assessed in the semi-continuous system. Semi-continuous system was chosen to avoid any sedimentation at the bottom of the reactors. Before the wastewater was collected from the reactors in the semi-continuous system, the main tanks were shaken adequately to ensure any sedimentation or attachment in the tank's wall removed. The system was also conducted in sterilized wastewater using 250 mL flask. The system was carried only for the most promising co-culture. This process was conducted using unsterilized (raw) wastewater after coarse particle removal by screening.

There were five hydraulic retention time (HRT)s that tested to the strains, with 20, 10, 5, 3.3, 2.5, and 2 days. The reactor was switched to semi-continuous by replacing the suspension in the reactor with the fresh wastewater that previously collected as much as the retention times requirement. The replacement of suspension was conducted daily. Based on the Verhulst kinetics model for the batch test (Equation 4), development of the model was made to suit the continuous mode (Ruiz et al., 2013). Briefly, the mass balance in the reactor is expressed in equation (11);

$$\frac{dX}{dt} = X_{in} - X_{out} + X_{product} \qquad (11)$$

where X is the biomass in (mg L^{-1}) and t is the time of measurements. After the reactor reached the steady state condition and the supply of wastewater into the reactors did not contain any significant amount of biomass, the equation (12) can be generated.

$$X_{out} = X_{product} \qquad (12)$$

After introducing the flowrate to the equation (10), equation (13) can be generated.

$$QX_{out} = V\left(\frac{dX}{dt}\right)_{reactor} \qquad (13)$$

Q is the flowrate (mL day^{-1}) and V is the volume of the reactor. Substituting the Verhuslt kinetic model in equation 9 to equation 4, the function of actual biomass to the growth rate (μ, day^{-1}) and maximum biomass (X_{max}, mg L^{-1}) is expressed in equation 14.

$$X = X_{max}\left(1 - \frac{1}{\mu t}\right) \tag{14}$$

Productivity is of the main parameters in this study. To calculate the productivity in semi-continuous system, equation 15 was employed.

$$P = \frac{X_{out}}{V} = \frac{QX}{V} = \frac{X}{t} \tag{15}$$

By combining equation 14 and 15, the equation 16 was generated.

$$P = X_{max}\left(t^{-1} - \frac{1}{\mu}t^{-2}\right) \tag{16}$$

In the equation 14, it is clear that the relationship of the growth rate and the maximum productivity can be determined. To obtain the maximum productivity (the highest productivity during same period of time), equation 17 was generated.

$$\frac{dP}{dt} = X_{max}\left(-t^{-2} - \frac{2}{\mu}t^{-3}\right) \tag{17}$$

Focusing on the optimum productivity, optimum HRT can be generated from equation 15. It is shown that the highest productivity can be achieved by combining the HRT and growth rate. The relation can be expressed in equation 18.

$$t_p = \frac{2}{\mu} \quad (18)$$

Based on this assumption, the growth of the microalgae in the raw wastewater treatment was measured. Later, the model was tested to observe the possibility of this model to be used in the co-culture inoculum. Similarly, the nutrient removals based on the steady state condition were also calculated as the function of the HRT.

3.4 Harvesting and Lipid Quantification

3.4.1 Harvestability of the cultivated co-culture

There are many harvesting methods available nowadays. However, coagulation-flocculation is one of the preferable harvesting methods in many recent studies (Mennaa et al., 2015; Oliveira et al., 2020). Harvestability of microalgae is one of the main bottlenecks to industrialized algal-based biofuel. Smaller scale coagulation and flocculation tests are feasible with limited amounts of culture and more replications in harvesting experiments (Mennaa et al., 2015). Here, a similar approach was employed to examine these four coagulants. Calcium chloride ($CaCl_2$), starch (S), iron (II) sulfate ($FeSO_4$) and ferric chloride ($FeCl_3$) at various level were used in this study to determine the harvestability of the cultures in a raw wastewater-based medium. These coagulants were compared with the self-sedimentation of the co-culture. Here, the main objective was to describe the effect of co-culture and the potency to replace the common coagulants that nowadays widely used. After cultivation, treated wastewater was separated from the biomass suspension by the sedimentation process. Comparison was made to be similar with the previous self-sedimentation process and the results were calculated using the equation 10.

3.4.2 Lipid and Composition of Fatty Acid Content

Oil content was measured at the end of the experiment. Up to 100 mL of the suspension was centrifuged (5000 rpm, 15 min) and the sediment was collected. Oil extraction was conducted by suspending the dried biomass in 15 mL of a 2:1 ratio (v/v) of dichloromethane:methanol. The suspension was sonicated for 15 min, using a cycle of 30 sec on followed by 30 sec off to ensure complete oil extraction. The dichloromethane/methane mixture was separated and centrifuged for 10 min at 8000 rpm. Solvent then evaporated at room temperature until the level of the residual solution (oil) was stable. Total lipid was measured using a simple carbonization method

based on chromatography. It was done by adding concentrated H_2SO_4 followed by carbonization at 200 °C for 15 min (Mercado et al., 2020).

The fatty acid profile was analyzed using the fatty acid methyl ester (FAME) method. Lipid from the previous extraction was boiled with a reagent consisting of a solution containing a 1:1 ratio of 15 g of NaOH in 100 mL methanol:water for 30 min. A methylation reagent (1:1.18 methanol:6 N HCl) was added to the solution at 80° C for 20 min. A 1:1 (v/v) solution of hexane:anhydrous diethyl ether was added after the second boiling period was finished and it cooled to ambient temperature decreased. The upper aqueous phase was removed and washed with 3 mL of a 1.2% (w/v) NaOH solution. FAME was measured using gas chromatography (Agilent 7890A; Agilent Technologies, Santa Clara, CA, USA), with a CP-Sil88 column for FA methyl esters (100 m x 0.25 mm, Chrompack, Inc., Middelburg, Netherlands). FAME properties were calculated based on a standard solution (a 37 component FAME mix, Supelco, Sigma-Aldrich). Further analyses of the degree of unsaturation (DU), kinematic viscosity (**U**i, $mm^2\ s^{-1}$), specific gravity (SG, kg^{-1}), cloud point (CP, °C), cetane number (CN), iodine value (IV, g I_2 100 g^{-1} oil) and higher heating value (HHV, MJ kg^{-1}) were conducted based on Oliveira et al. (2021). All the values are calculated from DU value that obtained based on Equation (19):

$$DU = \sum N \times Mf \tag{19}$$

where N is the number of carbon–carbon double bonds in fatty acid constituent and Mf is the mass fraction of fatty acid constituent. Based on DU value, several parameters in Eq (20-25) for assessing the biodiesel product quality were calculated.

$$\upsilon i = -0.6313\ DU + 5.2065 \tag{20}$$

$$SG = 0.0055\ DU + 0.8726 \tag{21}$$

$$CP = -13.356\ DU + 19.994 \tag{22}$$

$$CN = -6.6684\ DU + 62.876 \tag{23}$$

$$IV = 74.373\ DU + 12.71 \quad (24)$$

$$HHV = 1.7601\ DU + 38.534 \quad (25)$$

where υ i is kinematic viscosity, SG is specific gravity, CP is cloud point, CN is cetane number, IV is iodine value and HHV is the higher heating value.

3.5 Analysis of Energy Improvement

The analysis of energy improvement in this study was based on the extrapolation of the experimental data. The comparison data were retrieved from the previous databases previously demonstrated in Lardon et al. (2009). Briefly, the biomass production, total biomass, and the content of biomass per area of cultivation (or the volume employed for the cultivation to generate 1 kg of biodiesel stated as fatty acids methyl ester were calculated. As the basic data, *Chlorella vulgaris* that cultivated in the bench scale was the base of extrapolation. Nevertheless, the improvement only focused of the single culture and selected co-culture. This comparison aimed to avoid any bias from the different development and laboratory experiments between this study and the previous study.

3.6 Data analyses

All the experiments were conducted in triplicate. Comparison between the values was done using the Analysis of Variance (ANOVA) test with the Duncan Multiple Range Test (DMRT). The data are displayed as mean values ± standard deviations, with different letters indicating variation among these tests. Principal component analysis (PCA) was also conducted to clarify the extensive data set. These analyses were conducted using SPSS (IBM® SPSS® Statistics Version 20). The Microsoft Excel Solver Data Analysis Tool Pack was used to develop the growth rate and removal rates models.

CHAPTER 4
RESULTS AND DISCUSSION

4.1 Isolation and Screening of Co-culture

4.1.1 Characterization of the wastewater

Characterization of wastewater by measurements of crucial parameters was conducted to the wastewater treatment system (Table 4.1). Cassava biogas effluent wastewater (CBEW) was produced from the anaerobic digestion reactor, where the pulp and downstream waste of cassava starch is digested. The cassava starch production process also incorporates a considerable liquid (Watthier et al., 2019). After a sequence of processes, namely hydrolysis, acidogenesis, acetogenesis, and methanogenesis, most of the easily degradable contents are removed throughout the processes (Jiraprasertwong et al., 2019). Nevertheless, cell debris, remaining undegraded nutrients, and other components can still be found in the effluent.

Cassava biogas effluent is in agro-industrial waste that contains relatively low levels of nitrogen (N) and phosphorus (P), which is different from biogas process effluents that use animal manure as a substrate. These wastewaters from the animal manures contain high amounts of N and P (Tuszynska et al., 2020). Conversely, high chemical oxygen demand (COD) is usually found in this wastewater since the C/N ratios in agricultural crop wastes are higher than those in manure (Wandera et al., 2018). Relatively low nitrogen removal has been previously reported in biogas generation systems (Zeppilli et al., 2017). COD in these effluents may come from extracellular polymeric substances and residual COD from the influent, up to 92% removed (Jiraprasertwong et al., 2019) from materials with initial COD concentrations 4200-7000 mg L^{-1} (Colin et al., 2007). Nutrients in these effluents remain because biogas generation does not effectively remove phosphorus (Lin et al., 2015). Thus, these parameters may result in effluents unsuitable for direct discharge to the environment before further treatment. Several studies have reported levels of phosphate removal in terms of total phosphorous and orthophosphate in the wastewater (Tuszynska et al., 2020).

Table 4.1 Critical parameters of cassava biogas effluent wastewater.

Parameters	Value	Discard Standard	Units
COD	205 ± 12.3	<120	mg L^{-1}
BOD	75 ± 9.52	<20	mg L^{-1}
TP	37.26 ± 2.05	<6	mg L^{-1}
PO_4-P	23.53 ± 1.70	-	mg L^{-1}
TKN	54.1 ± 3.21	<10	mg L^{-1}
NO_2	0.08 ± 0.02	-	mg L^{-1}
NO_3	16.43 ± 0.69	-	mg L^{-1}
NH_4	31.24 ± 1.67	<10	mg L^{-1}
pH	7.6 ± 0.03	5.5-9.0	-
DO	3.21 ± 2.4	>3	mg L^{-1}
Conductivity	2699 ± 43.60	-	mS cm^{-1}
Alkalinity	700 ± 32.57	-	mg L^{-1} as $CaCO_3$
Salinity	2.2 ± 1.3	<5	parts per thousand (PPT)

Standard values are generated from Kayode et al. (2018).

Based on the wastewater characteristics, several parameters still need to be improved. Ammonium (NH_4), total nitrogen (TN), chemical oxygen demand and biological oxygen demand (COD & BOD), and total phosphate (TP) was still above the standard discharge. Nevertheless, several parameters have already fulfilled the standards of wastewater discharge in Thailand based on Kayode et al. (2018), namely pH, dissolved oxygen, and salinity.

Nitrogen content in CBEW was still higher than the allowable level. Most nitrogen species were found in the inorganic forms (NH_4 and NO_3). This situation can lead to detrimental effects on the ecosystem and human health (Fulazzaky et al., 2015). However, the low organic nitrogen in the system indicates the removal of organic matter in the system was sufficiently achieved (Krasner et al., 2009). The state of the amino nitrogen in the system as the subtraction result of TKN and inorganic nitrogen species was low and still acceptable. However, discarding this actual CBEW into the environment would need a large amount of non-polluted water to dilute it. Based on this result, the nitrogen treatment in the autotrophic condition would be suitable for removing the excess inorganic nitrogen.

Similarly, total phosphorus was found in significantly higher concentrations than the standard. Interestingly, most of the portion of phosphorus was in the form of orthophosphate than the polyphosphate. It is common to have orthophosphate, the simplest form of phosphate, in the effluent of aerobic/anaerobic treatments since polyphosphate are usually utilized in the biological process than mainly under heterotrophic condition (Fuhs & Chen, 1975). The abundance of orthophosphate (PO_4) in CBEW required treatment with the sufficient intake of this simple phosphate. It is also important to note that removing phosphate in this form can be slower and more complex than nitrogen (Chevalier & de la Noüe, 1985).

The organic matters expressed as COD and BOD values were higher than the expected standards. However, the concentrations of these parameters were relatively low compared to other parameters. Organic matters are often effectively utilized in biogas generation from cassava waste and wastewater. Although anaerobic digestion of the waste widely needs organic carbon sources, nitrogen and phosphorous in inorganic forms are still essential to complement the ratio of the substrate during the process (Lin et al., 2021). However, as the excess amount of TN and TP in the influent created a low C:N:P ratio, the effluent of the biogas process contained low organic carbon yet high inorganic nutrient contents. Nevertheless, the contents have still exceeded the limit. A large removal of COD was still needed for this particular CBEW. Based on these results, algal treatment is concluded as a suitable treatment for CBEW.

4.1.2 Algae isolation and screening

Microalgae have been reported to be versatile and practical remediation agents in wastewater treatment (Guldhe et al., 2017; Xie et al., 2018). Both engineered, and indigenous strains of microalgae can be used. Each has its advantages and disadvantages. Engineered strains have been developed throughout long and rigorous processes. Thus, oil production and substrate intake can be remarkably high. A mixotrophic-engineered strain that overexpresses endogenous RuBisCO activase to produce a high amount of lipid by genetic engineering of microalgae for this particular objective has recently been achieved (Benedetti et al., 2018). Nevertheless, the compatibility of the strains with use in wastewater is not high.

Conversely, isolated strains are well suited to the conditions of wastewater, which can change dramatically over a short period (Wollmann et al., 2019). Several isolated strains from extreme environments have been reported to possess high lipid contents (Sadvakasova et al., 2019). Similarly, it has been found that isolating strains from an environment where the substrate or similar environment occurs, e.g., wastewater is a promising way to obtain highly tolerant and adaptable strains (Cheng et al., 2019). To achieve high removal efficiency of nutrients in wastewater, getting candidate algal strain from a similar environment is among the most promising methods (Cui et al., 2020; Pandey et al., 2019). Numerous strains have been isolated from various sources of wastewater. Among the isolated, the *Chlorella* genus stands as one of the naturally occurred strains in the wastewater (Choi et al., 2018; Ferro et al., 2018). These strains are widely known for their fast growth rate, a wide range of substrate utilization, and high nutrient removals (Eladel et al., 2019; Tait et al., 2019; Wen et al., 2017).

Table 4.2 Isolated microalgae strain from the area around a CBEW pond and its growth on several carbon sources.

No	Strain name	Carbon source				
		Glucose	Sucrose	Fructose	Mannitol	Galactose
1	WB1DG	+++	+++	+++	+	++
2	P 21	+++	++	++	+	+++
3	BP 3	+++	+++	++	-	+++
4	RS 1	+++	+++	+++	-	++
5	RS 2	+++	+++	++	-	+
6	*RS 3*	+++	+++	++	-	+
7	RS 4	+++	+++	++	-	+
8	RS 5	+++	+++	++	-	+
9	RS 6	+++	+++	++	-	-
10	P21 TCB	+	++	+	-	+
11	BET 1 F	-	-	-	-	-
12	LD 1	-	-	-	-	-
13	NL 1	-	-	-	-	-
14	NL 2	-	-	-	-	-

Table 4.2 Isolated microalgae strain from the area around a CBEW pond and its growth on several carbon sources. (Continued)

No	Strain name	Carbon source				
		Glucose	Sucrose	Fructose	Mannitol	Galactose
15	PDG 1	-	-	-	-	-
16	P11	-	-	-	-	-
17	PRO 1	-	-	-	-	-
18	RCA 2	-	-	-	-	-
19	RCB 1	-	-	-	-	-
20	RCO 3	-	-	-	-	-
21	SW 1	-	-	-	-	-
22	UN 1	-	-	-	-	-
23	CEB 1	-	-	-	-	-
24	SP22	-	-	-	-	-

- means no growth (Initial A_{680} - A_{680} Final <= 0)), + = low growth (Initial A_{680} - A_{680} Final = 0 - 0.25), ++ means normal growth (Initial A_{680} - A_{680} Final = 0.25-0.05), and +++ means abundant growth (Initial A_{680} - A_{680} Final > 0.5)

It is well-established that indigenous strains of wastewater have shown remarkable removal activity in vast niches compensating for the environmental conditions of the wastewater. Thus, proper application of these indigenous strains can fasten their application with a minor adjustment for commercial applications. Alternatively, the introduced strain will first need to have the required properties such as fast growth, efficient removal of wastewater nutrients, adaptation to significant changes of environmental conditions, and capability to grow simultaneously with indigenous bacteria (Wang et al., 2016). Here, isolation of indigenous microalgae was demonstrated. Twenty-four different strains were isolated based on their morphology from six sites around wastewater effluent treatment systems. The early separation and labeling of microalgae were based on their presence at several sampling sites and morphological characteristics. After obtaining pure cultures, all strains were tested for growth under mixotrophic conditions. Only ten strains could successfully grow under mixotrophic conditions from all the isolated strains. They grew optimally using the organic carbon sources present and in the absence of light (Table 4.2). Among the strains, P21 and WB1DG were found to grow using all of the carbon sources tested in this study.

Identification of several district strains has been conducted. Phylogenetic tree construction was also used to confirm the BLAST strain classification to conserve the genome and draw relationships among the isolated strains or represented traits (Ferro et al., 2018). In this study, 18s rRNA was selected to analyze the phylogenetic traits of strains chosen based on their ability to utilize carbon sources. This approach is widely used to identify green microalgae, and it has been employed to identify the species level of microalgae (Friedl, 1997; Zou et al., 2018). Nucleotide sequences from reference *Chlorella* spp., *Miractinium* spp., *Dictyosphaerium* spp., and *Chlorellaceae* spp. were used to construct a phylogenetic tree to determine the relationships of microalgae strains among the members of the Chlorellaceae. Several strains of *Scenedesmus* spp. and *Symbiochloris pauciautosporica* were chosen as an outgroup of the root of the phylogenetic tree (Figure 4.1).

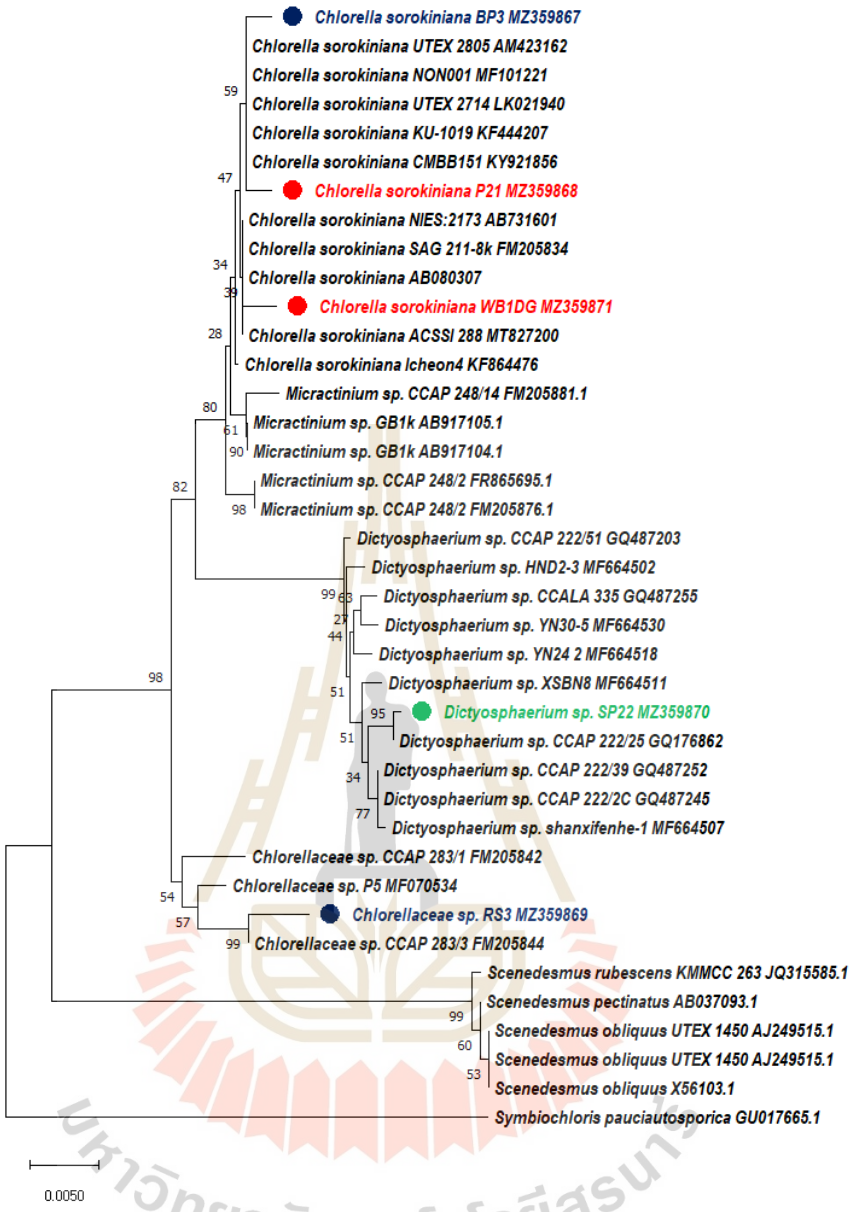

Figure 4.1 Phylogenetic tree of represented strains with various levels of organic carbon utilization. The colors of the dots indicate high (●), moderate (●), and no carbon utilization (●). The tree was constructed using the neighbor-joining method in MEGA X software using values based on 1000 replications. The bar at the lower left of the figure indicates the substitution length per site.

Based on the 18s rRNA gene sequence similarity, it has been found that the chosen strains have the capability to use organic carbon. These strains are distributed among several genera of the Chlorellaceae family. The high carbon utilizing strains, WB1DG (MZ35987) and P21 (MZ359868), are *Chlorella sorokiniana* species. Furthermore, organisms of moderate organic carbon source utilization include RS 3 (MZ359869) of the Chlorellaceae family, while BP 3 (MZ359867) is *C. sorokiniana*. Last, SP22 (MZ359870) as the representative strain with no organic carbon utilization is a *Dictyosphaerium* sp. Nevertheless, there is no substantial evidence to support the hypothesis that carbon utilization capability is based on evolutionary traits as SP 22 (no carbon utilization strain) was closer to the two representatives of high carbon utilizing strains (WB1DG and P21) while in *C. sorokiniana*, is among the moderate and high carbon utilization algae.

Further experiments focused on the P21 and WB1DG strains based on their carbon utilization results. Morphological observation revealed that the P21 and WB1DG strains have round and unicellular shapes (Figure 4.2), where WB1DG was found to be larger (4-6 µm) than P21 strain (3-5 µm). Isolation of microalgae in wastewater for bioremediation has been done for years. However, not all of the strains are suitable for the mixotrophic conditions in which removal for certain nutrients can be expected to be optimal. However, similar isolations from different kinds of wastewater have resulted in the isolation of various strains of this species. *Chlorella* has been widely recognized as one of the most common microalgae used to treat wastewater (Cui et al., 2020). *C. sorokiniana* has been successfully isolated from many sources of wastewater, such as in palm oil mill wastewater (Ding et al., 2020), secondary effluent of municipal wastewater (Eladel et al., 2019), dairy wastewater (Asadi et al., 2020), chicken farm flushing wastewater (Cui et al., 2020) and urban wastewater (Izadpanah et al., 2018).

C. sorokiniana has been reported to have the ability to grow using various carbon sources in mixotrophic culture (Cecchin et al., 2018; León-Vaz et al., 2019). The ability to grow under mixotrophic conditions is essential to achieve higher biomass yields than possible under autotrophic conditions. However, various structures of organic carbon can affect the structure and composition of *C. sorokiniana* (Chai et al., 2018). Glucose, sucrose, fructose, mannitol, and galactose are among the small and easily degraded carbon molecules for microbial growth under mixotrophic and heterotrophic conditions. The ability of the microalgae to grow and utilize various carbon sources is expected to be a function of the carbon sources utilized by microalgae cells (Perez-Garcia & Bashan, 2015).

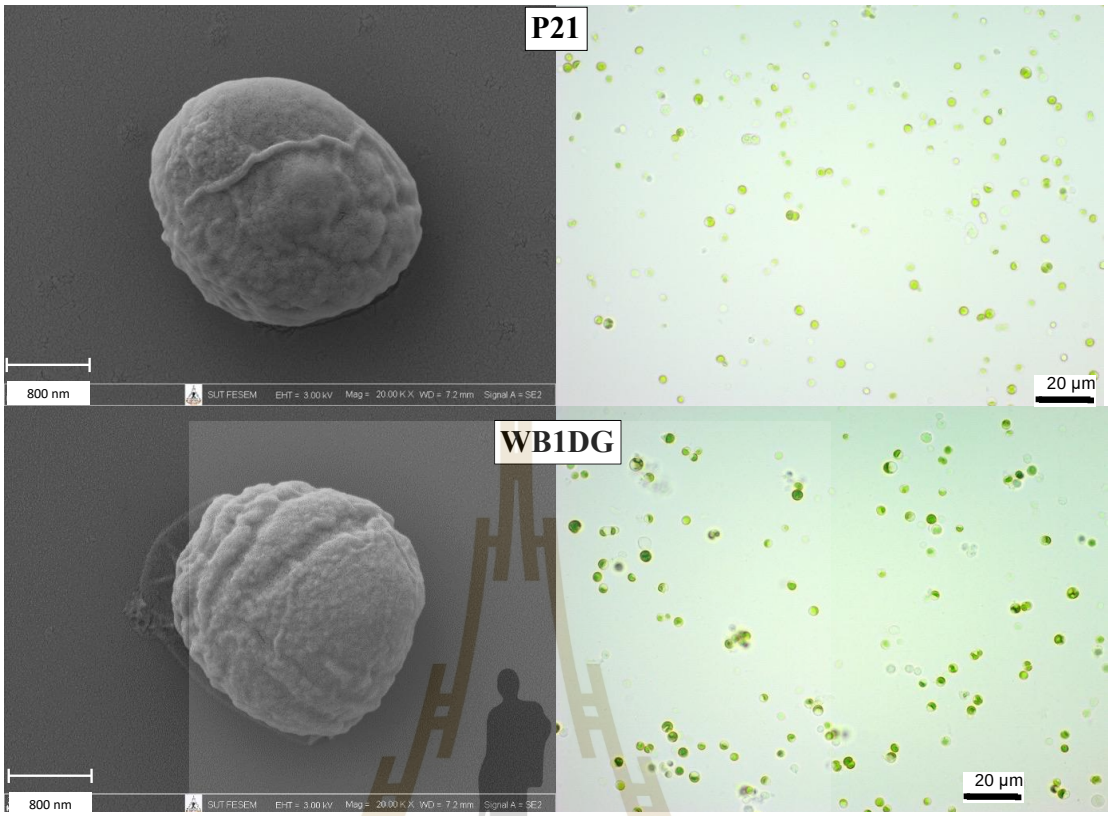

Figure 4.2 *Chlorella sorokiniana* strains P21 and WB1DG. left: SEM micrographs images, right: light microscopy.

Natural communities of microalgae were also taken from the wastewater. This community was often used to compare the microalgae treatments system with the native strains. Several algal communities were obtained from various sedimentation ponds for biogas effluent wastewater. During the sampling period, the four main ponds (P1 through P4), which exhibited a greenish color, were chosen as the source of the inoculums (Figure 3.1). The algal communities from the different dilution ponds in the CBEW treatment system were collected. Four algal community suspensions were obtained from four various lagoons. The lagoons were chosen based on their availability during the sampling periods. Apart from the abundance of microalgae in the selected lagoons, the composition of the microalgae in the algal bloom in unsterilized CBEW was also different (CTRL) (Figure 4.3). Similarly, the natural bloom from CBEW wastewater also showed similar composition (Figure 4.3a). In the Pond 1 bloom (P1), *Chlorella* spp., *Scenedesmus* spp., and *Pseudopediastrum* spp. were the dominant algae species (Figure 4.3b). In contrast, a *Chlorella* sp. was

dominant in other inoculums, namely algal community in pond 2 (P2), algal community in pond 3 (P3), algal community in pond 4 (P4), and CTRL (Fig. 4.3c, d, and e).

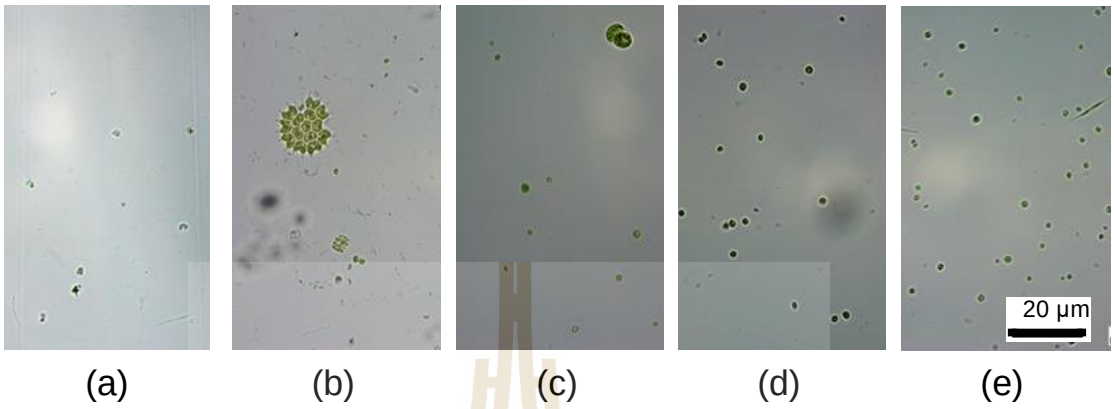

Figure 4.3 Microalgae community compositions from natural bloom (a) and ponds around cassava biogas effluent wastewater ponds P1, P2, P3, and P4 (b-e).

Commercial strains were also purchased from the Thailand Institute of Scientific and Technological Research Culture Collection, namely *Chlorella* sp., TISTR 8411, *Coelastrum* sp. TISTR 8508, *Scenedesmus obliquus* TISTR 8522, *Chlorella vulgaris* TISTR 8580, and *Chloroccocum* sp. TISTR 8583 to comprehensively study the possibility of all combinations. Microscopic observation showed that the algae were available in single-cell forms and did not form any floc or colony in the basal medium (BG 11) (Figure 4.4). Furthermore, the comparison of the native strains, commercial strains, and natural blooms was conducted to obtain the most suitable strains for the CBEW treatments.

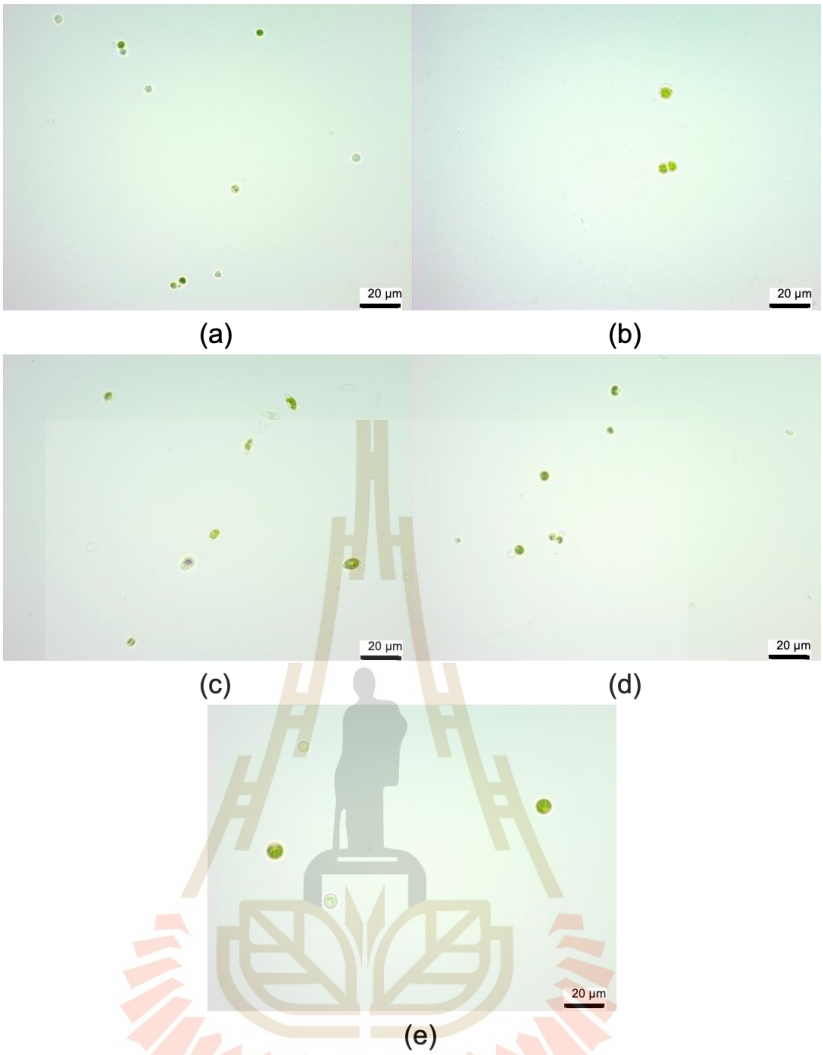

Figure 4.4 Commercial strains for CBEW treatment. (a) *Chlorella* sp., TISTR 8411, (b) *Coelastrum* sp. TISTR 8508, (c) *Scenedesmus obliquus* TISTR 8522, (d) *Chlorella vulgaris* TISTR 8580 (e) *Chloroccocum* sp. TISTR 8583.

All the inoculants were prepared in a similar condition before being applied to the sterilized wastewater. The growth of inoculates was in BG 11 medium until they reached the exponential phase. The washing and resuspension of the inoculate in DI water avoided the additional nutrients of the inoculates into the wastewater. The condition of wastewater was also slightly different after sterilization since an autoclave was used to create the sterile condition. The heat and pressure from the process changed and removed several nutrients that may affect the growth of the algae. The sterilized wastewater showed reduced media content to 191 ± 1.37 mg L^{-1} of COD, 22.53 ± 1.57 mg L^{-1} of PO_4, 40.15 ± 3.65mg L^{-1} of TN, 16.03 ± 1.25 mg L^{-1} of NO_3, and 25.47 ± 6.15 mg L^{-1} of TP, the pH range was 7.2 ± 1.08 while that of DO was 3.5 ± 0.17 mg L^{-1}.

All the inoculants were shown to grow in the sterilized wastewater after 13 days of algal cultivation (Figure 4.5). The period was determined after all the algal inoculants reached a stationary phase. The growth of the inoculants consisted of acclimatization in the lag phase, exponentially growing in the log phase, and linear growth at the early stationary phase (Lee et al., 2015). However, a relatively short lag phase was demonstrated by all algal inoculates, except for the control. It indicates the inoculates showed excellent adaptation in the sterilized wastewater. Differently, natural bloom in this study did not show exceptional growth when applied back to the bloom's origin. As the cultures were conducted in axenic conditions, the occurrence of bacteria was supposedly removed. Thus, as most natural blooms depend on their interaction between bacteria and other microbial communities in the wastewater, the axenic condition negatively affects the growth of natural bloom (Su et al., 2012).

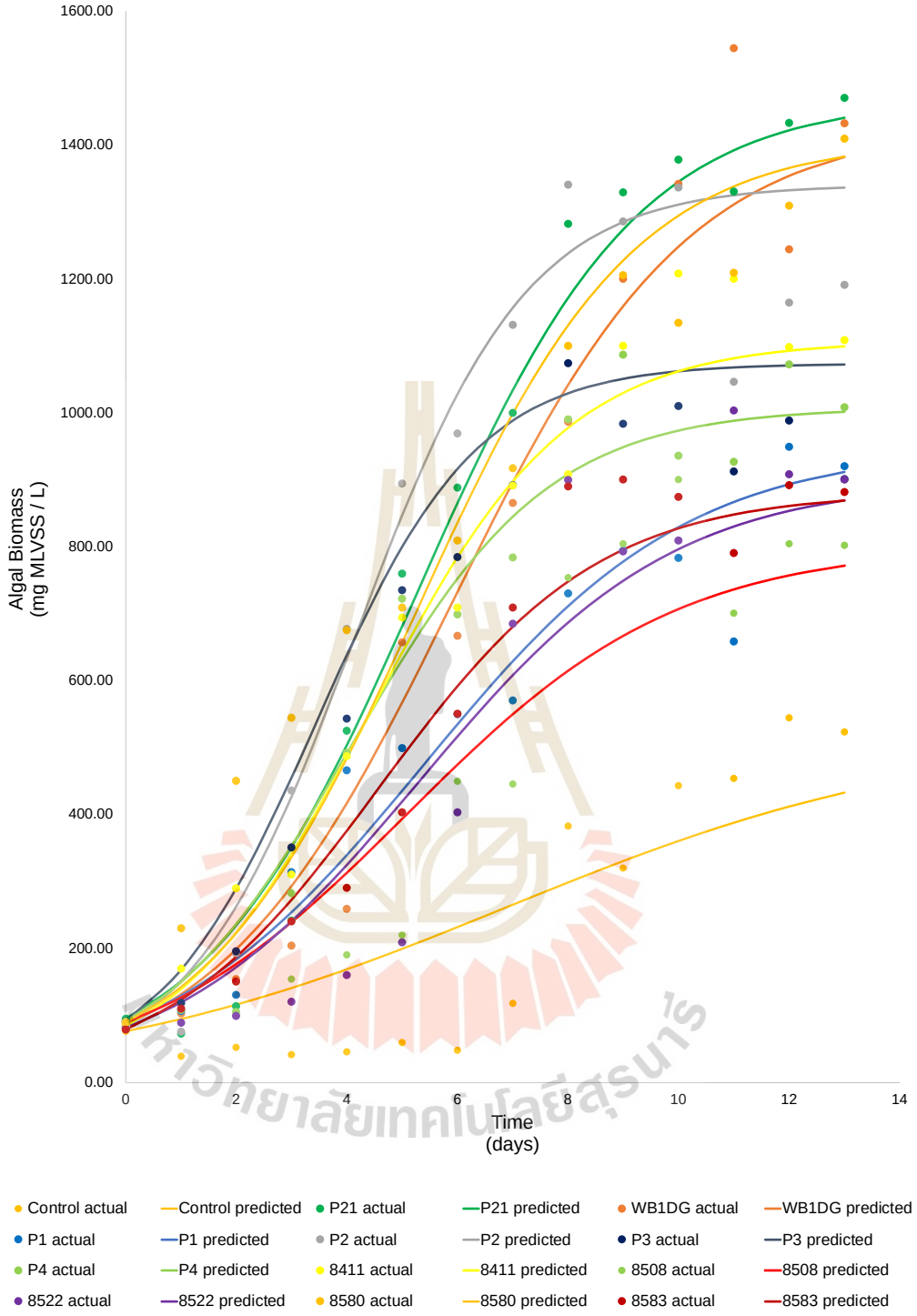

Figure 4.5 Biomass evolution in the wastewater with different inoculates of algae. Dots indicate the actual value from the measurements in the mean values, while straight lines are the predicted values using Verhulst biomass growth kinetics.

Based on the Verhulst kinetics model, several values were calculated to describe the inoculants' performances (Table 4.3) comprehensively. It was found that the P21 and 8580 strains showed the highest biomass production. However, the growth rates of P2 and P3 were found to be higher than other results. It indicates that these inoculants could grow in the CBEW conditions compared with the single isolated strains.

The isolated, natural communities and commercial strains showed relatively moderate growth in the medium than the previous study with similar conditions (Menna et al, 2015., Ruiz et al, 2013, & Leong et al 2018). However, most of the studies previously conducted were in the unsterilized condition. Mennaa et al. (2015) reported the evolution of *Chlorella vulgaris* (SAG 211-12), *C. kessleri* (SAG 211-11), *C. sorokiniana* (SAG 211-8k), *B. braunii* (SAG 30.81), *S. obliquus* (SAG 276-10) and *A. falcatus* (SAG-202-2), and *N. oleabundans* (UTEX-1185) showed growth rates of 0.10 - 0.52 day^{-1} in the urban wastewater. Differently, Ruiz et al. (2013b) reported the specific growth rates 0.78-0.38 day^{-1} of *C. vulgaris* secondary effluent of a conventional wastewater treatment plant with low carbon yet high ammonium and phosphorus. Domestic wastewater treated with the consortium of microalgae was also reported a growth rate of 0.74 day^{-1} without any other microbial growth and increased significantly to be 1.59-3.64 day^{-1} in the presence of different activated sludge (Leong et al., 2018).

The absence of a synergistic community in the system supports the evidence that the algal growth in the wastewater can be very dependent on the co-inoculants. Axenic condition of microalgae has been previously reported to show a relatively low growth rate and eventually affect the maximum biomass production (Halfhide et al., 2014). Another proposed reason for the algae's wide range of specific growth rates was the possibility of additional acclimatization (Japar et al., 2021). Nevertheless, the significant differences between algal communities (P2 & P3) and single cultures (P21) may indicate that these communities were well-adapted to similar environments.

Table 4.3 Kinetics parameters of all the inoculants in the sterilized wastewater.

Parameters	Inoculates			
	Control	P21	WB1DG	P1
X_0 (mg L^{-1})	76.8 ±17.2^{na}	95.5±20.6 ^{na}	85.4±35.7 ^{na}	89.5±20.7 ^{na}
X_m (mg L^{-1})	432.9±50.3^{d}	1440.9±61.2^{a}	1382.3±12.8^{a}	911.9±134.0^{bc}
R^2	0.85±0.1	0.99±.0.0	0.97±0.0	0.93±0.0
μ (day^{-1})	0.26±0.0^{d}	0.50±0.0^{b}	0.47±0.0^{bc}	0.42±0.0^{c}
P (mg $L^{-1}day^{-1}$)	21.59±1.63^{c}	126.79±5.43^{a}	111.59±9.53^{ab}	70.35±16.8^{bc}
CR-COD (mg $L^{-1}day^{-1}$)	2.39±1.68^{c}	8.02±2.68^{a}	5.7±0.92^{ab}	3.74±0.54^{bc}
CR-N (mg $L^{-1}day^{-1}$)	0.07±0.03^{c}	0.18±0.03^{a}	0.14±0.06^{abc}	0.08±0.02^{abc}
CR-P (mg $L^{-1}day^{-1}$)	0.84±0.25^{b}	0.71±0.03^{a}	0.67±0.05^{ab}	0.75±0.04^{ab}
Parameters	**Inoculates**			
	P2	**P3**	**P4**	**8411**
X_0 (mg L^{-1})	83.6±6.6 ^{na}	91.8±11.6 ^{na}	91.4±9.3 ^{na}	84.0±10.4 ^{na}
X_m (mg L^{-1})	1336.6±119.2^{ab}	1072.5±65.6^{ab}	1001.6±68.1^{b}	1099.6±58.6^{ab}
R^2	0.96±0.0	0.98±0.0	0.97±0.2	0.98±0.0
μ (day^{-1})	0.65±0.0^{a}	0.69±0.0^{a}	0.56±0.0^{b}	0.56±0.0^{b}
P (mg $L^{-1}day^{-1}$)	150.37±12.0^{a}	133.40±49.8^{a}	102.94±14.5^{b}	110.40±17.4^{ab}
CR-COD (mg $L^{-1}day^{-1}$)	2.70±0.53^{c}	6.14±0.32^{abc}	4.98±0.26^{bc}	5.41±0.74^{bc}
CR-N (mg $L^{-1}day^{-1}$)	0.07±0.03^{ab}	0.18±0.03^{abc}	0.07±0.03^{bc}	0.18±0.03^{abc}
CR-P (mg $L^{-1}day^{-1}$)	0.22±0.09^{a}	0.12±0.10^{ab}	0.84±0.25^{ab}	0.71±0.03^{ab}
Parameters	**Inoculates**			
	8508	**8522**	**8580**	**8583**
X_0 (mg L^{-1})	89.3±31.1 ^{na}	81.3±9.8 ^{na}	89.3±19.0 ^{na}	79.3±35.7 ^{na}
X_m (mg L^{-1})	771.8±81.2^{c}	869.8±79.3^{c}	1382.9±119.6^{a}	869.14±46.7^{c}
R^2	0.91±0.1	0.93±0.0	0.96±0.0	0.97±0.0
μ (day^{-1})	0.41±0.0^{c}	0.43±0.0^{c}	0.51±0.0^{b}	0.50±0.0^{b}
P (mg $L^{-1}day^{-1}$)	59.02±12.3^{bc}	69.08±25.0^{bc}	123.04±7.3^{a}	79.93±5.0^{bc}
CR-COD (mg $L^{-1}day^{-1}$)	6.54±0.27^{ab}	4.92±0.24^{b}	7.00±0.04^{ab}	4.72±0.51^{bc}
CR-N (mg $L^{-1}day^{-1}$)	0.14±0.06^{abc}	0.08±0.02^{abc}	0.21±0.00^{ab}	0.08±0.02^{abc}
CR-P (mg $L^{-1}day^{-1}$)	0.67±0.05^{ab}	0.75±0.04^{ab}	0.82±0.07^{ab}	0.72±0.03^{ab}

X_0=initial biomass, X_m=maximum biomass production, μ=specific growth rate, P=biomass productivity, and CR=consumption rate of nutrients. Values are generated from fitting the triplicate results into one single Verhulst model growth line. Different letters indicate significant different results of the DMRT test (*α*=0.5, n=3).

Later, they only needed slight changes before entering the exponential phase. However, P21 and 8580 maximum biomass yields were still higher than these algal communities, showing their potential to adapt further and eventually reach the industrial system (Hernández et al., 2013). It is noteworthy that even though the biomass productivity of the P2 and P3 communities were higher than in the P21 and 8580 single strains, the other parameters resulted differently where the P21 and 8580 possessed higher scores. Nevertheless, the implication of algae single culture of the co-culture construction using well-adaptable strains was more suitable to control the phenomena of interactions with advantageous results in removal efficiency and the nutrient removals. Although the biomass productivity and growth rate of the single cultures P21 and 8580 were lower than P2 and P3 alga communities, the development of these strains was suitable for the co-culture by considering the removal activity and maximum biomass production.

Wastewater characteristics and nutrient removals were examined at the end of the cultivation period. Several results of the consumption rate based on the Verhulst kinetics were also showed in Table 4.3. Here, only three main parameters were observed. Characterization of the wastewater on a daily basis was discussed in the co-culture examination. Removal efficiencies of all nutrients from all the wastewater showed different responses. COD removal in the wastewater showed a possibility of removal using single strains (Figure 4.6). The highest reduction of COD was established in P21 and 8580. Removal of COD by the communities was significantly lower than other inoculants. The highest removal was only found in the wastewater treated by P3 inoculant. Meanwhile, the P2 inoculant, one of the highest biomass productions, had a very low removal (similar to CTRL).

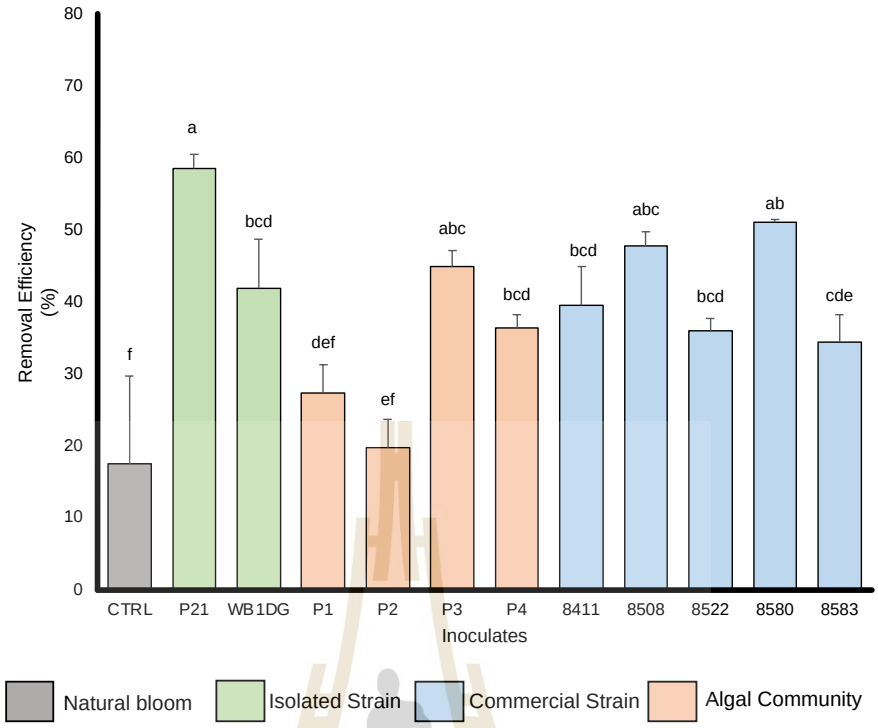

Figure 4.6 Chemical oxygen demand (COD) removal efficiency of inoculates in sterilized CBEW. Different letters indicate significant differences using DMRT (α=0.5, n=3).

COD represents the oxygen needed for carbon oxidization in the wastewater. It can be reflected as the carbon source for completing the required reactions for nutrient removal (Mujtaba et al., 2017). Here, the consumption of the COD was related to the wastewater organic content removal. Thus, the removal was more efficient in the mixotroph or heterotroph conditions, where the consumption of organic carbon is necessary for carbon uptake. A combination of the organic carbon utilization, growth rate, and total biomass from the algal communities was not observed in these algal communities (Venkata Subhash et al., 2014). Thus, the autotrophic condition might be the primary nutrient utilization in these communities. The high efficiencies showed by the single strains, especially in P21 and 8580, indicate that the mixotrophic ability in the early screening process of P21 was in line with the sterilized wastewater removal abilities. Similarly, microalga 8580 was previously reported to have sufficient capacity to grow under mixotrophic conditions (Yahampath et al., 2021).

Removal of nitrogen was also measured. Differently, the inorganic nitrogen was primarily removed throughout the autotroph condition. However, one of the main requirements for nitrogen removal in the state of nitrogen removal is the sufficient amount of CO_2 in the medium (Lee & Lee, 2002). The inorganic nitrogen was stated in the nitrate concentration rather than ammonium and nitrate (Lv et al., 2019; Wang & Lan, 2011). The nitrogenous nitrate was also reported to be the preferable nitrogen form for intake by the algae (Arumugam et al., 2013). Thus, the removal process only needed an efficiency of 30 % to fulfill the standard discharge (below 10 mg L^{-1}).

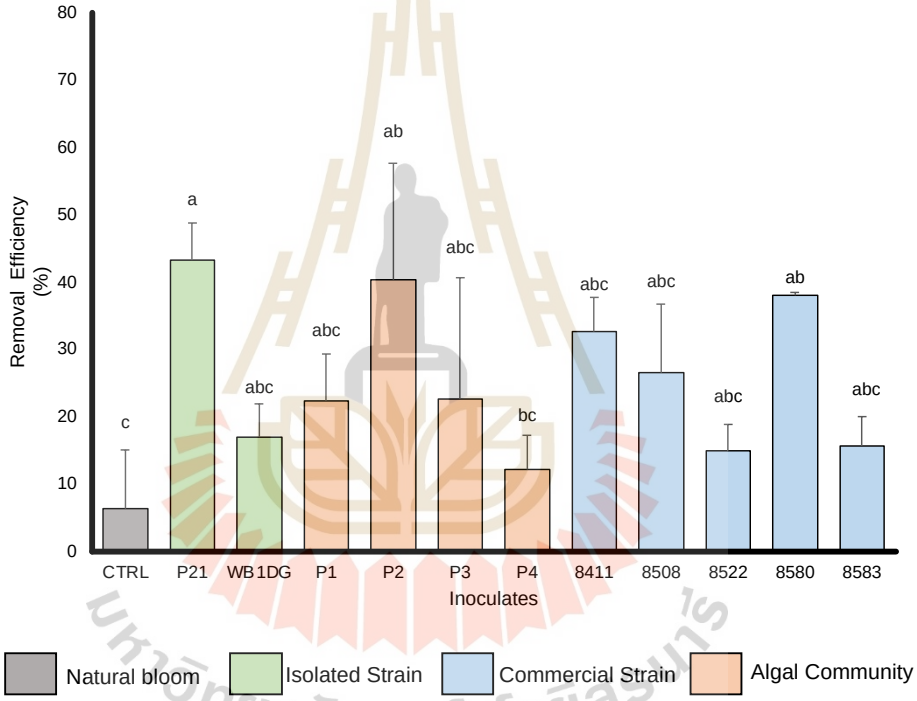

Figure 4.7 The nitrogen removal efficiency of inoculates in sterilized CBEW. Different letters indicate significant differences using DMRT (*a*=0.5, n=3).

It was shown that some of the inoculants could achieve the removal of >30 % (Figure 4.7). Algal community P2 and single inoculants of P21 and 8580 were found to have the highest removal efficiency in nitrate concentration. Meanwhile, single strains WB1DG, 8522, and 8583, together with the community P1, P3, and P4, removed less than 30 % nitrate concentration in the wastewater. Different inoculants respond differently to the nitrate concentration in the environment. While most media

contain nitrate as the nitrogen source, an insufficient amount of nitrate may lead to slow growth of microalgae but with high valuable compounds such as lipid or other metabolites (Xie et al., 2017). As the algal inoculants may utilize the nutrient under the mixotrophic condition as their main mechanisms, others may act autotrophic, leading to the state where biomass production is not related to some nutrient parameters (Seip, 1994).

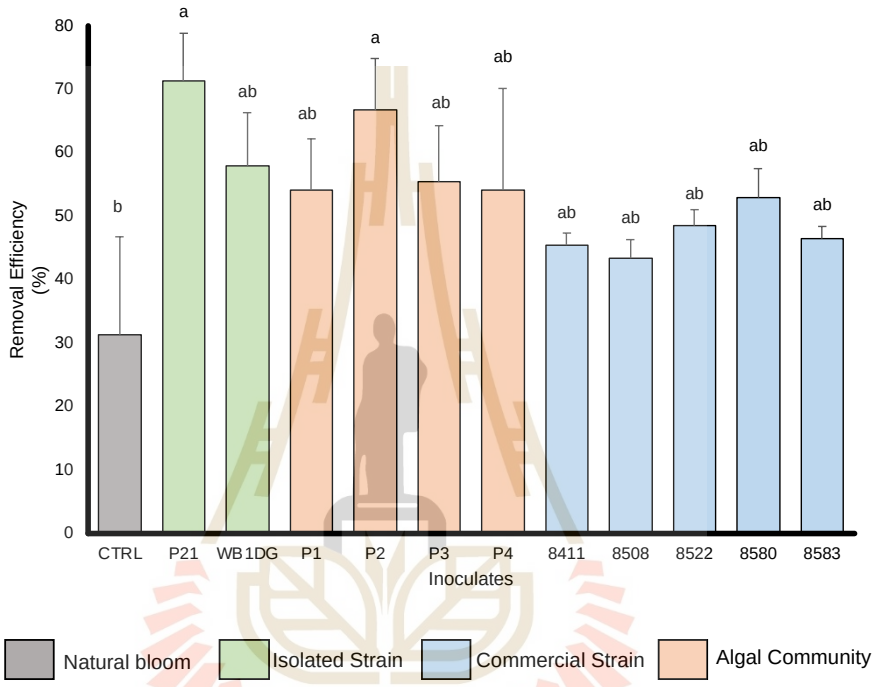

Figure 4.8 Phosphate (removal efficiency of inoculates in sterilized CBEW. Different letters indicate significant differences using DMRT (*α*=0.5, n=3).

Phosphate concentration achieved higher overall removal efficiency than the other two parameters. The highest efficiency was recorded in P21, WB1DG, P2, P3, P4, and 8580 inoculates. Although phosphate is the molecule that is necessary for a lesser amount than nitrogen or organic carbon (in heterotroph or mixotroph conditions), the removal of phosphate by the algae can be incredibly high due to the various available mechanisms of the phosphate intake by the algae (Whitton et al., 2016).

Biomass generation, growth rate, and nutrient removal are crucial parameters for developing algal-based wastewater treatments. Thus, comparing the results from these parameters needs similar bases. A simple normalization to

summarize the parameters results has been demonstrated by dividing the actual value of each seed with a maximum value obtained from all inoculants (Mennaa et al., 2015). Table 4.4 revealed *Chlorella sorokiniana* strain P21 and *C. vulgaris* TISTR 8580 were the most feasible inoculants for wastewater treatments based on the total normalization values.

Table 4.4 Phytoremediation scoring of various seeds of microalgae comparing several parameters.

Normalized Values	Inoculants					
	Control	P21	WB1DG	P1	P2	P3
N-X_m	0.30	1.00	0.96	0.63	0.93	0.74
N-μ	0.37	0.73	0.68	0.61	0.94	1.00
N-COD	0.30	1.00	0.72	0.47	0.34	0.77
N-NO_3	0.15	1.00	0.39	0.52	0.93	0.52
N-PO_4	0.44	1.00	0.81	0.76	0.94	0.78
Total	1.56	4.73	3.56	2.98	4.08	3.81
Normalized Values	**Inoculants**					
	P4	**8411**	**8508**	**8522**	**8580**	**8583**
N-X_m	0.70	0.76	0.54	0.60	0.96	0.60
N-μ	0.82	0.82	0.59	0.63	0.74	0.73
N-COD	0.62	0.67	0.81	0.61	0.87	0.59
N-NO_3	0.28	0.75	0.61	0.34	0.88	0.36
N-PO_4	0.76	0.64	0.61	0.68	0.74	0.65
Total	3.17	3.65	3.16	2.87	4.19	2.94

N (normalization), X_m (maximum biomass production), NO_3 (Nitrate removal efficiency), PO_4 (Phosphate removal efficiency), μ (Specific growth rate). All data are generated from the mean values in previous parameters. Normalization is based on the actual value divided by the maximum value in each parameter.

4.1.3 Actinomycete Isolation and Screening

Twenty-two isolates of actinomycetes were obtained from the isolation process based on their morphological appearance (Supplementary Figure 7). Apart from the filamentous morphology in most actinomycetes, the durability of actinomycetes to grow under many circumstances is one of the essential reasons for wastewater treatment purposes (Bhatti et al., 2017).

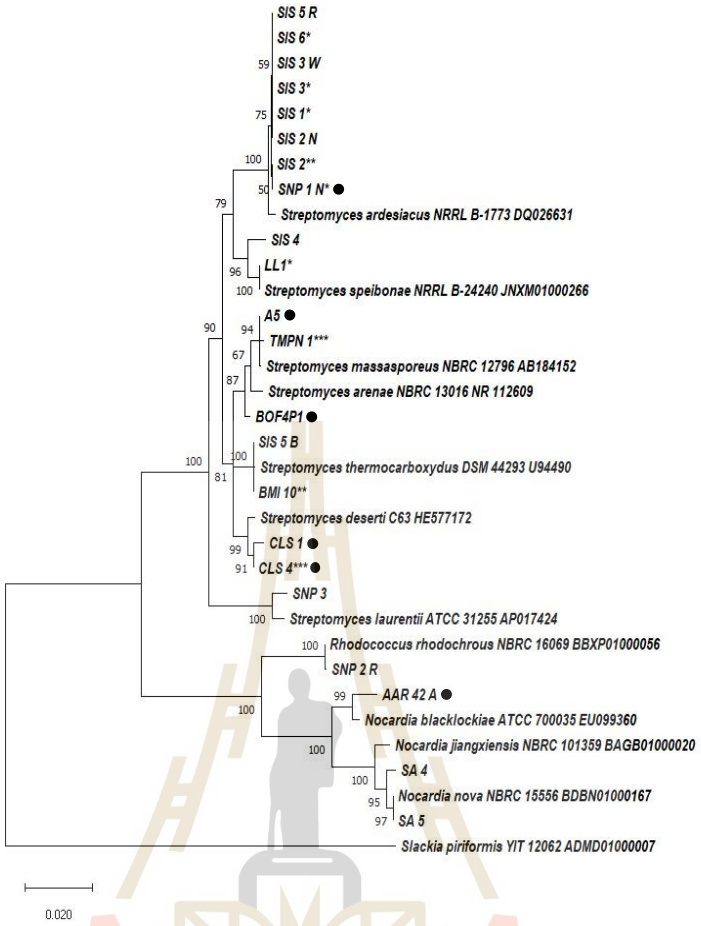

Figure 4.9 Actinomycetes isolated from cassava starch factory. Phylogenetic tree of represented strains with various indole-3-acetic acid (IAA) production and phosphate solubilization activity (●). The asterisks indicate high (***), moderate (**), and low (*) concentrations of IAA. The tree was constructed using the neighbor-joining method in MEGA X software using values based on 1000 replications. The bar at the lower left of the figure indicates the substitution length per site.

Table 4.5 Actinomycete strains and their plant growth-promoting abilities.

No	Strains	Closely related species	Accession Number	IAA production (mg mL^{-1})	PO_4 solubilizing
1	SNP 2 R	*Rhodococcus rhodochrous*	MZ389925	-	-
2	SIS 6	*Streptomyces ardeisacus*	MZ389923	0.001 ± 0.000^{e}	-
3	SNP 1 N	*Streptomyces ardeisacus*	MZ389924	0.016 ± 0.001^{c}	+
4	SIS 5 R	*Streptomyces arenae*	MZ389929	-	-
5	SIS 5 B	*Streptomyces thermorboxydus*	MZ389922	-	-
6	SIS 4	*Streptomyces speibonae*	MZ389921	-	+
7	LL 1	*Streptomyces speibonae*	MZ389916	0.001 ± 0.000^{e}	-
8	SIS 3 W	*Streptomyces ardeisacus*	MZ389920	-	-
9	SIS 2 N	*Streptomyces ardeisacus*	MZ389919	-	-
10	SIS 1	*Streptomyces ardeisacus*	MZ389928	0.010 ± 0.002^{d}	-
11	SIS 2	*Streptomyces ardeisacus*	MZ389918	0.026 ± 0.001^{b}	-
12	AAR42A	*Nocardia blacklokiae*	MZ389912	-	+
13	SA 4	*Nocardia nova*	MZ389917	-	-
14	BMI 10	*Streptomyces thermorboxydus*	MZ389913	0.039 ± 0.005^{a}	-
15	SA 5	*Nocardia nova*	MZ389927	-	-
16	CLS 4	*Streptomyces deserti*	MZ389915	0.041 ± 0.002^{a}	+
17	TMPN 1	*Streptomyces massasporeus*	MZ433282	0.042 ± 0.003^{a}	-
18	CLS 1	*Streptomyces deserti*	MZ433284	-	+
19	A5	*Streptomyces massasporeus*	MZ389911	-	+
20	SIS 3	*Streptomyces ardeisacus*	OK356613	0.019 ± 0.001^{c}	-
21	BOF4P1	*Streptomyces arenae*	MZ389914	-	+
22	SNP 3	*Streptomyces laurentii*	MZ389926	-	-

Different letters indicate a significant difference using DMRT (a=0.05, n=3). (+) demonstrates the solubilization ability while (-) shows no solubilization.

After molecular identification, a phylogenetic tree based on the 16s rRNA was constructed. The tree was constructed using neighbor-joining tree algorithms. Bootstrap analysis was conducted in 1000 repetitive (Dede et al., 2020). It has been found that the strains were distributed throughout several genera. All strains are closely related the *Rhodococcus*, *Streptomyces*, and *Nocardia* genera (Figure 4.9). Embley and Stackebrandt (1994) explained that the phylogenetic tree in actinomycete studies was formerly built and further interpreted for several purposes. The first one is to conclude the historic development for actinomycetes. Secondly, the tree was made to track the possibility of phenotypic characteristics from evolutionary traits. Lastly, it was built to bridge the genotypic and phenotypic characteristics. Commonly, these purposes were depicted in the actual phylogenetic tree combined with the phenotypic characteristics (Stackebrandt & Woese, 1981).

Nine actinomycetes isolates were found to have the ability to produce Indole-3-acetic acid (IAA) (Table 4.5). Auxin (IAA), which regulates many fundamental cellular processes, including cell division, elongation, and differentiation, is among the most utilized traits of actinomycetes for promoting plant growth (Bhatti et al., 2017). Interestingly, only strains from the genus *Streptomyces* were found to have IAA synthesis ability from the L-tryptophan supplementation. Narayana et al. (2007) previously reported that the *Streptomyces* genus is the dominant genus for IAA production. There are numerous pathways related to the production of IAA. However, for *Streptomyces*, indoles-3-acetamin formation was the common synthesis pathway of IAA (Ruanpanun et al., 2010). However, the ability to produce this phytohormone depends on nutrition and cultivation conditions (Myo et al., 2019). The amount of IAA production in the ISP II medium with L-tryptophan supplementation was shown a wide range of concentration (0.001-0.041 mg mL^{-1}). Similarly, this range was previously reported by Khamna et al. (2008), who obtained the degree of 5.5–144 μg mL^{-1} from several isolates of actinomycetes from the rhizosphere around Thailand.

The phosphate solubilizing activity test showed that six isolates could solubilize phosphate (Table 4.5). Differently, the process of phosphate solubilization depends on the acid production for most of the actinomycetes. Thus, a carbon source becomes essential to ensure organic acid production for solubilization (Bhatti et al., 2017). *Nocardia* and *Streptomyces* were the genera with the phosphate solubilization activity in this condition. Anwar et al. (2016) previously reported that the solubilization activity from actinomycetes was mostly found in the *Streptomyces* genus. Another critical parameter for the actinomycete is that although the solubilization in the system involves the acid secretion, actinomycetes cannot acidify the medium as much as the fungi (Kishore et al., 2015).

In this study, the traits of plant-growth-promoting activity (IAA production and phosphate solubilization) did not show any distinct distribution. There was no clear pattern or distinctive clade where the ability of growth-promoting plant in the isolated strains (Figure 4.9). Strains with positive activity in IAA production and phosphate solubilization were further proceeded for the synergistic test with the algae from the previous section. The selection was based on the positive categories to limit the study's scope. Previous studies were also employed these screening processes to test the potentially synergistic effect from the bacteria.

4.1.4 Fungal Isolation and Screening

Isolation and screening of plant-growth-promoting fungi showed two strains from the selected sampling areas, which were strain F2 and F5, with the plant growth-promoting activity. There was no IAA-producing activity detected in all strains. Nevertheless, F2 and F5 showed halo zones around the hyphae of the fungi. Growth and halo zone areas in the PKV agar medium showed that these fungi differed in their abilities to solubilize phosphate (Figure 4.10). Nevertheless, all the strains presented smaller clean halo zones than the mycelium area. It was previously described that *Aspergillus* spp., *Penicillium* spp., *Talaromyces* spp., and *Gongrorella* spp. could mobilize and increase the phosphate intake in plants through the phosphate solubilizing activity (Doilom et al., 2020b; Zhang et al., 2018). As the phosphate-solubilizing fungi excrete organic acids, the P bonds from minerals or organic chains containing P can be broken and released to the available state (Li et al., 2016; Zhang et al., 2018).

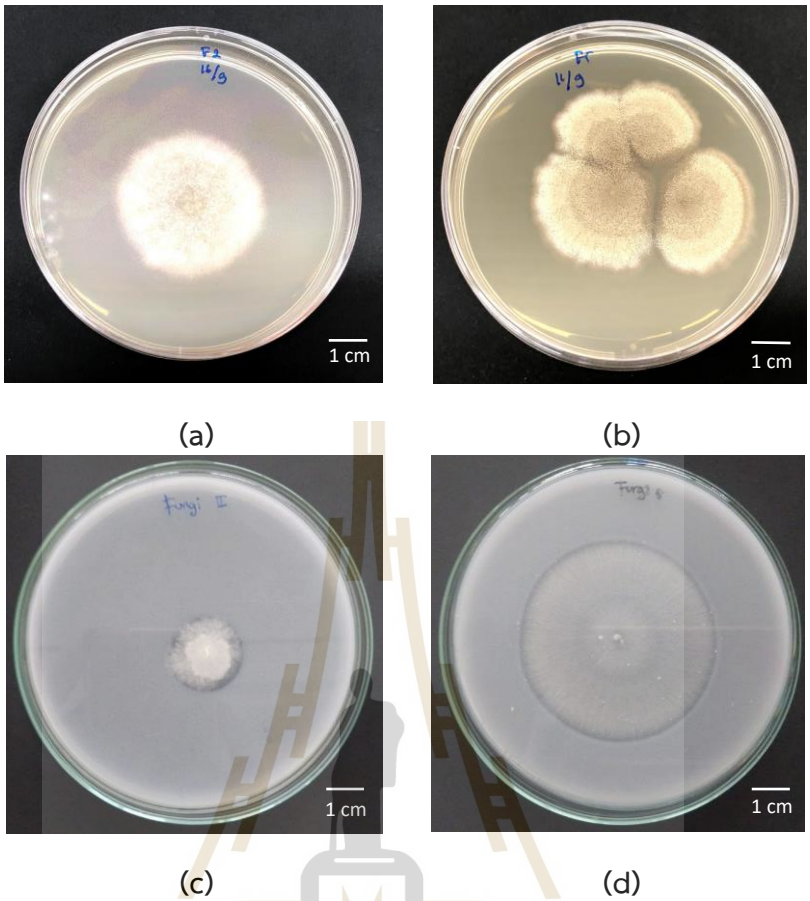

Figure 4.10 Phosphate solubilizing fungi on PDA (a) F2 and (b) F5, and on PKV medium (c) F2 and (d) F5.

The sequence of 18s rRNA from these fungal strains revealed that all strains belong to the *Aspergillus* genus (Figure 4.11). Strain F2 (Accession No. MZ359863) was closely related to *A. awamori* EU667995 (99.76% similarity), while F5 (Accession No. MZ359863) was closely related to *A. niger* MG889595 (99.99% similarity). *Aspergillus* is a well-known genus with versatile advantages in cropping systems. The solubilization of phosphate was very limited based on the halo zones of these strains. The solubilizing index for F2 and F5 were 2.12 ± 0.11% and 3.36 ± 1.12%, respectively. These values were similar to other isolates from the rhizosphere (Elias et al., 2016). Thus, the fungal isolates from CBEW also possessed the promising ability to solubilize phosphate for growth promoting purposes.

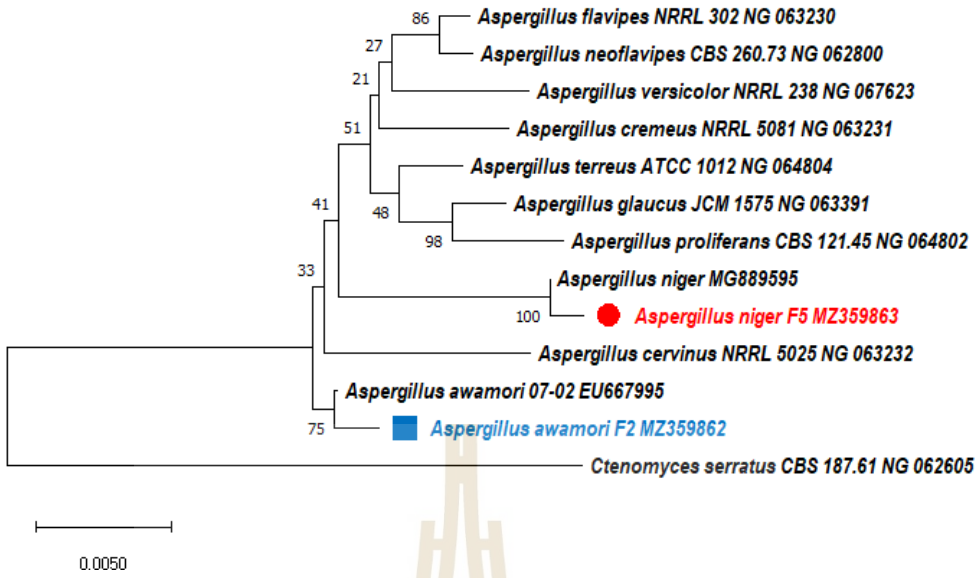

Figure 4.11 Phylogenetic tree of phosphate solubilizing isolated F2 and F5 fungi and closely related strains. The tree was constructed using the neighbor-joining method in MEGA X software using values based on 1000 replications. The bar at the lower left of the figure indicates the substitution length per site.

4.2 Construction of Co-cultures

4.2.1 Construction of alga-actinomycete co-culture

Construction of alga and actinomycete co-culture was performed by co-inoculate both microorganisms in the basal medium (BG 11 broth). Based on the normalization of the results in the algal isolation and screening process, *Chlorella sorokiniana* strain P21 was chosen as the algal inoculum to be further developed based on the overall score of optimum removal and biomass production (Table 4.4). Thus, this strain was tested to all the positive strains that possess the ability to promote the plant growth.

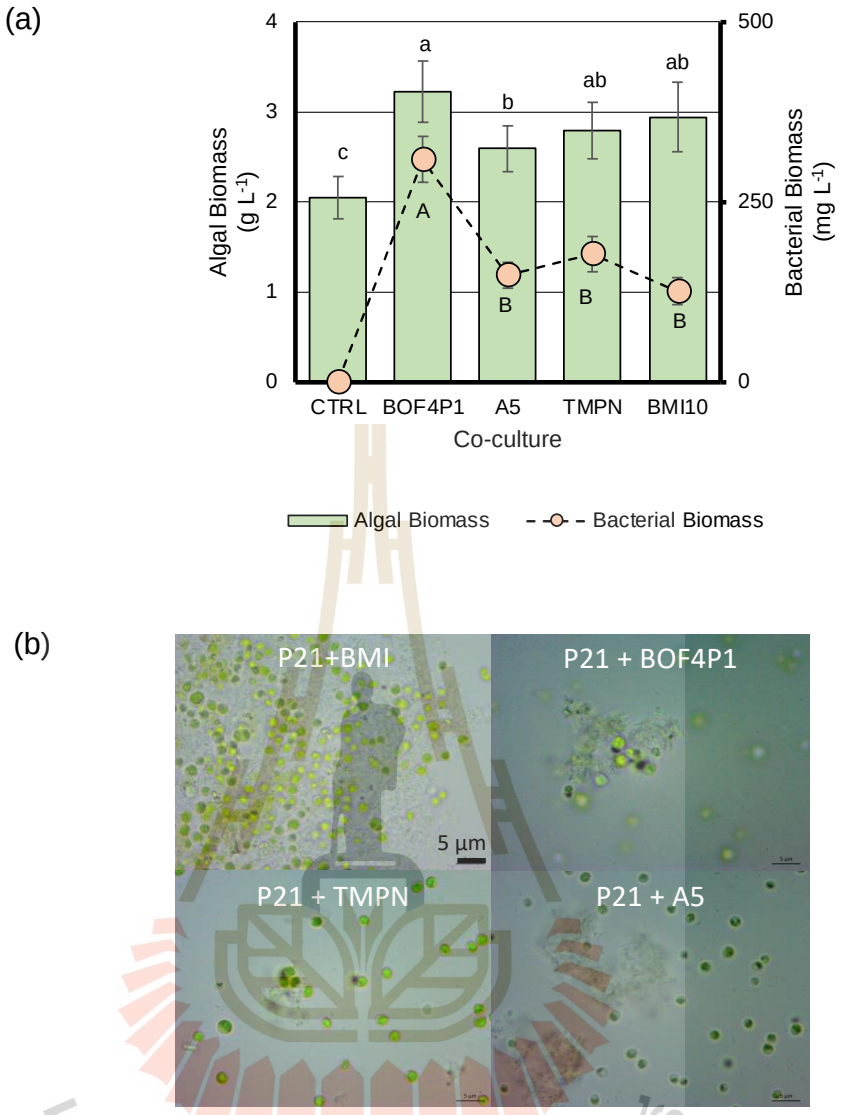

Figure 4.12 (a) Effects of various actinomycetes on algal biomass, and (b) Hyphae microalgae-actinomycetes in synergistic growth. CTRL showed final biomass of sole alga P21 as inoculant. Different letters on the same line indicate a significant difference by the DMRT (α=0.05, n=3). Lowercase letters indicate algal biomass, uppercase letters indicate bacterial biomass.

After the co-inoculation experiments, Streptomyces *thermocarboxydus* strain BMI 10, *S. messosporeus* strain TMPN, *S. arenae* strain BOF4P1, and *S. messosporeus* strain A5 showed positive effects on algal biomass by significantly increasing total biomass of alga P21 after the cultivation period over that of the control (with no bacteria added) (Figure 4.12a) Meanwhile the other strains showed similar or lower algal biomass than the control (Supplementary Figure 7). The co-culture also showed that BMI 10 and BOF4P1 hyphae contained more P21 cells whilst TMPN and A5 hyphae did not show any affinity for the algal cells (Figure 4.12b). Some of the isolates with plant growth-promoting abilities did not show positive effects on the growth of the algae. Many actinomycetes are pathogens and excrete toxic or inhibitory compounds that negatively affect algal growth (Zheng et al., 2013). These results also showed that some bacteria with plant-promoting activities might not be suitable for use in algal application (Wang et al., 2015). There may be a special or specific roles that some actinomycetes play to yield positive results and greater microalga P21 growth (Boivin et al., 2007). Only certain bacteria can synergistically grow with the microalgae in a particular consortium.

As there were four potential strains with different ability of algal growth-promoting mechanisms, all the combinations of positive strains of actinomycetes and the microalga *C. sorokiniana* P21 were employed in a syntrophic test. This test was done to identify actinomycetes that did not compete with microalgae to utilize nutrients. The direct intake of wastewater nutrients by actinomycetes can limit the available nutrients for microalgae, leading to competitive interaction (Park et al., 2008). In this case, the proportion of oil produced by the algae biomass is higher than that contained in the actinomycetes biomass in terms of percentage and total productivity (Kumsiri et al., 2021).

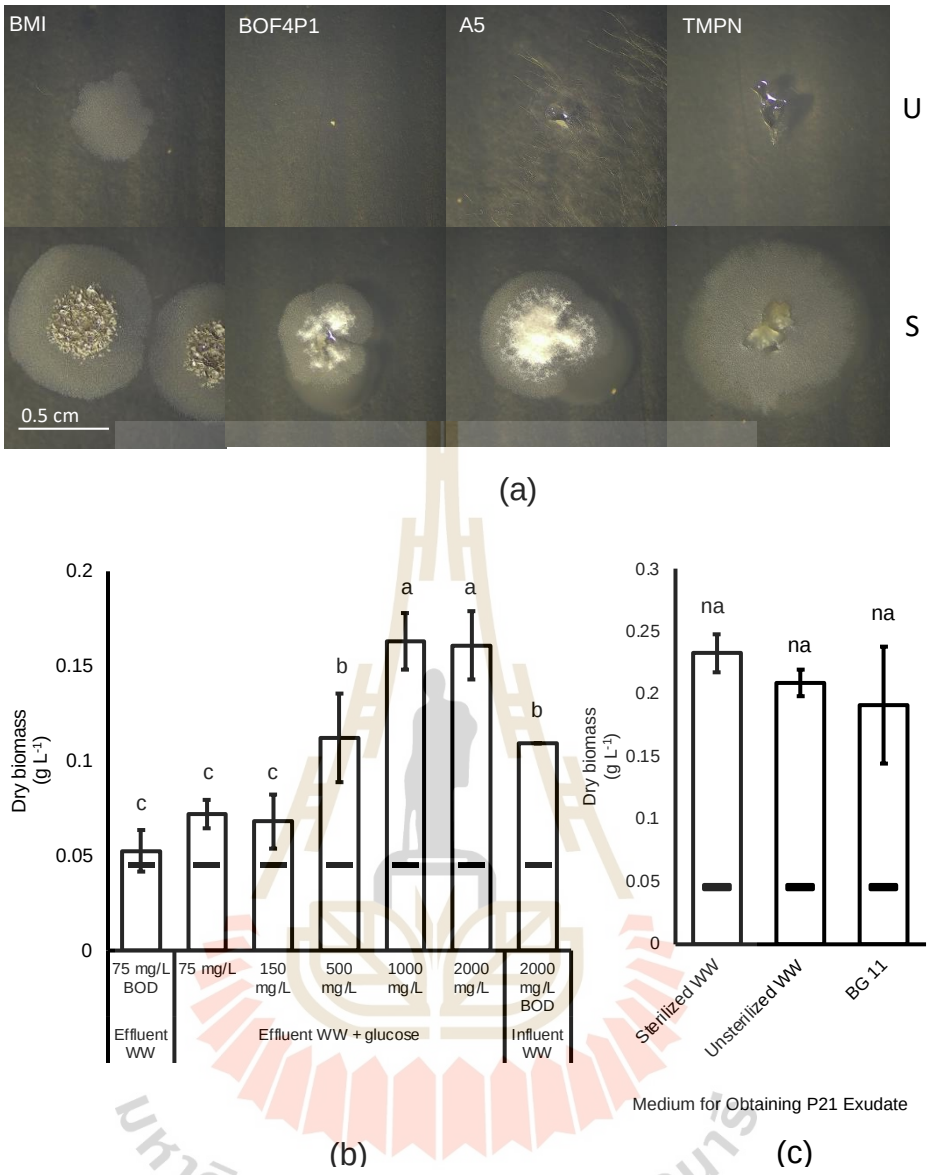

Figure 4.13 Syntropic test of positive co-cultures of actinomycetes and microalgal P21. (a) Spore germination of microalgae in P21 exudate cultivated in sterilized (S) and unsterilized (U) wastewater after a 7-day incubation, (b) Growth of BMI in influent and effluent wastewaters with supplementation of glucose, and (c) Growth of BMI in P21 exudate broth. Different letters on the same line indicate a significant difference by the DMRT (α=0.05, n=3). "na" letters means no significant difference.

In all positive co-cultures, only the BMI 10 strain showed germination in a P21-exudate agar medium from sterilized and unsterilized conditions while the A5, TMPN, and BOF4P1 strains only germinated on the P21-exudate medium under an axenic condition (Figure 4.13a). It was previously reported that the metabolites of algae could be excreted and become an endogenous source of bacterial growth substrate (Wang et al., 2015). However, in the P21 exudate from unsterilized wastewater, only BMI 10 showed germination while others did not. The exudate mainly contain carbohydrates and some vitamins in micro-concentrations (Natrah et al., 2014). Some simple exudate molecules were directly consumed by native strains in the unsterilized wastewater while the remaining nutrients were more difficult to utilize by the bacteria.

Further tests of the BMI 10 strain showed that the carbon source became a limiting factor for germination and growth. It is shown in Figure 4.13b that additional glucose in the wastewater, at a level of 500 mg L^{-1}, successfully initiated BMI 10 growth. Moreover, the composition of the P21 strain exudate, that is important for BMI 10 to grow, was not related to the P21 growth medium (Figure 4.13c). Here, the algal exudates are shown to support the growth of bacteria (Kim et al., 2014). This phenomenon is related to the photosynthetic activity of *C. sorokiniana*, where the exudate was primarily produced by an autotrophic mechanism. Thus, the exudate composition was similar when the macronutrients for photosynthesis are sufficient (Watanabe et al., 2006).

A set of experiments was conducted to confirm the effect of IAA to the P21 growth. BMI 10 was previously found to only possess the IAA production as trait for growth promoting activity with no phosphate solubilizing (Table 4.5). Nevertheless, there are numerous mechanisms of growth promoting that may involve in this co-culture. Co-culture of these two organisms were conducted to obtain the possibility of the mechanism involved in the system. BMI 10 and IAA from the standard solution were induced to the medium where alga P21 was grown. Both inoculations showed a similar pattern (Figure 4.14a and 4.14b). It has been shown that the positive effect of IAA was started to observe at concentration of 5 ng mL^{-1}. Similarly, positive effect of BMI 10 was observed in the 10^5 spores mL^{-1}. The effects then remained stable in the higher concentrations. Thus, it can be concluded that among the interaction between these organisms, the main mechanism of growth enhancement of the actinomycete BMI 10 to the growth of alga P21 was IAA signaling.

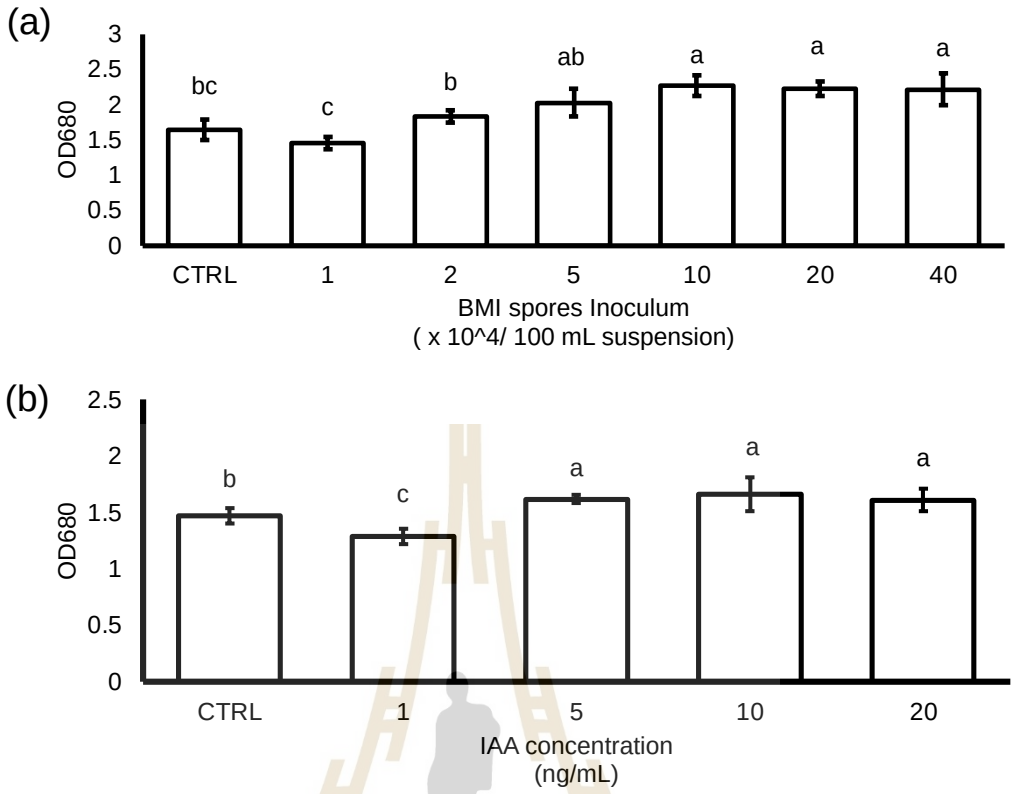

Figure 4.14 (a) Effect of different inoculum concentration, and (b) different IAA concentration. Different letters indicate significant difference among cultures using DMRT with $P < 0.05$.

It is well known that the effect of IAA on the algal growth is dose dependent where low concentration of IAA (Lin et al., 2022). The hormone was involved in several mechanisms of the growth promoting activity in higher plants such as which regulates various metabolic processes such as cell division, cell elongation, organ differentiation, and apical dominance (Guldhe et al., 2019). However, as the microalgae are not involved in the cell differentiation and organ formations, effects of IAA are mainly on induction of mitosis (Piotrowska-Niczyporuk & Bajguz, 2014) and cell maturation (Tarakhovskaya et al., 2007).

Co-culture of the microalga P21 and actinomycete BMI 10 was also conducted in the sterilized wastewater to ensure the co-culture has the ability to grow and possess similar effect in the wastewater. Maximum algal biomass concentration was significantly increased in the co-culture in sterilized condition. The increase was found up to 21% than single culture (Figure 4.15a). In the early stage of cultivation, it was demonstrated that there was no significant difference between mono-culture and

co-cultivation. However, after six days of cultivation in sterilized wastewater (Figure 4.15a), the concentration of P21 strain in the co-culture increased. Differently, the growth of BMI 10 biomass (stated as the total bacterial biomass in sterilized condition) was detected since the starting day of cultivation (Figure 4.15b). It can also be seen that the effect occurred in the early stage of stationary phase of the microalga P21 growth. After the microalga reached later phases of growth, metabolites of this alga can significantly increase the number of actinobacteria in wastewater (Udaiyappan et al., 2020).

The kinetics parameters from the single culture and the co-culture in the sterilized CBEW were also calculated. The content of sterilized wastewater was different from the raw CBEW. The sterilized wastewater showed reduced media content to 178 ± 4.64 mg L^{-1} of COD, 20.14 ± 0.64 mg L^{-1} of PO_4, 41.31 ± 6.21mg L^{-1} of TN, 14.23 ± 1.55 mg L^{-1} of NO_3, and 27.17 ± 1.67 mg L^{-1} of TP, the pH range was 7.03 ± 0.18 while that of DO was 3.9 ± 0.09 mg L^{-1}. The slight change of concentration was previously described as the effect of the heat and pressure in the autoclaving process.

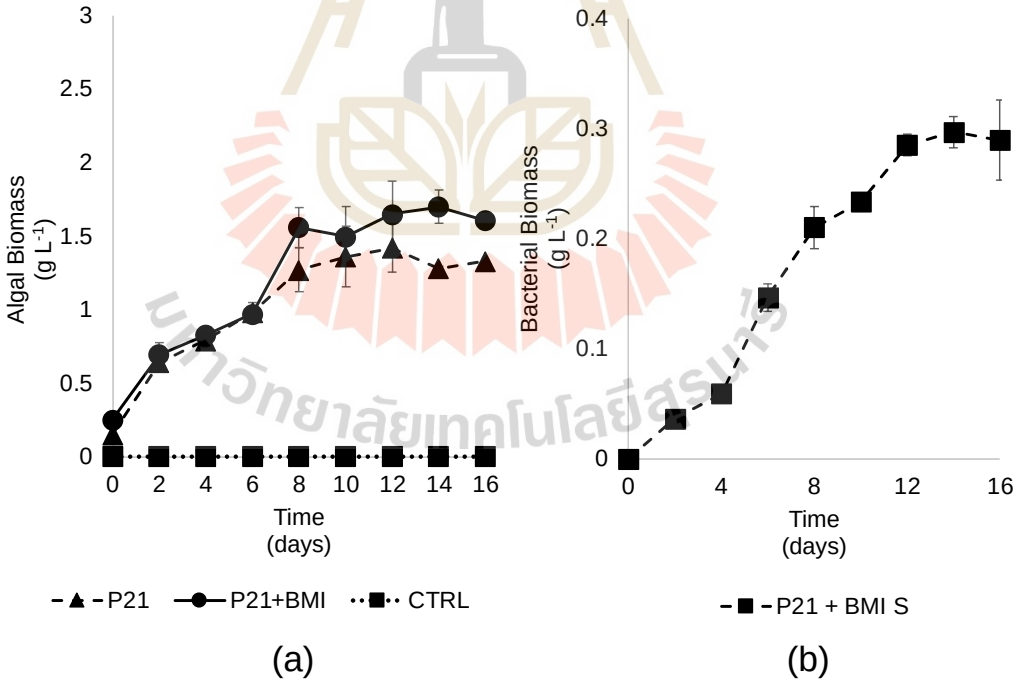

Figure 4.15 Biomass evolution of native algae (CTRL), *C. sorokiniana* (P21), and *S. thermocarboxydus* (BMI 10). (a) Co-culture in sterilized wastewater, and (b) Bacterial biomass in sterilized co-culture.

The growth rate of the co-culture in sterilized wastewater showed the converse result to that of the total yield biomass. The initial growth rate of the P21 was 0.34 day^{-1} in the single-inoculant cultivation. Addition of BMI 10 cells in the inoculum decreased the growth rate by 13 % in sterilized cultures (Table 4.6). The growth rate of the P21 was decreased 0.04 day^{-1} after additional of BMI 10. Most of the algal-bacterial co-cultures positively affected the growth rates that had a linear correlation with the total biomass obtained. When the algae can rapidly grow in the co-culture condition, the maximum biomass obtained increases (Kumsiri et al., 2021; Kumsiri et al., 2018). Other co-cultures resulted in decreased growth rates and less total biomass was obtained from the system (Wang et al., 2015). The inverse correlation between increased total biomass and decreased growth rate can be explained as an effect of syntrophic bacterial application.

Table 4.6 Kinetics Parameters of Biomass evolution in sterilized CBEW.

Kinetics Parameters	Algal Biomass		BMI Biomass
	P21-S	P21+BMI 10-S	P21+BMI 10-S
X_o (g L^{-1})	0.65	0.65	0.055
X_m (g L^{-1})	1.85	2.11	0.29
μ (d^{-1})	0.34	0.30	0.30
R^2	0.97	0.95	0.97

P21: Single culture of *Chlorella Sorokiniana* strain P21; P21+BMI: Co-culture of *C. sorokiniana* P21 and *Streptomyces thermocarboxydus*.

One possible mechanism is that the effect of the BMI 10 strain was only to extend the exponential phase without increasing the growth rate of the alga. This mechanism starts with the production of tryptophan or other precursors by the alga cells (González-González & de-Bashan, 2021). Nevertheless, the precursors for the algal growth promoting activity were mostly available in the exponential or stationary growth phases (Palacios et al., 2016a). Thus, the effects of co-culture to enhance the algal biomass are likely to occur at either the end of the exponential phase or the beginning of the stationary phase of growth (Lee et al., 2019). In the current study, BMI 10 enhancement on the microalga was only observed at the end of the exponential growth period of the P21 strain, supporting the explanation that an exudate containing a precursor for promoting activity was produced in a sufficient quantity during this period (Watanabe et al., 2006). However, IAA was not detected at the end of the

cultivation period (Figure 4.16). This phenomenon reflects the efficiency of IAA production in that it is only produced in the quantity required for sustaining the interaction (Palacios et al., 2016b). Therefore, it can be concluded that the first stage of alga growth was a commensalism condition with no effect on P21 growth. Later, the interaction became mutualistic, increasing the P21 strain biomass and supporting the growth of the BMI 10 strain.

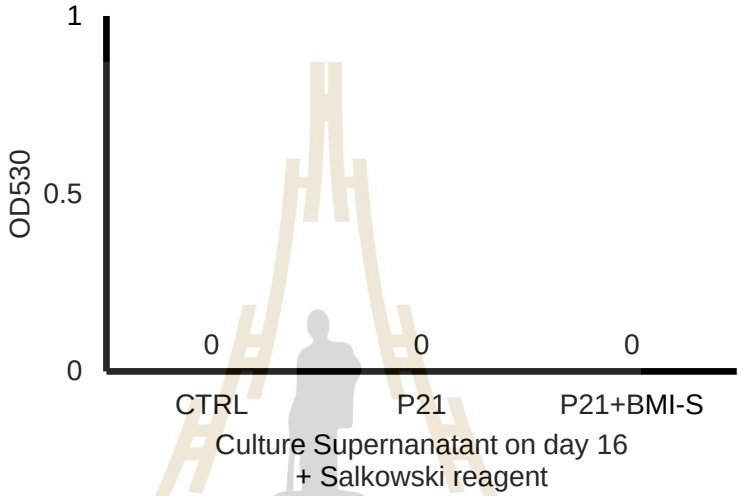

Figure 4.16 Final IAA concentration in the culture (n=3). "0" means not applicable/ below detection limit.

4.2.2 Construction of alga-fungi co-culture

Different approach was made to the fungi application to enhance the algal growth. *C. sorokiniana* P21 had the highest phosphate removal, indicating that there was no need for significant phosphate removal enhancement. This was proven by co-culturing alga P21 with fungi F2 and F5. It showed there was no significant increase in the growth of the P21 in the suspension (Supplementary Figure 8). Furthermore, there was no fungal isolates with the alga growth-promoting ability apart from the phosphate solubilization activity. Hence, phosphate solubilization would be redundant for P21 strain as the solubilization process would only be effective for increasing the TP removal in waste (Negi & Suthar, 2018). On the other hand, *C. vulgaris* 8580 also showed a high potency for wastewater treatment in all parameters (Table 4.4). Nevertheless, the phosphate removal in this strain was relatively low compared to other inoculants. Thus, this strain was chosen to be applied in the co-culture of alga and fungi in all parameters. The development of this co-culture was also slightly

different with the algal-actinomycete co-culture. The development of fungi would be based on the ability and capacity of fungal pellets to entrap the algal cells in the basal medium as the main trait to be developed for algal-fungal interaction in order to treat wastewater (Leng et al., 2021; Muradov et al., 2015; Wrede et al., 2014; Zhou et al., 2012a; Zhou et al., 2013). In the wastewater treatment section, the constructed algal-fungal co-culture was also examined in all removal activity.

A. niger F2 and F5 strains were examined for their ability to form pellets in a basal medium containing glucose (Muradov et al., 2015; Zhou et al., 2012a; Zhou et al., 2013). It was found that all the strains were able to form pellets. Pellet formation screening was conducted to obtain a minimal concentration of glucose necessary to form pellets. Glucose concentrations of 5, 10 and 15 g L^{-1} were supplemented into basal medium to investigate the effect of sugar content on pellet formation and size. It was found that the pellet size of the F2 strain became smaller as the concentration of glucose was increased. However, the F5 strain formed smaller sized pellets in 10 g L^{-1} of glucose followed by 15 g L^{-1} and 5 g L^{-1} (Table 4.7).

Fungal pellet formation was affected by the presence of organic carbon. It was suggested that a higher concentration of glucose is beneficial to obtain rapid pellet formation (Zhou et al., 2012a). Another study reported that formation of pellets was affected by adjusting the pH (Zhou et al., 2013). Form of organic carbon and the form that available in the medium also play an important role to the pellet formation (Leng et al., 2021). However, this method is not suitable for nutrient removal after pellet formation. The pH of the medium after pellet formation was also related to pellet size. Pellet size was greater at higher pH values resulted from the dilution of wastewater (Table 4.7). Higher pH tends to neutralize the surface charges of spores and mycelia, creating an isoelectric point so that the spores and mycelium did not repel each other (Dynesen & Nielsen, 2003).

Table 4.7 Effect of initial glucose concentration in BG 11 medium on fungal palletization after a three-day cultivation.

Strain	Glucose Concentration (g L^{-1})	pH	Pellet size (Ø mm)
F2	5	6.27±0.11[a]	4.20 ± 2.35[a]
	10	4.30±0.12[b]	3.50 ± 0.97[a]
	15	4.08±0.20[b]	3.40 ± 2.76[a]
F5	5	5.17±0.32[a]	6.80 ± 3.99[a]
	10	4.09±1.03[ab]	3.70 ± 1.16[b]
	15	4.06±0.92[b]	5.00 ± 2.31[ab]

Different letters indicate significant difference among the pellets from a single strain using DMRT test (a=0.05, n=3).

A series of dilutions ranging from 10-100% of wastewater supplemented with 1% glucose was conducted to obtain a potential substrate than can replace the BG 11 medium with CBEW as a source of minerals for pellet formation. One percent glucose was selected owing to the greater homogeneity of pellet size at this concentration (Table 4.8). It was found that the F2 and F5 strains only formed pellets in CBEW concentrations of 30-70% and 30-60%, respectively. The absence of pellets in higher concentrations of wastewater might have been caused by the alkalinity of the wastewater (Table 4.1) at higher concentrations. Although the alkalinity enhanced pellet formation, extremely high alkalinity could create a repulsive charges as negative ions were abundant at the surface of the mycelia (Dynesen & Nielsen, 2003). At lower concentrations, there were inadequate nutrients to support pellet formation (Liu et al., 2008).

Table 4.8 Size of pellets in various concentrations of wastewater supplemented with 10 g L^{-1} glucose after a three-day inoculation.

% Wastewater supplemented with 10 g L^{-1} glucose	Pellet size (∅ mm)	
	F2	F5
10	na	na
20	na	na
30	9.29 ± 6.63[a]	14.57 ± 3.64[a]
40	6.43 ± 2.37[b]	4.71 ± 2.43[c]
50	5.43 ± 1.62[b]	6.43 ± 2.30[b]
60	5.86 ± 2.73[b]	9.14 ± 0.90[b]
70	6.71 ± 1.80[b]	na
80	na	na
90	na	na
100	na	na

Different letters indicate significant difference among the pellets from a single strain using DMRT test (α=0.05, n=3). na (not applicable to produce pellets).

The processes of algal pellet formation were further observed using pellets formed in a basal medium at three glucose concentrations. This medium was used to avoid additional compounds in the wastewater, which can interfere with adsorption process. The removal efficiency of the pellets showed different patterns. In the first two hours, all the pellets showed high removal efficiencies, up to 80% using the F2 and F5 strains grown with 5 and 10 g L^{-1} of supplemental glucose (Figures 4.17a & b). However, algal cell removal by pellets grown in a medium containing 15 g L^{-1} of glucose only reached 78% efficiency after the same time intervals (Figure 4.17c). Removal efficiency is also related to the specific surface area and zeta potential of the fungal mycelium. As the individual fungal pellets increased in size, they had a larger surface areas per pellet. So, their capacity to adsorb and remove the algal cells from the suspension was higher. The early stage of removal was typically related to physical mechanisms, where the algae attached to the surface of the mycelium or became entrapped in spaces formed in the mycelium (Li et al., 2020b).

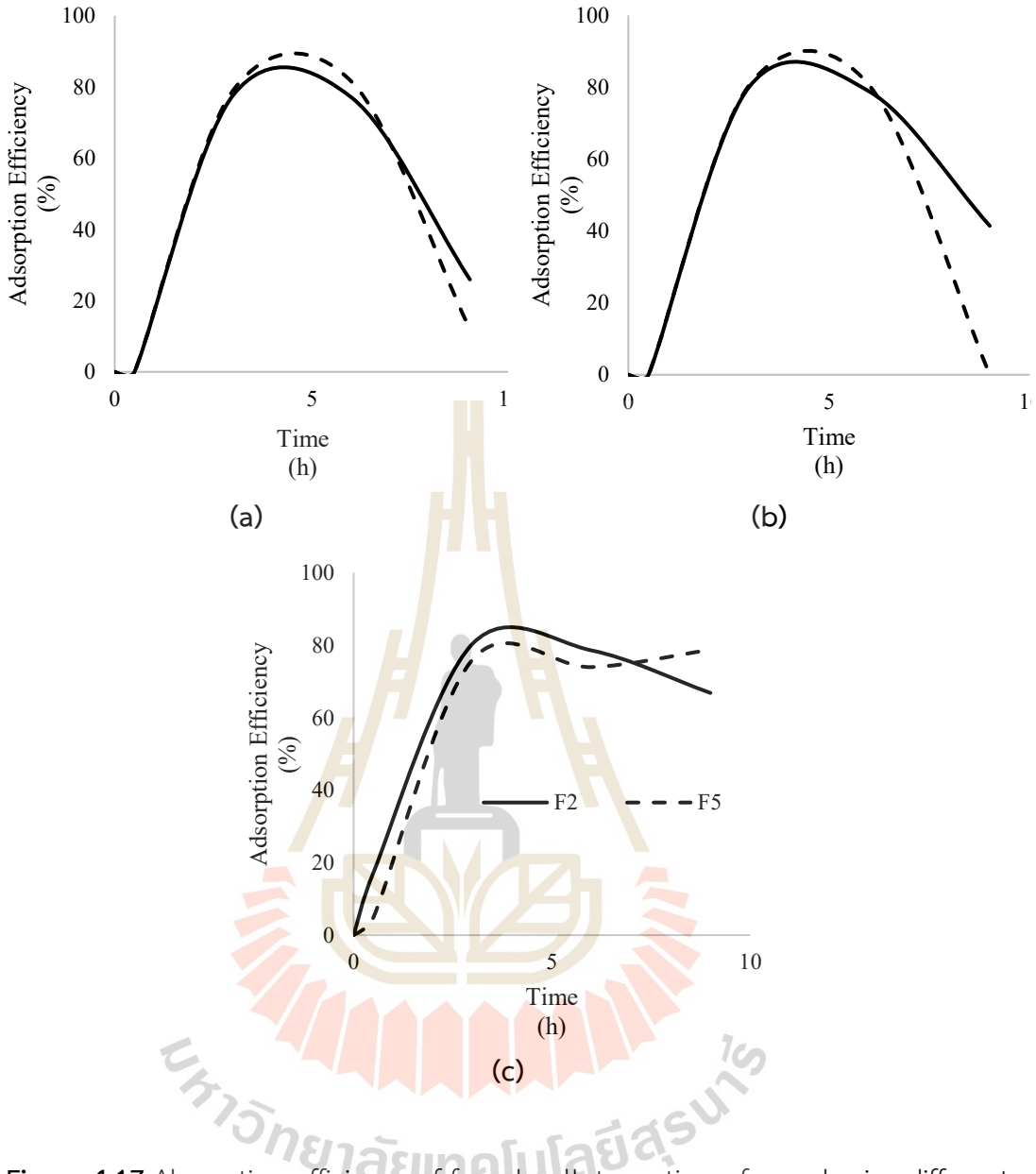

Figure 4.17 Absorption efficiency of fungal pellets vs. times formed using different concentrations of glucose, (a) 5 g L^{-1} glucose, (b) 10 g L^{-1} glucose, and (c) 15 g L^{-1} glucose.

Another factor for consideration is zeta potential. As this value is strictly related to pH (Liu et al., 2013) and the pH of the algal culture was nearly neutral (7.3 ± 1.00), the surfaces of the fungal pellets determined the adsorption capacity in this early stage. Material with low pH was reported to have high zeta potential that can aid in attachment (Pei et al., 2021). Comparing the pH values from media with three concentrations of glucose, it was found that pellet size was inversely proportional to pH. Thus, a higher zeta potential did not positively affect the adsorption. So, removal

was greatly affected by pellet size. It is notable that optimum removal in the current study was achieved within 4-6 hours of mixing. Several previous studies that reported the optimal removal could be achieved within 12 hrs (Bhattacharya et al., 2017; Pei et al., 2021). After a six-hour shaking period, only the pellets formed with 15 g L^{-1} of glucose supplementation showed stability after removal (Figure 4.17c). The other two pellet types exhibited release of algal cells. It is well established that the physical mechanisms by which attachment occurs in the early stages are weak, and thus this release phenomenon is possible (Li et al., 2020b).

The use of algal-fungal pellets was further studied in the sterilized wastewater. To develop the most suitable combination, the strain with more solubilizing index was chosen. As demonstrated previously, F5 had a solubilization index of 3.36 ± 1.12%. Thus, this strain was chosen for application in sterilized wastewater conditions. Alga *C. vulgaris* s was chosen as it showed great potency in CBEW treatment and biomass generation with relatively low P removal that compatible with advantageous trait of fungus. The results of biomass evolution showed a positive effect on the growth of *C. vulgaris* 8580 (Figure 4.18a). It showed that the growth of microalga 8580 with the F5 inoculant at the first stage of exponential phase directly enhanced from the single culture. After day 10 the culture was showed a stable concentration where the stationary phase of growth begun. Higher result of the growth of the microalga 8580 in this case may be caused by the occurrence of the phosphate solubilizing activity of F5. As it was previously stated that the dynamic of phosphate, including its composition can determine the growth of alga (John & Flynn, 2000). Co-inoculant of F5 has elevated the maximum biomass achieved from the sterilized wastewater. The growth of the pellet of fungus F5 was higher in the co-culture condition than in the single F5 culture (Figure 4.18b). Here, the process of co-culture showed the ability of fungus F5 to grow in the wastewater and utilize the nutrient source in the wastewater. However, the co-culture also showed the increase of F5 biomass compared with single F5 inoculant. This phenomenon indicated the synergistic effect of the alga and fungus co-culture where the fungus utilized additional carbon sources on algal cell walls or other secretion products from the alga (Leng et al., 2021).

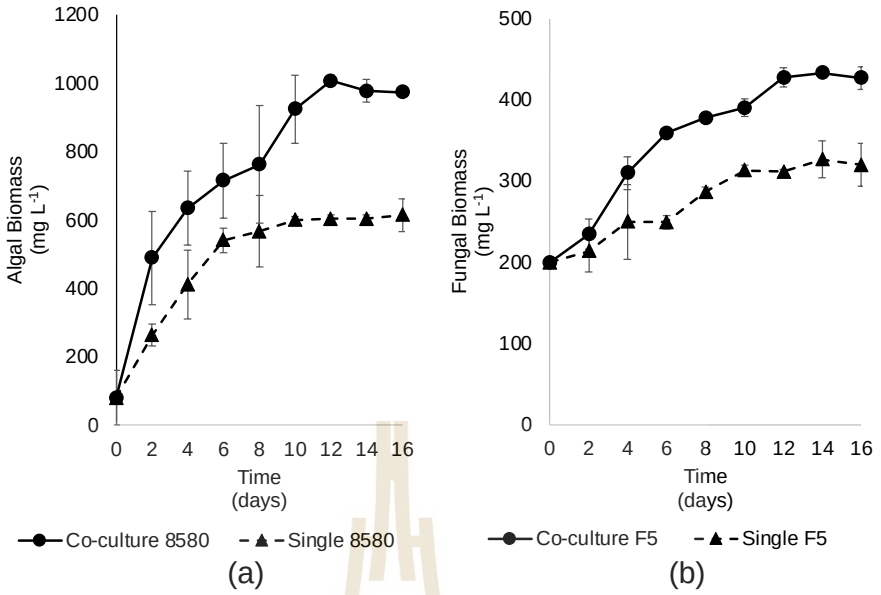

Figure 4.18 Biomass evolution of single and co-culture of *C. vulgaris* 8580 and *A. niger* F5 in sterilized wastewater (a) Algal biomass, and (b) Bacterial biomass.

The application of co-culture of algae and fungus for wastewater treatment was previously reported to achieve maximum biomass levels of as much as 4770 mg L^{-1} on a reactor scale (Guo et al., 2017). In the current study, the highest result only reached 1073 mg L^{-1}. CO_2 limitation might have played an essential role in elevating the maximum biomass concentration in the co-culture process. However, the significant increase of the biomass could be a positive indicator of co-culture advantages in algal biomass production using wastewater as a substrate.

4.3 Removal Efficiency of the Co-culture

4.3.1 Removal of Nutrients by Alga-Actinomycete Co-culture

The various nutrient contents showed a similar decrease of the P21 strain in mono- and co-cultures in sterilized CBEW condition (Figure 4.19). Slight increases in removal efficiencies in all parameters were found at the end of the cultivation period. Sterilized culture of P21 showed the removal of 69.51 % while the co-culture showed the removal of 86.55 % in phossphate removal. Interestingly, there was a fluctuation in the control condition. This phenomenon indicated there was another mechanism that affected the phosphate concentration. Nevertheless, the change of phosphate concentration was not significant in daily basis. Differently,

nitrogen removal of the alga P21 was also relatively similar after additional of BMI 10 as a co-culture with the more stable increase of removal found in the co-culture. The increase of COD removal was also found where the removal was enhanced from 56.54 % to 72.68 % after additional BMI 10 in the co-culture. The removal of COD in the co-culture was relatively slower than in the single culture. However, the final removal efficiency showed higher removal in the co-culture after dramatical increase between day 6 to day 8.

All nutrient concentrations met the permissible discharge standards at the end of the cultivation period (Mohsenpour et al., 2021). Interestingly, COD removal was noticeably higher in co-culture than in the single P21 culture under this sterilized condition. A high COD removal was previously reported in the co-culture of bacteria and algae in municipal wastewater (Foladori et al., 2018). This result showed that the BMI 10 strain combined with the native bacterial community successfully utilized the carbon source provided in the form of algal exudates (Natrah et al., 2014). This mechanism was confirmed by the increase of biomass of both organisms while the removal of COD was higher in the end of cultivation period. The removal of nutrients in all cultures was primarily found to have similar pattern with the change of dissolved oxygen (DO), pH, and biomass growth (bacteria and P21) (Figure 4.19 & 4.20). There was a positive correlation of these abiotic factors with biomass growth and a negative correlation with the various nutrient levels. This pattern confirmed that 1(Foladori et al., 2018). Alternatively, the amount of microbial biomass produced was also strongly related to the growth of the P21 strain and the removal of nutrients from the wastewater (Figure 4.15 and 4.19).

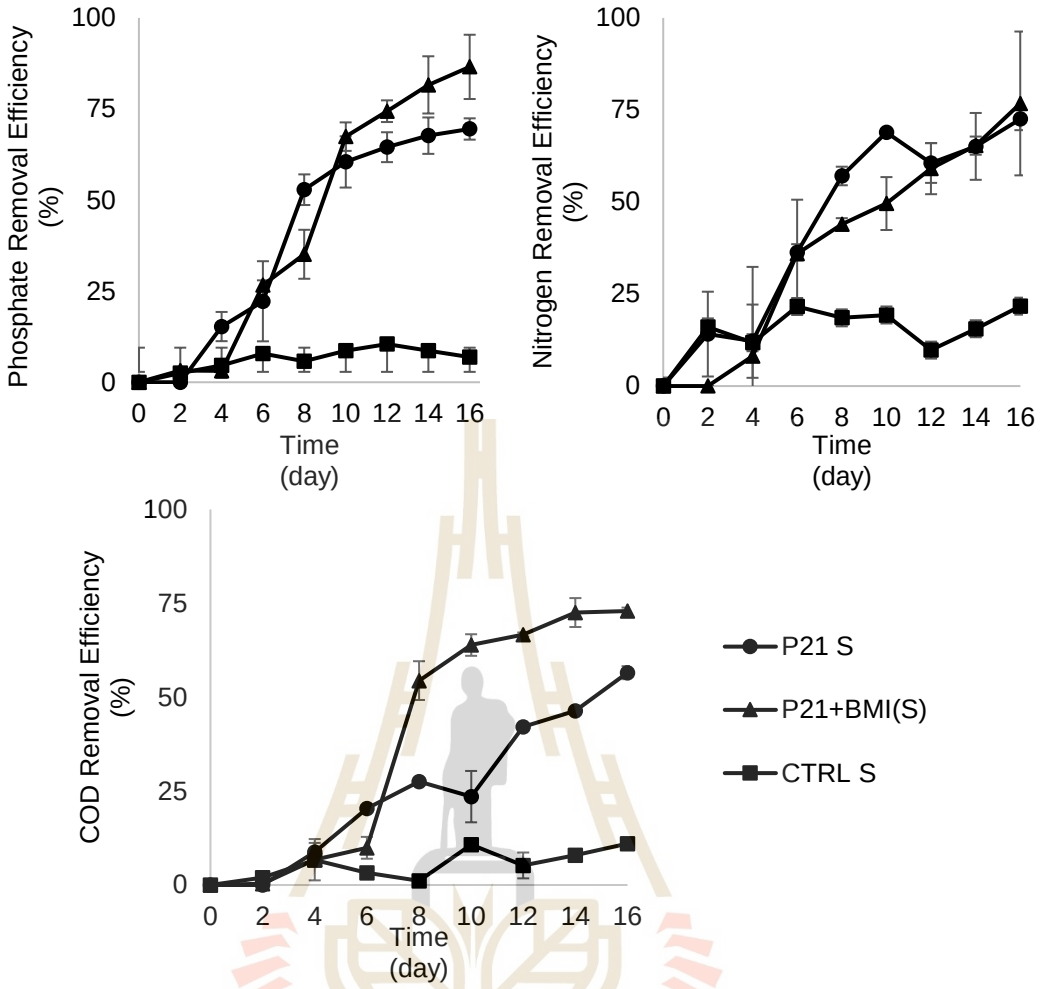

Figure 4.19 Effects co-culture of alga P21 and actinomycete BMI 10 on phosphate, nitrogen, and phosphorus of sterilized CBEW.

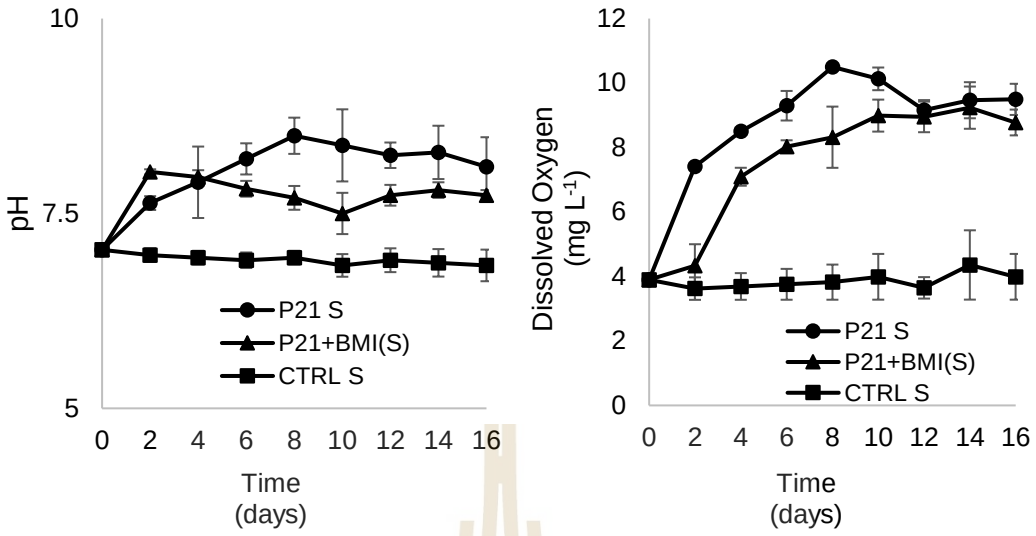

Figure 4.20 Evolution of pH and Dissolved Oxygen in wastewater during the cultivation of microalga P21 and actinomycete BMI 10.

4.3.2 Removal of Nutrients by Alga-Fungus Co-culture

Four different schemes were tested to elucidate the effects of fungal pellets, microalga, and combinations of these organisms (Figure 4.21) as the co-culture relied on the phosphate solubilizing activity. These schemes were tested based on the fact that the fungus F5 only had the ability to solubilize phosphate without other mechanisms such as IAA production. Thus, the optimization of the application was solely focused on the solubilizing phosphate and the harvestability. The mechanisms were based on the separated and combined processes of co-culture for optimum removal and harvesting with the further examination of the removal efficiencies.

Treatment I only utilized alga 8580 inoculum throughout the cultivation period. Treatment II used the F5 fungus with no additional alga throughout the cultivation period. Treatment III was done by adding fungus F5 after growth of the alga 8580 in the treatment reached the stationary phase. Treatment III was prepared in a similar manner as Treatment I in early stage of this treatment. After reaching the stationary phase of algal growth (less than 10% of additional growth), pellets were introduced into the wastewater to adsorb algal cells. The treatment was continued until the biomass reached a stable concentration (stationary). Treatment IV used alga 8580 and fungus F5 in pellet form from the beginning of treatment. Treatments I and II were run to obtain a baseline removal by each organism. Treatments III and IV were later done based on the results of Treatments I and II to obtain comparable results.

Addition of fungal pellets in Treatment III must be done after the stationary phase of TP removal. In Treatment IV, the pellets were earlier prepared.

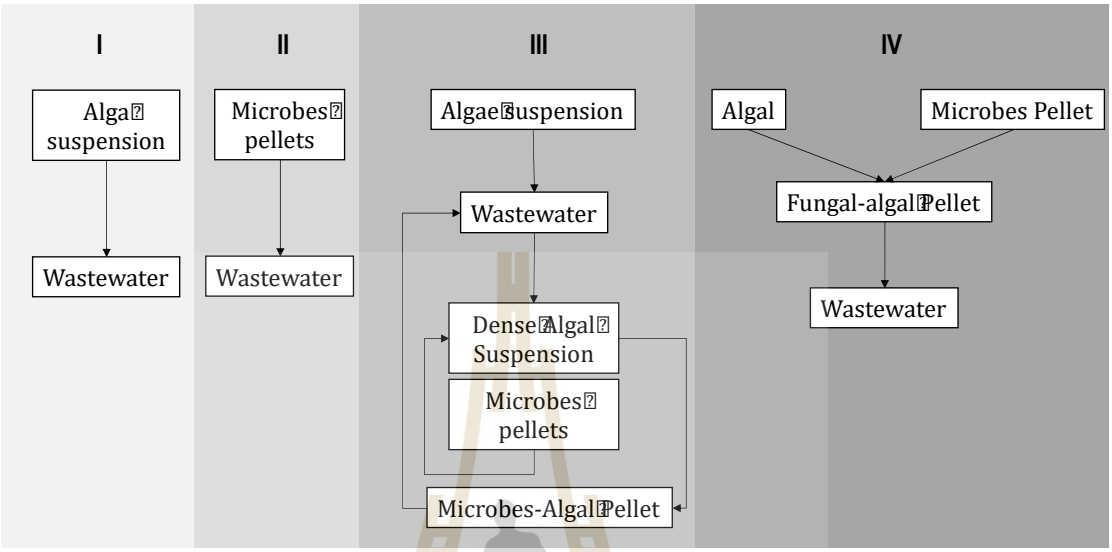

Figure 4.21 Application of co-culture in the sterilized CBEW.

Treatment I, using *C. vulgaris* 8580 as an inoculum, showed removal of up to 52% of TP (Figure 4.22). Interestingly, the removal of insoluble phosphate (a difference between total P and PO_4) was only 44 %. Treatment II, in contrast, showed less than 25 % removal of TP. Addition of fungal pellets to adsorb the algal cells showed that insoluble P removal increased to 90.58 % (Figure 4.22) in Treatment III. However, in the same treatment, the removal of PO_4 was relatively similar to that of Treatment I. However, Treatment IV greatly removed insoluble P from the wastewater, with a complete P removal. Using the algae, *C. vulgaris*, and the fungus, *Aspergillus* sp., it has been reported that the treatment achieved an 85% removal using diluted wastewater from a swine facility (Zhou et al., 2012a). Similarly, using 25% diluted swine facility wastewater, *A. fumigatus* and *C. vulgaris* showed a 69% of phosphate uptake in a co-cultivation process (Wrede et al., 2014). Another study reported removal of 85% of total phosphorus using a combination of the fungus *G. lucidum* and the alga *C. vulgaris* (Guo et al., 2017).

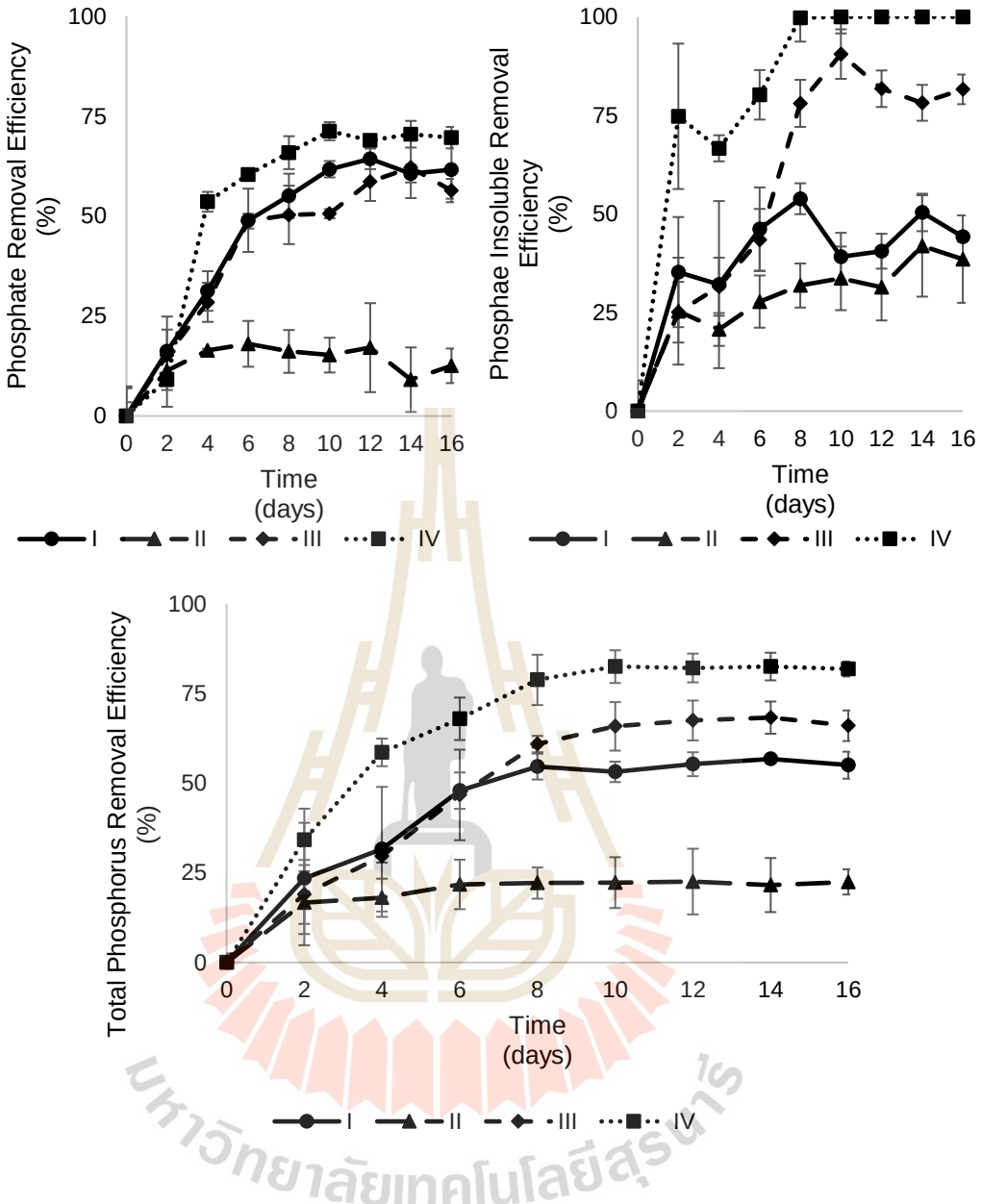

Figure 4.22 Total phosphorous (TP), phosphate (PO_4), and phosphate insoluble (IP) profiles during cultivation period.

Overall, co-cultivation of algae and fungi in pellet form showed higher phosphorous removal than other treatments. Treatments III and IV showed the highest removal efficiencies (66.00 and 81.82%, respectively), while the removal by an algal monoculture was only 51.01%. A fungal monoculture was found to remove less than 35 % of phosphorous. *C. vulgaris*, as a mixotroph culture, showed a capacity to remove TP after a considerable time. Phosphorus is a nutrient that is commonly removed using an algal treatment system since these microorganisms have a phosphate uptake

mechanism (Bunce et al., 2018). However, the remaining phosphorus in the water after *C. vulgaris* reached the stationary phase of removal was still considerably high, above the discharge standard. Here, additional pellets in the stationary phase of phosphorous removal increased removal significantly, almost reaching the removal using the co-culture in Treatment IV. Addition of the fungus, *A. niger*, in the system allowed algae to utilize phosphorus in the wastewater that was not assimilated in the algal monoculture. Phosphate solubilizing fungi are known to be affected by their carbon source availability (Seshadri et al., 2004). As the carbon in the CBEW consisted of hard-to-degrade carbon sources, extracellular polymeric substances (EPS) from the algae were abundant to support the solubilization activity of the fungi. As preferring extracellular carbon energy (Xiao & Zheng, 2016), algal EPS was also available in the co-culture of *A. niger*.

The chemical oxygen demand (COD) profile was also observed during the treatments (Figure 4.23). The COD profile showed fluctuation of the total organic components during treatment. Removal of COD did not significantly occur in Treatment II. Treatment I, similarly, did not effectively remove COD from the wastewater. Additional pellets rendered a dramatic decrease of COD concentration after day 6. Moreover, the removal of COD in Treatment IV was significantly higher than in other treatments. Treatment IV had a removal efficiency of 68.07%, followed by Treatments III, I, and II with removal efficiencies of 51.26, 39, and 33%, respectively. A COD removal of up to 70% was previously shown using *Aspergillus* sp. and the alga, *C. vulgaris* (Zhou et al., 2012a). As much as 79.74% COD removal was reported in treated swine facility wastewater using *G. lucidum* and *C. vulgaris* (Guo et al., 2017). It has been reported that the removal of sugar from a medium was improved in the co-cultivation system of algae and fungi, and the removal by a fungal monoculture did not occur due to insufficient degradable carbon (Xie et al., 2013).

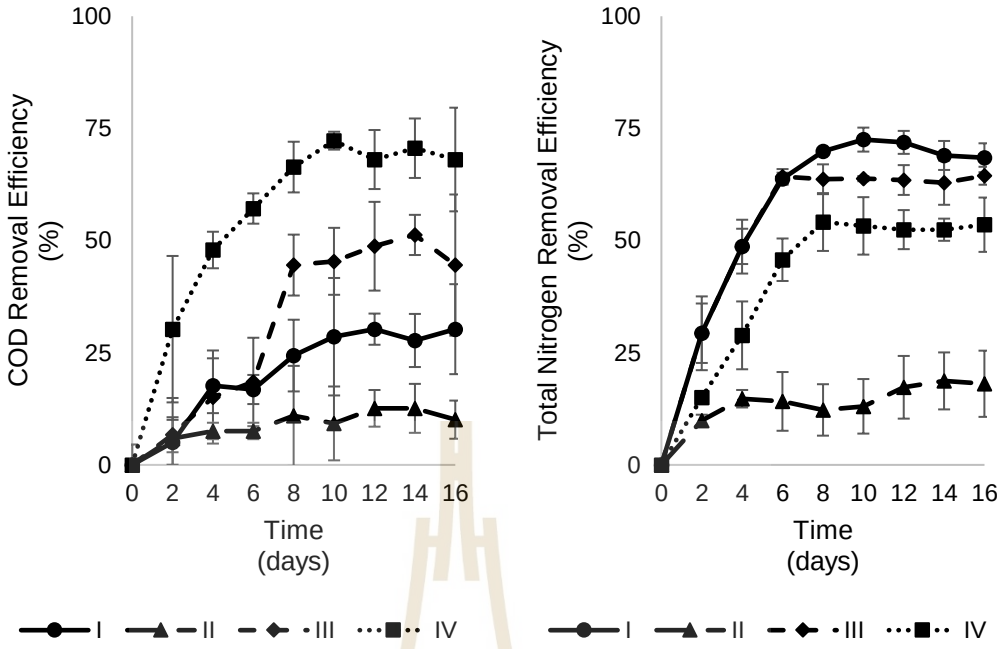

Figure 4.23 Chemical oxygen demand (COD) and total nitrogen (TN) profiles during the cultivation period. I-IV indicate the treatment I to treatment IV.

Nitrogen removal during these four treatments varied. Treatment II did not show any significant removal of nitrogen (18.08 %). Interestingly, Treatment IV showed lower TN removal activity than Treatments III and I. Addition of fungal pellets on day six also inhibited TN removal (Figure 4.23). The highest removal was found for Treatment I, 68.46%, followed by Treatments III, IV, and II with removal efficiencies of 64.45, 52.42, and 18.08%, respectively. It has been reported that the use of *A. fumigatus* co-cultured with *Thraustochytrid* sp. presented 86% removal of NH_4 in 25% swine facility wastewater (Wrede et al., 2014). Co-culture of *G. lucidatum* with *C. vulgaris* demonstrated 74.28% removal of TN in a similar wastewater (Guo et al., 2017). Concurrently, a combination of *C. vulgaris* and *A. niger* removed only 23% of NH_4 in a diluted swine facility wastewater.

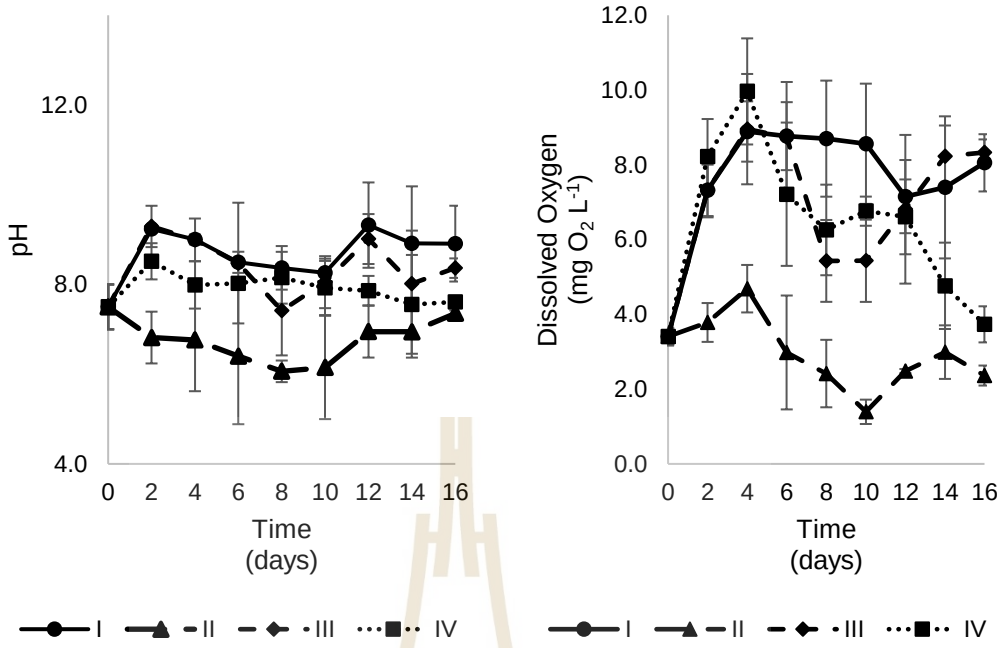

Figure 4.24 Abiotic conditions in four different inoculant methods of algal-fungal pellets for CBEW treatment. I-IV indicate the treatments result.

Interestingly, removal efficiencies of a co-culture of *C. vulgaris* and *A. niger* were lower than in a monoculture of *C. vulgaris*. Moreover, removal of TN in Treatment II tended to become constant after *A. niger* was introduced into the culture (Figure 4.23). This indicates that there was an inhibition of nitrogen uptake when these two organisms are co-cultured. The inhibition might be due to a reduced pH and an increase of DO (Figure 4.24) after addition of pellets that changed nitrogen uptake. It was reported that the optimum pH for nitrogen removal in green single-cell algae ranges from 7.5 to 8.5 (Ullrich, 1983). As the pH did not meet the requirement for ion exchange and direct assimilation for available nitrogen (Figure 4.24), the removal of nitrogen in the presence of *A. niger* was lower than in a monoculture of *C. vulgaris* (Treatment I).

4.4 Harvestability

4.4.1 Harvestability of Alga-actinomycete co-culture

Harvestability of the single and co-culture was also assessed at the end of the cultivation period. As it mentioned previously that the inoculants of microalga were prepared in the exponential period, the inoculants of actinomycete BMI 10, on the other hand, were prepared in the pellets form from the spores (Figure 4.25). After

the cultivation there were two mains formation of the actinomycete hyphae. Some hyphae were found in the filamentous suspension, whilst the others were found in pellets (Figure 4.25 & 4.26).

It was shown that P21 harvestability was significantly improved in the co-culture than in the single inoculant form (Figure 4.27). Here, the biomass recovery based on physical attachment was observed via algal-algal attachment in both P21 and P21+BMI 10 cultures. The process of algal-algal attachment was initially possible when the surface of algal where containing less charge which can cause the repellent effect to algal cells. This phenomenon is often called charge dispersion mechanism (Rawat et al., 2011). The surface of algal cell has negative charge as a result of dissociation or ionization from the functional surface groups (Pragya et al., 2013). Thus, the coagulation of the algal cells in the suspension of P21 single culture can indicate that there was organic compound or other positively charge substance that bridge the negatively charge surface of algal cells (Li et al., 2017b). Nevertheless, the filamentous bacteria such as *S. thermocarboxydus* BMI 10 was proven to initiate the flocculation better than the sole ionic bridging between the algal cells, as it can entrap the algal cells between the hyphae and fasten the sedimentation process (Leng et al., 2021).

Figure 4.25 Actinomycetes BMI 10 pellet and its adsorption of microalga P21

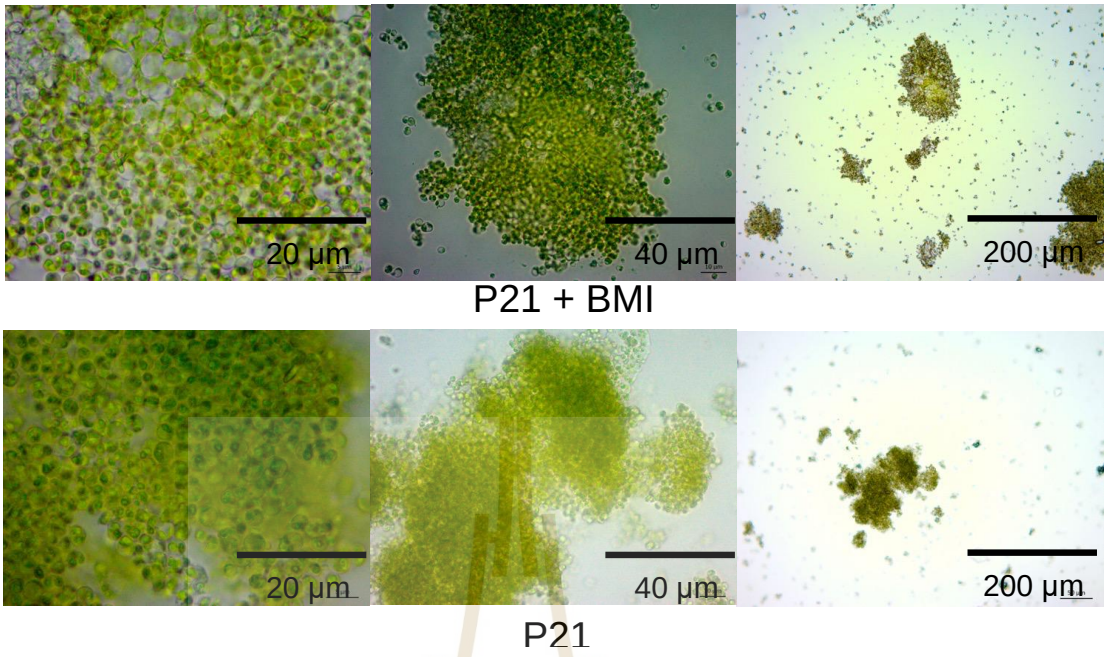

Figure 4.26 Microscopic observation result of single P21 and co-culture with BMI in sterilized CBEW.

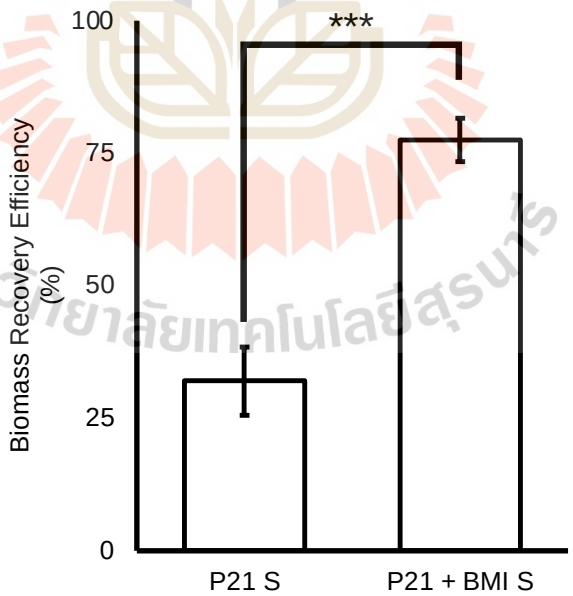

Figure 4.27 Harvestability of microalga P21 in single culture and co-culture with actinomycete BMI 10. *** means there is a significant difference using T-test at the 99.9 % confidence level.

4.4.2 Harvestability of Alga-Fungus co-culture

Harvestability of the single alga and co-culture was also measured in all treatments that previously described in the removal efficiency section. Self-sedimentation test was conducted similar to the test with the combination of actinomycete and alga. It has been found that there was no significant difference between the removal of solids in the suspension in all treatments where the removal ranged from 65 to 75 % (Figure 4.28). All the treatments showed the relatively similar decrease based on the difference between the absorbance just after the agitation stopped and the absorbance after 30 min sedimentation. It is important to note that there was a significant difference between these four treatments in terms of biomass contained in the suspension.

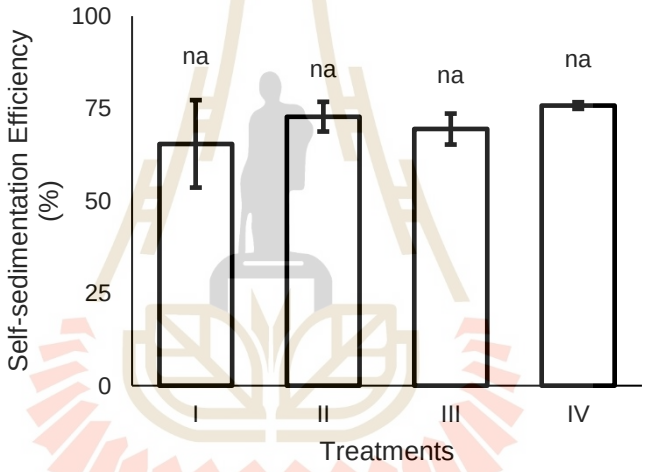

Figure 4.28 Self-sedimentation of four different types of treatments in CBEW by alga *C. vulgaris* 8580 and fungus *A. niger* F5.

To explore the possibility of the difference between the treatments, the biomass removal efficiency of the pellets was shown in the absorbance measurement of all treatments throughout the cultivation period. It was found that even though there was only slight increase of fungus in Treatment II, the A_{680} of this culture increased and then fluctuated throughout the cultivation period (Figure 4.29). The additional pellets in Treatment III affected the A_{680} significantly, reducing it to 0.129. However, after a further cultivation, the suspension A_{680} increased to 0.353. Absorbance decreased as the culture time increased in Treatment III, indicating that the capacity of the pellets to hold algal cells was constant while the number of algal

cells increased (Figure 4.18). The algal cells in Treatment IV outgrew the capacity of the pellets to hold them. As a result, the A_{680} in Treatment IV increased gradually, in line with the increased SS. This phenomenon was also reported previously where the adsorption capacity of pellets was lower than the available algal cells since the algae kept growing (Xie et al., 2013).

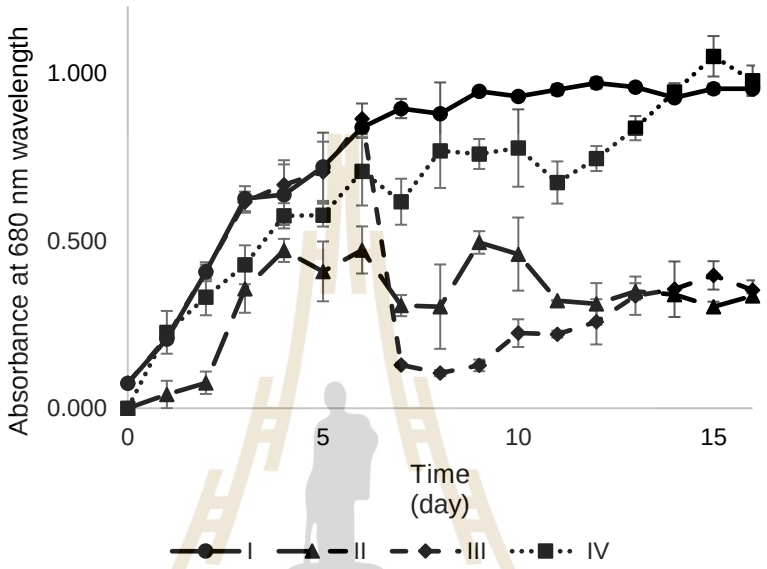

Figure 4.29 Absorbance of the suspension throughout the cultivation period of four different treatments during the cultivation period. I-IV indicate the treatment I to treatment IV.

Overall, the removal activity of pellets was lower than in previous studies. It has been reported removal efficiencies of 50-90% using isolated fungi (Muradov et al., 2015). Another study also reported a removal efficiency of almost 100% by *A. oryzae* (Zhou et al., 2013) as well as by *Cunninghamella echinulate* (Xie et al., 2013) and *Penicillium* sp. (Chen et al., 2018). Using *Pleurotus ostreatus*, as much as 64.86% algal removal was achieved to produce an edible product (Luo et al., 2019). Although the results of algal adsorption varied depending on many factors, such as the strain used and physicochemical properties, the additional removal of phosphorus with comparable removal using *A. niger* F5 to harvest *C. vulgaris* was remarkable.

To further examine the possibility of attachment mechanisms in the algal-fungal pellets, set of experiments were conducted. Using the formed pellets of the F5 using 15 g L^{-1} glucose supplementation and the suspension of alga 8580, the adsorption was carefully conducted. It was previously intended to be observed under

compound microscope. At the first step of observation, the fungal pellets were successful to adsorb the algal cells (Figure 4.30). However, this form was permanent in the system (similar with the co-culture examination, Figure 4.17) and thus to observe the attachment of the fungi to the wastewater other method needed to be employed since it could not be observed under compound microscope. Thus, the observation was conducted different from the alga-fungus suspension.

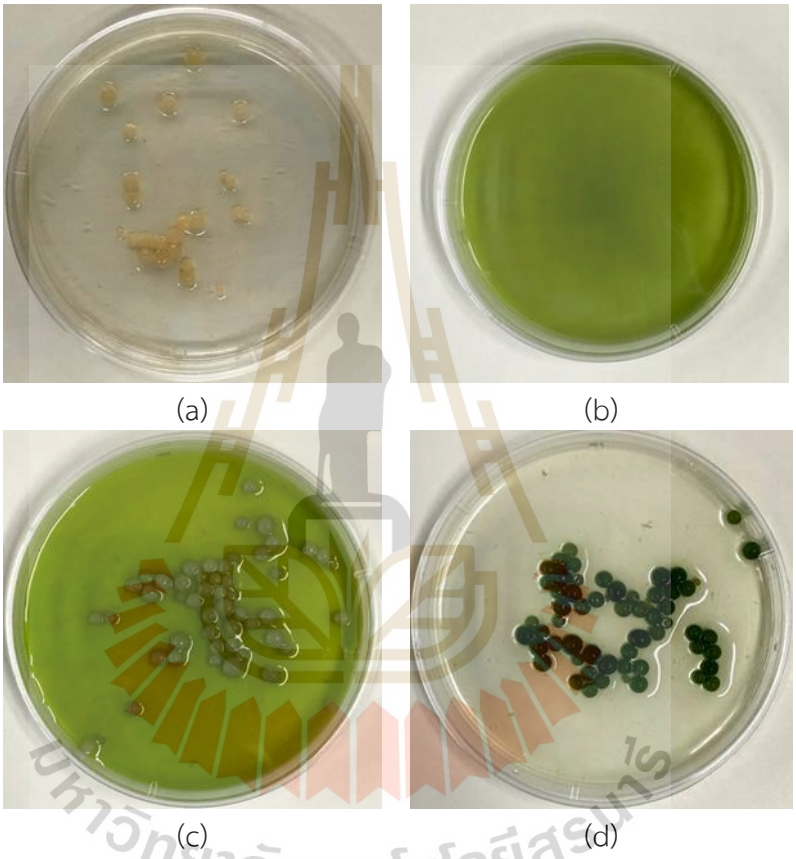

Figure 4.30 The adsorption process of microalgal suspension into fungal pellets (a) fungal pellets, (b) microalgal suspension, (c) early stage of adsorption, and (d) saturated stage of adsorption.

Scanning Electron Microscope (SEM) was employed, and the images revealed several mechanisms that affect adsorption. Direct attachment of algal cells to mycelial surfaces was detected in some observations (Figure 4.31a). However, evidence indicates that some algal cells were trapped between other algal cells, indicating no strong repulsion between the cells (Figure 4.31b). This phenomenon also

demonstrated that the zeta potential on the algal cell surfaces was not strong (positive or negative). Alga-alga cell attachment may be due to the neutral or acidic states on the contact surface (Ozkan & Berberoglu, 2013) or an alkaline state that generates auto-flocculation mechanisms (Zhu et al., 2018).

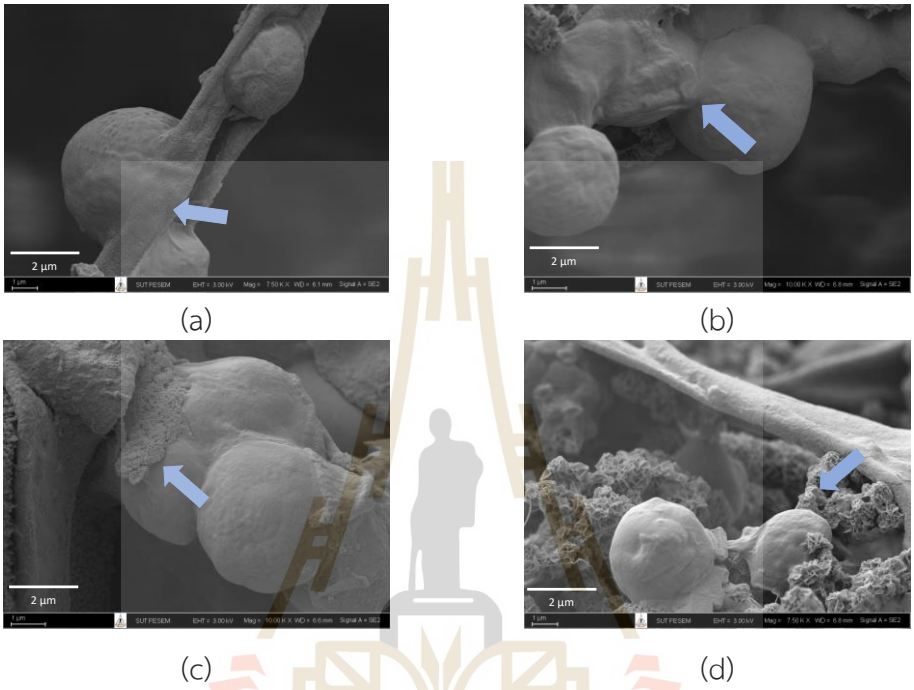

Figure 4.31 Attachment steps of microalgal cells into filamentous pellets of fungi, (a) direct attachment, (b) algal-algal attachment, (c) saccharide-assisted attachment, and (d) polypeptide-assisted attachment.

Apart from physical attachment, biological attachments were also found on most surfaces where the algae attached. Two molecular species were identified during these observations. The first was a sponge-like structure with a porous surface (Figure 4.31c), which was prominent on most surfaces. The other was a granular structure (Figure 4.31d). Here, porous, or wrinkled, structures were tentatively identified as peptides or their derivatives, while homogenous granules were expected to be saccharides, as previously shown in SEM imaging studies (Mann et al., 2010; Sousa et al., 2017). These structures might have been derived from extracellular polymeric substances (EPS) of the fungi since they were abundant in pellets formed with 15 g L^{-1} of glucose. Fungal EPS has been reported on pellet surfaces of many studies. This material commonly consists of polysaccharides and polypeptides (Dang et al., 2018)

with high polarity (Zhang et al., 2020b). Surface protein interactions and exopolysaccharide adhesion were also reported as alternative mechanisms of attachment of algal in pellets (Leng et al., 2021). EPS plays an essential role in the adsorption capacity of algae onto fungal pellets where attachment due to EPS reached levels of as much as 88 %. In comparison, the other two factors, zeta potential and surface area, only accounted for 11 % and 5 %, respectively, of attachment (Li et al., 2020b).

4.5 Normalization for Batch to Semi-continuous systems, Oil Production, and Life Cycle Assessment

4.5.1 Comparison of fungus and actinomycete for CBEW treatment

To compare the coupling of CBEW wastewater treatment potency and algal biomass production, the normalization of the obtained values from each experiment of co-culture was made (Table 4.9). It was shown that the combination of the alga P21 and the actinomycete BMI obtained the highest normalized number for the parameters measured. It was followed by 8580+F5 using treatment IV and treatment III respectively. However, the application of P21 and 8580 showed similar results with the previous normalization in the Table 4.4. Nevertheless, the difference in the application of the wastewater caused by the different preparation of wastewater that may cause the slight change in the total score of the inoculants.

Table 4.9 Normalization critical parameters of alga-actinomycete and alga-fungus co-culture for wastewater treatment and algal biomass generation.

Parameters	P21	P21+BMI 10	8580	8580+F5 (III)	8580+F5 (IV)
μ	0.67	0.59	1.00	0.75	0.81
Xmax	0.88	1.00	0.28	0.43	0.59
N-removal	0.85	1.00	0.93	0.83	0.69
P-removal	0.82	1.00	0.72	0.90	0.95
COD-removal	0.74	1.00	0.49	0.65	0.91
Harvestability	0.40	0.98	0.66	1.00	0.85
Total	4.36	5.57	4.09	4.56	4.80

Values are the normalization from the actual triplicate data of the experiments.

Although the results were obtained using slightly different approaches, values of biomass yield and nutrients removal with the harvestability were compared using the simple normalization calculation. Here, it was demonstrated that the computational-based analysis is an important tool for deciding the wastewater treatment system that presumably the most suitable for given condition. It was also previously demonstrated for similar purpose (Kalbar et al., 2016). Normalization is among the methods required for the complicated and further analysis. This data transformation can be easily done and keep the important information in the transformed results (Franco et al., 2019). Based on the calculated values, further application of the co-culture was focused on the P21+BMI as the most promising combination. Based on the calculated values, it has been decided to further develop co-culture of microalga P21 with alga

4.5.2 Co-culture batch cultivation in raw wastewater

As the leading combination of algal co-culture, alga *Chlorella sorokiniana* strain P21 and actinomycete *Streptomyces thermocarboxydus* strain BMI 10 were tested to co-cultivate in the raw CBEW system. Without any sterilization, native microorganisms in the wastewater were preserved in the system and thus combined with the co-culture. Similar with the result in sterilized condition (Figure 4.15), the cultivation of co-culture did have similar pattern with the single P21 culture in the early stage of cultivation (Figure 4.32). Meanwhile, the difference of P21 and P21+BMI 10 started to occur after 8-day period of cultivation. Chlorophyll *a* was also detected in the unsterilized culture as a sign of native pigmented organisms (Figure 4.32a), indicating that the raw wastewater contained considerable amount of native green microalgae.

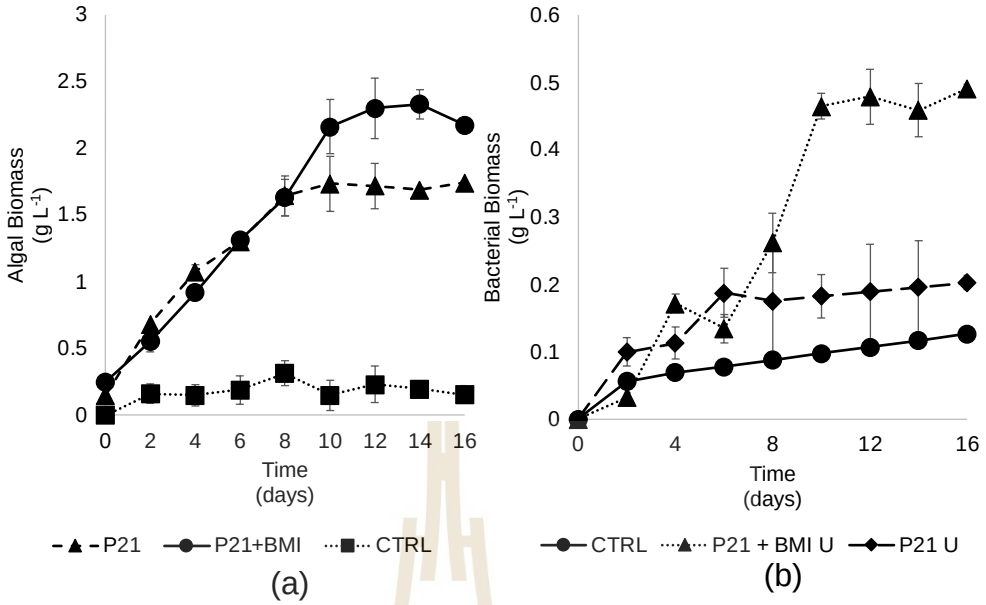

Figure 4.32 Biomass evolution of native algae and bacterial (CTRL), *C. sorokiniana* (P21), and *S. thermocarboxydus* (BMI 10). (a) Algal biomass in raw wastewater cultivation, and (b) Bacterial biomass in raw wastewater cultivation.

The growth of bacterial biomass in the system was higher in the occurrence P21 and the control (Figure 4.32b). Interestingly, the growth of the total bacteria of the P21+BMI 10 co-culture dramatically increased and had higher total bacterial biomass than the wastewater system with only P21 as the single inoculant. However, the differences can only be seen after eight days. These variations can be caused by the presence of native bacteria that compete with the BMI strain to obtain organic carbon from the P21 strain (Petrini et al., 2020). While the increase of wastewater bacterial biomass P21+BMI 10 can be explained as the increased of total bacterial together with BMI 10 biomass that inoculated as the effect of exopolysaccharides (EPS) from alga P21 (Lupi et al., 1994).

The phenomenon of higher EPS results in a higher bacterial growth rate and greater biomass in the BMI 10 co-culture of BMI 10 and native strains (0.29 day^{-1}) wastewater compared to a native strain only (0.19 day^{-1}) (Table 4.10). Therefore, it can be surmised that there was no competition between the native strain and those in the co-culture (Grover, 2000). Additionally, there was no inhibition of the native wastewater strain by the actinomycetes (Salah El-Din Mohamed & Zaki, 2019). The latter reason was also supported by the negative results in the antagonistic test of the BMI 10 strain and native bacteria (Supplementary Figure 9).

Table 4.10 Kinetics Parameters of several wastewater treatments in the system.

Kinetics Parameters	Algal Biomass		Bacterial Biomass		
	P21	P21+BMI 10	P21+BMI 10	P21	CTRL
X_o (g L^{-1})	0.65	0.65	0.070	0.015	0.015
X_m (g L^{-1})	1.74	2.17	0.49	0.12	0.13
μ (d^{-1})	0.35	0.25	0.29	0.46	0.19
R^2	0.97	0.93	0.90	0.95	0.98

P21: Single culture of *Chlorella Sorokiniana* strain P21; P21+BMI: Co-culture of *C. sorokiniana* P21 and *Streptomyces thermocarboxydus*; CTRL: native bacteria.

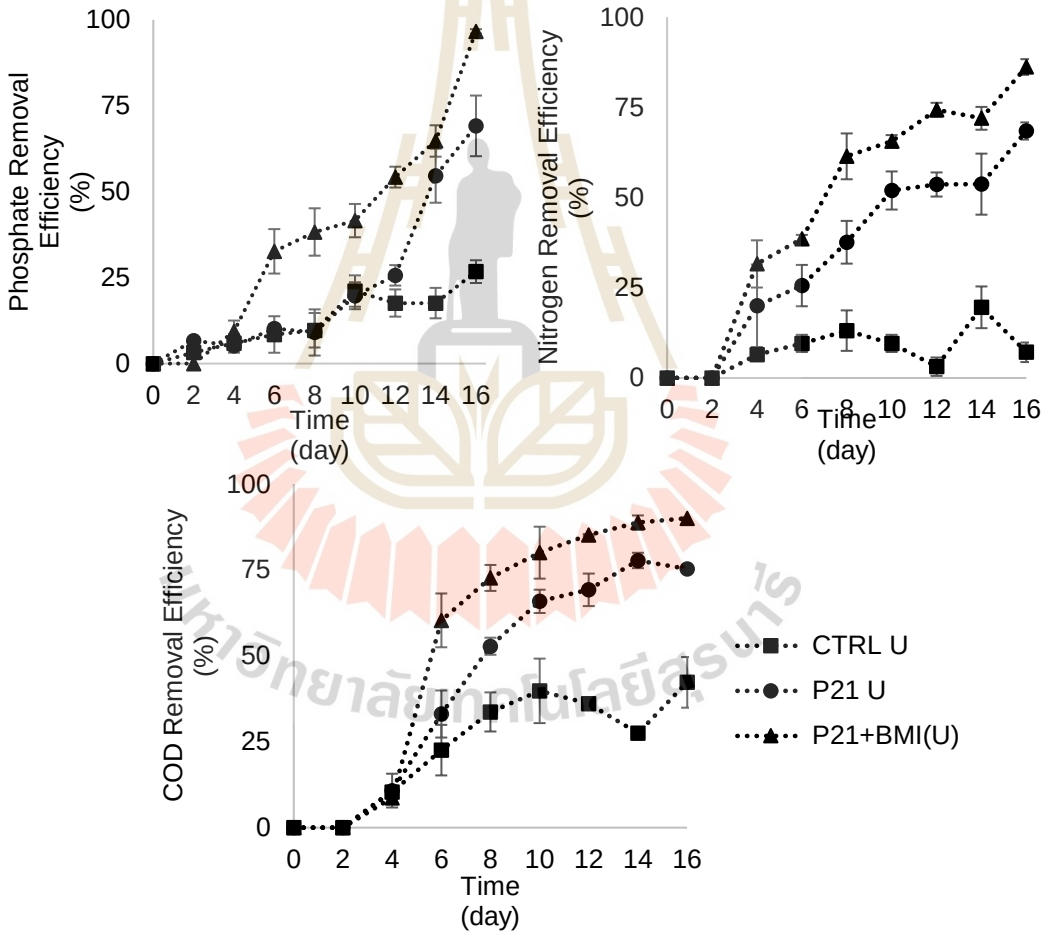

Figure 4.33 Effects co-culture of alga P21 and actinomycete BMI 10 on wastewater nutrient content parameters in raw wastewater condition.

The enhancement of nutrient removal efficiencies of phosphate, nitrogen, and COD was also found in the unsterilized culture. All these important contaminants were completely fulfilled the standard for the discard to the environment in the combination of BMI 10 as a co-inoculant for alga P21. All parameters also showed an increase of removal rate in the presence of BMI 10 (Figure 4.33). These results are in line with the batch culture in the sterilized condition that previously demonstrated and explained previously (Figure 4.19). However, a slight wider gap between P21 inoculant and P21+BMI 10 inoculant in the raw wastewater was found indicating that the occurrence of wastewater's native microbial communities can be beneficial for the water treatment.

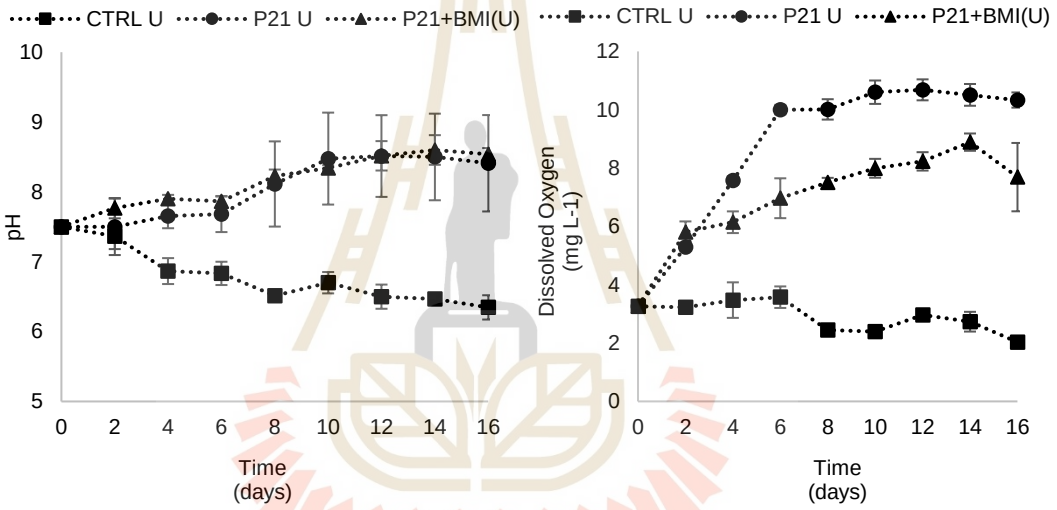

Figure 4.34 Evolution of pH and Dissolved Oxygen during the cultivation of microalga P21 and actinomycete BMI 10 in raw CBEW condition.

COD removal was significantly increased from 75.3 % to 90.47 % in the BMI 10 co-inoculation. Here the increased of COD removal was likely related to the increase of the organic compound consumption by microalgae and other microbes in the system (Su et al., 2012). The increase of nitrogen removal was also found from 68.45 to 86.19 % in the co-culture. Here, there are two main mechanisms of the removal. Firstly, the main removal by the autotrophic mode of the P21 and small number of native strains algae. Secondly, other process called nitrate/nitrite respiration in anaerobic conditions may take place to enhance the nitrogen consumption by the native bacteria (Paddock et al., 2020). Lastly, phosphate removal also increased from

69.15 % to be 96.63 %. As the value of pH was below 9.5 (Figure 4.34), the phosphate precipitation can be negligible (Ferguson & McCarty, 1971). Similar with the COD removal, phosphate removal activity may come from the increased number of algae which increased the number of phosphate capacity. It also may come from the removal of phosphate from the native bacteria. Combination of both removals is also possible such environment.

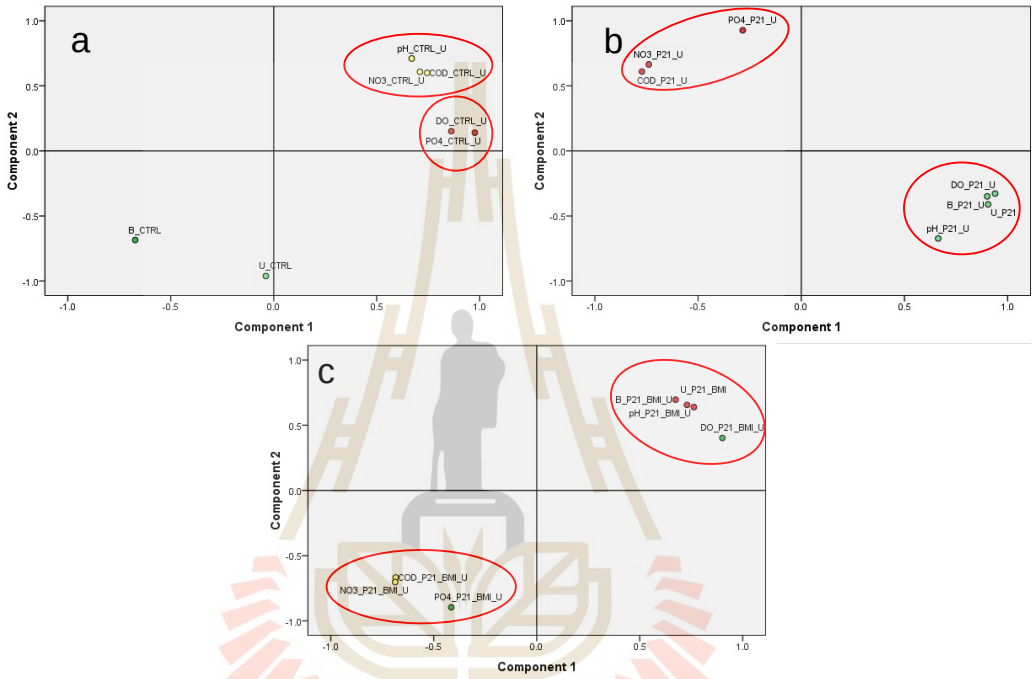

Figure 4.35 Principal Component Analysis (PCA) of co-culture result to the wastewater nutrient removal and wastewater characteristics. (a) native microorganisms' condition, (b) P21 in unsterilized wastewater condition (c) Co-culture of P21 and BMI in unsterilized wastewater condition. PCA was tested using two components (70-95 % data explained in component 1, 5-30 % data explained in component 2).

Principal component analysis of the co-culture in the CBEW revealed the process of the removal and biomass generation had strong correlation each other. In the control system, native microbial acted as the only biological agent for the removal and the nutrients concentration parameters with the wastewater condition were affected by the biomass increase (Figure 4.35a). Differently, phosphate, nitrogen, and COD were negatively correlated with the biomass, pH, and DO in the single culture

and co-culture systems (Figure 4.35b&c). This indicates that the wastewater was mainly treated by the algae with small amount portion of direct interaction between native bacteria and removal of the wastewater. The patterns of the pH and DO which drew different area in native strains PCA (Figure 4.35a) may indicate that the precipitation or air sparging could take place as other mechanisms to remove the contaminants (Urum et al., 2005).

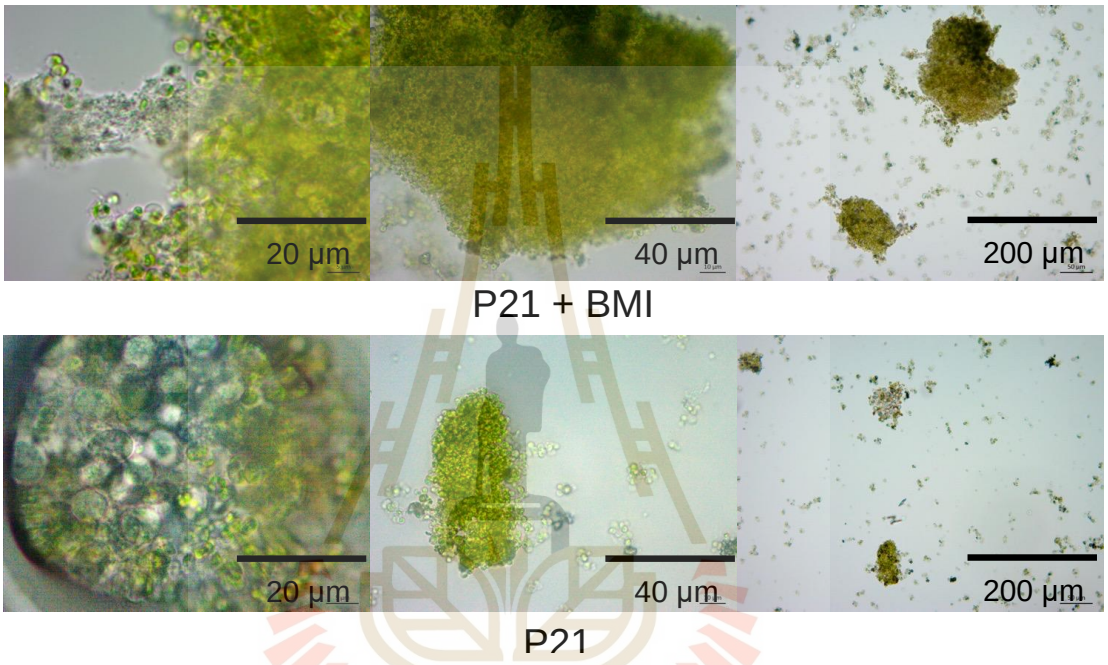

Figure 4.36 Microscopic observation result of single P21 and co-culture with BMI in raw CBEW.

The size of flocs formed in the P21+BMI 10 were larger than in the P21 single inoculant in raw wastewater (Figure 4.36). However, the raw wastewater condition showed a slight increase of P21 self-sedimentation ability in the occurrence of BMI 10 as a co-inoculant (Figure 4.37). It is important to note that the removal of the wastewater in the single and co-culture of raw wastewater was only slightly increased compared to the dramatical increase of additional BMI in the sterilized CBEW (Figure 4.27). Here, the occurrence of native bacterial or additional BMI 10 was found to enhance the harvestability, apart from the mycelial form of the bacteria. Additional coagulants and flocculant have various impacts upon sedimentation in P21 single culture. Among all the coagulants-flocculants that added, only $FeCl_3$ could remove up

to 90%. In contrast, other flocculants showed removal effectiveness of 82% for $CaCl_2$, and 74% for $FeSO_4$. However, starch showed removal that was even worse than with no flocculant (52%) (Figure 4.37).

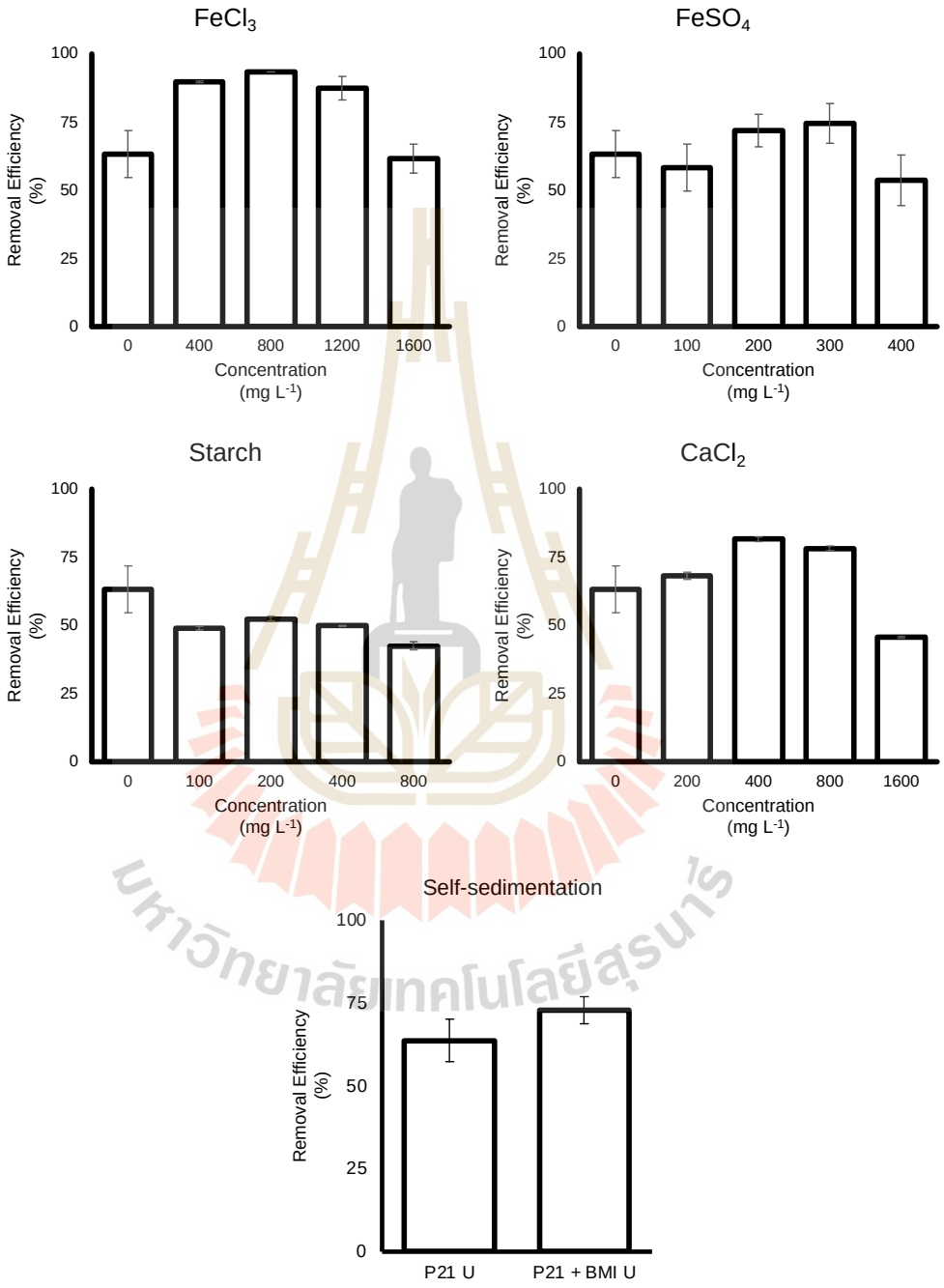

Figure 4.37 Harvestability of microalga P21 in single culture in several coagulant-flocculants and co-culture with actinomycete BMI 10 in raw CBEW wastewater.

$CaCl_2$ is widely used in microbial harvesting processes since it contains a sufficient amount of positively charge groups in its calcium ions. However, several studies have reported the feasibility of using this coagulant for algal harvesting. The optimal concentration of $CaCl_2$ in an *Arthrospira maxima* culture of 2 g L^{-1} was reported as 200-300 mg L^{-1} at a high pH (Caetano et al., 2020). $CaCl_2$ was also used as a flocculant aid for enhanced harvesting by bioflocculation of bacteria. It was also previously reported that 70 mg L^{-1} was optimal for enhancing the removal of algal from a suspension using *Streptomyces* sp (Li et al., 2017b). The mechanism of calcium-binding in algal and other microorganisms in culture might result in removal of 74% of the biomass. Here, the removal of $CaCl_2$ through a calcium-binding mechanism was assumed to take place (Branyikova et al, 2018).

Starch was reported to require 20-40 mg L^{-1} doses for >80% removal (Vandamme et al., 2010). Similarly, 20-30 mg L^{-1} doses of maize starch reduced turbidity in an algal suspension from >100 NTU to <20 NTU (El-Naggar et al., 2018). However, incomplete removal of starch has also been reported (Gerde et al., 2014). This result may be related to insufficient cationic groups in the starch. Higher doses of starch can increase the turbidity of water. The formed flocs were not as compact as when other flocculants were used (Supplementary Figure 11). Moreover, the P21 strain was found to settle more quickly in the absence of starch as a flocculant. This result is similar to the use of *Scenedesmus rubescens*, which exhibits considerable self-sedimentation (Vergini et al., 2016).

Utilization of $FeCl_3$ for examining the harvestability of microalgae has been demonstrated in previous studies. $FeCl_3$ was reported to recover algal biomass by applying as much as 15 mg L^{-1} dose into 55 mg L^{-1} algal suspension (Chekli et al., 2017). Mennaa et al. (2015) also reported obtaining high removal efficiency (>90%) in various cultures of microalgae, including a blooming algal seed at a concentration of 60 mg L^{-1}. $FeCl_3$ is a well-known coagulant for wastewater treatment systems and works suitably with the extracellular components of algae (Agbovi & Wilson, 2017). Another reason for the high removal activity displayed by $FeCl_3$ is the ability of Fe^{+2} ions to capture extracellular polymeric substances (Dong et al., 2015) that contribute to the turbidity of the culture where colloids are found in the suspension (Supplementary Figure 11).

Conversely, $FeSO_4$ is an economical metal coagulant for wastewater. It was reported to obtain removal efficiencies of 57-86% in *C. vulgaris* culture with an initial concentration of 1.12 g L^{-1} (Zhu et al., 2020). With a 2.5 mg L^{-1} algal biomass in a urine supplemented culture, 300 mg L^{-1} of $FeSO_4$ was reported to remove 65% of the biomass present (Behera et al., 2020). Although $FeCl_3$ and $FeSO_4$ rely on Fe ions to

neutralize charges (Branyikova et al., 2018), the lower removal of $FeSO_4$ than $FeCl_3$ is related to the sensitivity of $FeSO_4$ flocculation to high pH values (>9) (Liu et al., 2021). Moreover, a high concentration of $FeSO_4$ can generate a yellow-brown color caused by corrosion (Behera et al., 2020). However, the optimal concentration of $FeSO_4$ was found to be relatively lower than that of $FeCl_3$ since the sulfate salt of ferrous sulfate can act as a coagulant via a charge neutralization mechanism.

Meanwhile, the improvement of self-sedimentation of BMI 10 is in line with a previous study that reported the enhancement of harvestability in co-culture or xenic conditions (Lakshmikandan et al., 2021; Sivasankar et al., 2020). Similar recovery was achieved in presence of bacteria showing that the main improvement factor in self-flocculation was related to the number of total bacteria in the system, which is usually in proportion with the level extracellular polymeric substances (EPS) in the culture. It was also previously found that the physical mechanism in the hyphae for algal removal was only significant in the early stage of recovery, while 88.2% of the successful recovery was determined by the level of EPS components in the medium (Li et al., 2020b). However, the current co-culture presented higher removal than a previous application using *Streptomyces* for bio-flocculation of *Chlorella* sp. (70%) (Li et al., 2017b), but slightly lower than the result of Lakshmikandan et al. (2021), who obtained more than 90% removal after 24 hours.

Based on these results in coagulation and flocculation experiment, it can be concluded that the removal of this self-sedimentation was still lower than the additional of coagulant and flocculants in several conditions. $FeCl_3$ and $CaCl_2$ were proven to have higher removal efficiencies than the self-sedimentation using P21+BMI 10. However, P21 self-sedimentation after addition of BMI 10 in the inoculant was higher than the sedimentation of starch and $FeSO_4$ (Figure 4.37). Farooq et al (2015) was stated that the application of iron-based coagulants may result biodiesel product that contain metals ion. This phenomenon may reduce the biodiesel quality since it can lower engine performance and change oxidation stability of the biodiesel (Jain & Sharma, 2014). Here, some coagulation aids may still be needed to complete harvestability of the P2+BMI 10. However, improvement can still be important to reduce the utilization of coagulant and ease the dewatering process of the biomass.

4.5.3 Semi continuous system for co-culture

Application of BMI 10 in the algal co-culture was developed from batch to semi-continuous system. The system was chosen based on several considerations. It eased the system to completely stirred and ensured that there was no sedimentation or other unmixed biomass in the reactors' wall (Ashokkumar et al., 2014). The complete mixing and scrubbing before the discharging the certain amount of the suspension in

the reactor were conducted daily. This system was previously applied by da Silva et al. (2009), where the closed photobioreactor was employed to examine the growth of *N. oleoabundans* and *S. obliquus*. Thus, the total biomass from daily sampling can represent the actual condition without reduction from the sedimentation process.

Development of the Verhulst model for batch culture was done to predict the system in this co-culture system based on Ruiz et al. (2013a). In that previous study, a flat panel photobioreactor with the working system of 4.5 L was used to treat raw wastewater. Meanwhile, in this study, the working volume of the reactor was approximately 5 L (without evaporation). Based on the batch data, Verhulst kinetic models for biomass growth were successfully projected in the continuous system. Here, a similar approach was also demonstrated.

Furthermore, to comprehensively draw the difference between batch and semi-continuous systems, a comparison also made in the flask of sterilized conditions using the same system. The previous culture of P21 and BMI 10 in the sterilized condition was further assessed in a semi-continuous system. The HRT was adjusted to be similar to the reactors' raw wastewater. Nevertheless, biomasses of the alga and bacteria were the only parameters measured in the sterilized condition as the cultivation in the flask volume was not sufficient for complete measurements of nutrient removals and their evolution through the experiment period.

The batch culture was used as the starting point of the experiments. Based on the growth curve in the batch system, it has been decided that the semi-continuous system would be started on day 16 of the batch cultivation (Figure 4.32 and 4.33), where all the cultures reached the end of the exponential phase. The treatment process of raw and sterilized wastewaters was conducted every day, and the suspension that discarded and fresh wastewater that supplied was based on the HRT. The steady-state condition was measured as the standard deviation of growth or biomass reduction was less than 10 % (Silva et al., 2019). Different from most of the semi-continuous studies that were previously conducted using several cycles based on the number of HRT (Salgueiro et al., 2018; Zheng et al., 2021), the cultivation in this study was conducted only one cycle for each HRT with the triplicate of each HRT (Álvarez & Otero, 2020). The biomass evolution data obtained from the wastewater treatments were obtained by daily measurements and combined to calculate the productivity (Supplementary Figure 12).

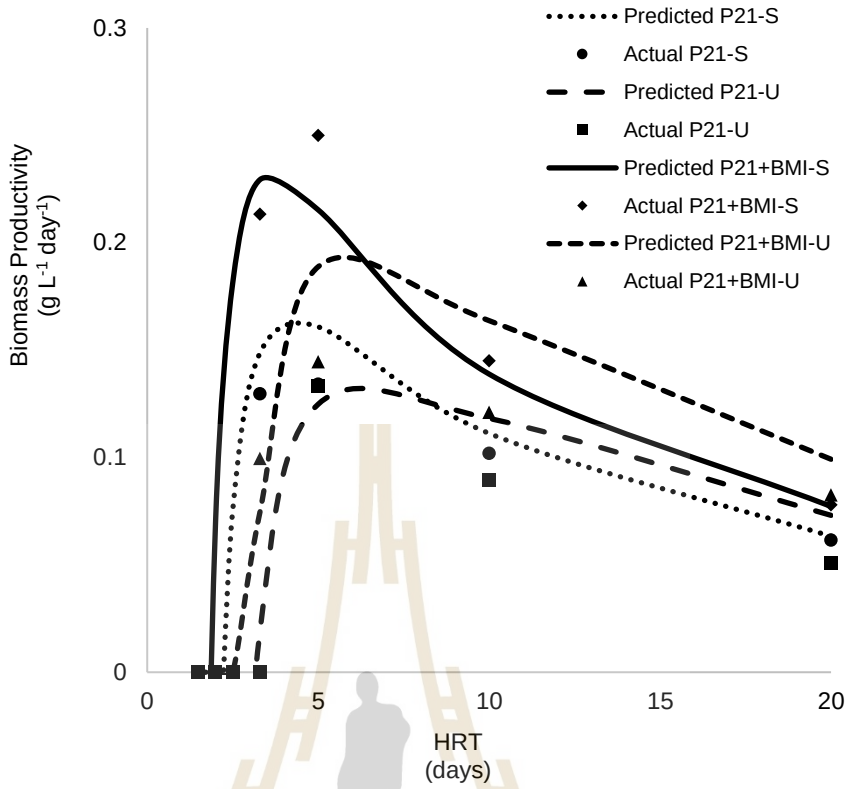

Figure 4.38 Biomass Productivity from different inoculants of alga P21 and Actinomycete BMI 10 in sterilized and unsterilized conditions.

A semi-continuous system from P21+BMI 10 showed different results in biomass generation in sterilized and raw conditions. Daily biomass productivity was higher in the sterilized condition than in the raw condition (Figure 4.38). The differences found in the P21 and P21+BMI results in sterilized and raw wastewaters were previously reported in several studies. A similar result from sterilized and unsterilized cultures was also reported before.

A study by Odjadjare et al. (2018) showed no significant difference between sterilized and unsterilized conditions. That study also pointed out that the nutrient contents in the wastewater were essential to determine the difference in biomass production, removal efficiency, and biomass contents. Nevertheless, Arora et al. (2020) also emphasized the possibility of different results from sterilized and raw wastewaters. Thus, the test using raw wastewater in a bench scale was essential to conduct in parallel with the sterilized wastewater treatment experiment. Here, the dynamic of the wastewater community where the competition to utilize the limited carbon source may happen (Zhang et al., 2020a). Unlike the batch experiment of raw

wastewater treatment by P21+BMI in Section 4.21, the semi-continuous system seems to require constant adaptation. The acclimatization, including the increase of pH and DO before the log phase, could not be provided in the continuous system. At the same time, bacterial loads and native microbial have favorable conditions in the constant supply of raw wastewater (González-Camejo et al., 2018). Slight degradation of wastewater content which eased other organisms to utilize the nutrient in the sterilized condition, may also affect the reduction of algal biomass productivity in this semi-continuous system for raw wastewater treatment system (Marjakangas et al., 2015). However, the co-culture results were still higher than in the single inoculant of algal P21. This result proved the feasibility of the co-culture to adapt not only in sterilized CBEW but also in raw CBEW conditions.

The results also showed that the model previously developed by Ruiz et al. (2013a) was correctly suited for the wastewater treatment system using the co-culture as an inoculant. Here, the application of the model showed the R^2 of 0.90-0.99 in four treatment systems (single and co-culture in sterilized and raw CBEW). Although the previous model was developed for the raw wastewater condition containing native strains, co-culture application by combining two different organisms in the inoculant forms was also proved to suit this model. It is also important to note that the low HRTs (1.5-3.3) caused a complete washout in the system (Figure 4.38). These HRTs were affected by the lower growth rate previously discussed in the batch system, and thus the washout could be found in the lower HRTs (Section 4.21).

Based on the productivities of five different HRTs, five days HRT appeared to produce the highest biomass per day. Sterilized P21+BMI 10 showed the highest productivity of 0.25 g L^{-1} day^{-1}, followed by P21+BMI 10 with biomass productivity of 0.15 g L^{-1} day^{-1}. Meanwhile, the sterilized and raw wastewater conditions of P21 showed relatively similar productivities on five days HRT which were 0.129 and 0.133 g L^{-1} day^{-1}, respectively. This result indicated that the main effect of additional BMI 10 was detected in sterilized wastewater. Nevertheless, the raw wastewater condition was enhanced but not as high as the sterilized condition. The productivity of the culture is also relatively low compared to previous studies. Utilizing aquaculture wastewater with a low carbon source and additional CO_2 injection, Gao et al. (2016) showed incredibly high productivity of *C. vulgaris* with 42.6 mg L^{-1} d^{-1} within only one day HRT. Ruiz et al. (2013a) reported that the productivity of *S. obliquus* reached 0.23 g L^{-1} day^{-1} using secondary effluent from the WWTP with the injection of 5 % of CO_2. Differently, some studies had shown lower productivity in the higher HRTs and only ambient air bubbling. Solís-Salinas et al. (2021) reported the steady-state of the natural algal community using HRT ranging from 6 to 10 days only resulted in 0.05 g L^{-1} day^{-1}

in a primary effluent from a wastewater treatment plant treating sewage. Although these studies were conducted separately using the different substrate, conditions, and inoculants, the composition of nutrients and other crucial factors were relatively similar, except the supplementation of CO_2. Thus, CO_2 supplementation may play a pivotal role in increasing productivity and lowering the optimum HRT to obtain the maximum yield (Min et al., 2011a).

The results of biomass productivity in Figure 4.38 were consistent with the nutrient removal of the wastewater in a semi-continuous system. Their washout conditions were found in the low HRT (1.5 and 2.5 days), and it was similar to the removal of nutrients where there was no daily removal found in the wastewater (Figure 4.39). Similarly, the daily removal patterns of COD as the function of HRT in all conditions were found to be different for both sole P21 and P21+BMI inoculants. This daily removal demonstrates that the alga *C. sorokiniana* P21 and actinomycete *S. thermocarboxydus* BMI 10 utilize soluble organic carbon in the wastewater to generate biomass. COD represents the oxygen needed for carbon oxidization in the wastewater. It can be reflected as the carbon source for completing the reactions required for nutrient removal (Mujtaba et al., 2017).

Moderate COD removal from wastewater is related to microalgae's unbiodegradable carbon, as CBEW comes from a long hydrolysis process called methanogenesis. Most of its easily degradable carbon is utilized, and the biodegradable carbon in the form of BOD was only 37.5% (Table 4.1). Similar to the batch culture using sterilized and raw wastewaters, daily removal by the co-culture was higher in phosphorus and nitrogen than in COD. It is common to have higher inorganic nutrient removals than organic removal since the COD can be very difficult to assimilate by the algae (Gupta et al., 2016; Nagi et al., 2020). Another scheme that might be possible is that degradation of nitrate and other nitrogen forms in wastewater that require organic matter are released in the system to incorporate nitrogen (Tsioptsias et al., 2016). Thus, organic matter as COD could not be removed entirely from the system.

Daily phosphate removal revealed that the most optimum HRT for the co-culture (P21+BMI-U) was five days with the daily removal of 1.397 mg L^{-1} day^{-1}. Meanwhile, phosphate removal in the single alga inoculant (P21-U) showed the most optimum HRT was ten days with the daily removal of 1.1205 mg L^{-1} day^{-1}. The removal was relatively higher than previous studies reported using a similar system. Solís-Salinas et al. (2021) reported the daily phosphate removal of 0.46 mg L^{-1} day^{-1} after reaching steady-state condition in the continuous culture by the native algal community. McGinn et al. (2012) also showed lower daily removal of *Scenedesmus* sp. with 0.97 mg L^{-1} day^{-1} of secondary effluent from the wastewater treatment plant. Because pH

was below 9.5 during most of the culture period (Supplementary Figure 12), it can be concluded that the removal via precipitation of insoluble phosphorous was low (Czerwionka et al., 2012). Thus, apart from the precipitation of this insoluble removal, a possible mechanism is by converting the most labile portion of particulate phosphorous into PO_4 for further utilization by algal cells (Patel et al., 2012).

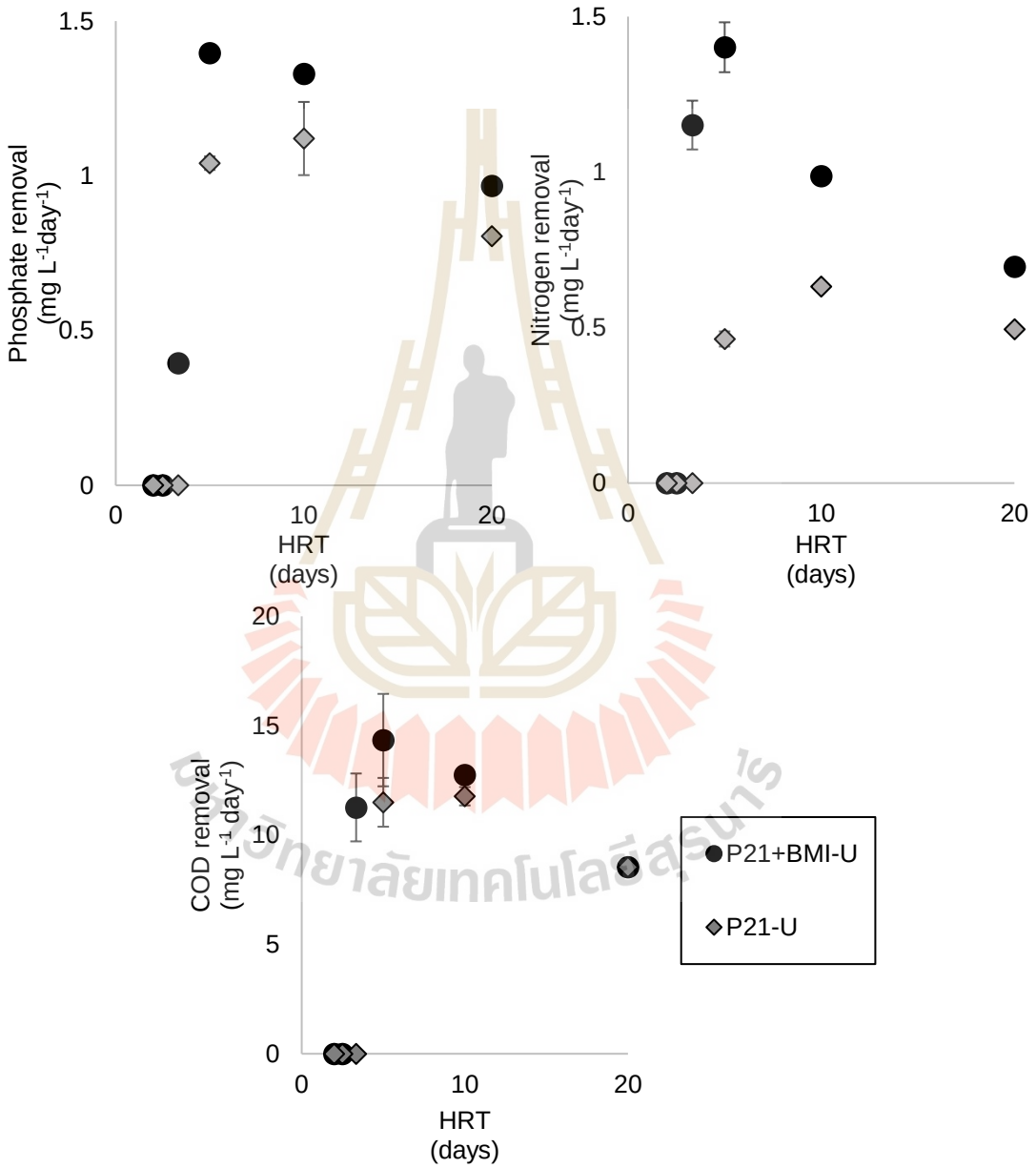

Figure 4.39 Daily removal of the nutrients in Phosphorus, Nitrogen, and COD as Hydraulic Retention Times (HRT) functions.

Dissolved nitrogen plays an essential role in biomass generation. Lack of available nitrogen for assimilation can lead to a lower biomass generation. In the current experiments, the daily removal of nitrogen was 1.4 mg L^{-1} day^{-1} using five days HRT by P21 and BMI as the co-culture. On the other hand, the highest daily removal in P21 single inoculant was found in the HRT of 10 days, removing 0.6 mg L^{-1} day^{-1} of nitrogen. In the continuous system, it can be seen that the daily removal was increased more than two times. This result showed higher removal than several previous studies. Application of natural microalgal bacteria in the high-rate algal pond was reported to remove 0.87 mg L^{-1} day^{-1} of dissolved inorganic nitrogen (Sutherland et al., 2020). Similarly, *C. vulgaris* culture utilized for dairy manure treatment showed daily removal of 0.93 mg L^{-1} day^{-1} using 20 days HRT (Wang et al., 2010b).

Nitrate has been reported to be a nitrogen source that microalgae can quickly assimilate (Leong et al., 2021). Similarly, ammonium is also preferable for assimilation since it requires less energy (Yang, 2011), even though ammonium was also toxic to microalgae at higher levels (Salbitani & Carfagna, 2021). Thus, it was expected that P21 and P21+BMI results could be predicted based on the batch culture reactors. The significant increase of N removal in the semi-continuous system was in line with the batch culture system of P21 and BMI (Section 4.3.1). Nevertheless, a high amount of dissolved oxygen in the system (Supplementary Figure 13) emphasizes that denitrification was not the primary removal mechanism in the present study (Xiao et al., 2012). This phenomenon supports the concept that algae consume ammonium simultaneously during nitrification, rather than a single nitrification process of NO_2-N into NO_3-N (Leong et al., 2021). Similarly, several strains of *Chlorella* were reported to survive and take up ammonium in a pond scale reactor (Ayre et al., 2017). Nitrogen in ammonium is preferred for assimilation by microalgae because it is more efficient for nitrogen reduction into organic nitrogen. Ammonium also can be directly converted into amino acids by glutamine synthetase (GS)-glutamate synthase (GOGAT) enzymes (Salbitani & Carfagna, 2021).

4.5.4 Oil Production

Production of oil in sterilized and raw wastewaters was measured in this study. The focus was to compare the oil produced and its quality based on the standard parameters. The amount of the oil produced from an unsterilized medium using the P21 strain and a sterilized medium to grow P21 + BMI 10 was not significantly different (Figure 4.40). The increase of microalgal biomass was in proportion with the oil yield, indicating sufficient nutrients in the system for algal growth (Hernández-García et al., 2019) and no nutrient limitations, enhancing lipid production (Piligaev et al., 2018). Here, the lipid percentage from unsterilized co-culture was 16.73 %, followed

by unsterilized single culture of P21 by 16.68 %, sterilized co-culture of P21 + BMI 10 16.47 %, and 10.93 % in P21 using sterilized culture. These values fall in the median range of lipid percentage from microalgal biomass (Table 2.1). Several important factors have been determined as the success keys for total lipid accumulation in the algal biomass, namely salinity (Pandit et al., 2017), temperature (Chaisutyakorn et al., 2018), nutrient limitation (Li et al., 2020c), and other environmental as well as metabolic and genomic factors (Singh et al., 2016).

Among the oleaginous microorganisms, algal was reported to have the lowest NO_x emission in the engine operation and even better than petroleum diesel (Wahlen et al., 2013). Thus, it is also essential to maintain the lipid level from the co-inoculant of this co-culture (BMI 10 biomass) to not interfere with the primary portion of algal lipid as the main source of lipid from the co-culture biomass. Here, the low concentration of lipid detected from a single strain of *S. thermocarboxydus* in the single inoculant condition may be advantageous since the lipid can only come from the alga.

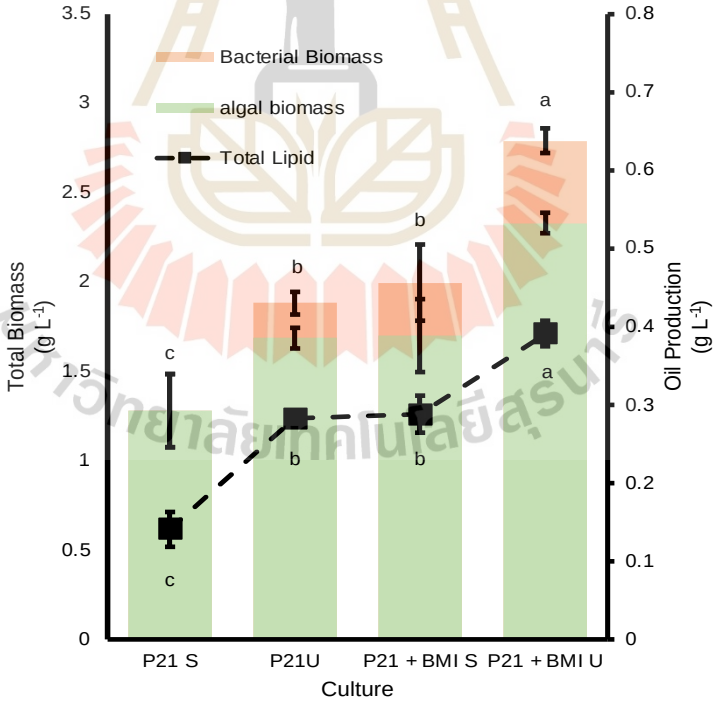

Figure 4.40 Lipid production by microalga *C. sorokiniana* P21 and its co-culture with actinomycete *S. thermocarboxydus* BMI 10. Different letters on the same line indicate significant differences by the DMRT ($p < 0.05$).

Materials flow analysis was depicted to track the analyzed nutrients conversion and the oil conversion into the biodiesel. Simple material flow was showed based on the total nutrients that removed from the wastewater, total biomass utilized per volume unit, and the final oil product for each unit volume of substrate (CBEW). Extracted oil from the biomass was then proceed into the transesterification process after saponification process using sodium hydroxide. The total process resulted the percent of oil that converted into biodiesel as much as 96.179 %. However, there was the side product including the glycerol that does not contain fatty acids. Based on the removal of nutrients, the percent of nutrients that utilized as biomass was also presented in Figure 4.41. It has been showed that the total nutrients utilized was still lower than the total biomass generated (only 9.6 % of the total biomass), indicating the process of autotroph was dominant where the inorganic carbon utilized and converted as biomass building blocks in the photosynthesis process. It can be concluded that the reduction of nutrients was coupled with the carbon sequestration in the form of CO_2 to complete the biomass synthesize process.

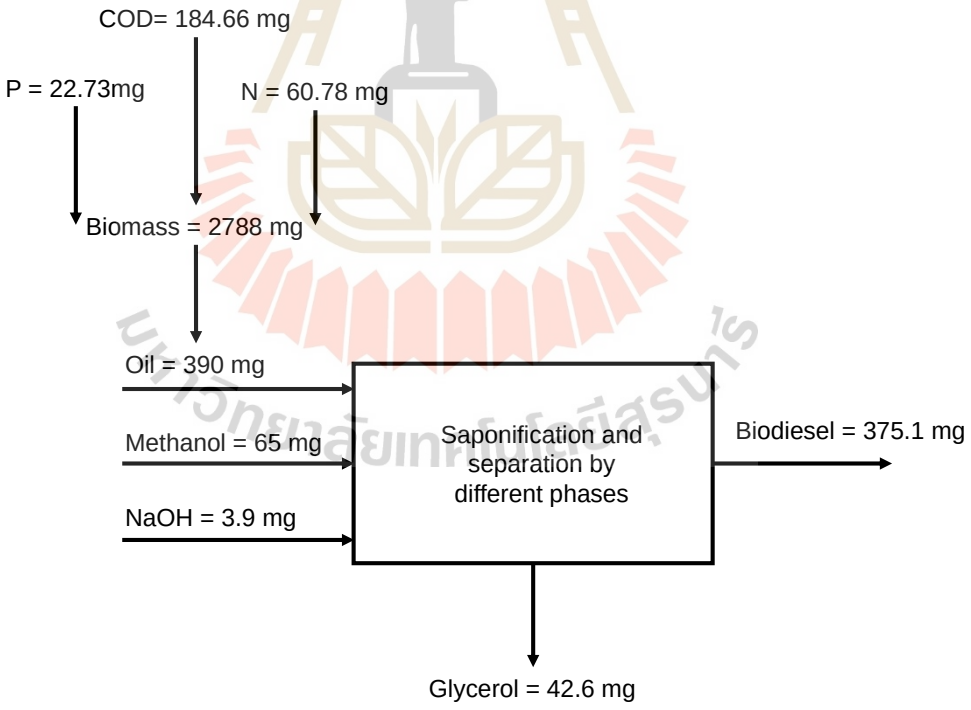

Figure 4.41 Flow of materials in the process of biodiesel generation.

Examination of fatty acid components produced by co-culture reveals that the percentage of saturated fatty acids (SFAs) was between 54-62% in all cultures, indicating that the FAs in the saturated and unsaturated forms were relatively equal. Mono-unsaturated fatty acids (MUFAs) were found to increase when the BMI 10 strain was co-inoculated, whereas tri-unsaturated fatty acids (TUFAs) were decreased (Table 4.11). Interestingly, polyunsaturated fatty acids (PUFAs) were not produced in any significant amount in any of the cultures. Previously, co-cultivation of *Tetradesmus obliquus* and *Actinomycete* isolate WA3 was reported to dramatically decrease SFA levels (from 80.66 to 31.64%) (Kumsiri et al., 2021). Wang et al. (2015) conversely reported slight changes in SFA levels as an effect of co-culture. The slight changes of FA composition from the co-culture in sterilized and unsterilized conditions may indicate no effect of the BMI 10 strain on the expression of fatty acid biosynthesis-related genes in P21 as an IAA inducing effect in algae (Kumsiri et al., 2021). It was also previously reported that the composition of fatty acids in algae can also be affected by the carbon source in the media and its trophic condition, contaminants, light intensity, and nutrient composition (Nzayisenga et al., 2020; Oliveira et al., 2021; Wang et al., 2015).

Fatty acid composition is crucial to biodiesel quality since it is directly related to its flow properties and oxidative stability (Knothe, 2005). The composition of SFAs was appropriate since SFAs are important for biodiesel density and viscosity with higher calorific values. However, excessive SFAs can cause problems in cold flow (Jeong et al., 2008). PUFAs were not detected in any of the cultures, except in P21+BMI 10-S (0.94%). This composition is a positive indication of suitability for biodiesel fuel since this value should be low since PUFAs can reduce oxidative stability (Knothe, 2008).

Values of several essential parameters were calculated based on the simple equation derived from the fatty acids' profiles (Table 4.12). Moreover, a comparison with previous studies with different strains, co-culture, and communities was also provided. It was found that the quality of biodiesel after the co-culture was not affected. FAME analysis also showed that most of the parameters were acceptable for biodiesel production. Interestingly, the values of **U**i, SG, CP, CN, IV, and HHV were not significantly different between the co-culture and single culture. In the previous study of Kumsiri et al. (2021), co-culture of *T. obliquus* AARL G022 with actinomycete *Piscicoccus intestinalis* showed noticeable changes in the cetane number (CN) and the degree of unsaturation (DU) parameters of the biofuel generated from this co-culture.

Table 4.11 Fatty Acids composition of single *C. sorokiniana* strain P21 and its co-culture with *S. thermocarboxydus* strain BMI in sterilized and unsterilized wastewater.

Fatty Acid	% Composition			
	P21-S	P21-U	P21+BMI-S	P21+BMI-U
(C6:0)	0.00±0.00^{b}	0.00±0.00^{b}	0.94±0.87^{a}	0.00±0.00^{b}
(C8:0)	0.00±0.00^{b}	4.73±1.73^{a}	4.70±1.67^{a}	2.58±1.58^{ab}
(C10:0)	0.00±0.00^{b}	2.84±1.54^{a}	1.51±0.45^{ab}	1.32±0.27^{ab}
(C11:0)	0.00±0.00^{c}	10.58±0.64^{a}	11.30±0.92^{a}	5.45±2.81^{b}
(C12:0)	16.44±13.91^{a}	26.42±5.21^{a}	11.53±2.42^{a}	14.49±10.56^{a}
(C13:0)	0.00±0.00^{a}	0.00±0.00^{a}	1.63±0.32^{a}	1.09±1.90^{a}
(C14:0)	0.35±0.32^{b}	4.52±2.72^{ab}	4.74±1.61^{a}	3.43±2.98^{ab}
(C15:1)	0.22±0.09^{a}	0.35±0.61^{a}	2.38±2.19^{a}	2.12±1.07^{a}
(C16:0)	28.25±6.69^{a}	7.06±3.37^{b}	14.84±4.83^{b}	5.82±3.00^{b}
(C18:0)	12.06±3.98^{a}	0.00±0.00^{c}	1.12±1.04^{bc}	6.27±4.57^{b}
(C18:1n9c)	2.51±2.50^{ab}	0.00±0.00^{b}	4.36±1.46^{a}	0.36±0.62^{b}
(C18:2n6c)	24.93±7.07^{a}	13.65±2.17^{b}	20.87±0.76^{a}	8.15±1.54^{b}
(C18:2n6t)	0.00±0.00^{c}	4.26±1.59^{b}	4.72±1.12^{b}	10.80±0.55^{a}
(C18:3n3)	4.31±1.76^{a}	3.93±1.56^{a}	4.25±2.74^{a}	2.49±1.76^{a}
(C20:2)	0.00±0.00^{a}	0.00±0.00^{a}	0.40±0.69^{a}	0.13±0.22^{a}
(C20:3n6)	0.00±0.00^{c}	8.31±1.02^{a}	2.30±1.59^{b}	0.00±0.00^{c}
(C20:4n6)	0.00±0.00^{a}	0.00±0.00^{a}	0.94±1.28^{a}	0.00±0.00^{a}
(C21:0)	0.00±0.00^{b}	0.00±0.00^{b}	0.00±0.00^{b}	1.45±0.49^{a}
(C22:0)	0.00±0.00^{b}	5.36±1.16^{a}	1.54±0.46^{b}	5.79±3.55^{a}
(C22:1n9)	1.23±0.11^{a}	0.46±0.80^{ab}	0.13±0.23^{b}	0.00±0.00^{b}
(C24:0)	0.00±0.00^{b}	0.00±0.00^{b}	0.26±0.44^{b}	12.60±2.84^{a}
(C24:1n9)	0.00±0.00^{c}	4.68±3.64^{b}	0.00±0.00^{c}	10.90±1.52^{a}
PUFA	0.00±0.00^{a}	0.00±0.00^{a}	0.94±1.28^{a}	0.00±0.00^{a}
TUFA	4.31±1.76^{b}	12.24±0.55^{a}	6.54±3.44^{b}	2.49±1.76^{b}
DUFA	24.93±7.07^{a}	17.91±3.75^{a}	25.99±2.14^{a}	19.08±1.32^{a}
MUFA	3.96±2.56^{b}	5.49±4.96^{b}	6.87±1.81^{a}	13.39±2.38^{b}
SFA	57.10±4.29^{a}	61.52±5.56^{a}	54.11±3.68^{a}	60.29±2.73^{a}
TOTAL	90.30±9.78	97.17±3.09	94.45±0.58	95.25±0.53

PUFA: Poly-unsaturated fatty acid, TUFA: Tri-unsaturated fatty acid, DUFA: Di-unsaturated fatty acid, MUFA: Mono-unsaturated fatty acid, SFA: Saturated fatty acid. Data presented as mean ± standard deviation (n = 3). Different letters on the same line indicate a significant difference by the DMRT ($p < 0.05$).

Table 4.12 Biodiesel properties of the lipid from P21 and P21+BMI 10 based on their fatty acid composition.

Inoculants/ condition	Parameters									References
	DU	*vi*	SG	CP	CN	IV	HHV	C18:3(%)	PUFA (wt%)	
P21-S	0.67±0.21[a]	4.79±0.13[a]	0.88±0.00[a]	11.08±2.84[a]	58.42±1.42[a]	62.35±15.80[a]	39.71±0.37[a]	4.31±1.76[a]	0.00±0.00[a]	This Study
P21-U	0.78±0.10[a]	4.71±0.06[a]	0.88±0.00[a]	9.57±1.32[a]	57.67±0.66[a]	70.75±7.34[a]	39.91±0.17[a]	3.93±1.56[a]	0.00±0.00[a]	
P21+BMI-S	0.82±0.12[a]	4.69±0.08[a]	0.88±0.00[a]	9.01±1.64[a]	57.39±0.82[a]	73.88±9.12[a]	39.98±0.22[a]	4.25±2.74[a]	0.94±0.64[a]	
P21+BMI-U	0.59±0.05[a]	4.83±0.03[a]	0.88±0.00[a]	12.11±0.64[a]	58.94±0.32[a]	56.61±3.59[a]	39.57±0.08[a]	2.49±1.76[a]	0.00±0.00[a]	
C. vulgaris	0.58	-	-	10.81	61.83	52.63	-	1.57	-	(Nascimento et al., 2013)
Choricystis minor	1.38	4.33	0.88	1.47	53.63	115.84	40.97	11.28	8.47	(Oliveira et al., 2021)
T. obliquus+ Actinomycete WA3	0.83	4.68	0.88	5.99	70.51	97.73	39.99	4.31	-	(Kumsiri et al., 2021)
T. obliquus+ Actinomycete WA3+L-tryptophan	0.53	4.87	0.88	37.16	57.16	56.07	39.46	1.76	-	
Chaetoceros sp.	-	4.72 ± 0.01	-	-	55.56 ± 0.49	79.34 ± 2.74	36.19 ± 0.26	0.39 ± 0.03	5.61	(Chaisutyakorn et al., 2018)
T. suecica	-	4.56 ± 0.02	-	-	57.79 ± 1.42	104.58 ± 2.85	30.11 ± 0.67	14.43 ± 0.47	5.54	
Nannochloropsis sp.	-	4.73 ± 0.02	-	-	55.26 ± 1.71	94.88 ± 6.63	34.56 ± 0.45	16.69 ± 4.40	-	

Table 4.12 Biodiesel properties of the lipid from P21 and P21+BMI 10 based on their fatty acid composition. (Continued)

Inoculants/ condition	Parameters									References
	DU	*vi*	SG	CP	CN	IV	HHV	C18:3(%)	PUFA (wt%)	
ASTM D6751	-	1.9-6.0	0.85-0.90	-	Min. 47	-	-	Max. 12	-	Retrieved from Oliveira et al. (2021)
EN 14214	-	3.5-5	-	-	51-120	Max. 120	-	Max. 12	Max. 1	

DU (Degree of Unsaturation, dimensionless), **U**i (kinematic viscosity, $mm^2\ s^{-1}$), SG (specific gravity, kg^{-1}), CP (cloud point, °C), CN (cetane number, unitless), IV (iodine value, g I_2/100 g oil), and HHV (higher heating value, MJ kg^{-1}). All data are shown as mean±SD (n=3). Different letters indicate significant differences using the DMRT test α=0.05)

Overall, all the quality parameters of the biodiesel were still in the range of acceptable values from the International (ASTM D6751) and European (EN 14214) standards (Oliveira et al., 2021). Additionally, CP and HHV parameters were not included in these standards. However, the CP can be a helpful parameter for minimum temperature where the quality increases when this values decreases and higher HHV values are suitable for biodiesel quality (Knothe, 2008). Thus, this application of co-culture successfully increased the amount of oil ideal for biodiesel production while maintaining oil quality from the microalgal P21 strain.

4.5.5 Total Energy Demand

The current study demonstrated several improvements. However, a comparison based on the total input and output energy has to be made to sum up these enhancements. There are numerous methods to compare the bench test results similar to this study. The calculation process was mainly based on the input and output of the bench scales. Here, the analysis method was adopted from Lardon et al. (2009). This method was chosen since it contains a comprehensive but straightforward calculation for many factors affecting biodiesel production from algal biomass. Nevertheless, normalization and conversion of the bench scale to the actual or industrial scale were based on Tejido-Nuñez et al. (2020). It was stated that although the change of the condition from bench to pilot scales could be significant, the average difference was detected to be less than10 %. Thus, in this calculation, a 15 % reduction was employed. It was summed from 10 % typical reduction in pilot-scale and 5 % reduction to allow any difference in the system approach from the previous study.

Cumulative energy demand and energy production associated with 1 MJ of biodiesel production were calculated and described in Table 4.13. It was shown that the total biomass required for 1 kg of biodiesel was higher in P21 and P21+BMI 10 (10.08 and 8.07 kg, respectively) than the control, a single culture of *C. vulgaris* in synthetic medium (5.93 kg). This value was caused by the low lipid percentage from the P21 cultures compared with *C. vulgaris* as the control value. CO_2 and nitrogen sources were absent in this study's single and co-culture inoculants. Nevertheless, energy consumption from drying and oil extraction was also higher in wastewater-based cultivation. The optimum concentration was higher than the control (0.5, 1.57, and 1.96 g L^{-1} in control, P21, and P21+BMI 10, respectively). Here, the increase of total biomass seems to be a disadvantage to the system's development. However, the calculation of the total potency of generated energy from the same culture volume showed that the P21 and P21+BMI 10 had the higher production potencies, where the co-culture also increased the potency as much as 54.51 % from the single inoculant of P21.

The Life Cycle Assessment (LCA) is often calculated based on the energy and material inputs and outputs for the process involved in algae biodiesel production and consumption (Passell et al., 2013). Similar approaches have been previously calculated from different studies. A mixotroph combination of the microalgae in the closed photobioreactor and open pond showed increases of efficiency as much as 75 and 76 %, respectively (Adesanya et al., 2014). The rise of efficiency and energy surplus from the system here was merely generated from the increase of oil production per unit volume of medium available. The absence of an additional nutrient or other supplementation in the system could also reduce the total cost of production. Besides, wastewater removal was also an essential part of the enhancement. However, the values were not applied in the system as the current technology for the CBEW treatment was solely open and sedimentation ponds, in which the operational energy demand was deficient. An increase in the energy gap between cost and production after applying P21 as a selected strain combined with the BMI 10 as the co-culture can open promising opportunities for the biodiesel from the wastewater.

Table 4.13 Flow generated from biodiesel derived from algal biomass in mass culture.

Parameters	Control	P21	P21+BMI	Remarks
Algae Culture and harvesting				
Algae (kg)	5.93	10.08	8.07	Combining co-culture and other contaminants required higher total biomass for the same amount of algal biomass.
CO_2 (kg)	10.4	-	-	The additional CO_2 was not used in the development process of the single and co-culture of alga P21.
Electricity (MJ)	7.5	5.53	4.07	In the control value of the model, the oil content was 38.5 % of the algal suspension with a density of 0.5 g L^{-1}. The cultivation in this study obtained a higher biomass value per volume reactor. Thus, this factor can be lower.
Nitrogen Source (g NO_3)	273	-	-	No use of additional N as the system utilized wastewater.
Drying				
Heat (MJ)	81.8	307.57	456.12	The total biomass required for the same amount of lipid was increased as the co-culture contained the biomass of bacteria and contaminants.
Electricity (MJ)	8.52	32.04	47.51	As the total biomass per volume unit increases, the energy demand for drying significantly increases.

Table 4.13 Flow generated from biodiesel derived from algal biomass in mass culture. (Continued)

Parameters	Control	P21	P21+BMI	Remarks
Oil Extraction				
Heat (MJ)	7.1	9.63	12.03	a similar method was employed with the lower percentage of total oil per total biomass increased the heat for energy demand.
Electricity (MJ)	1.5	2.03	2.54	
Hexane loss (g)	15.2	20.62	25.75	Hexane demand increases as the total biomass increases.
Oil transesterification				
methanol (g)	114	114	114	a similar method was employed. Thus, there was no difference per unit of biodiesel.
heat (MJ)	0.9	0.9	0.9	
Total Energy				
Consumption (MJ)	106.4	356.81	522.27	Total of Algal cultivation, harvesting, and post-harvest processes energy consumption.
Production (MJ)	103.8	747.37	1125.91	The detail of the calculation is provided in Supplementary Table 1.
Balance (MJ)	-2.6	390.66	603.64	The control value was minus as the *C. vulgaris* did not show significant biomass or lipid content to cover the energy consumption. Development has been made in the same study using low-N medium

Values and flow systems are based on (Lardon et al., 2009) with minor modification. The control value was based on *C. vulgaris* culture in the synthetic medium.

It can be highlighted from Table 4.13 that electricity can contribute a significant amount to the process of biomass generation. Among the burdening factor of energy demand, electricity may cost the most in the culture as the substrate cost can be negligible and co-culture can be recirculated. It is expected that the electricity in the aquaponic operational system can increase total operational expenditure (Boxman et al., 2016). Interestingly, the wastewater utilization in this study can eliminate the factor of global warming causes and acidification (Papadaki et al., 2017). Carbon sequestration possessed by this system can also create a deficit in carbon production. It is also crucial to fulfilling the Life cycle impact assessment (LCIA) factor. Although the co-culture in the wastewater was designed to be in the mixotrophic system, most of the mechanisms take place in the autotroph condition, reflecting the ratio of C:N:P in the CBEW. Thus, the contribution of the carbon footprint of this system can be counted as one of the most critical improvements for the environment (Tan et al., 2017).

4.6 General Remarks, Limitation, and Future Prospects

4.6.1 Alga and Actinomycete Co-culture

The application of alga and actinomycete co-culture has been successfully demonstrated. The results had shown the increase of removal with the consistency of harvestability in the self-sedimentation test of *C. sorokiniana* P21. Application of *S. thermocarboxydus* BMI 10 has been chosen as the co-culture based on the condition of the wastewater that possesses moderate carbon sources. Different from the algal-fungal co-culture, additional screening of syntrophic traits from all the alga growth-promoting actinomycetes was conducted to counteract the condition of moderate carbon source. Here, several key findings can be pointed out.

i. Traits of the algae growth-promoting bacteria can vary from phosphate solubilizing activity to the production of the growth hormones. However, these traits may not relate to the evolutionary process. It was found that there was no significant clade that possessed specific abilities or effects on the algal growth in the isolated actinomycetes. This finding was contrary to the previous result that stated the clade among the isolated strains might strongly relate to the physiological traits in the algal-bacterial interaction (Goecke et al., 2013).

ii. This study demonstrated that the mixotrophic activity of the microalgae was crucial to determine the trait of the actinomycete co-culture. With numerous attributes of actinomycetes, the suitability to the mixotrophic became an essential factor in selecting the proper co-inoculant strains. Stoecker (1998) has stated

that in the chlorophyte, commonly known as green algae, mixotrophic or phagotrophic was not the preferable pathway of nutrient utilization. They only become mixotroph in the limitation of light and abundance of the organic matter in the substrate. Thus, it has been proven here that the wastewater condition can be a crucial factor in switching the mode from autotroph obligative to be mixotroph facultative.

iii. Syntrophic activity of the BMI 10 is considerably a novel mechanism of the strain isolated from wastewater. It is well-known that the actinomycetes may have difficulties growing in a medium with low carbon content. However, the syntrophic mechanism may lead to different pathways of communication. As previously described by Wang et al. (2015), the interaction of alga-bacterium can vary from competition, inhibition, mutualistic, and commensalism. However, this study was the first to demonstrate the possibility of dependency of one *Streptomyces* strain on alga's exudate or metabolism activities.

Related to the latter point, the interaction of the organisms during the utilization of the wastewater can be depicted in Figure 4.42. While the alga P12 acted as the mixotrophic organism, actinomycete BMI 10 relied on the exudate or photosynthate products from the algae. This interaction has been indirectly depicted in Kumsiri et al. (2021). However, this study confirms the possibility of the mechanism where algae act as the bioagent to produce an intermediate product (exudate that is still detected as remaining COD). The actinomycete will further utilize this intermediate product to enhance the removal, and as feedback, the growth-promoting activity of the actinomycete can also be activated.

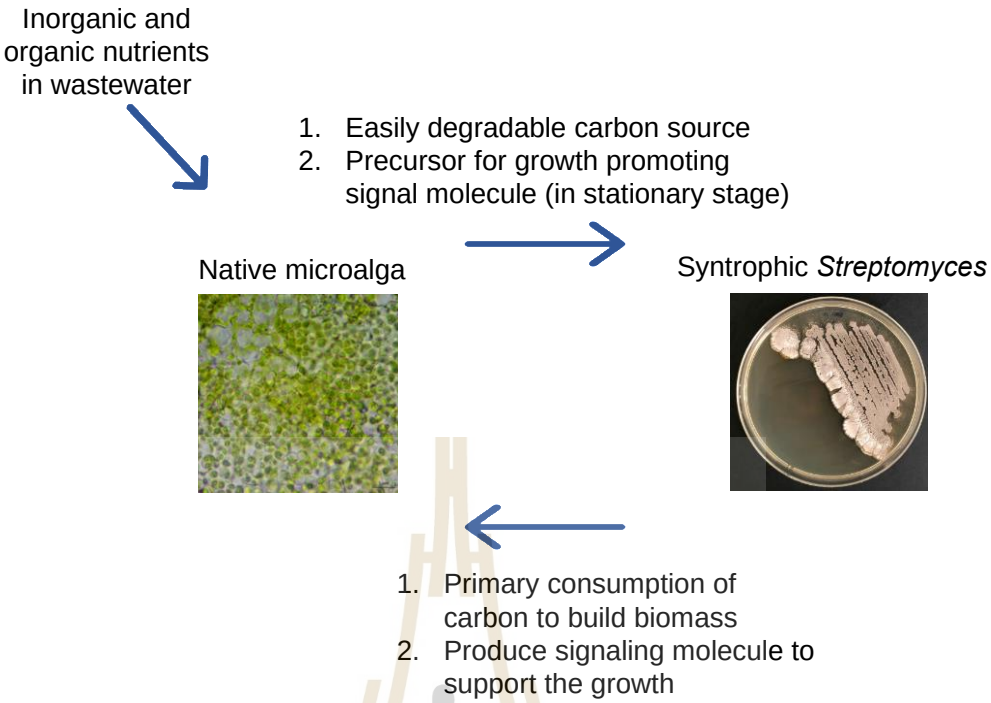

Figure 4.42 Proposed mechanism of the co-culture in the CBEW treatment in *C. sorokiniana* P21 and *S. thermocarboxydus* BMI 10.

Several aforementioned critical findings in this study also raise the limitation and further possibilities of developing this treatment method. Followings are the main drawbacks and prospects for developing this microalga actinomycete co-culture.

i. One of the critical parameters of wastewater treatment in a biological system is the growth rate. It helps the design and adjusts substrate utilization, pre-treatments, and other operational parameters for optimum efficiency. However, additional *S. thermocarboxydus* BMI 10 has increased the maximum biomass yield but reduced the algal growth rate. The proper decision between reaching maximum biomass yield and maintaining a sufficient growth rate shall be reconsidered to optimize the system on a larger scale (Vasumathi et al., 2012). It is essential since the extension of the logarithmic phase without any growth rate enhancement can be a critical drawback for this method to be applied.

ii. The introduced strain, *S. thermocarboxydus* BMI 10, and native strain did not show any antagonistic growth. However, this conclusion was constructed based on the application of the 5 L stirred tank reactor. Numerous types of reactors may result in converse interaction between the native and introduced strains, including

their composition and relative abundance (Sánchez-Zurano et al., 2021). Thus, a more significant scale examination and comprehensive calculation of the total biomass product shall be made in the future.

iii. Different systems of cultivation can also produce different compositions of biomass production. It has been found that the composition of fatty acids in sterilized and unsterilized conditions was slightly different. However, the significant switches in some fatty acids did not affect the final quality of the potential biodiesel product. Nevertheless, different reactors with different scales may result differently, as their operational parameters can be highly different from each other (Powtongsook & Nootong, 2019). As the application of wastewater to cultivate algae is mainly targeted to be in an open pond for economic reasons (Lardon et al., 2009), the further development or pilot scales shall be in this reactor/pond.

Application of *Streptomyces thermocarboxydus* BMI 10 was previously reported to be employed as a bioagent for remediation (Desjardin et al., 2003; Passari et al., 2019; Qin et al., 2021). Nevertheless, one of the most important factors to be tested is the pathogenicity of this strain. Based on the previous applications of this strain, the possibility of application is widely open. However, further confirmation still needs to be conducted.

4.6.2 Alga and Fungus Co-culture

After the cultivation period, the wastewater treatment results using co-culture of alga and fungi have shown improvement from the single-strain treatment results. Biomass growth and nutrient removal among the *Chlorella vulgaris* TISTR 8580 and *Aspergillus niger* strain F5 combinations treatments showed different removal traits and disparate growth, nutrient removal efficiency, and harvestability. As the application of *A. niger* at the stationary phase of *C. vulgaris* phosphate removal occurred to be the most optimum for removal, several key findings and prospective applications are as follows.

i. The decision to choose *C. vulgaris* 8580 after *C. sorokiniana* P21 was based on the fact that these two strains had relatively similar potencies to generate biomass and remove the wastewater nutrients. It was primarily caused by the trait of alga P21 that mainly needed the improvement in the biomass generation since the removal of phosphate was already high. Another reason is that there was no significant growth after adding fungi F2 and F5 to the alga P21 culture. It might be uneven to develop different strains and later compare them with the same parameters. The development of other phycoremediation based on their traits has also been demonstrated by Ren et al. (2017), who developed different applications of algal strains based on their abilities and specifications. It showed remarkable results

with various removal depending on the cultivated strains and targeted contaminants. Thus, this decision-making process was previously demonstrated and successfully addressed the aims of the treatment.

ii. Application of the solubilizing activity as a primary mechanism of growth-promoting activity of fungi to the algae shares a new perspective of co-culture algae and fungi. Previously, most co-culture studies focused on the growth-promoting signaling and growth hormones produced by the fungi to enhance algae growth (Kumar et al., 2021). Here, the solubilizing activity was proven to have competitive results for increasing the removal of phosphate and biomass yield.

iii. Most fungi-solubilizing phosphates created extreme acid conditions through organic acid secretion mechanisms (Rawat et al., 2021; Turan et al., 2006). The acidic environments often lead to unfavorable conditions for algae to grow. However, the application of *A. niger* did not show the extreme change in the wastewater medium that could be harmful to the alga. This situation may have resulted from the low availability of degradable carbon sources available for the fungus. Thus, the substrate with a limited carbon source for the fungi and proper nutrient concentration for the alga is essential to support the synergistic interaction between the organisms.

iv. Numerous previous studies emphasized the additional sugars on the wastewater to create filamentous pellets with high adsorption capacity (Zhou et al., 2012a; Zhou et al., 2013). The extra sugar for fungi would only be essential to form the pellets in this study. It is proven that the hyphae that developed during the co-culture (outside the pellets) do not need any additional sugars to enhance the removal. Thus, this advantageous trait of fungi can overcome the feasibility limitation of fungi as co-culture of the alga.

v. The findings did not cover the actual wastewater content that may differ from sterilized wastewater. However, the positive effect of the co-culture by adding fungi at the early stage of the stationary phase of phosphate removal indicates the promising result to be applied in the actual wastewater content. The successful growth in sterilized wastewater can demonstrate the promising applicability of the system and the interaction between these organisms (Mann et al., 2010).

The combination of the *C. vulgaris* 8580 with *A. niger* F5 can be adopted to the wastewater treatment with the excess phosphorus concentration. Thus, these particular experiments' main contribution is the applicability of phosphate solubilizing organisms with microalgae to enhance phosphorus removal and increase the harvestability of the algae. In other words, this concept is also feasible to be applied in the system with fully established consortia (Yuan et al., 2012). Eventually, this

enhancement is also essential to elevate the affordability of biological removal of phosphorus by the algae in the wastewater compared with other established methods (Wu et al., 2020).

Overall, coupling the algal harvesting process with the additional removal of phosphorus is demonstrated in the particular experiments. Except for nitrogen removal, all parameters were enhanced by adding *A. niger* F5 pellets in the stationary phase of *C. vulgaris* 8580. Several vital points that need to be addressed further are highlighted based on the results.

i. The results show the benefits of algal-based wastewater treatment for removing phosphorus. This removal is often an obstacle as low removal efficiency is typical to find in algal-based treatment (Bunce et al., 2018). However, comparing the removal between the current results and well-established consortia for phosphate removal, the cultivation period becomes one of the significant issues. The removal period was still lower than the combination of microalgae and suspended sludge (Mujtaba & Lee, 2017) or activated sludges (Ge et al., 2018) for nutrient removals, which only require less than a week for optimum removal. Here, the acclimatization process for the alga may reduce the lag phase of the culture and eventually shorten the treatment period (Rezaei et al., 2019).

ii. The use of fungi for additional treatment positively affected the growth of algal cells, even though the TN removal was slightly reduced. Here, pH adjustment and other operational parameters can be further explored to improve all removal parameters simultaneously, including TN (Chu et al., 2021).

iii. The stability of the combination in unsterilized wastewater and its application in the continuous system is essential to be addressed further. Here, application in the actual wastewater with a continuous system will be necessary for development at the pilot and actual scales (Paulo et al., 2021). Microbiome analysis for system stability can also address the exact mechanisms of algae-microbial interactions in the community (De Sotto & Bae, 2020).

iv. The formation of pellets using glucose is still not economically viable for industrial-scale, and thus, affordable carbon sources for practical utilization can be further tested. Among the sources, cassava wastewater from the starch extraction can be further tested for this purpose as it was previously reported to contain high degradable carbon contents (Wadjeam et al., 2019).

v. The biomass generated can be analyzed to assess the chemical conversions of the process better. This knowledge is essential to decide how the biomass generated in such a system can be further utilized (Naaz et al., 2019).

Additionally, the economic value of generated compounds from the biomass can significantly reduce this co-culture application's total cost.

4.6.3 Selection of the most suitable co-culture for batch and semi-continuous system reactor

From two attempts of co-culture using different algae with fungus and actinomycete, two different results were obtained. The reasoning to choose the most optimum combination was based on the normalization of the critical parameters, such as wastewater nutrient removal efficiency, total biomass generation, and harvestability. Previously, this simple normalization has been employed to decide the most suitable algal inoculant for urban wastewater with similar parameters (Mennaa et al., 2015). However, this study was the first to determine the more suitable co-culture with the slightly different approaches for development. The algal-fungal co-culture focused on attachment, while the algal-actinomycete co-culture concentrated on the syntrophic mechanism. The differences between these two co-cultures approaches and examination may lead to incomparable results. Although the method might be incomparable, Ren et al. (2017) previously stated that normalization of the values from numerous factors aims to eliminate the interference of data property among the treatments. It can help clarify the patterns of advantages. Thus, the different approaches and results of the actinomycete- and fungal- algal co-culture can still be comprehensively comparable.

The semi-continuous system in this study was also explained as the limitation of the reactors, which often contained the biofilm that disrupted the light to penetrate the suspension. This phenomenon is called algal wall-growth, where the light penetration disruption may adversely affect the overall biomass productivity (Plengsakul et al., 2021). With the reasons mentioned above in the semi-continuous part, the limitation of the complete continuous system may be overcome by careful operation and frequent change of wastewater. On the other hand, the results of both experiments were still similar or even higher than previous studies with similar approaches.

4.6.4 Biomass yield and feasibility study

Biomass yield from the microalga P21 and BMI 10 with different conditions (sterilized and raw) was analyzed and compared. The results showed the switch of the fatty acids composition. However, as this study focuses on the potency of biofuel production from algal biomass, the other products were not examined, but they may also be evaluated in the future. This analysis is also beneficial since the carbohydrate and other components in the biomass may also be utilized for economic purposes (Wang et al., 2015). Furthermore, a feasibility study based on the life cycle

analysis is also an essential part of this study purpose. However, the simple calculation based on the energy values in each step could only describe the enhancement of operational features, such as cultivation, oil extraction, and biofuel production—the results of 35 % enhancement for overall operating cost based on electricity consumption. Nevertheless, it is also important to note that the capital costs from other steps such as maintaining culture and construction of the system can also be the burdening factors (Sutherland et al., 2020).

By incorporating the wastewater parameters and the content of elements in the wastewater content, nutrient utilization in several conditions has been successfully described. Figure 4.43 shows the proposed mechanisms that possibly happened during the cultivation in the raw wastewater process. The COD removal rate was lower than inorganic nutrients removal such as N and P. This may be caused by the common undegradable or hardly degradable organic carbon (Gupta et al., 2016; Nagi et al., 2020). Another scheme that might be possible is that degradation of nitrate and other nitrogen forms in wastewater that require organic matter are released in the system to incorporate nitrogen (Tsioptsias et al., 2016). Hence, organic matter as COD could not be removed entirely from the system.

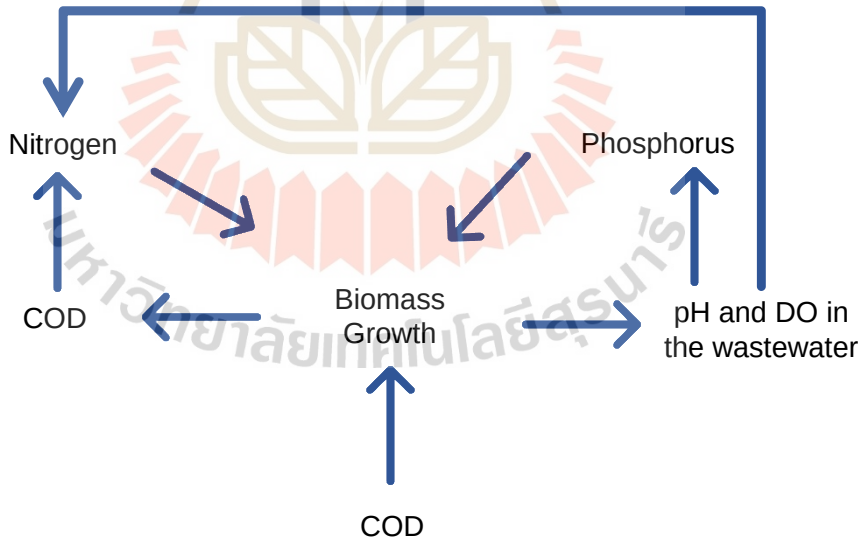

Figure 4.43 Proposed nutrient flow in the CBEW treatment in *C. sorokiniana* P21 and *S. thermocarboxydus* BMI 10.

Differently, nitrogen available in ammonium (NH_4) and nitrate (NO_3) forms were assimilated in an efficient percentage. It was expected to have such high removal from this nutrient since NO_3 can quickly be incorporated (Leong et al., 2021), and NH_4 also requires less energy to assimilate (Yang, 2011). These two intakes were also possible since the DO was considerably high. It eliminates the possibility of one-way intake where all the NH_4 shall undergo a single nitrification process of NO_2 into NO_3 (Leong et al., 2021). Similarly, the pH parameter also determined the main mechanisms in wastewater treatment using this co-culture. pH below 9.5 during the cultivation period revealed that phosphorous precipitation was low (Czerwionka et al., 2012). Here, luxury phosphorus uptake was suspected to be the primary mechanism of phosphorus removal (Brown & Shilton, 2014). Eventually, these mentioned mechanisms were confirmed based on the supporting data from the cultivation condition.

The mechanisms, as mentioned above, can be coupled with other minor complementary processes that help the removal reach the reported values. Mass balance analysis may be needed to deeply understand the side mechanisms and the possibility of managing the high cellular density during the wastewater treatment scaling up (Barros et al., 2017). However, the laboratory system demonstrated in this study might be different from the actual scale industry (Grobbelaar, 2012). Therefore, the mass balance calculation based on this study's reactor may differ and fail to be widely applied on larger scales (Barros et al., 2017). Thus, more details of these comprehensive calculations may be further calculated based on the cultivation's upscaling. The open pond application can be suitable for further development with the adjustment of several factors to confirm the feasibility of this result for actual application (Lardon et al., 2009).

CHAPTER 5
CONCLUSION

Based on the experimental results and comparison of literatures, these conclusion points are made as follows.

i. Microalga *Chlorella sorokiniana* strain P21 and actinomycete *Streptomyces thermocarboxydus* strain BMI 10 were found to have synergistic effect in sterilized wastewater through syntrophic mechanism. Meanwhile, combination of *C. vulgaris* strain TISTR 8580 and fungus *Aspergillus niger* strain F5 has shown promising enhancement in both microorganisms' biomass by phosphate solubilizing activity of the fungus F5 with several attachment mechanisms of alga and fungal hyphae.

ii. Combination of alga P21 and actinomycete BMI 10 showed removal efficiency of chemical oxygen demand (COD), nitrogen (N), and phosphorus (P) as much as 72.68, 72.52, 84.55 %, respectively. Similarly, cultivation of alga 8580 and fungus F5 resulted the removal of COD, N, and P, as much as 68.07, 53.54, and 81.82 %, respectively. Total algal biomass yielded was found to be 1606 mg L^{-1} in the algal-actinomycete co-culture while the combination of alga and fungus resulted 1073 mg L^{-1}. Harvestability of algal-actinomycete showed 77.37 % biomass recovery using self-sedimentation while the combination of fungus F5 and alga 8580 reached up to 75 % recovery efficiency. Thus, the actinomycete *S. thermocarboxydus* BMI 10 and microalga *C. sorokiniana* P21 co-culture has been found to be the most promising consortium for application in actual CBEW treatment.

iii. Co-culture of microalga *C. sorokiniana* P21 and actinomycete *S. thermocarboxydus* BMI 10 resulted the total algal biomass of 2173 mg L^{-1}. Furthermore, application in the raw wastewater using semi-continuous system also resulted an increase of total algal biomass production as much as 0.145 g L^{-1} day^{-1} using 5 days hydraulic retention time (HRT). This HRT was also proven to be the most optimum time for daily removal of COD, N, and P with satisfactory results of removal. The co-culture showed slight improvement of self-sedimentation in the raw wastewater cultivation. Fatty acid analysis showed the switch of several fatty acids' portions. However, the generated biodiesel in the forms of fatty acids methyl ester showed those characteristics that met the international standards.

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APPENDIX

APPENDIX A
SUPPLEMENTARY MATERIALS

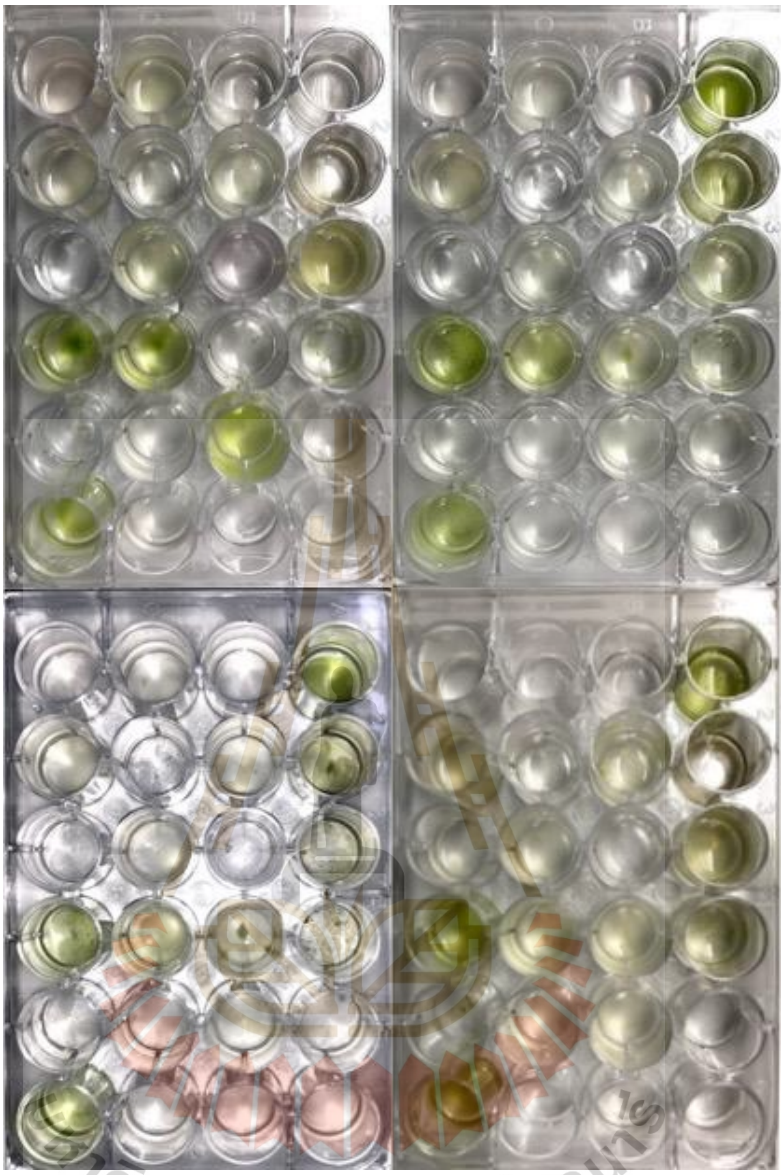


Figure 1A Heterotrophic test of isolated microalgae using 24-well plates.

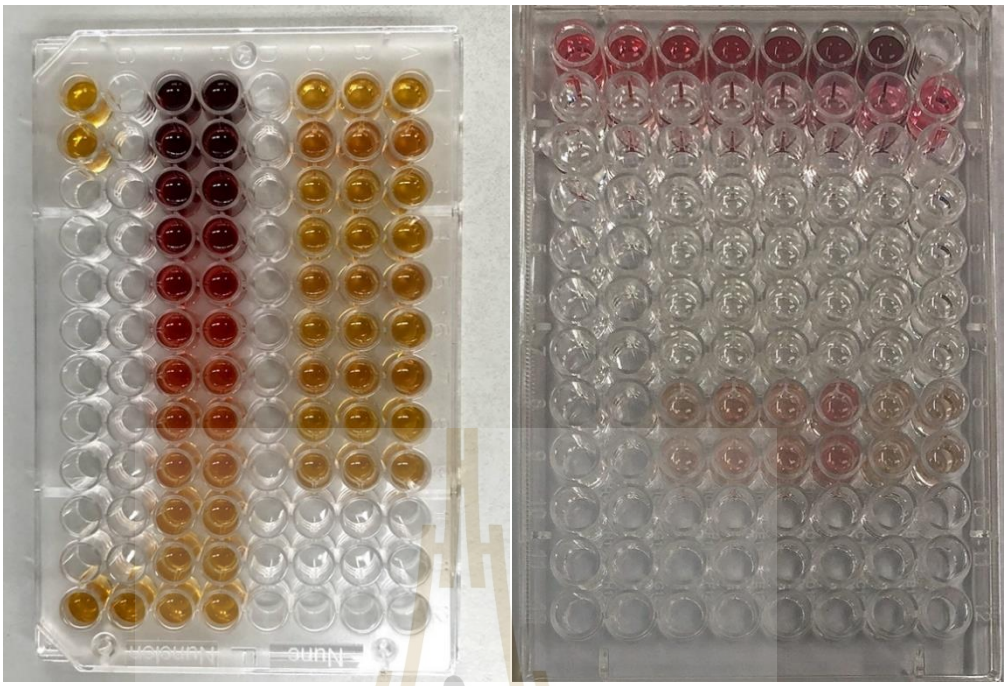


Figure 2A Detection of IAA and the measurements for the isolated strains.

Figure 3A Halo area from the Pikovskaya Agar medium.

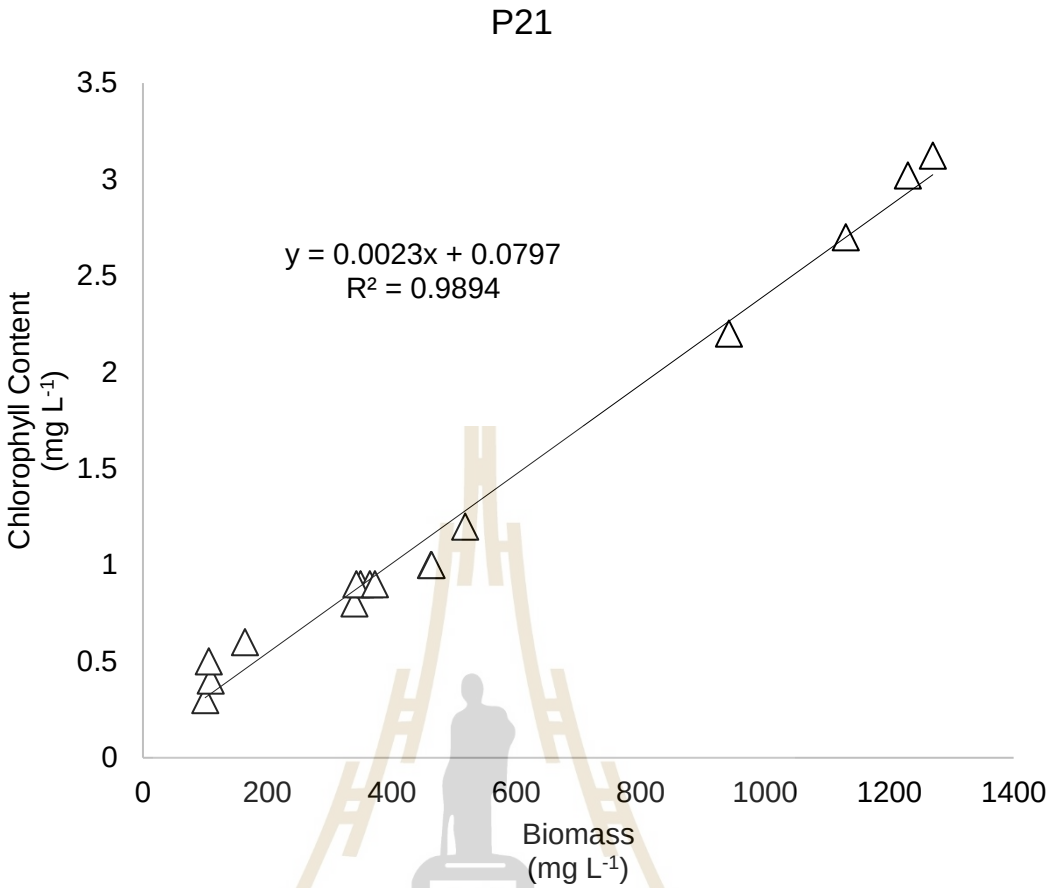


Figure 4A Standard curve of algal biomass determination based on its chlorophyll content.

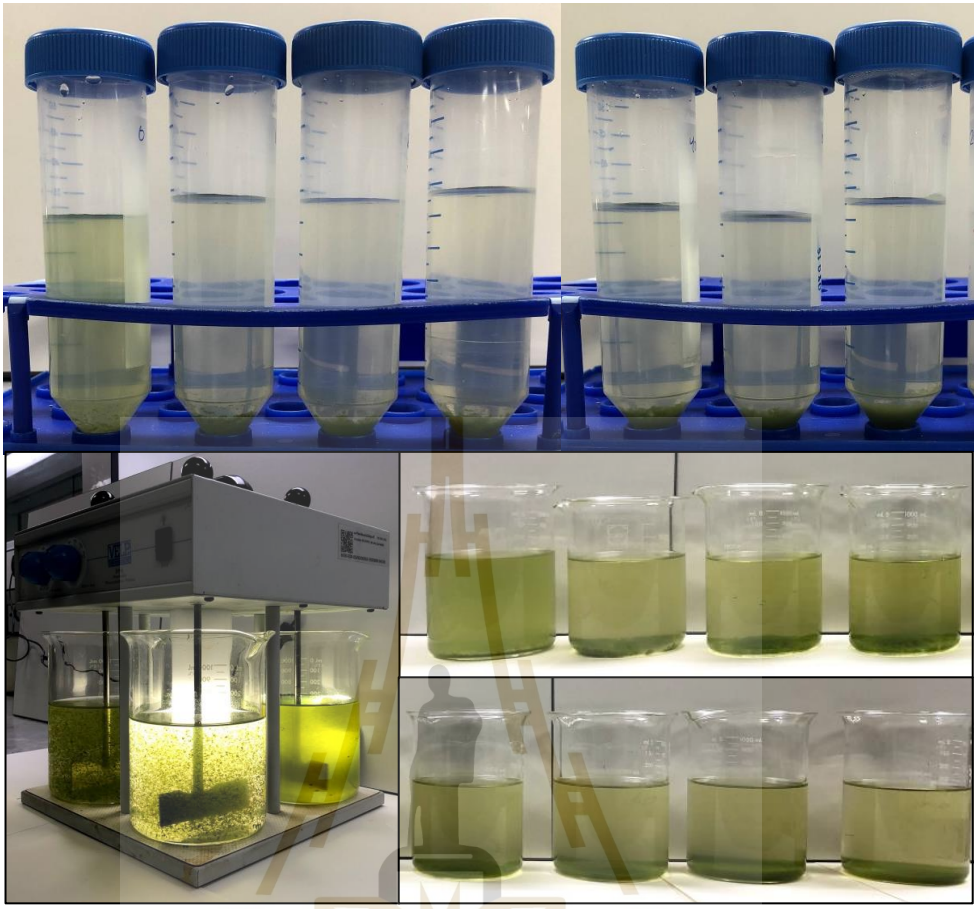
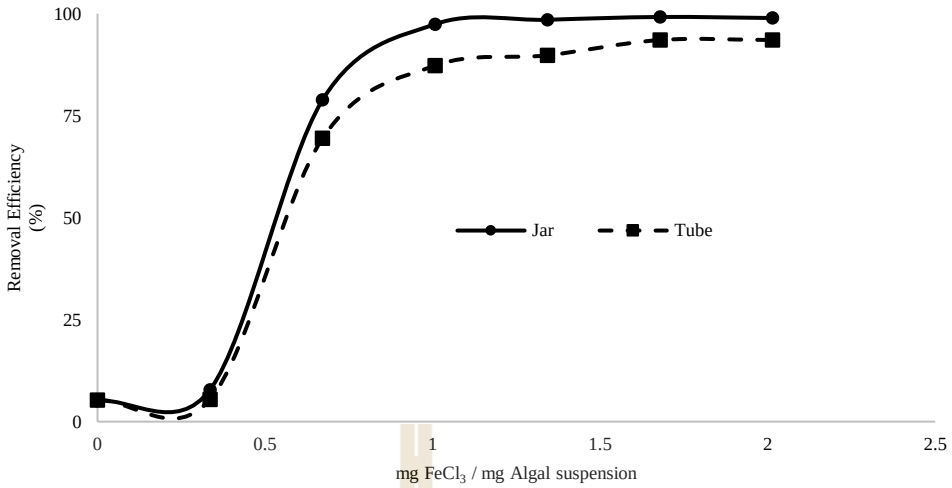
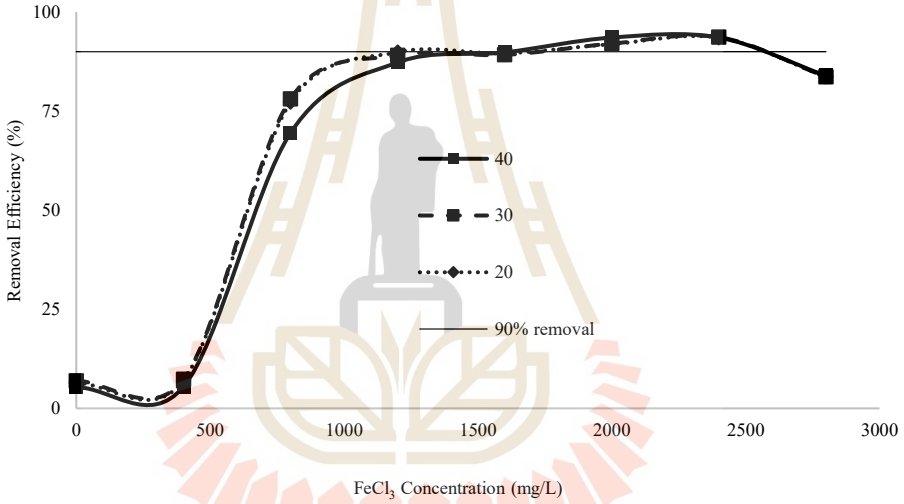


Figure 5A Installation of Jar test and Micro jar test (tube test).



(a)



(b)

Figure 6A Efficiency comparison between $FeCl_3$ applied in (a) microscale jar test and actual jar test, (b) different levels of water sampled from 50 mL tube (40, 30, and 20 indicating water level in mL based on the scale in the tube). —90% removal is the standard.

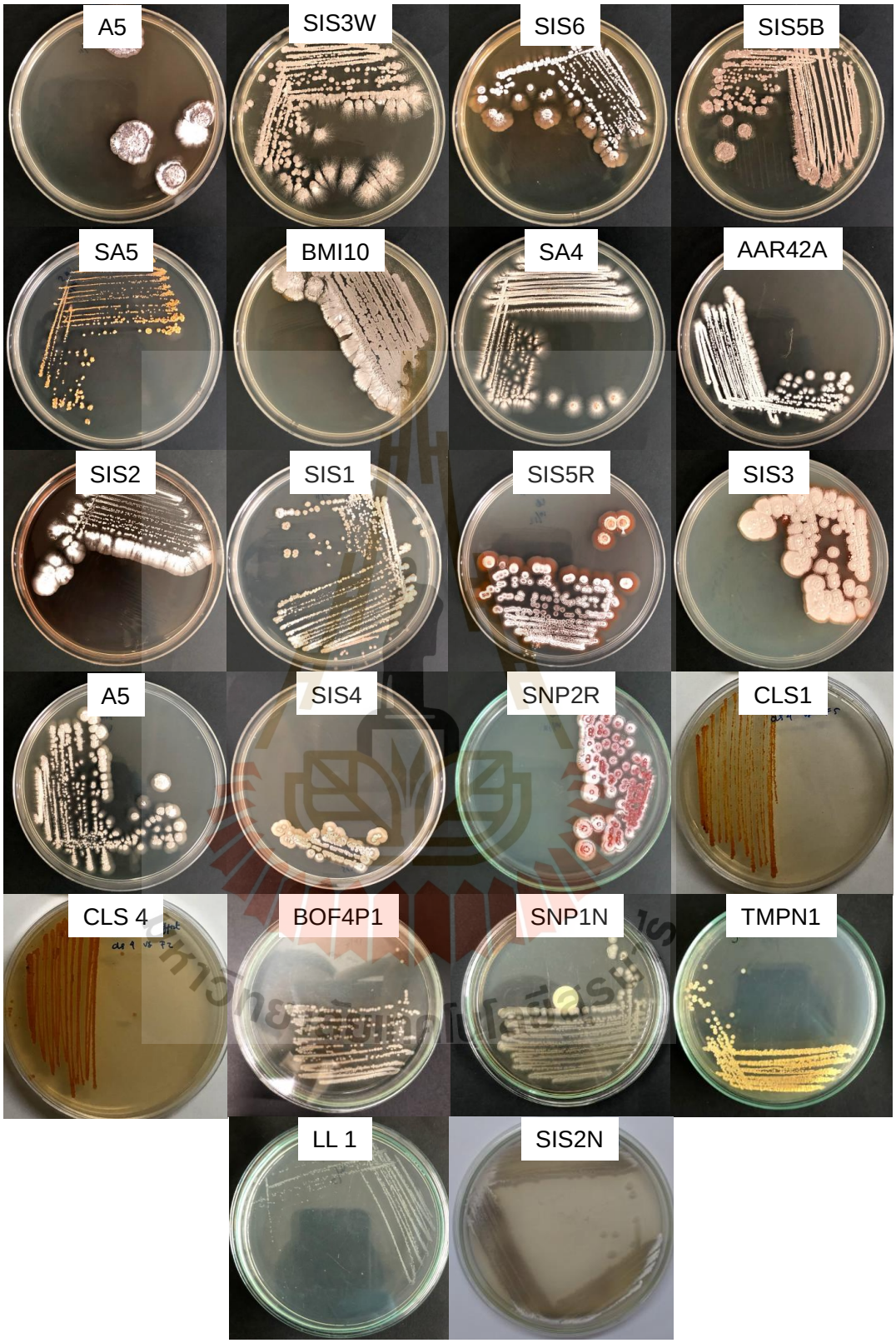


Figure 7A Isolated actinomycetes from Cassava Starch Industry Area.

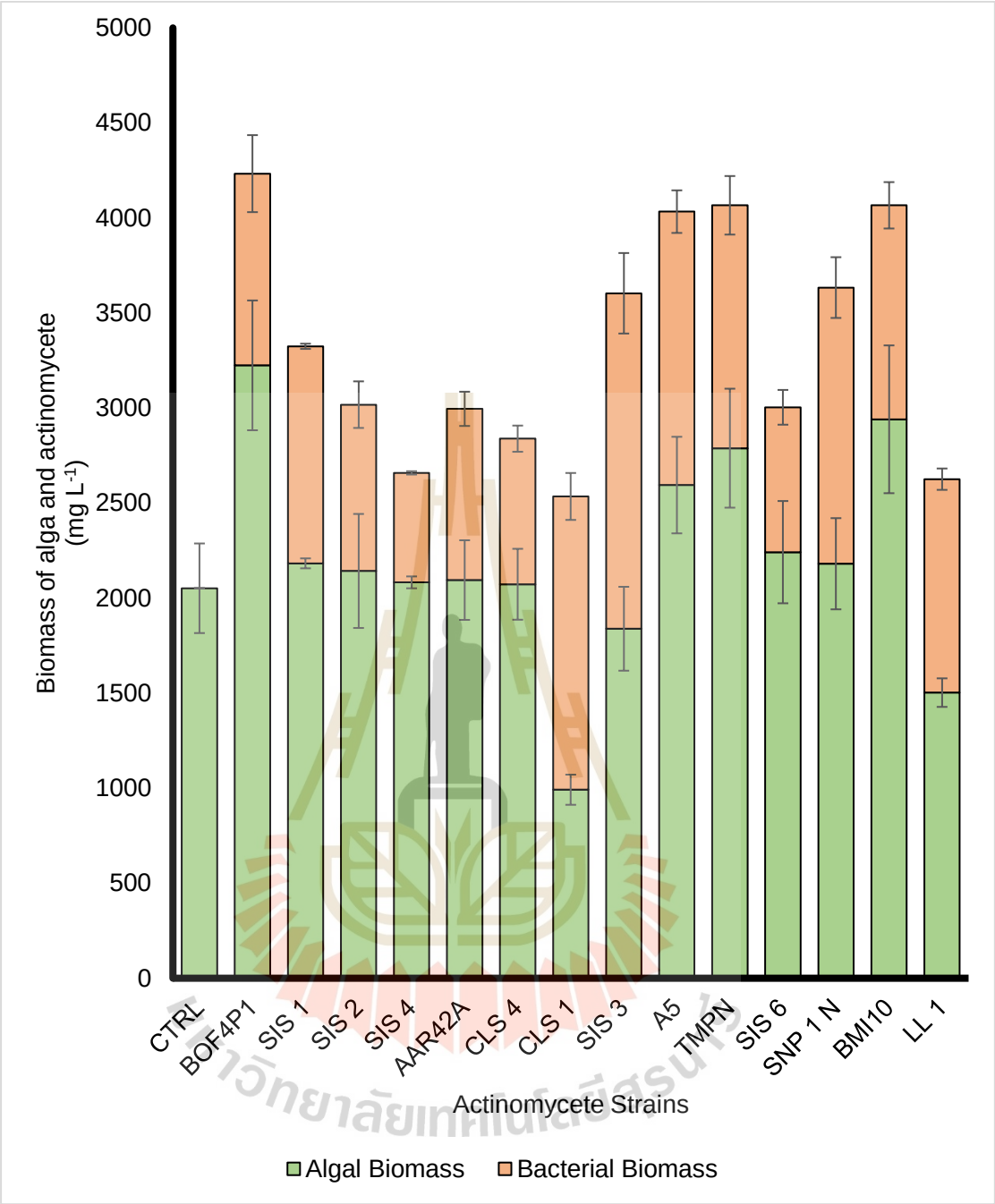


Figure 8A Effect of Actinomycetes co-culture to the total biomass from microalga P21.

Figure 9A Effect of *Aspergillus niger* F5 and *A. awamori* F2 to the microalga P21.

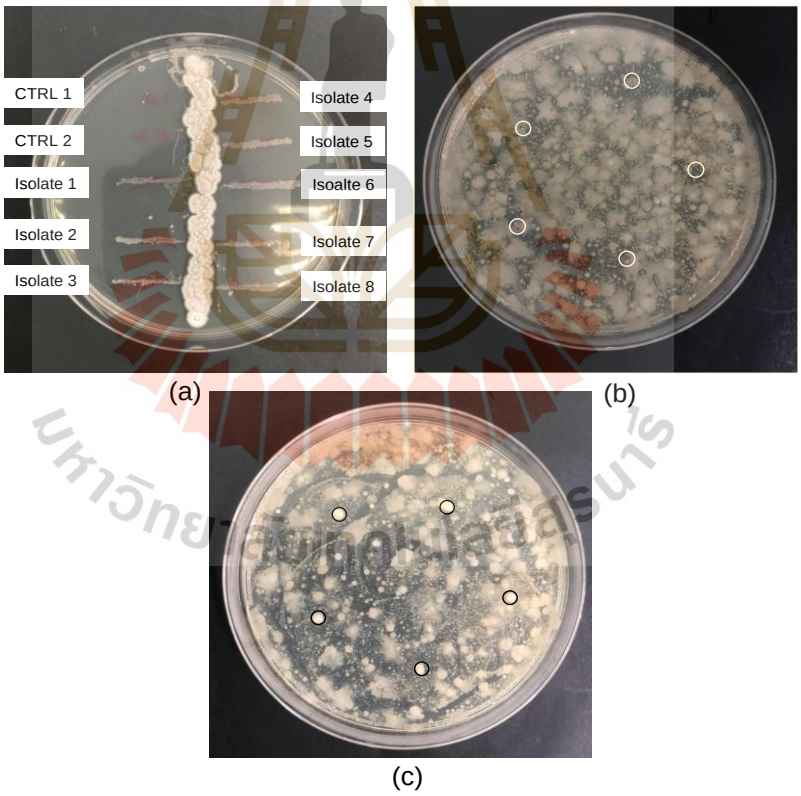


Figure 10A Antagonistic test between BMI and cultivable native bacteria in cassava wastewater. (a) Perpendicular test using several strains, (b) Spread and tested by spores, and (c) Spread and tested by hyphae. The circles indicate the place where BMI spores and hyphae inoculated.

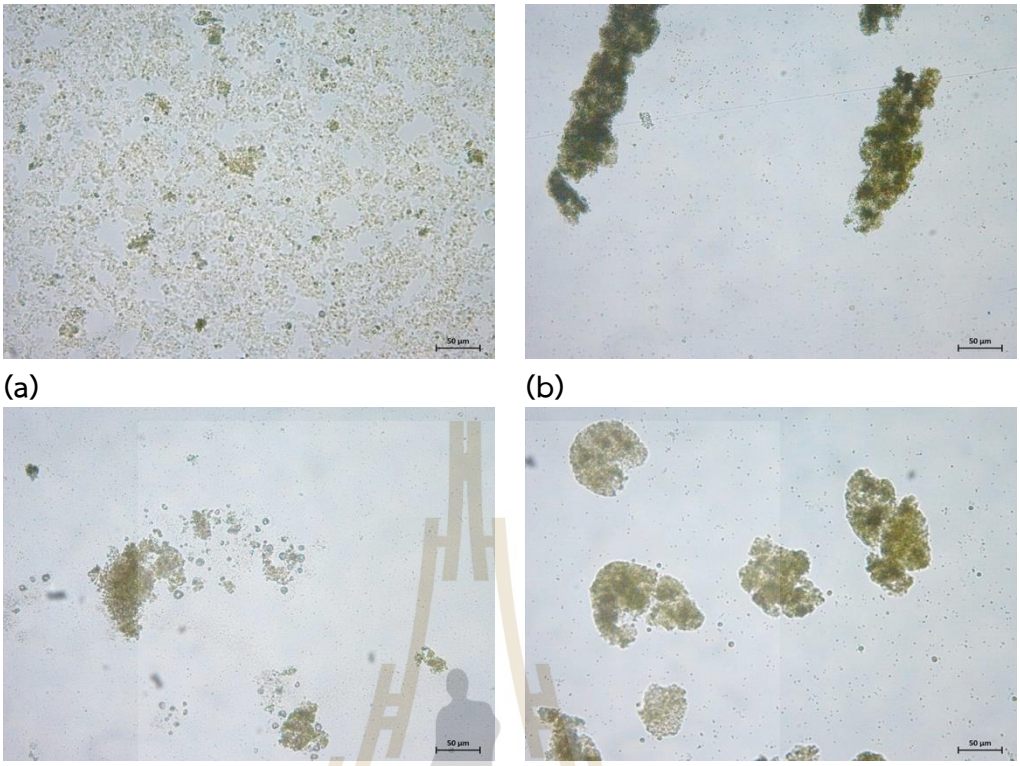


Figure 11A Various structures and sizes of algae flocs of four different coagulant-flocculants applied in sufficient concentrations for harvesting microalga *C. sorokiniana* P21 after cultivation in CBEW. (a) $FeCl_3$, (b) $CaCl_2$, (c) Starch, and (d) $FeSO_4$.

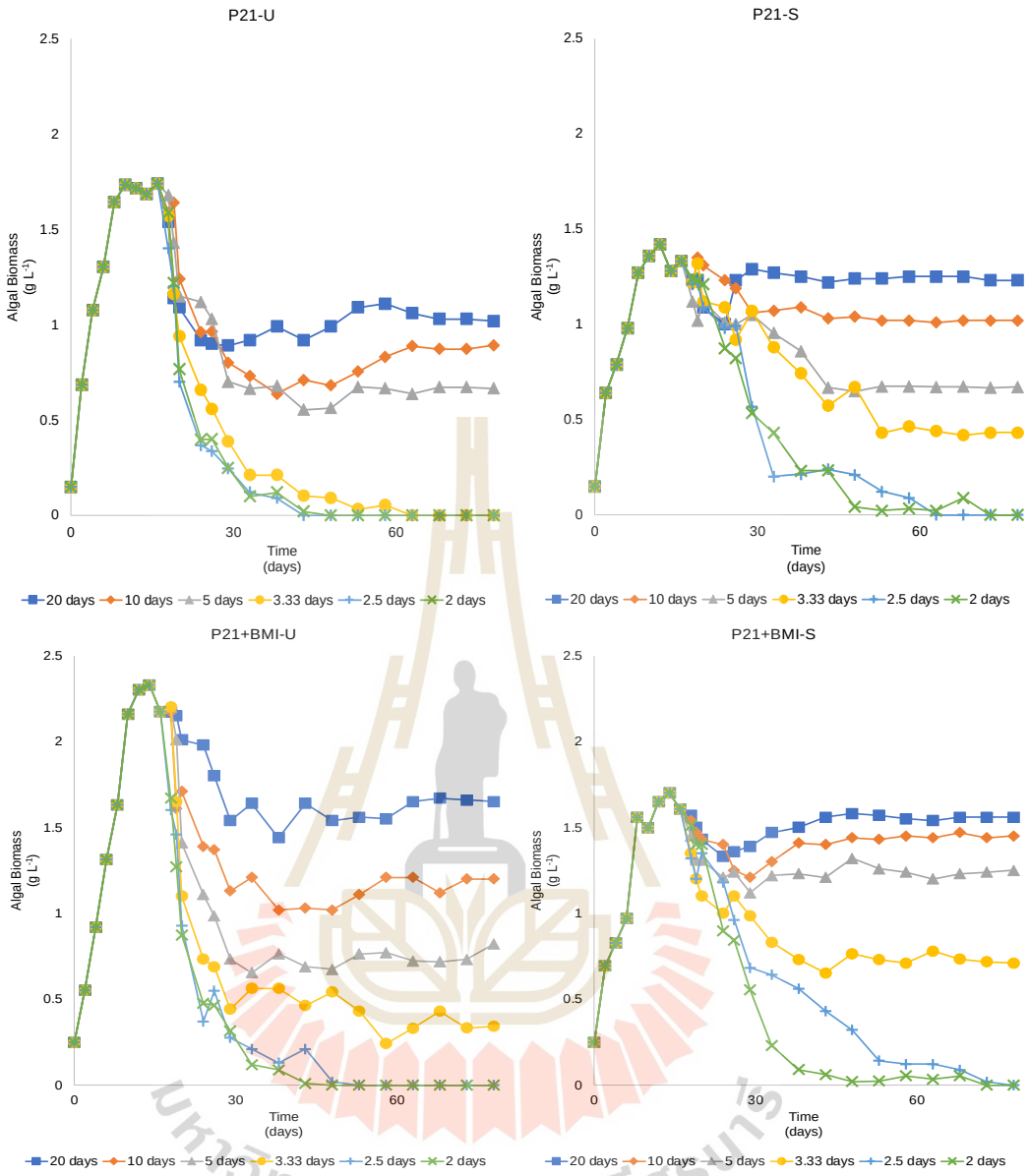


Figure 12A Algal biomass evolution in the semi continuous system with Different HRTs and condition.

P21 = *Chlorella sorokiniana* P21 single inoculant

P21+BMI = *C. sorokiniana* P21 co-cultured with *Streptomyces thermocarboxydus* BMI 10

S = Sterilized wastewater condition

U = Raw wastewater condition

Data were mean of triplicate experiments. the experiments were conducted until all the biomass reached the steady state condition.

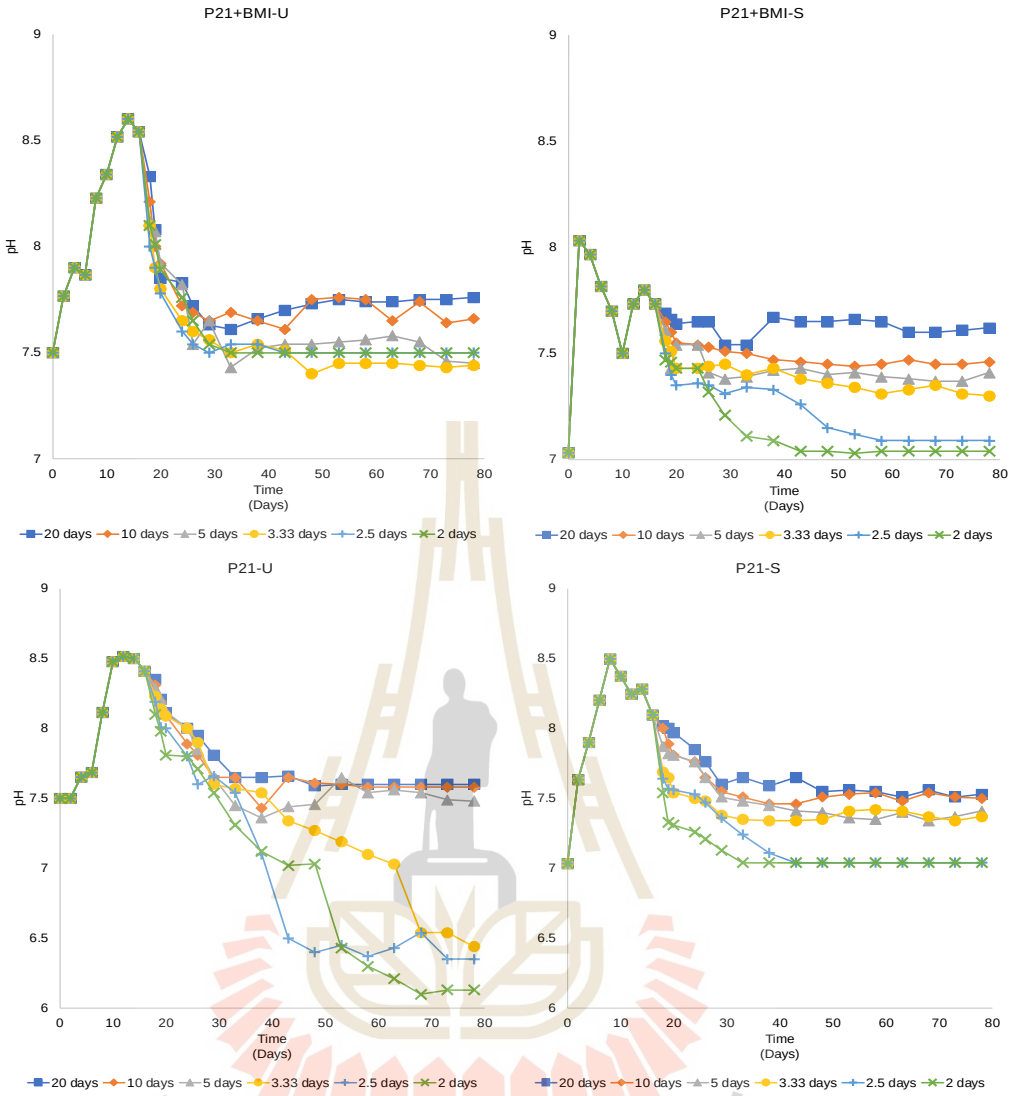


Figure 13A pH evolution of the sterilized and raw wastewaters during batch and semi-continuous system.

P21 = *Chlorella sorokiniana* P21 single inoculant

P21+BMI = *C. sorokiniana* P21 co-cultured with *Streptomyces thermocarboxydus* BMI 10

S = Sterilized wastewater condition

U = Raw wastewater condition

Data were mean of triplicate experiments. the experiments were conducted until all the biomass reached the steady state condition.

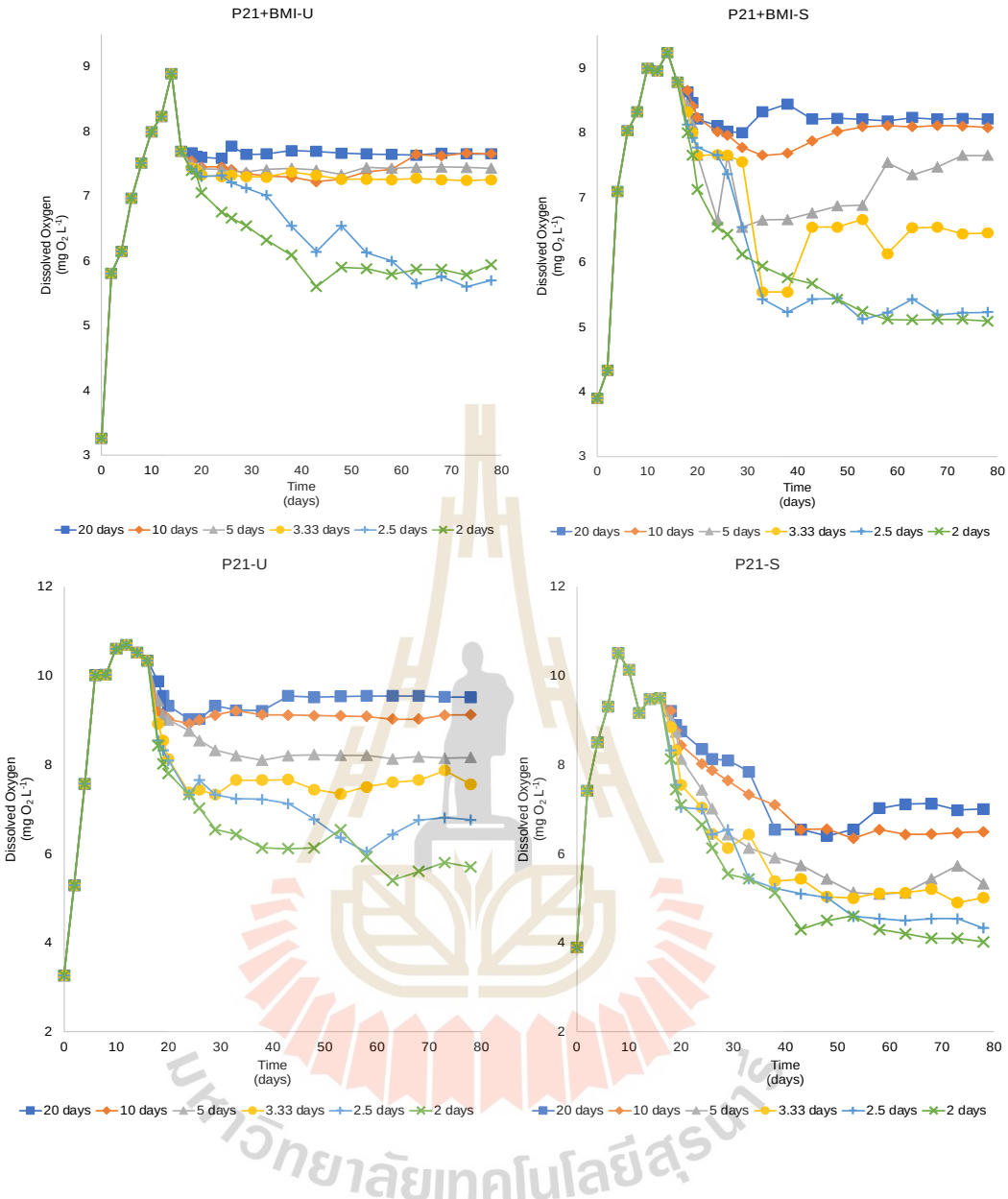


Figure 14A Dissolved oxygen evolution of the sterilized and raw wastewaters during batch and semi-continuous system.

P21 = *Chlorella sorokiniana* P21 single inoculant

P21+BMI = *C. sorokiniana* P21 co-cultured with *Streptomyces thermocarboxydus* BMI 10

S = Sterilized wastewater condition

U = Raw wastewater condition

Data were mean of triplicate experiments. the experiments were conducted until all the biomass reached the steady state condition.

Table 1A Distribution of cumulative energy production per unit volume reactors.

Parameters	CTRL	P21	P21+BMI 10
Biodiesel (MJ kg^{-1})	37.8	37.8	37.8
Cake (MJ kg^{-1})	64.12	200.82	250.45
Total (MJ kg^{-1})	101.92	238.62	288.25
Density of Algae (g L^{-1})[1]	0.5	1.57	1.95
Factor of total algal biomass (unitless)[2]	1	3.13	3.91
Total Potential Energy production based on algal density (MJ unit $volume^{-1}$)[3]	101.92	747.37	1125.92

1) Based on the density in the bench scale yield. The values were reduced from the bench scale (multiply by 0.9).

2) Factor where the total algal biomass that yield if the single strain of P21 and P21+BMI 10 were applied in the control model.

3) The total energy production multiplied by the factor of total algal biomass.

APPENDIX B
LIST OF PUBBLICATIONS

List of Publications

Followings are the publications based on this thesis contents.

Padri, M., Boontian, N., Teaumroong, N., Piromyou, P., & Piasai, C. (2022). Application of Aspergillus niger F5 as an alternative technique to harvest microalgae and as a phosphorous removal treatment for cassava biogas effluent wastewater. *Journal of Water Process Engineering*, *46*, 102524. https://doi.org/10.1016/j.jwpe.2021.102524

Padri, M., Boontian, N., Teaumroong, N., Piromyou, P., & Piasai, C. (2022). Co-culture of Microalga Chlorella sorokiniana with Syntrophic Streptomyces thermocarboxydus in Cassava Wastewater for Wastewater Treatment and Biodiesel Production. *Bioresource Technology*, 126732. https://doi.org/10.1016/j.biortech.2022.126732

Padri, M., Boontian, N., Teaumroong, N., Piromyou, P., & Piasai, C. (2021). Application of Two Indigenous Strains of Microalgal Chlorella sorokiniana in Cassava Biogas Effluent Focusing on Growth Rate, Removal Kinetics, and Harvestability. Water, 13(17), 2314. https://doi.org/10.3390/w13172314

Padri, M., Boontian, N., Piasai, C., & Phorndon, T. (2020). Coupling Wastewater Treatment with Microalgae Biomass Production: Focusing on Biomass Generation and Treatment Efficiency. Engineering Journal, 24(6), 11-29. https://doi.org/10.4186/ej.2020.24.6.11

Padri, M., Boontian, N., Piasai, C., & Tamzil, M. S. (2021). Construction of co-culture of microalgae with microorganisms for enhancing biomass production and wastewater treatment: a review. In *IOP Conference Series: Earth and Environmental Science* (Vol. 623, No. 1, p. 012024). IOP Publishing. https://doi.org/10.1088/1755-1315/623/1/012024

Padri, M., Boontian, N., Piasai, C., & Phorndon, T. (2021). Cultivation process of microalgae using wastewater for biodiesel production and wastewater treatment: a review. In *IOP Conference Series: Earth and Environmental Science* (Vol. 623, No. 1, p. 012025). IOP Publishing. https://doi.org/10.1088/1755-1315/623/1/012025

Padri, M., Boontian, N., & Piasai, C. (2020). *The Potency of Municipal and Industrial Waste and Wastewater as Substrate for Microalgae* [Paper Presentation]. SUT International Virtual Conference on Science and Technology, Nakhon-Ratchasima, Thailand. 28th August 2020. https://ivcst.sut.ac.th/2021/

Padri, M., Boontian, N., & Piasai, C. (2020). *Microalgae Harvesting Methods: A State of Art, Bottleneck, and Further Possibilities.* [Paper Presentation]. SUT International Virtual Conference on Science and Technology, Nakhon-Ratchasima, Thailand. 28th August 2020. https://ivcst.sut.ac.th/2021/

BIOGRAPHY

Mr. Mohamad Padri was born on June 29th, 1993 in Dili, Indonesia. He graduated with a Bachelor Degree of Biology in Universitas Negeri Makassar in 2015. Later then he continued his master in environmental science in Naresuan University, Thailand in 2015-2017. He received SUT-ASEAN Scholarship Phase-II in 2017 to continue study Master-Doctoral degree combined under supervised of Assistant Prof. Dr. Nittaya Boontian. His topic research was "Selection and Optimization of Biomass Production from Microalgal Consortium using Biogas Effluent Wastewater for Potential Biodiesel Generation."