STUDY ON THE EFFECT OF NITRITE AND FREE NITROUS ACID ON CULTIVATION OF MICROALGAE

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Project report submitted in partial fulfilment of the requirement for the degree of

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CHAN JYH LOONG

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LIST OF ABBREVIATION

AOM	Algal organic matter
APHA	American Public Health Association
BBM	Bold's Basal Medium
BCA	Bicinchoninic Acid
COD	Chemical oxygen demand
Total-P	Total phosphorous
COD	Chemical oxygen demand
EPS	Extracellular polymeric substances
EDTA	Ethylenediamine tetraacetic acid
FNA	Free nitrous acid
LKIM	Fisheries Development Authority of Malaysia
MIDA	Malaysian Investment Development Authority
MLSS	Mixed liquor suspended solids
MLVSS	Mixed liquor volatile suspended solids
NOB	Nitrobacter
NTU	Nephelometric Turbidity unit
OD_{682}	Optical density at wavelength of 682nm
PN	Protein
PS	Polysaccharide
PBR	Photobioreactor
TP	Total phosphorus

TN	Total nitrogen
TNN	Total nitrite nitrogen

KAJIAN TENTANG KESAN NITRIT DAN ASID NITRAT BEBAS TERHADAP KULTIVASI MIKROALGA

ABSTRAK

Chlorella vulgaris adalah mikroalga yang mengasimilasikan nutrient yang berpotensi khas untuk penyingkiran jumlah nitrogen dan jumlah fosforus yang tinggi. Kajian telah dikaji untuk mengkaji kesan penambahan nitrit pada kultivasi dengan memperkenalkan 0 mg/L, 50 mg/L, dan 100 mg/L nitrit ke dalam reaktor masing-masing, menyiasat kesan FNA pada kultivasi mikroalga dengan mengawal keadaan kultivasi pada pH 4, 6, dan 8, dan menilai pengaruh kepekatan asid nitrat bebas pada rembesan zat polimer ekstraselular dari mikroalga dengan melakukan pengekstrakan EPS serta analisis protein dan polisakarida. Hasilnya menunjukkan bahawa 50 mg / L nitrit yang dimasukkan ke dalam penanaman mikroalga mempunyai kadar pertumbuhan tertinggi akibat penghambatan oksigen telah dihilangkan, sementara 100 mg/L nitrit dimasukkan ke dalam mikroalga penanaman mempunyai kadar pertumbuhan terendah kerana konsentrasi FNA yang tinggi ditunjukkan. Selain itu, kultivasi mikroalga dengan penambahan 50 mg / L nitrit dan keadaan kultur pH 4 menunjukkan kadar pertumbuhan terendah, dan kadar pertumbuhan mikroalga yang dikultivasi pada pH 8 sedikit lebih tinggi daripada mikroalga diusahakan pada pH 6. Dari aspek protein dan polisakarida yang dilepaskan dalam EPS, titik puncak kepekatan protein (PN) yang dilepaskan dengan keadaan pH 4, 6, 8 adalah 156.06 ug/mL (pada hari pertama), 171.35 ug/mL (pada hari ketujuh), dan 173.71 ug/mL (pada hari kesembilan), sementara polisakarida (PS) yang dilepaskan dengan pH 4 meningkat hingga 91.02 ug/mL pada hari ketiga dan berkurang menjadi 13.43 ug/mL pada hari ketujuh yang hampir sama dengan sistem perkultivasi yang lain. Secara keseluruhan, penanaman mikroalga dengan penambahan 50 mg / L N dan keadaan kultur pH lebih dari 6 dapat meningkatkan kultivasi mikroalga.

STUDY ON THE EFFECT OF NITRITE AND FREE NITROUS ACID

ON CULTIVATION OF MICROALGAE

ABSTRACT

Chlorella vulgaris was widely used as potential nutrients elimination microalgae in wastewater treatment plant due to its high removal efficiency of total nitrogen (TN) and total phosphorous (TP). The aim of this study was to study the effect of nitrite addition on microalgae cultivation by introduce 0 mg/L, 50 mg/L, and 100 mg/L of nitrite into batch reactors respectively, to investigate the effect of free nitrous acid (FNA) on microalgae cultivation by controlling microalgae cultivation condition in pH 4, 6, and 8 with addition 50 mg/L of nitrite into batch reactors respectively, and to evaluate the influence of FNA concentration on the extracellular polymeric substances (EPS) secretion from microalgae by conducting EPS extraction as well as proteins and polysaccharides analysis. The results show that 50 mg/L of nitrite introduced into microalgae cultivation had highest growth rate in the result of oxygen inhibition had been eliminated, while 100 mg/L of nitrite introduced into microalgae cultivation had lowest growth rate because of high FNA concentration presented. Moreover, microalgae cultivation with addition 50 mg/L of nitrite and pH 4 culturing condition show the lowest growth rate, and the growth rate for microalgae cultivated at pH 8 had slightly higher than microalgae cultivated at pH 6. From the aspects of proteins and polysaccharides released in EPS, the peak points for protein (PN) concentration released with condition of pH 4, 6, 8 were 156.06 ug/mL (at first day), 171.35 ug/mL (at seventh day), and 173.71 ug/mL (at ninth day), while polysaccharide (PS) released with pH 4 was up to 91.02 ug/mL at third day and reduced to 13.43 ug/mL at seventh day which almost similar trend with others cultivation systems. In overall, microalgae cultivation with addition 50 mg/L of N and pH culturing condition more than 6 could enhance the microalgae cultivation.

CHAPTER 1 INTRODUCTION

1.1 Project Background

Generally, wastewater is mostly produced and discharged from municipal, agricultural, industrial establishment and it contains high load of oxygen demanding wastes, suspended organic materials, nutrients, inorganic chemicals and minerals, and sediments that could cause series of critical environmental and human health issues if the wastewater is discharged without any treatment (Sonune & Ghate, 2004). Recently, series of water pollution issues that occurred at Malaysia, such as Kim Kim river (Noh, 2020), had prompt the awareness of Malaysian society towards cleaner water environment. It became a driving force to develop high efficient wastewater treatment process strategies.

In the view of wastewater that produced from fishery sectors (agricultural establishment), according to Fisheries Development Authority of Malaysia (LKIM) stated in 2017, processing seafood was Malaysia's largest export in the seafood and fisheries industry, and valued at more than RM1.2 billion. While, Malaysian Investment Development Authority (MIDA) stated that the total export of fish and others seafood in Malaysia had exceed RM2.5 billion per year with 80.5% of the production came from marine catchments and 19.5% from aquaculture (ADMIN, 2021). The intensive of the fishery sector's production had causes a series of environment issues, as some of the fish processing factories have discharge partially treated or untreated effluents into the receiving streams and river since high operating costs and frequently maintenance of the treatment system are required to treat the high organic loading and salt content of the wastewater. The wastewater will deplete the oxygen in these receiving water bodies that would starve aquatic life of the oxygen required and anaerobic

decomposition of organic matter may occur and leads to the decompose of proteins and other nitrogenous compounds, releasing hydrogen sulphide, methane, amines, diamines, and ammonia which will damage to the ecosystem and aquatic life (Ching & Redzwan, 2017). In summarize, the discharging of wastewater in to receiving water bodies will lead to eutrophication and oxygen depletion of the receiving water bodies, as well as these receiving water bodies will polluted by toxic compounds generated. (Ching & Redzwan, 2017). Thus, an effective and low operating cost of wastewater treatment technology is needed to overcome the issues.

Among the existing wastewater treatment process, wastewater treatment by using microalgae had been widely studied and investigated since microalgae have demonstrated a high capability to reduce organic and inorganic nutrients in particular of $NH_3 - N$, P, and PO_4^{3-} which refer to TP compounds, these nutrients act as 'food' sources for microalgae, from wastewater. For the type of microalgae proposed, *Chlorella vulgaris* was proposed and widely used as potential nutrients elimination microalgae since it had relatively rapid growth rate, resist to unknown contaminants, and high content of nutritious component such as proteins, pigments and lipid which bring to high valuable biomass yield (Nguyen et al., 2019). In addition, *Chlorella vulgaris* strain had high efficiency to remove $NH_3 - N$ and Total-P up to 79% and 82% (Megharaj et al., 1992). Moreover, the from the process could be used as biomass feedstock for biofuel production and valuable product such as animal feeds, fertilizers and raw material for 3D painting (Cheng et al., 2020).

In another perspective, microalgae use is limited by high operating costs that consist of cultivation system design and construction, nutrients supply, as well as biomass harvesting operation (Iasimone et al., 2018). In addition, microalgae production technology had not been developed in Malaysia due to its hot and dry environment as well as the actual biomass

growth is relatively lower than its theoretical maximum biomass growth rate, it would led to addition nutrient required and longer cultivation period to achieve certain biomass concentration for secondary wastewater treatment, while photosynthetic of oxygen was a major problems to inhibit the microalgae cultivation (Bilanovic et al., 2016). In addition, the photosynthetic of oxygen or in others term as photorespiration could be happened when the supplied of carbon dioxide (CO_2) was not sufficient to carried out photosynthesis of microalgae. This was because the atmospheric concentration of CO_2 (0.03 to 0.04%) was not enough for efficient photosynthesis and most microalgae species had lower CO_2 binding activity than oxygen (O_2) in rubisco enzymes, the excess O_2apparently was competed with CO_2 for rubisco binding sites, leading to insufficiency of photosynthetic process (Chang et al., 2017). Thus, an alternative path was proposed such as introduce nitrite into microalgae culture to provide efficient CO_2capturing for microalgae and eliminate of oxygen inhibition that substantially increases production of microalgae biomass. Hence, a lab-scale experiment was conducted to determine suitable amount of nitrite addition to enhance the cultivation of microalgae.

1.2 Problem Statement

Currently, wastewater treatment via microalgae cultivation is still primitive due to its low biomass production or low biomass growth rate (Bilanovic et al., 2012). One of the major factors is the photosynthetic oxygen which could act as an inhibitor on microalgae cultivation (Carvalho et al., 2006). Fortunetely, addition of nitrite into the microalgae cultivation was found as a non-intrusive solution to eliminate oxygen inhibition (Bilanovic et al., 2016). However, the presence of free nitrous acid (FNA), produced from protonation of nitrite introduced, can cause inhibition to anabolic processes and hence it affect the growth related activities and limits the biomass growth capability (Vadivelu et al., 2006). Thus, this study is conducted to determine the effect of nitrite addition and presence of FNA on microalgae cultivation. In addition, extracellular polymeric substances (ESP), mainly composed of proteins (PNs) and polysaccharides (PSs), secreted by microalgae act as a protective barrier against xenobiotics (Chen et al., 2015). However, presence of FNA will cause release of EPS and reduce its structure stability (Tang et al., 2021). So, analysis of PNs and PSs is conducted to evaluate the influence of FNA concentration on the EPS secretion by microalgae.

1.3 Objective

1. To study the effect of nitrite addition on microalgae cultivation.
2. To investigate the effect of free nitrous acid on microalgae cultivation.
3. To evaluate the influence of FNA concentration on the EPS secretion by microalgae.

CHAPTER 2 LITERATURE REVIEW

2.1 Photobioreactor (PBR)

Photobioreactors (PBR) is a closed cultivation systems and used as a reactor that made of transparent glass in which the culture was agitated by air-lift and usage of light as an energy source to perform a photobiological reaction (Eze et al., 2017). In particular of microalgae cultivation, a continuous and controlled supply of specific environmental condition could be achieved by using PBR and it contribute low risk of contamination and hence PBR could support the production of monocultures. In addition, PBR could provide better gas diffusion rate, efficiency mixing, and higher gas utilities efficiencies especially for CO_2 utilities. However, the drawback of PBR is the accumulation of oxygen generated from photosynthesis of microalgae in the dissolved form which could inhibit growth of microalgae when the level of dissolved oxygen excess the air saturation since the oxygen cannot freely escape to atmosphere (kazbar et al., 2019).

In specifically for lab-scale experiment, column photobioreactor was one of the types of photobioreactors, it provide sufficiency high mass transfer, compact and well mixing with low shear stress, and prone to sterilize to avoid microalgae being contaminated. **Figure 2.1** shows the column photobioreactor that orientated in vertical direction to allow the gases flow from the bottom to the top of the column (Tan et al., 2018).

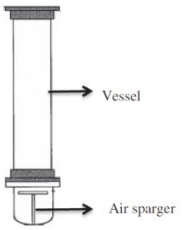

Figure 2. 1 Column photobioreactor (Tan et al., 2018)

2.2 Cultivation of microalgae on wastewater treatment

It was well known that microalgae was a type of microorganisms which contain simple cellular structure and it was a valuable substance to became biomass feedstock for biofuel production, animal feeds, and fertilizers. For cultivation of microalgae, microalgae require sources of carbon dioxide, nutrient, and suitable intensity of light and temperature to carry out photosynthesis. Based on Raouf et.al (2012), microalgae cultivation was one of the efficiency ways to carbon fixation since 1.8Kg of CO_2 is required to produce 1Kg of microalgae biomass. Generally, livestock wastewater, industrial wastewater, and agricultural wastewater were used as cultivation medium since they contains large amount of nutrients such as $NH_3 - N$ and P (Tan et al., 2018). Moreover, the efficient temperature for microalgae cultivation were range with 25℃ to 28℃, the higher temperature might limit the photosynthesis process in the result of decrese the growth rate of microalgae (Nguyen et al., 2019). Furthermore, *Chlorella vulgaris* was prefered to used and grow in wastewater since the microalgae had high growth rate, resist to toxic and unknown contaminates especially when cultivated in an open pond system, and high lipid content which could act as a valuable product for biomass feedstock. **Table 2.1** show the chemical composition of microalge species. Theoretically, the growth of microalgae consists of 4 phases which are lag phase, exponatial phase, stationary phase, and death phase. However, according to Nguyen et al., (2019), microalgae might not reach stationary phase and rapidly declined in death phase due to the presence of bacteria that will compete with microalgae to consume nutrients. **Figure 2.2** show the growth rate of *Chlorella vulgaris* that contaminated by bacteria under fluorescent illumination and seafood wastewater as cultivation medium.

Table 2. 1 Chemical composition of microalgae species on dry matter bases (%) (Demirbas & Demiebas, 2011)

Species	Proteins	Carbohydrates	Lipids	Nucleic acid
Chlorella Vulgaris	51-58	12-17	14-22	4-5
Chlorella pyrenoidosa	57	26	2	-
Spirulina maxima	60-70	13-16	6-7	3-4.5

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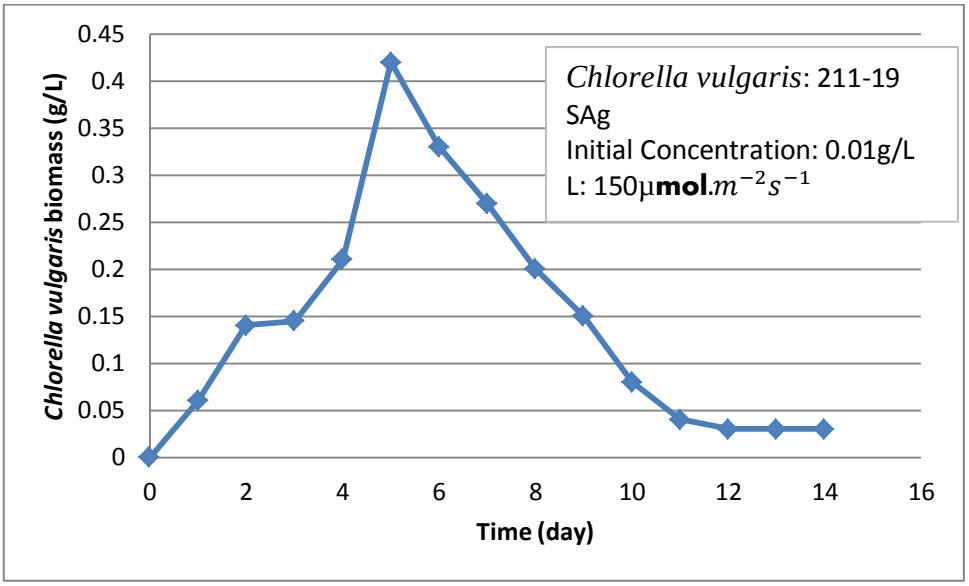

Figure 2. 2 Growth rate of *Chlorella vulgaris* that contaminated by bacteria under fluorescent illumination and seafood wastewater as cultivation medium (Nguyen et al., 2019).

2.3 The nutrient supply for cultivation of microalgae

In a lab-scale experiment, microalgae cultivation was conducted by using tap water which didn't contain required nutrient for the microalgae to grow. Thus, the culture was supplied with nutrient rich Bold's Basal Medium (BBM) stock solution and it fed continuously to maintain the nitrate content (NO3-) in the reactor within the range of 10-15 mg/L. **Table 2.2** show the composition normal BBM stock solution for the cultivation medium for *Chlorella vulgaris* species. In additional information, 20ml of stock A, 2ml of stock B and 0.2ml of stock C is supply into 1L of microalgae for cultivation at 2 to 3 days once. The scale up of cultivation of microalgae is allowable by increase the nutrient supply by multiplying the required nutrient supply for 1L with the scale up ratio of cultivation medium (Iasimone et al., 2018).

Table 2. 2 The composition of normal BBM stock solution (Iasimone et al., 2018)

No.	Chemical name	Formula	Amount
Stock A			
1	Di-potassium hydrogen orthophosphate	K_2HPO_4	7.5 g/L
2	Potassium di-hydrogen orthophosphate	KH_2PO_4	17.5 g/L
3	Magnesium sulphate	$MgSO_4.7H_2O$	7.5 g/L
4	Sodium nitrate	$NaNO_3$	25 g/L
5	Calcium chloride	$CaCL_2.2H_2O$	2.5 g/L
6	Sodium chloride	$NaCl$	2.5 g/L
Stock B			
7	EDTA tetra-sodium salt	$EDTA - Na_4$	50 g/L
8	Potassium hydroxide	KOH	31 g/L
9	Ferrous sulphate	$FeSO_4.7H_2O$	4.98 g/L
10	Concentrated sulphuric acid	H_2SO_4	1ml
11	Boric acid	H_3BO_3	11.42 g/L
Stock C			
12	Zinc sulphate	$ZnSO_4.7H_2O$	14.12 g/L
13	Manganese chloride	$MnCl_2.4H_2O$	2.32 g/L
14	Cupric sulphate	$CuSO_4.5H_2O$	2.52 g/L
15	Cobaltous nitrate	$Co(NO_3)_2.6H_2O$	0.8 g/L
16	Sodium molybdate	$Na_2MoO_4.2H_2O$	1.92 g/L

2.4 Effect of light intensity on growth of *Chlorella vulgaris*

Fluorescent light was usually used to supplies energy since fluorescent light was a stable and continuous lighting source (Blanken et al., 2013). **Figure 2.3** shows the effect of light intensity on the growth *Chlorella vulgaris* with the range of 2000 to 10000 lux. The growth rate of *Chlorella vulgaris* increased when the cultivation light intensity was increased from 2000 to 8000 lux, while the decrement of the growth rate is observed when the light intensity supplied up to 10000lux due to photo-inhibition effect and the light intensity was overloaded (Cheirsilp & Torpee, 2012). In addition, Microalgae will not undergo photosynthesis process when the light intensity less than light saturation point (Tingting, 2014).

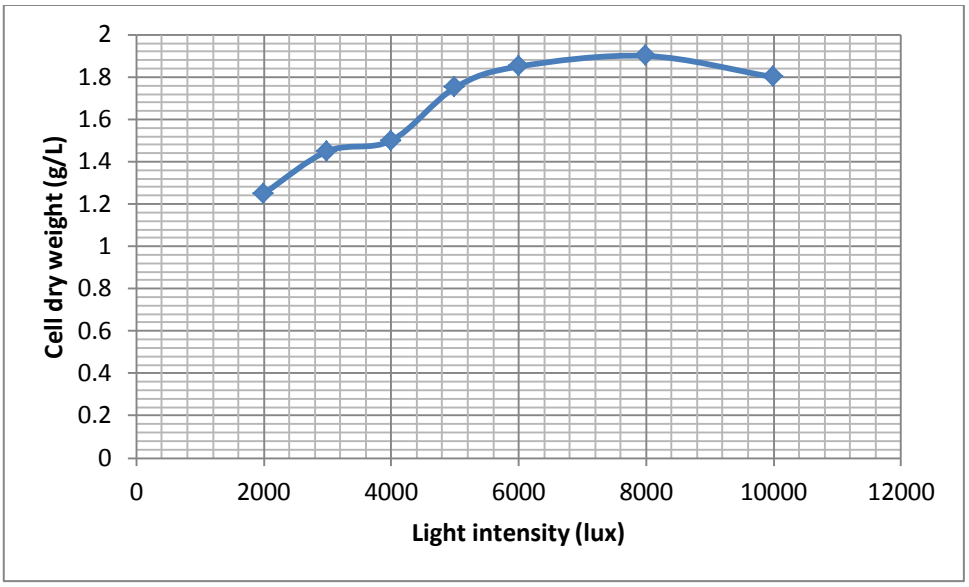

Figure 2. 3 Effect of light intensity on the growth *Chlorella vulgaris* with the range of 2000 to 10000 lux (Cheirsilp & Torpee, 2012)

2.5 Algal organic matter (AOM)

During the cultivation of microalgae in photobioreactor, microalgae will excrete large amount of AOMs (60 to 80$mgCL^{-1}$) (Hulatt & Thomas, 2010). For information, AOMs contains major fraction of neutral and charged polysaccharides, protein, nucleic acids, and lipids. The negatively charged of carboxyl group at extracellular polysaccharides will compete with microalgae to interact the positively charged metal such as Ca and PO_4^{3-}in the results of reduce removal efficiency of microalgae flocculation (Vandamme et al., 2012). In others words, the presence of AOMs during the cultivation could cause the increment of electrostatic repulsion and hydrophobicity with negatively charged of microalgae cells in the result of decrease of removal efficiency of microalgae flocculation. Rashid et.al (2019) had demonstrated an experiment to estimate the effect of AOM and pH on removal efficiency of microalgae collected in exponential phase and the result was shown in **Figure 2.4**. **Figure 2.4** show that the removal efficiency of microalgae cultured with AOMs is higher than microalgae cultured without AOMs when pH less than 9 and the removal efficiency of microalgae cultured with AOMs is relatively lower than microalgae cultured without AOMs when pH more than 9. The reason behind the trend was AOMs can behave as flocculants to increase the removal efficiency for the harvesting process at low concentrations and act as inhibitor at high concentration. Furthermore, the lowest influence of AOMs on flocculation at pH 13 had been observed due to the high ionic strength of microalgae.

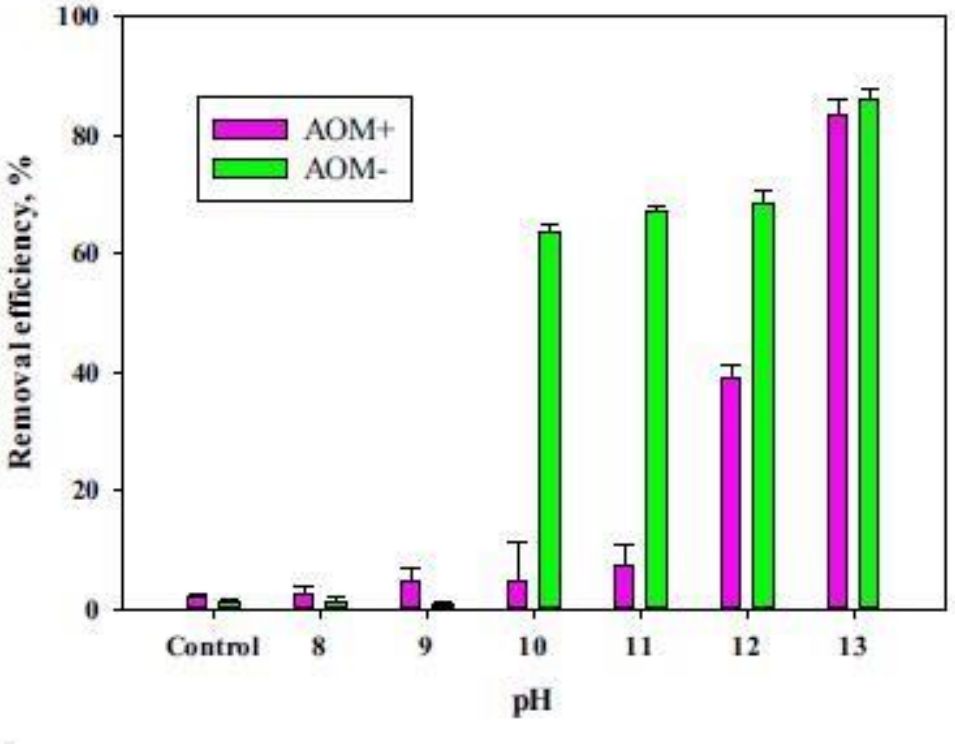

Figure 2. 4 Effect of AOM and pH on removal efficiency of microalgae collected in exponential phase (Rashid et al., 2019)

2.6 Effect of photosynthetic oxygen on microalgae cultivation

During the microalgae cultivation, microalgae will consume CO_2 and produce O_2. However, high concentration of dissolved oxygen can easily build up in closed PBR when the microalgae cultivation is exposure to high light intensities for a long period and it lead to loss of biomass productivity due to result of photosynthetic oxygen occurrence. In addition, the photosynthetic of oxygen or in others term as photorespiration could be happened when the supplied of CO_2 was not sufficient to carried out photosynthesis of microalgae. This was because most of the microalgae species had lower CO_2 binding activity compared to O_2 in rubisco enzymes, and the excess O_2 apparently was competed with CO_2 for rubisco binding sites, leading to insufficiency of photosynthetic process (Chang et al., 2017).

2.7 Introduced of sodium nitrite into microalgae culture

A suitable nitite concentration could be introduced into the culture to reduce the effect of photosynthetic oxygen, since nitrite promoting a better carbon dioxide capture and enabling high levels of CO_2 fixation by photosynthesis by decrease the local concentration of photosynthetic oxygen at the surface of microalgae cells. Substantially, the growth rate of microalgae could be improved (Bilanovic et al., 2016). However, the accumulate of nitrite ion NO_2^- could be protonated to form free nitrous acid (FNA), a weak monobasic acid which is mostly stable at pH more than 5, which would act as inhibitor to the microalgae culturing by disrupt the cell wall of microorganism (Bai et al., 2015). **Equation 2.1** show the equation of protonation of NO_2^- .

$\boldsymbol{NO_2^-} + \boldsymbol{H^+} \longleftrightarrow$ HONO (pKa = 3.3) **(2.1)**

2.8 Effect of FNA on microalgae cultivation

The presence of FNA at certain ppm level could influence significantly on EPS during the microalgae culturing. While, the effect of FNA could be identified by analyse the proteins and polysaccharides released from EPS. **Figure 2.5** shows the release of proteins and polysaccharides from EPS as a function of FNA concentration. From **Figure 2.5,** it could be realized that the protein being released when explore to low FNA concentration, while polysaccharides will released exponentially when explore to high FNA due to the deep penetration into chloroplasts and membrane lipids (i.e. phospholipids) (Bai et al., 2015).

According to Vadivelu et al (2006), the presence of FNA concentration of 0.011 mg N/L could cause inhibition of the anabolic processes of NOB while the NOB growth was completely stopped when the FNA concentration was apparently similar or higher than 0.023mg N/L on the cultivation, while at concentrations of FNA up to 0.05 mg N/L, it did not show any inhibitory effect on the catabolic processes. Hence, FNA had been identified that it was more inhibitory on the anabolism than on the catabolism of microalgae culture, anabolism refer to the processes carried out by microalgae for the synthesis of cellular component from carbon sources, while catabolism refer to the processes involved in oxidation of substrates or use of sunlight in order to obtain energy, as FNA does not primarily affect the energy generation capacity of NOB, but more immediate effects on growth related activities and limits the biomass growth capability.

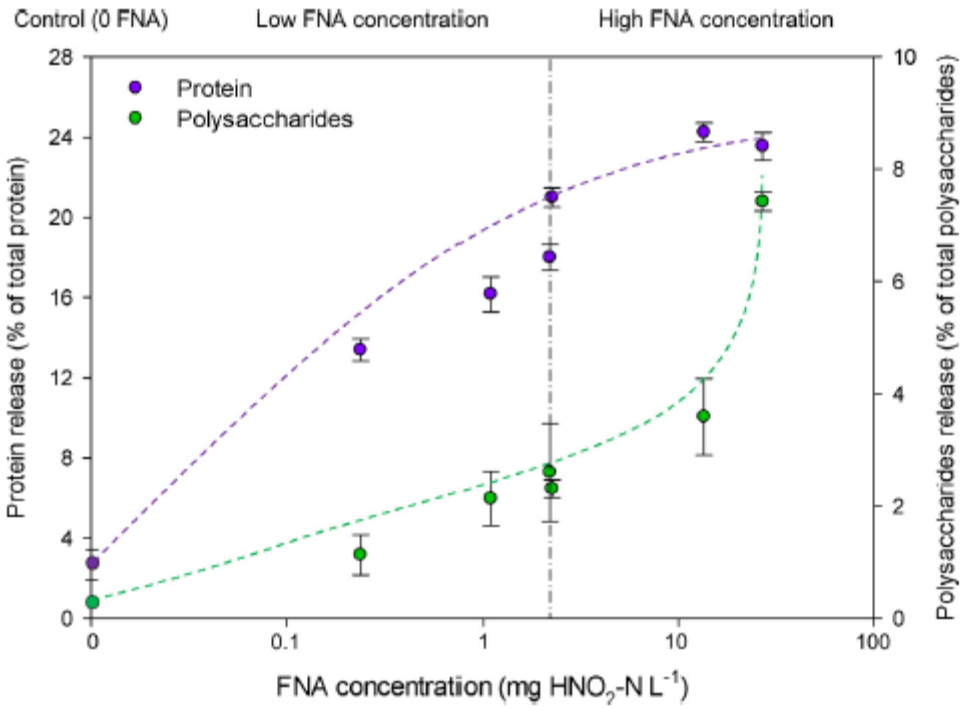

Figure 2. 5 Release of proteins and polysaccharides as a function of FNA concentration (Bai et al., 2015)

2.9 Role of EPS on microalgae cultivation and harvesting of microalgae

EPS is a kind of the macromolecular compound secreted outside the cell during microalgae cultivation which mainly composed of PNs and PSs (Chen et al., 2015). EPS has abundant functional groups including carboxyl, amide, hydroxyl, carbonyl group and it can bind with many exogenous organic compounds and inorganic ions to form a protective barrier against environmental stresses or xenobiotics (Chen et al., 2015). Moreover, due to its various functional groups, it will positively affect the adsorption and transmission of material to EPS in the result of variation in the hydrophilicity and hydrophobicity of EPS (Chen et al., 2015). Thus, the role of EPSs is to maintain structure stability by resist to xenobiotics as well as enhance transferring and storing nutrients activities (Tang et al., 2021). Commonly, EPS can be considered as a dynamic double-layered structures consisting of soluble-EPS (SB-EPS), loosely-bound EPS (LB-EPS), and tightly-bound EPS (TB-EBS) (Henderson et al.,

2008). Next, LB-EPS has highest Pb(II) adsorption abilities compared to TB-EPS and while TB-EPS have much higher flocculating abilities than LB-EPS (Babiak & krzeminska, 2021). In the aspect of role of EPS on harvesting microalgae, EPS has high ability to carried out flocculating activities due to its high molecular weight, the weight of a polymer extracted from *Chlorella vulgaris* is $9.86\ x\ 10^3 g/mol$, long chains molecules, and large amount of acidic functional groups that could partly neutralize the negative charge of the cell surface (Babiak & krzeminska, 2021). **Table 2.3** show the potential flocculating properties of EPS for *Chlorella vulgaris* where the chlorella vulgaris was cultivated under 11 hours of 25 $\mu mol.\,m^{-2}s^{-1}$ light intensity supplied and 13 hours under dark condition, BBM stocks solution was supplied 2 to 3 days once to the culture, and the culture temperature at 28 °C (MA et al., 2014).

Table 2. 3 Culture conditions and flocculation efficiency (FE) of *Chlorella vulgaris* (MA et al., 2014)

Species	**Culture Conditions**	**Flocculating efficiency (%)**	**Settling time (min)**
Chlorella vulgaris	BBM medium, 28 ℃, 11/13 hours for light/dark cycle, light intensity 25 $\mu mol.\,m^{-2}s^{-1}$	76.3	30

2.10 Extraction of extraction of extracellular polymeric substances (EPS)

Extraction of extracellular polymeric substances (EPS) were a metabolic products that consist of proteins and polysaccharides and it usually accumulate at the external surface of cells and in the interior of microbial aggregates to form a protective layer whereby the microorganisms were able to adapt in extremely adverse environmental conditions (Lv et al., 2019).

To qualitatively and quantitatively analyse characteristics of EPS especially for protein and polysaccharide analysis, several method of extraction EPS had been studied such as centrifugation, cation exchange resin, heating, alkaline, EDTA, sulfide and sodium hydroxide (NaOH) methods. In an ideal case for extraction of EPS, it should able to extract as much as possible of proteins and polysaccharides (EPS yield) without destroy or bring damage to the cells and the influence of leakage of intracellular substances on the property and content of EPS should avoided. From **Figure 2.6** that shows the process of different EPS extraction methods, centrifugation method was act as a control method because of it was the less degradative technique and there is a common step of centrifugation in all other protocols investigated which normally have the lowest extraction efficiency. While, **Figure 2.7** show the chemical composition and amounts of EPS extracted from different methods. From the **Figure 2.7**, the control method which was centrifugation method show a least efficiency from extraction of proteins and polysaccharides, while NaOH method contribute the highest yields of proteins and polysaccharides coupled with a large amount of nucleic acids. It means that the NaOH method was not an appropriate method to this extraction because the presence of nucleic acids could act as indicator to cause a serious cells lysis and disruption of cells (Dai et al., 2016).

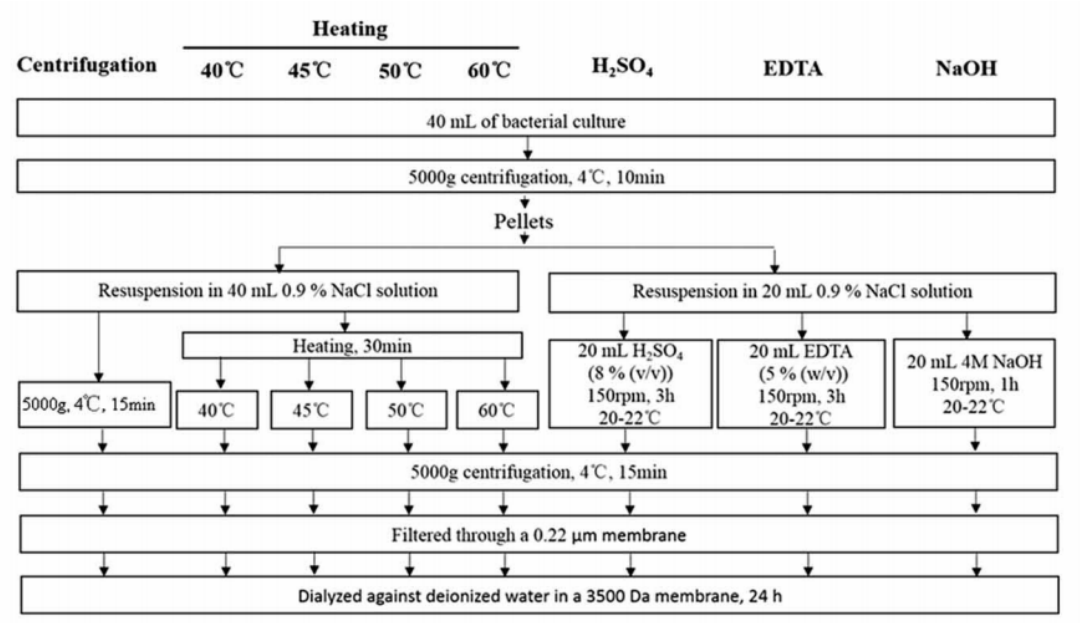

Figure 2. 6 Process of different EPS extraction methods (Dai et al., 2016)

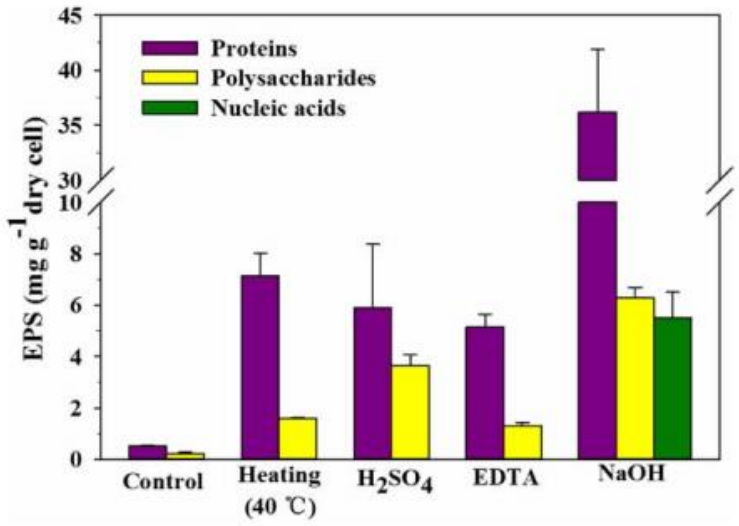

Figure 2. 7 Chemical composition and amounts of EPS extracted from different methods (Dai et al., 2016)

Chapter 3 METHODOLOGY

3.1 Research Methodology

Figure 3.1 shows the overall activities of the research.

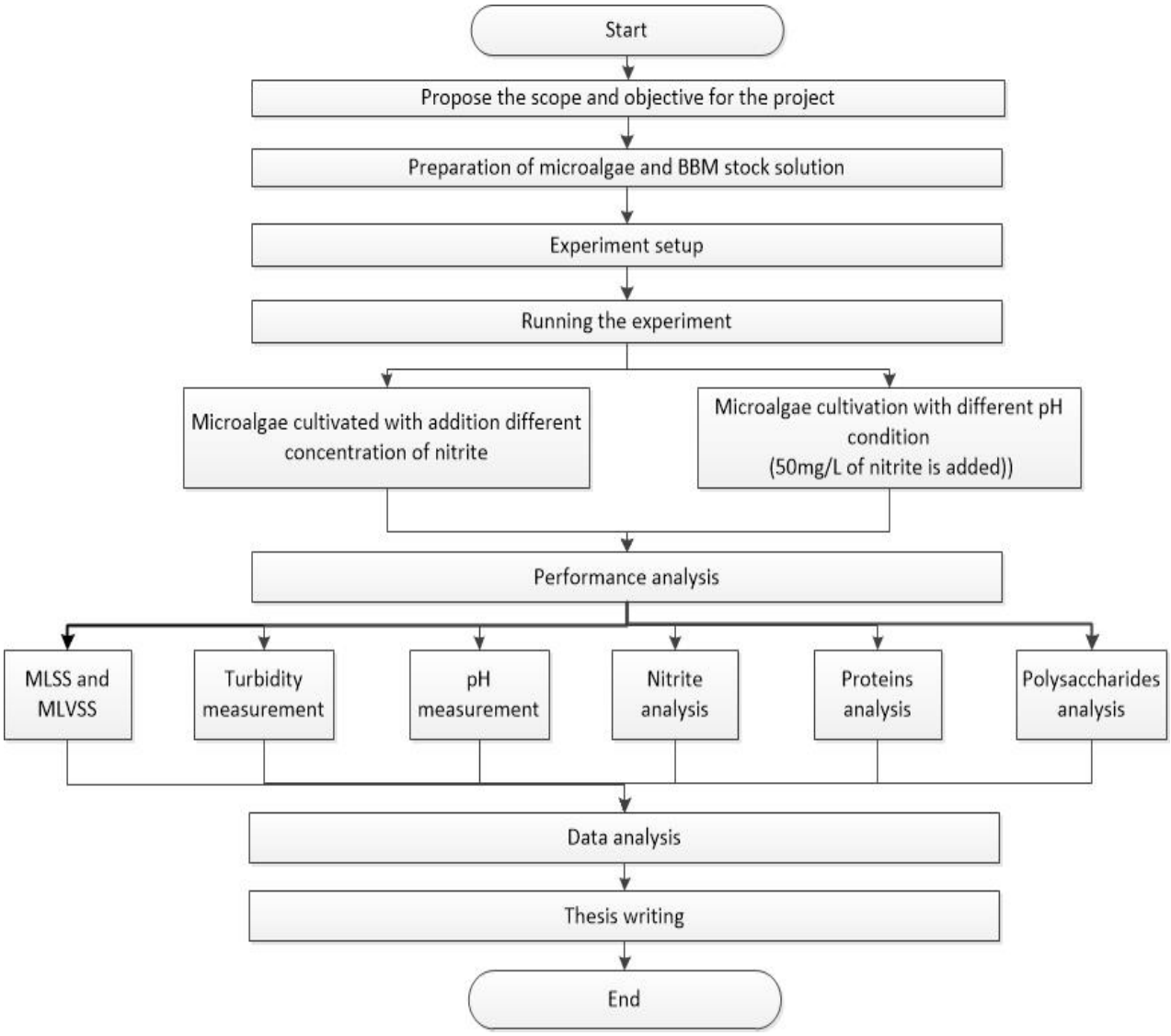

Figure 3. 1 Flow diagram of the overall research activities

3.2 Materials and Equipment

The required materials and equipment in conducting the experiments for the project are listed in **Table 3.1** and **Table 3.2** together with their respective purposes.

Table 3. 1 List of materials required.

Material	Purpose
Chlorella vulgaris species microalgae	Study microalgae for this project
Normal BBM stock solution	Act as a nutrient to feed the microalgae
Sodium nitrite ($\boldsymbol{NaNO_2}$)	To provide nitrite ion through the cultivation
NitriVer 2 Reagent Powder	To analyse the nitrite content in the sample
Bicinchoninic Acid (BCA) protein assay solution	To analyse the protein in extracellular polymeric substances (EPS)
4% Cupric sulphate solution	
Concentrated sulfuric acid	To analyse the polysaccharide in EPS
4% Phenol solution	
Sodium hydroxide	To control the pH of the microalgae culture
Hydrochloric acid	

Table 3. 2 List of apparatus and equipment required

Apparatus and Equipment	Purpose
Filter paper	To filter the out the microalgae sample in order to conduct mixed liquor suspended solid (MLSS) and mixed liquor volatile suspended solids (MLVSS)
Vacuum filter	
Pump	
Pipette	To carry out a measured volume of liquid
Column photobioreactor	A column to cultivate microalgae
Oven	To dry the sample and apparatus
Furnace	To remove the volatile material from the sample
DR 6000 Spectrophotometer (HACH)	To test the nitrite content from the sample
Centrifugator	To extract the EPS
Sonicator	
Water bath heater	
UV-Vis Spectrophotometer	To analyse the protein and polysaccharide in EPS
pH meter	To measure the pH value
Turbidity meter	To measure the turbudity

3.3 Experimental Procedure

The experimental procedure of the research is divided into sections such as preparation of normal BBM solution, enrichment of microalgae culture, experiment set up, MLSS and MLVSS measurement, analysis of nitrite content of the sample, extraction of EPS, analysis of proteins in EPS, analysis of polysaccharides in EPS, and preparation of standard curve for protein and polysaccharide.

3.3.1 Preparation of normal BBM solution

By referring to **Table 2.2** in chapter 2, the normal BBM solution was divided into 3 stocks which were stock A, B, and C. Stock A is prepared by adding 7.5 g K_2HPO_4, 17.5 g KH_2PO_4, 7.5 g $MgSO_4.7H_2O$, 25 g $NaNO_3$, 2.5 g $CaCL_2.2H_2O$, 2.5 g $NaCl$ into 1 L of distilled water and mixed together. While stock B is prepared by adding 25 g $EDTA-Na_4$, 15.5 g KOH, 2.49 g $FeSO_4.7H_2O$, 0.5 ml of H_2SO_4 and 5.71 g H_3BO_3 into 500 mL of distilled water and mixed homogeneously as well as stock is prepared by adding 0.824 g $ZnSO_4.7H_2O$, 0.464 g $MnCl_2.4H_2O$, 0.504 g $CuSO_4.5H_2O$, 0.16 g $Co(NO_3)_2.6H_2O$ 0, and 0.384 g $Na_2MoO_4.2H_2O$ into 200 mL of distilled water and mixed homogeneously. The stocks could be store at refrigerator at 4 $°C$ (Iasimone et al., 2018). **Figure 3.2** shows the prepared BBM stock solution.

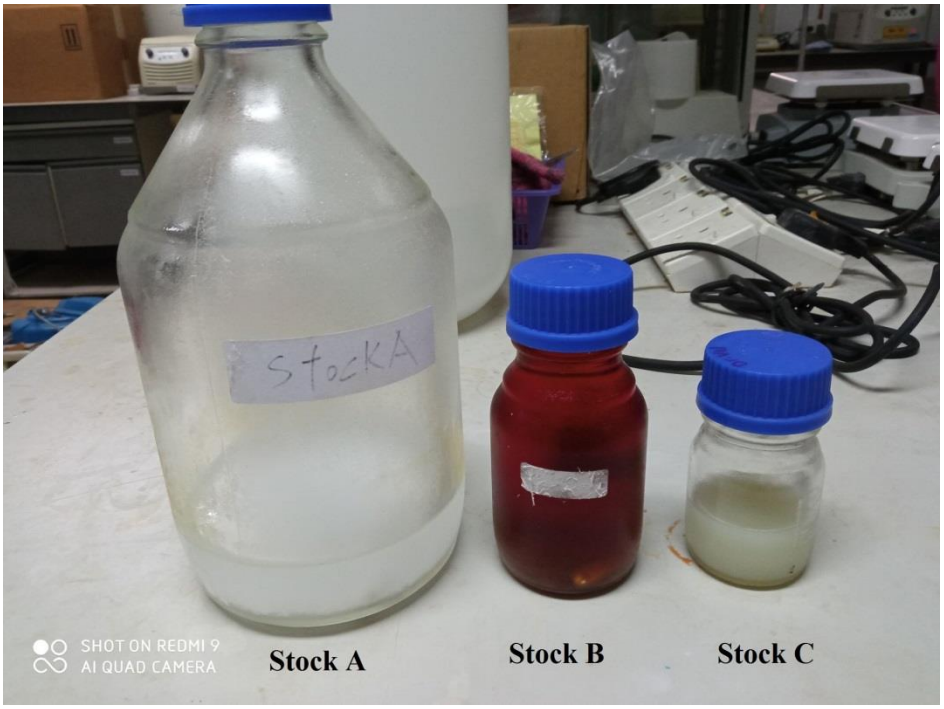

Figure 3. 2 Prepared BBM stock solution

3.3.2 Enrichment of microalgae culture

A *Chlorella vulgaris* sp. microalgae culture was used as inoculum for the preparation of enriched mixed microalgae feedstock. The microalgae culture was cultivated in photobioreactor columns. Moreover, 2L of microalgae culture was cultivated in by using tap water. The schematic of cultivating batch reactor was shown in **Figure 3.3**. The microalgae culture was cultivated under continuous aeration (2L/min) and fluorescent light (white light) supplies. The room temperature was 29.8 ºC. The nutrient from normal BBM solution was supplied 2 to 3 days once to the microalgae culture with the ratio of stock A:B:C equal to 100:10:1 and the total amount of BBM solution for 2L of microalgae culture was 22.2mL (Nguyen et al., 2019).

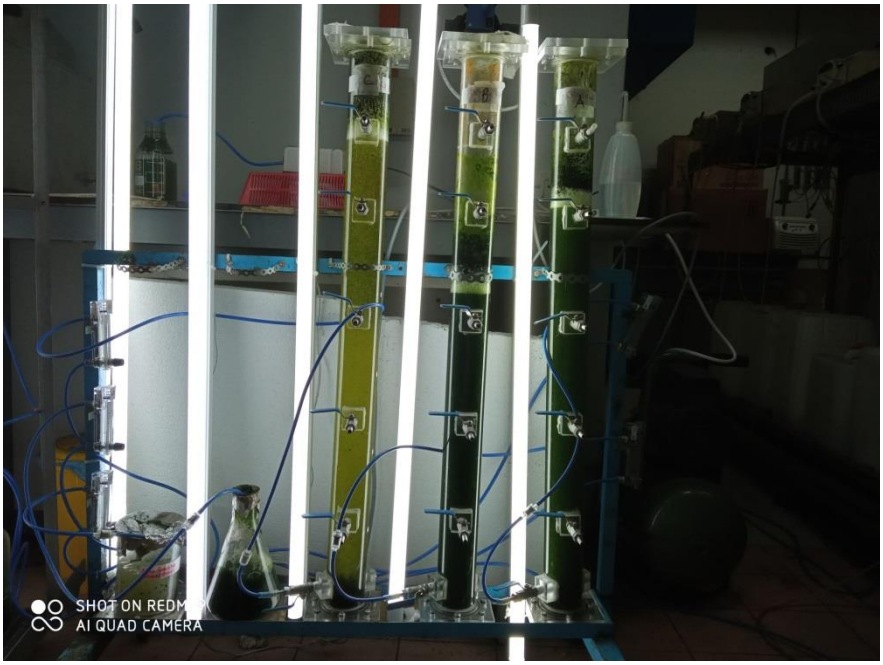

Figure 3. 3 Photobioreactor column used to conducting the experiment at Sustainable Environmental Research Laboratory (USM)

3.3.3 Experiment set up and procedure

Enriched microalgae culture of 250 mL was transferred from the parent reactor and cultivated in a 2L batch reactor by adding 750mL of tap water. The microalgae cultivation was under continuous aeration (1 L/min) and fluorescent light (white light) supplies (Nguyen et al., 2019).

3.3.3.1 Effect of nitrite concentration on microalgae cultivation

To study the effect of nitrite on the microalgae culture, a concentration of 0mg/L, 50mg/L, and 100mg/L of nitrite is added into each 1L of microalgae culture. The nutrient from normal BBM solution was supplied 2 to 3 days once to the microalgae culture with the ratio of stock A:B:C equal to 100:10:1 and the total amount of BBM solution for 1L of microalgae culture was 11.1mL. The biomass concentration in terms of MLSS and MLVSS (mg/L), turbidity measurement (NTU), pH value measurement were performed throughout