MICROALGAE SEPARATION THROUGH SILICA-AIDED-SEDIMENTATION METHOD FOR FISHPOND WATER TREATMENT AND BIOFUEL PRODUCTION

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By

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ABSTRACT

MICROALGAE SEPARATION THROUGH SILICA-AIDED-SEDIMENTATION METHOD FOR FISHPOND WATER TREATMENT AND BIOFUEL PRODUCTION

Tan Kor Yin

In this research, the silica microparticles is used to harvest microalgae to avoid nanotoxicity. Silica-aided-sedimentation (SAS) method is proposed and carried out through embedding-flocculation and immobilized-on strategies respectively. In embedding-flocculation strategy, at optimum dosage of chitosan and poly(diallyldimethylammonium chloride) (PDDA), 7 mg/L respectively, with 1 g/L of silica, the optimum cell separation efficiencies are achieved at approximately 100 % respectively and the sedimentation rate are 557.21 cm/h and 19.35 cm/h respectively. The chitosan flocculation is mainly driven by bridging mechanism A which formed bigger flocs and led to faster sedimentation. In immobilized-on strategy, the cell separation efficiencies up to 99.89 ± 0.22 % and 99.61 ± 0.16 % are achieved by 1 g/L of chitosan- and PDDA-coated silica respectively. The flocs formed by the chitosan-coated silica are much larger which led to the faster sedimentation rate (501.48 cm/h) compared to PDDA-coated silica (15.38 cm/h). The embedding-flocculation strategy promoted by chitosan is preferably chosen due to the high separation efficiency and sedimentation rate. The silica microparticles is found not toxic to the microalgae, as study model, as the cell density, total lipid, carbohydrate

and protein contents of microalgae have no significant changes after 7 days of cultivation with the presence of silica. Furthermore, it promoted cell separation efficiency and sedimentation rate up to 99.78 ± 0.76 % and 324.95 cm/h respectively at 1 g/L of silica with 2 mg/L of chitosan and able to polish the fishpond wastewater by removing 95.45 % of ammoniacal nitrogen, 95.60 ± 1.90 % of nitrate, 94.76 ± 2.42 % of ortho-phosphate, 98.61 ± 0.33 % of turbidity, 98.75 ± 1.77 % of biochemical oxygen demand (BOD), 81.04 ± 0.90 % of chemical oxygen demand (COD), and 36.36 % of total suspended solid (TSS) from the fishpond wastewater. Moreover, around 60 % of unsaturated fatty acids (UFA) which is suitable for the generation of biofuel is successfully extracted from the microalgae by the SAS method through embedding-flocculation strategy. This strategy did not affect the composition of fatty acid from microalgae biomass also proving the reliability for biodiesel production purpose.

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APPROVAL SHEET

This dissertation entitled "**MICROALGAE SEPARATION THROUGH SILICA-AIDED-SEDIMENTATION METHOD FOR FISHPOND WATER TREATMENT AND BIOFUEL PRODUCTION"** was prepared by TAN KOR YIN and submitted as partial fulfillment of the requirements for the degree of Master of Engineering Science at Universiti Tunku Abdul Rahman.

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SUBMISSION OF DISSERTATION

It is hereby certified that **Tan Kor Yin** (ID No: **17AGM05691**) has completed this dissertation entitled "MICROALGAE SEPARATION THROUGH SILICA-AIDED-SEDIMENTATION METHOD FOR FISHPOND WATER TREATMENT AND BIOFUEL PRODUCTION" under the supervision of Dr. Toh Pey Yi (Supervisor) from the Department of Petrochemical Engineering, Faculty of Engineering and Green Technology, and Dr. Chng Lee Muei (Co-Supervisor) from the Department of Petrochemical Engineering, Faculty of Engineering and Green Technology.

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Yours truly,

_____

(Tan Kor Yin)

DECLARATION

I, Tan Kor Yin hereby declare that the dissertation is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.

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(TAN KOR YIN)

Date _____

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS AND ABBREAVIATIONS

Symbols/Abbreviations

$^1O_2$	Singlet Oxygen
3N-BBM+V	Bold Basal Medium with 3-fold Nitrogen and Vitamins
ABS	Absorbance Intensity
ANOVA	Analysis of Variance
AOM	Algogenic Organic Matter
APS	(3-aminopropyl)trimethoxysilane
ASP	Aquatic Species Program
BCA	Bicinchoninic Acid
BOD	Biochemical Oxygen Demand
C	Carbon
CCAP	Culture Collection of Algae and Protozoa
COD	Chemical Oxygen Demand
-COOH	Carboxylic Functional Groups
DHA	Docosahexaenoic Acid
DNA	Deoxyribonucleic Acid
DOE	Department of Energy
EPA	Eicosapentaenoic Acid
FAME	Fatty Acid Methyl Ester
FAO	Food and Agriculture Organization of the United Nation
FEGT	Faculty of Engineering and Green Technology
FESEM	Field Emission Scanning Electron Microscope

Symbols/Abbreviations

FSc	Faculty of Science
FTIR-ATR	Fourier Transform Infrared Spectroscopy - Attenuated Total Reflectance
GC-FID	Gas Chromatography-Flame Ionization Detector
H	Hydrogen
H_2O_2	Hydrogen Peroxide
HCl	Hydrochloric Acid
IEA	International Energy Agency
IUPAC	International Union of Pure and Applied Chemistry
N	Nitrogen
$-NH_2$	Amino Functional Group
NH_4^+	Ammonium Ion
NO_2^-	Nitrite
NO_3^-	Nitrate
O_2^-	Superoxide
P	Phosphorous
PDDA	Poly(diallyldimethylammonium chloride)
PO_4^{3-}	Phosphate
ROS	Reactive Oxygen Species
SAS	Silica-Aided-Sedimentation
SiO_2	Silicon Dioxide
Si-OH	Silanol Group
TAG	Triacylglycerol
TEM	Transmission Electron Microscopy
TEOS	Tetraethyl Orthosilicate

Symbols/Abbreviations

TSS	Total Suspended Solid
UFA	Unsaturated Fatty Acids
U.S.	United States
USM	Universiti Sains Malaysia
UTAR	Universiti Tunku Abdul Rahman
UV	Ultraviolet
UV-Vis	Ultraviolet-Visible
d	Distance between Charges
g	Gravitational Constant
k_e	Coulomb's Constant
q	Signed Magnitude of Charges
r	Particle Radius
ρ_l	Density of Fluid
ρ_s	Density of Particle
η	Fluid Viscosity

CHAPTER 1

INTRODUCTION

1.1 Background

Microalgae are a diverse type of prokaryotic and eukaryotic organisms that have simple cell structure and being classified into unicellular, diatoms and multicellular forms (Li et al, 2008). They can grow easily and rapidly under light together with the supply of carbon dioxide and nutrients, such as nitrogen, phosphorous and potassium (Dragone et al, 2010). Microalgae convert light energy, carbon dioxide and nutrients into biomass through photosynthesis. Commonly, domestic, municipal, agricultural, aquacultural, industrial wastes and wastewaters consist of enormous organic and inorganic compounds such as nitrogen (ammonium (NH_4^+), nitrate (NO_3^-)) and phosphorous (phosphate (PO_4^{3-})) which serve as nutrients for microalgae (Markou and Georgakakis, 2011; Shanab et al., 2012; Velichkova et al., 2014).

The global aquaculture industry has increased in average 5.8 % annually. It is known as one of the fastest growing food production sectors (FAO, 2016). However, the expansion of the aquaculture industry has increased the quantity of wastewater which consists of nutrients such as nitrogen and phosphorus, and carbon source, which are commonly originated

from fish excretion, fish food and decaying organic matter (Huang et al., 2010; Perez-Garcia et al., 2011). According to Kawasaki et al. (2016), approximately 100 to 200 kg of nitrogen and 10 to 15 kg of phosphorus will be released into waterways with wastewater per fishpond annually. Nutrients will initiate the algal blooms with the present of sunlight, especially during summer or in tropical country like Malaysia. Microalgae assimilate the nutrients from water body to build their cells (Markou and Georgakakis, 2011; Abdel-Raouf et al., 2012). The water that crowded with microalgae will affect the productivity of fish because the microalgae tend to compete with fish on the dissolved oxygen supply at night. Fish will suffocate when the concentration of dissolved oxygen less than 2 mg/L (NT Fisheries Group, 2004; Kangur et al., 2005; Santhosh and Singh, 2007; Demirbas, 2010). Hence, the wastewater generated from aquaculture industry always saturated with nutrients and microalgae. Eutrophication will happen if the nutrient-rich wastewater that crowded with microalgae is simply discharged to environment without further treatment (Nasir et al., 2015; Wuang et al., 2016). In point of fact, the water quality of aquaculture wastewater tends to improve if the microalgae were being removed from the wastewater after sufficient retention duration for the growth of microalgae. This is because the growth of microalgae helps to remove the excessive nutrients from wastewater, which means the microalgae will contribute in treating wastewater (Ansari et al. 2017). If without the removal of microalgae from treated wastewater before discharging, the downstream water body will be contaminated again after the lysing of microalgae cells. Therefore, the microalgae should be removed from the aquaculture wastewater before discharge to surrounding in order to avoid secondary pollution or for

reuse in aquaculture.

From literature review, microalgae are recognized as a potential third generation biofuel resource. The rapid growing of world's population has increased the demand of fuel energy. However, the depletion of fossil fuel resource, increasing prices of petroleum based fuel, and the environmental problems such as greenhouse gas emission associated with utilizing of non-renewable fossil fuels have obviously shown that human dependency on fossil fuel resource as energy source is ultimately unreliable and unsustainable (Goldemberg and Guardabassi, 2009). Therefore, biofuels are being produced and found to have high potential and opportunity throughout the world to replace the fossil fuels. Biofuel is a renewable and environmental friendly fuel resource. It is a fuel that derived from biomass such as food crops, non-food crops and algae (Harun et al., 2010b; Ho et al., 2010a). The biofuels derived from food and non-food crops are found ultimately unsustainable due to certain drawbacks such as competition for arable lands, high energy consumption, low concentration of free fatty acid and the fuel versus food feud (Lee and Lavoie, 2013). The selection of biomass in the production of biofuels is directly related to greenhouse gas emissions, environmental and economic sustainability (Cerri et al., 2017).

Microalgae-based biofuel production gets much concerned and attentions as they have high growth rate and high potential to store high amount of lipids, carbohydrates and proteins in their cells (Lam and Lee, 2012). They do not need land and freshwater for cultivation, and non-limit to

seasonal condition. Furthermore, they are non-edible and not affecting the food chain (Park and Lee, 2016; Pandey, 2017). They are eco-friendly, non-toxic and have high ability to fix global carbon dioxide. Microalgae able to capture the carbon dioxide that releases from the fuel consumption activity to meet the zero carbon cycle and hence able to control the emission of greenhouse gases (Klinthong et al., 2015). A report showed that 1 kg of algal biomass able to fix 1.83 kg of carbon dioxide (Gendy and El-Temtamy, 2013). Therefore, the aquaculture wastewater treatment system that employed microalgae in biological treatment process will become more green and sustainable due to the potential to produce biofuel as by-product.

The microalgae cells are microscopic spherical cells in micron-size which generally range from 2-20 μm. They are also growing in low cell density culture medium (mass concentration less than 1 g/L) (Gultom and Hu, 2013). Generally, only if when the microalgae occur in massive form, which is known as algal blooms, and suspend on the surface of river or pond and turn the clear water into a blue, brown, green or orange liquid mass, or else, human cannot see the microalgae by naked eyes (Wolkers et al., 2011). Owing to the very dilute and small size of microalgae cells, large volumes of water must be handled to harvest microalgae biomass and thus cause the harvesting process becomes energy-intensive and challenging (Singh et al., 2013). Furthermore, the negative charge on the cell surface which derived by carboxylic (-COOH) and amino ($-NH_2$) functional groups (Pugazhendhi et al., 2019) and the negatively charged algogenic organic matter (AOM) which consists of carbohydrates and proteins that excreted by cells causes the cells to be very

stable in dispersed state during growth phase (Shelef et al., 1984; Henderson et al., 2008), this has further increased the difficulty of cell harvesting process. The difficulties in microalgae harvesting cause the process to consume much energy and increase the cost of the process which contributes about 20-30 % of the total production cost of microalgae-based biofuel production (Gudin and Thepenier, 1986). The preference of harvesting technology depends on microalgae species, growth medium, end product and production cost benefit. Therefore, further study onto the separation method of microalgae from culture medium is important for biofuel production as well as to complete the method of microalgae treatment onto aquaculture wastewater.

1.2 Problem Statement

The harvesting of microalgae from treated wastewater is necessary to prevent eutrophication and secondary pollution. However, the volume of the water medium that needs to be handled is enormous and the relative small size of microalgae cells are the reasons to cause the high capital expenditure and energy consumption of microalgae harvesting process (Grima et al., 2003; Pahl et al., 2013). Likewise, there are bottleneck in producing microalgae-based biofuel, which are the microalgae biomass harvesting and dewatering from cultivation broth (Uduman et al., 2010; Jihar et al., 2014). Currently, there is no superior technique that can harvest the microalgae at high efficiency while also meet the time and cost effectiveness (Singh et al., 2013). The drawbacks of the conventional separation methods that enable high yield

of biomass are in high capital cost, high energy consumption or high content of contaminant (Uduman et al., 2010). The readily developed microalgae harvesting methods are centrifugation, filtration, flotation, flocculation, sedimentation and magnetophoretic separation (Uduman et al., 2010).

Conventionally, centrifugation and filtration are common used to harvest microalgae, however, these energy-intensive processes make the process to be cost-ineffective (Xu et al., 2013). In centrifugation, high energy input is required to produce centrifugal force and accelerate the separation process (Barros et al., 2015). Pressure is applied in the case of filtration in order to force the fluid to pass through the filter medium and increase the efficiency of the process. Furthermore, the clogged filter membranes have to be changed to maintain the effectiveness. Moreover, flotation is originated from mineral industry and it is found to be effective in harvesting of microalgae (Ndikubwimana et al., 2016). However, flotation can have high investment and operational costs, and high energy requirement especially when small bubbles are required to induce efficient separation (Milledge and Heaven, 2013).

Generally, gravity sedimentation is used for separation of microalgae in order to reduce the capital cost. The gravity sedimentation utilizes the gravitational force to aid the auto-sedimentation of microalgae cells but the separation of the microalgae cells, which is in micron-size and at density similar to that of water, by sedimentation method is very time consuming and therefore not practical for harvesting purpose (Shelef et al., 1984; Milledge

and Heaven, 2013). The gravity sedimentation has long settling duration (at least 10 hours) and low total solid contents (2-3 %) (Pahl et al., 2013). This process yields a wet and voluminous sludge due to its poor compaction and slow settling rate (Jihar et al., 2014). The flocculation is an ideal way that can be applied to assist the gravity sedimentation. In flocculation, the flocculants with surface charge opposite to microalgae cells are added into the microalgae cell suspension to destabilize and neutralize the surface charge of cells for the cells to aggregate into larger bodies and the cell flocs can be easily harvested from medium through gravity sedimentation (Muylaert et al., 2017). According to the theory described by Stokes' Law, the sedimentation rate of microalgae cells can be increased by increasing the microalgae cell dimension and the compaction between cells (Shelef et al., 1984). The larger bodies of microalgae cells may increase the settling velocity and hence increase the efficiency of this process (Grima et al., 2003). Toh et al. (2018) employed two flocculants which were chitosan and poly(diallyldimethylammonium chloride) (PDDA) to enhance the sedimentation of *Chlorella* sp. microalgae respectively. These two flocculants were proven to be effective agents to enhance the cell separation efficiency and sedimentation rate of microalgae through electrostatic patch flocculation. The positively charged PDDA at 4.196 ± 0.094 μmcm/Vs and chitosan at 4.913 ± 0.085 μmcm/Vs interacted well with negatively charged *Chlorella* sp. at -2.116 ± 0.054 μmcm/Vs. The cell separation efficiency up to 96 % can be achieved at 30 mg/L of chitosan. Also, at 30 mg/L of PDDA, 98 % of cell separation efficiency was achieved. The self-sedimentation rate of 3.66 μm *Chlorella* sp. microalgae was 0.12 m/day, after adding chitosan and PDDA, the sedimentation rate increased to

0.53 m/day (cell flocs was 14.31 μm) and 0.24 m/day (cell flocs was 10.47 μm) respectively. The sedimentation rate was proven to be directly proportional to the size of microalgae cells as increasing of microalgae cell dimension can speed up the sedimentation rate.

A simple and rapid microalgae cell separation technique by in situ magnetic separation has been introduced in the mid-1970s (Bitton et al., 1975; Yadida et al., 1977). The method of magnetophoretic separation is revisited after around 30 years and has been extensively studied since 2011 (Xu et al., 2011). Based on the research of Lim et al. (2012), the removal efficiency of *Chlorella* sp. can achieve up to 99 % within 3 minutes by attaching the iron oxide nanoparticles onto microalgae cells in the presence of cationic polyelectrolyte PDDA as a binder. Toh et al. (2014a) has proven that the surface functionalization of negatively charged iron oxide nanoparticles by two different cationic polyelectrolyes, chitosan and PPDA, can work effective in microalgae harvesting through magnetic separation. The chitosan and PDDA coated on the surface of iron oxide through adsorption. The cell separation efficiency of the *Chlorella* sp. promoted by 300 mg/L chitosan and PDDA surface functionalized iron oxide nanoparticles were 99 % and 98 % respectively. The magnetophoretic separation technology offers a significant probable for time and energy saving solution (Xu et al., 2011). However, the nanotoxicity of iron oxide nanoparticles that use in wide range of application has been investigated as well.

There are several studies reported that the nano-sized particles have high degree of toxicity towards microalgae. A report showed that, at the concentration of 250 µg/mL titania nanoparticles, the toxicity towards *Chlorella reinhardtii* microalgae increased with the decreasing of particle size (from average particles diameter 145 nm to 25 nm). The cell viability has reduced with the decreasing of titania nanoparticles size after incubated in both dark conditions and in ultraviolet (UV) light for 6 hours. This is because the smaller particles have more packed conditions to attach to the cells than that of larger particles (Al-Awady et al., 2015). Furthermore, from the study of Toh et al. (2015), the iron oxide nanoparticles were found toxic to *Chlorella* sp. microalgae when in concentration more than 20 mg/L. The suspending iron oxide nanoparticles has blocked the light to reach the microalgae cells and hence retarded the growth of microalgae. Also, the biochemical components of microalgae which include the total lipids, proteins and carbohydrates were affected by iron oxide nanoparticles. The total lipids, protein, carbohydrate yields decreased with the increasing of iron oxide nanoparticles concentration. From the review of Demir et al. (2015), both alpha- and gamma-iron oxide nanoparticles (10-90 nm and 10-80 nm respectively) at 1 mg/L tended to decrease the sizes of marine microalgae species, *Nannochloropsis* sp. and *Isochrysis* sp., from day to day. They agglomerated iron oxide nanoparticles covered the surface of both microalgae brought adverse impact on photosynthetic or respiratory processes (Sadiq et al., 2011). This physical nanotoxicity decreased the sizes of the microalgae (Demir et al., 2015). According to the study of Ayatallahzadeh Shirazi et al. (2015), they found that the aluminium oxide nanoparticles tended to release aluminium ions into the

culture medium and changed the morphology of microalgae cells and caused shrinkage of microalgae cells. From the studies above, the size of attaching agent is being proven to be one of the key factors that confirm the toxicity of nanoparticles. Owing to the nanotoxicity of nanoparticles towards microalgae, the application of nanoparticles as attaching agent is not realistic in large scale environmental application. Therefore, the selection of the size of attaching agent is crucial important to realise the separation technology in environmental application.

In order to avoid the nanotoxicity, micro- or macroparticles is being considered as attaching agent for the separation of microalgae cells (Markides et al., 2012). The magnetic property of the attaching agent can be eliminated when the micro- or macro-sized particles is used because sedimentation can be easily promoted by the gravity force. In this study, silica in micron-size is chosen as sedimentation aiding agent to enhance the microalgae separation process. The silica is abundantly available which can be formed by oxidation of silicon surface (Cash, 2015). In addition, they are non-reactive, biocompatible and easy for modification and processing (Zhuang et al., 2010; Deng et al., 2011). The toxicity effect of the micro-sized silica towards microalgae will be analyzed in terms of cell density and biochemical composition in this research. A method named silica-aided-sedimentation (SAS) is proposed to enhance the cell separation efficiency and sedimentation rate as a result of microalgae flocs size and weight increments, and decreasing of colloidal stability of microalgae in the suspension (Zheng et al., 2012a). The silica is embedded with the microalgae cells through the flocculation and

sedimentation harvesting process and this strategy is named as embedding-flocculation. In embedding-flocculation strategy, the negatively charged silica is premixed with mutual charged microalgae cells and followed by flocculation by positively charged flocculants. The silica-cell flocs is harvested through sedimentation. The performance of embedding-flocculation in terms of cell separation efficiency and sedimentation rate is compared with immobilized-on strategy. The immobilized-on strategy has outperformed the attached-to strategy and is more preferable in magnetic separation which the particles will undergo surface functionalization before adding into microalgae suspension. The surface functionalized iron oxide nanoparticles have better distribution and colloidal stability compared to naked iron oxide nanoparticles in the case of attached-to strategy (Lim et al., 2012; Toh et al., 2014c). Hence, the performance of immobilized-on strategy in SAS method has to be investigated.

The freshwater *Chlorella vulgaris* microalgae are employed in this research. Due to its spherical shape and low settling rate (lower than 0.54 cm/h) (Jonasz and Fournier, 2011; Tiron et al., 2017), it is suitable to be used as study model as the cell separation efficiency and sedimentation rate promoted by SAS method can be observed easily. There are two flocculants will be used in this study, which are chitosan and PDDA. The preference of flocculants is based on the properties of microalgae especially the charges on the cell surface (Singh et al., 2013). The chitosan and PDDA were proven to be promising agents as flocculants and binders in conventional flocculation and immobilized-on strategy respectively (Toh et al., 2014c; Toh et al., 2018).

Hence, their performances in embedding-flocculation strategy will be determined and compared with immobilized-on strategy. The mechanism of silica-to-microalgae cell interaction promoted by the outperform combination of strategy and flocculant will be studied and its feasibility on real aqueous environmental and biofuel production will be demonstrated.

1.3 Research Objectives

The goal of this research is to apply the micro-sized silica onto microalgae separation so that it will act as an agent to enhance the cell separation process through flocculation and sedimentation. The specific objectives in this research are as followed:

1. To compare the performance of embedding-flocculation and immobilized-on strategies of SAS method in terms of cell separation efficiency and sedimentation rate.
2. To study the mechanism of silica-to-microalgae cell interaction involved that dictates the successful application of the SAS method for harvesting microalgae cells.
3. To investigate the toxicity effect of silica towards aquatic life by employing microalgae as study model.
4. To demonstrate the effectiveness and feasibility of the SAS method for real environmental system application and also for biofuel production.

1.4 Outline of Study

In first chapter, the potential of microalgae in biomass and wastewater treatment are introduced. The difficulties in microalgae harvesting and problem statement about nanotoxicity by using nano-sized particles as attaching agent for harvesting the microalgae cells are stated. The new harvesting method named SAS is suggested. The research objectives are presented.

In the following chapter, the characteristics and the potential of microalgae are studied. Moreover, the potential of *Chlorella vulgaris* for wastewater treatment and biofuel production are summarized as well since it is the study model in this research. Besides, there are reviews on different harvesting methods, such as flocculation, filtration, flotation, centrifugation, gravity sedimentation and magnetophoretic separation. In this study, silica microparticles is used to enhance the cell separation efficiency and sedimentation rate, hence, the properties and application of silica are reviewed.

In chapter three, a flow diagram of the experiment that uses to briefly explain the procedures of the experiment is shown. It involves the cultivation of microalgae, characteristics study, toxicity study, mechanism study and application of SAS method until the step to enhance the cell separation efficiency and sedimentation rate of microalgae cells. Besides, the materials and equipment used are also shown. The methodology is stated in details as

well.

In next chapter, the optimum concentration of chitosan and PDDA that required for flocculation of microalgae are determined. The performances study of the microalgae separation by SAS method through embedding-flocculation and immobilized-on strategies are investigated and compared. Furthermore, the mechanism of silica-to-microalgae cell interaction involved that dictates the successful application of the SAS method for harvesting microalgae cells is studied. Lastly, the effectiveness and feasibility of the SAS method for real environmental system application and also for biofuel production are demonstrated.

In last chapter, the results obtained are displayed and the discussion on the results was well explained. The conclusion from the research was made. Lastly, the several recommendations are suggested for further improvement.

CHAPTER 2

LITERATURE REVIEW

2.1 Characteristics of Microalgae

Microalgae are called as phytoplankton by biologists. They are one of the oldest living microorganisms on Earth (Song et al., 2008). Also, they are a diverse group of microorganism which comprise of prokaryotic cyanobacteria and eukaryotic photoautrophic protists. Almost half of the global photosynthetic activity is estimated to be performed by these microbes (Andersen, 1996). Microalgae contribute to nutrient cycling and energy conduction in aquatic systems. They can transform inorganic nutrient such as carbon, nitrogen and phosphorous into organic form (Murdock and Wetzel, 2009). There is an estimation which states that there are around 300,000 to few million species of microalgae where the diversity is more than land or terrestrial plants (around 250,000 species) (Norton et al., 1996; Scott et al., 2010). The growth rate of microalgae is hundred times quicker than land plants. Microalgae also have the ability to double their biomass within one day (Tredici, 2010). They can be found at almost everywhere and can live in marine, brackish and freshwater where they act as the source for most food chain. Microalgae are well-known primary producers in the ocean (Bark, 2012). A report stated that, microalgae are the origin of food chain for over

two third of the biomass in the world (Wiessner et al., 1995). On the other hand, most of them are microscopic spherical cells in micron-size which generally range from 2-20 μm. They are also growing in low cell density culture medium (mass concentration less than 1g/L) (Gultom and Hu, 2013). Commonly, most of the microalgae are not noticeable as single specimen, they are observable when become large population (microalgae blooms) which turn the clear water into blue, brown, green or orange liquid mass (Wolkers et al., 2011). The charge on the microalgae surface is created due to ionizable functional groups present on the cell wall or in the extracellular AOM attached to cell surface. Microalgae often excrete AOM which mainly consists of polysaccharides and proteins into growth medium. The surface charge of microalgae is typically electronegative and the AOM originating from different microalgae is found to be predominantly hydrophilic with negative zeta potential (Henderson et al., 2008).

As a photosynthetic microorganism, microalgae suspend within a water body as their growth medium and use solar energy to combine carbon dioxide with water to generate biomass (Mohammadi and Azizollahi-Aliabadi, 2013). The growth medium for microalgae must contain essential nutrient such as nitrogen (usually in form of nitrate) and phosphorous (usually in form of orthophosphate) (Dragone et al, 2010). These nutrients are used to hit the promising growth rate and obtain large quantity of biomass. Phosphorous is known as an important limiting agent for microalgae growth. It helps to transfer energy and biosynthesis of nuclei acids, phospholipids, deoxyribonucleic acid (DNA) etc., and can affect the microalgae biomass

composition. Commonly, microalgae prefer to absorb inorganic orthophosphate (ionic form of phosphorous) as source of phosphorous and the absorption depends on energy. Orthophosphoric acid also can provide inorganic phosphorous such as dyadic phosphate or dihydrogen phosphate for microalgae growth (Salazar, 2015). Nitrogen is another limiting agent for microalgae growth. The nitrogen is found to affect the protein content, lipid content and fatty acid profile of microalgae (Piorreck, et al., 1984). According to the data presented by Grobbelaar (2003), the minimal nutritional requirements for cultivation of microalgae can be calculated by the approximate molecular formula of the microalgae biomass which is $CO_{0.48}H_{1.83}N_{0.11}P_{0.01}$.

Most of the microalgae are photoautotrophic and consist of chloroplasts which are similar to plants cells. The chloroplast consists of chlorophyll molecule at the core which makes the photosynthesis possible. Through a complex series of biochemical reaction, chlorophyll in microalgae cells uses the carbon dioxide and light energy to produce sugar glucose and lipid, which means they produce their own food and generate stored energy through photosynthesis process. Furthermore, ribosome, a small organelle that is active in protein synthesis also exist in microalgae cells. Microalgae cells possess Golgi apparatus which acts like “cell gland”, they provide material for structuring and maintenance of the cell and cell wall membrane, and ships proteins and other materials for other parts of cell. Mitochondria in the microalgae cells burn substances for respiration. Microalgae do consist of vacuoles that occupy most of the space in cell and exert large pressure to

maintain cell structure and shape. They do not have roots, stems or leaves but they exhibit similar characteristics like cellular organelles in higher plants. Microalgae cells have membrane-bound organelles such as nuclei which contains DNA the genetic information of the cell. The biochemical composition of cell membrane functions as a selective barrier for material to pass through. Also, microalgae have rigid cell wall with porous outside the membrane and layered structure at outer surface of cells. There are plasmodesmate (passageways) that connect cell to cell through the wall and membrane. Microalgae do not have similar reproductive structure like plants, thus, they do not need to use energy to generate support for reproductive structure. They allocate more of their energy for trapping and converting light energy and carbon dioxide into biomass (Singh and Saxena, 2015). Through photosynthesis, microalgae generate energy straight away from the Sun's radiation. Microalgae have high photon conversion effectiveness and able to produce large amount of carbohydrate biomass (Melis and Happe, 2001; Harun et al., 2010b). They can convert around 6 % of total incident radiation energy into fresh biomass (Odum, 1971).

The main contents in microalgae biomass are carbohydrates, proteins and lipids. The carbohydrate and protein content in microalgae cells are considerably high (up to 50 % of dry weight) and the maximum lipid content are around 40 % on wt. basis (Singh and Gu, 2010). Due to these high quality contents, microalgae are used for a wide range of production (Harun et al., 2010a, Brennan and Owende, 2010) such as source of food supplements for pharmaceuticals, nutrient for livestock and biofuel like biodiesel and

bioethanol. Unlike fish, microalgae can self-produce omega-3 fatty acid. This makes the process of extracting omega-3 fatty acid for the production of food supplements and biomaterials to be very straightforward and economical (Belarbi et al., 2000). Several studies have been carried out to examine the biochemical composition, nutrition and toxicology of microalgae for their suitability for livestock feed production. Some of the microalgae are found have low number of calories, low fat content, non-toxic effect and high concentration of minerals, vitamins, and proteins hence they are suitable to be used as aquaculture feed, feed supplement in metabolism of chickens, food additives, etc. (Belay et al., 1996; Ginzberg et al., 2000; He et al., 2002; Humphrey, 2004; Thajuddin and Subramaniyan, 2005; Spolaore et al., 2006; Dhargalkar and Verlecar, 2009). Microalgae are evaluated as potential source for biodiesel production as they possess high growth rate, not compete with land crops and contains high quality lipid (neutral lipids with low degree of unsaturation) (Song et al., 2008). Microalgae can convert atmospheric carbon dioxide into glucose. The glucose that remains after the consumption can be used to form triglycerides. Transesterification of triglycerides converts the triglycerides into fatty acid methyl ester (FAME) (source of biodiesel) (Cravotto et al., 2008; Ranjan et al., 2010). The oil produced by microalgae has physic-chemical characteristics similar to vegetable oil (FAO, 1997). The protein and carbohydrate contents of several microalgae have been investigated and showed that the microalgae are promising substrate that rich in carbohydrates and proteins and can be used as carbon sources for fermentation to produce bioethanol. The carbon dioxide generated from fermentation process can be recycled as carbon sources for microalgae growth

which can in turn reduce the greenhouse gases emissions (Singh and Saxena, 2015).

Generally, microalgae will go through four phases during growing. Firstly, the microalgae will undergo lag or adaption phase. At this phase, the microalgae are getting used to the environment especially the culture medium and will not reproduce. Hence, there is no increase of cell concentration. After a few days, the microalgae reproduce and multiplies rapidly in short period of time, this phase is known as exponential growth phase. During this phase, the division rate reaches maximum and the cell concentration increases rapidly. Later, the growth of microalgae reaches stationary phase as there are insufficient space and nutrient for microalgae to grow and microalgae will stop reproducing. The population growth ceases and the concentration of cell stops increasing. In the middle of this phase, is the optimum time to harvest the microalgae. The microalgae will move to death phase at where the microalgae start to die as they have no more space and nutrients for their growth. Hence, the concentration of cell and the number of viable cells decrease. The duration of each phase vary with the microalgae species and cultivation condition (Cruz et al., 2018). There are several factors that may influence the growth characteristics and composition of microalgae, including nutrients, pH, light intensity, temperature, initial density and type of cultivation (Bark, 2012).

2.2 Cultivation of Microalgae

There are few common types of cultivation which are phototrophic, heterotrophic, mixotrophic and photoheterotrophic (Amaro et al., 2011). Under phototrophic cultivation, photosynthetically, the microalgae use light (for example sunlight) as energy source and carbon dioxide as inorganic carbon source to produce energy (Huang et al., 2010). Hence, by using this cultivation method, generally the microalgae will be cultivated in open pond system in real industrial. The microalgae can absorb the atmospheric carbon dioxide for microalgae growth and reduce the contribution to greenhouse gases. Therefore, phototrophic growth is the most general procedure for microalgae cultivation (Yoo et al., 2010). When cultivate phototropically, the lipid content of the microalgae are range from 5 % to 60 %. However, there are problematic questions using this cultivation type in open pond microalgae cultivation system which are the insufficient supply of inorganic carbon dioxide due to low carbon dioxide concentration in atmosphere, the irregular distribution of light intensity and photoperiod, and the possibility of contamination of open pond system. The limitation of light brings adverse impact on biomass productivity (Mata et al., 2010; Azma et al., 2011; Zheng et al., 2012b).

Some microalgae species are not only able to grow under phototrophic condition, they also can grow under heterotrophic cultivation. For heterotrophic cultivation, the microalgae can grow in the absence of light, they use organic carbon dioxide as both energy source and carbon source (Xiong et

al., 2008; Huerlimann et al., 2010). This cultivation method avoids the problem associated with phototrophic cultivation, which are the limitation of light. It can produce higher microalgae biomass yield than phototrophic cultivation and generally culture in conventional fermenter (Huang et al., 2010). After changing the cultivation type from phototrophic to heterotrophic, an increase of 40 % lipid content of *Chlorella protothecoides* has been observed (Xu et al., 2006). Nonetheless, the drawback of this method is that the addition of organic carbon source may increase the cultivation cost and therefore making the microalgae-based biofuel production unviable (Feng et al., 2011; Liang, 2013).

In mixotrophic growth, the microalgae submitted to photosynthesis and use both organic carbon and inorganic carbon dioxide compound as carbon source, the microalgae in this case grow under both phototrophic and heterotrophic conditions. The use of organic carbon source may release carbon dioxide via respiration, and the released carbon dioxide may be absorbed and used as inorganic carbon source in mixotrophic cultivation (Mata et al., 2010). Similar with heterotrophic cultivation, the use of organic carbon source leads to high cost of cultivation process. Also, the presence of light as energy source may require a large-scale photobioreactor which in turn leads to high operation cost (Suali and Sarbatly, 2012).

In photoheterotrophic cultivation, microalgae use light as energy source and organic carbon as carbon source. Under this cultivation condition, some of metabolites that regulated by light intensity may increase in

production, however as in case of mixotrophic cultivation, a large-scale of photobioreactor is required due to the condition with the presence of light as energy source. Also, the organic carbon source may result in high cultivation cost. Hence, the application of photoheterotrophic cultivation for biodiesel production is very rare (Suali and Sarbatly, 2012).

In this study, *Chlorella vulgaris* is cultured using phototrophic cultivation method. *Chlorella vulgaris* is green algae that possesses high photosynthetic ability. The continuous illumination at 2000 lux was provided as energy source to avoid the irregular distribution of light intensity and photoperiod caused by sunlight. The carbon dioxide is supplied through continuous aeration by air pump as inorganic carbon source to ensure the sufficiency of carbon dioxide for microalgae growth. The aeration also prevents the precipitation of microalgae during cultivation period (Daliry et al., 2017).

2.3 Aquaculture Wastewater

In the 1970s, the world aquaculture production reached around 3 million tonnes of fishes and the amount increased to 80.1 million tonnes by 2017. In 2017, the Asia-Pacific region contributes to 91.9 % of global aquaculture production. Food and Agriculture Organization of the United Nation (FAO) recognizes the aquaculture as the fastest growing food-producing sector globally which contributes around 50 % of world's fishes as

food (FAO, 2020). This phenomenon may bring adverse impacts towards environment as the development of aquaculture industries will increase the production of aquaculture wastewater that rich in nutrients. Aquaculture wastewater contains huge amount of nutrients such as nitrogen, phosphorous and carbon source that needed for microalgae growth. The principal forms of pollutants are NH_4^+, NO_2^- (nitrite), NO_3^- and PO_4^{3-} (Mook et al., 2012; Lananan et al., 2014; Nasir et al., 2015; Wuang et al., 2016). These overloaded pollutants in wastewater would lead to eutrophication by stimulating the growth of microalgae (microalgae blooms) and severe social, environmental and health problems if proper treatments are not carried out before discharging into water body (Nasir et al., 2015).

Eutrophication is a phenomenon characterized by the excessive algal growth as a result of the increasing of limiting growth factors (sunlight, carbon dioxide and nutrients) required for photosynthesis (Schindler, 2006). The microalgae uptake the nutrients for their growth and prevent the overloading of nutrients in water medium. This helps to maintain the water quality in a stable condition. Also, the organism such as fishes can hide underneath the microalgae to prevent direct penetration of sunlight. Conversely, the oversupplying of nutrients will make the aqua system to be imbalance due to the algal blooms. High microalgae concentration will increase the oxygen source through photosynthesis. During night time, the microalgae will consume the oxygen through respiration and start to release carbon dioxide. These can kill fishes when the dissolved oxygen is less than 2 mg/L, due to suffocation. When the algae bloom extremely fast, the dense microalgae will

block the sunlight from entering and plants beneath the algal blooms cannot get sunlight for photosynthesis and a death zone will form. Eventually, the algae will die and sink to the bottom of water body. The microorganism or bacteria will start to decompose the remains, this will use up the oxygen that the aquatics use for respiration and release carbon dioxide. The oxygen depletion may lead to fish kills and eventually affect the fish production and the aquaculture industries (NT Fisheries Group, 2004; Kangur et al., 2005; Santhosh and Singh, 2007; Demirbas, 2010). Therefore, the treatment before discharging of wastewater is vital to avoid secondary pollutions. Furthermore, in economic and sustainability prospects of aquaculture industries, the treatment of aquaculture wastewater is important for discharging to water body or reusing.

According to several reviews, aquaculture wastewater consists of chemical oxygen demand (COD), ammonia, nitrates and phosphates in range of 100-150 mg/L, 3-7 mg/L, 2-110 mg/L and 2-50 mg/L respectively (Lowrey et al., 2014; Gao et al., 2016). These intermediate ranges of nutrients are suitable for direct microalgae cultivation in aquaculture wastewater (Ansari et al., 2017). The microalgae absorb the nutrients in aquaculture wastewater for their growth and convert the nutrient into biomass. Cultivation of microalgae in aquaculture wastewater is an emerging wastewater treatment technology. This biological technology provide several advantages which are (1) reducing cost in wastewater treatment and microalgae production, (2) producing valuable microalgae biomass, (3) generated biomass can be processed into aquaculture feed directly, (4) minimizing the use of freshwater and fertilizer

for microalgae cultivation, and (5) reusing the treated water in aquaculture industries (Gao et al., 2016). Table 2.1 showed the studies of the nutrient removal, productivity and the biochemical composition of biomass microalgae grown using aquaculture wastewater.

Table 2.1: The Productivity, Biochemical Composition and Nutrient Removal of Microalgae Biomass Cultivated in Aquaculture Wastewater

Microalgae	Biomass productivity (mg/L/d)	Lipid P = productivity (mg/L/d) C = content (%)	Protein P = productivity (mg/L/d) C = content (%)	Carbohydrate P = productivity (mg/L/d) C = content (%)	Ammonium Ci = initial concentration (mg/L) R= removal (%)	Nitrate Ci = initial concentration (mg/L) R= removal (%)	Orthophosphate Ci = initial concentration (mg/L) R= removal (%)	References
Chlorella sp.	-	-	-	-	C_i = 0.91 R = 98.70	-	C_i = 2.6 R = 92.20	Nasir et al., 2015
Platymonas subcordiformis	-	-		-	-	C_i = 47.8 R = 98.00	C_i = 8.8 R = 99.00	Guo et al., 2013
Chlorella vulgaris	42.6	-	-	-	-	-	C_i = 0.42 R = 82.70	Gao et al., 2016
Chlorella	-	-	-	-	C_i = 5.59 R = 97.71		C_i = 6.75 R = 49.73	Lananan et al., 2014

Table 2.1 Continued: The Productivity, Biochemical Composition and Nutrient Removal of Microalgae Biomass Cultivated in Aquaculture Wastewater

Microalgae	Biomass productivity (mg/L/d)	Lipid P = productivity (mg/L/d) C = content (%)	Protein P = productivity (mg/L/d) C = content (%)	Carbohydrate P = productivity (mg/L/d) C = content (%)	Ammonium Ci = initial concentration (mg/L) R= removal (%)	Nitrate Ci = initial concentration (mg/L) R= removal (%)	Orthophosphate Ci = initial concentration (mg/L) R= removal (%)	References
Gracilaria birdiae	-	-	-	-	C_i = 8.04 µmol/L R = 34.00	C_i = 0.44 µmol/L R = 100	C_i =1.54 µmol/L R = 93.50	Marinho-Soriano et al., 2009
Chlorella sorokiniana	107.85	P = 34.35 C = 31.85	P = 31.07 C = 35.43	P = 38.21 C = 28.81	C_i = 5.32 R = 98.21	C_i = 40.67 R = 75.76	C_i = 8.82 R = ~100	Ansari et al., 2017
Scenedesmus obliquus	89.61	P = 27.65 C = 30.85	P = 17.50 C = 35.05	P = 31.41 C = 19.52	C_i = 5.32 R = 88.70	C_i = 40.67 R = 77.70	C_i = 8.82 R = ~100	Ansari et al., 2017
Ankistrodesmus falcatus	160.79	P = 57.72 C = 35.90	P = 49.18 C = 33.88	P = 54.47 C = 30.59	C_i = 5.32 R = 86.45	C_i = 40.67 R = 80.85	C_i = 8.82 R = 98.52	Ansari et al., 2017

2.4 Microalgae in Wastewater Treatment

Biological treatment by using microalgae cultures offer an elegant solution due to the ability of microalgae to uptake inorganic nitrogen and phosphorus for their growth (Mook et al., 2012; Lananan et al., 2014; Nasir et al., 2015; Wuang et al., 2016). Several microalgae species provide efficiency higher than 80 % in the removal of ammonia, nitrate, and total phosphorous. During microalgae growth phase, nutrients in wastewater can be removed by microalgae at high rate as microalgae are able to store excess nutrients in their cell for future use for cell synthesis (Rawat, et al., 2016). Furthermore, they may also remove heavy metals (Rai et al., 1981) and toxic organic compound (Redalje et al, 1989). Hence, this biological treatment would not cause secondary pollution (De la Noue and De Pauw, 1988).

In the early 1950s, the wastewater bio-treatment by using microalgae was started in United States (U.S.). Cultivation of microalgae on wastewater was evolved when the microalgae based wastewater treatment was found useful and effective (Oswald and Gotaas, 1957). At the end of 1950s, high rate algal ponds were used for microalgae based wastewater treatment (Oswald and Golueke, 1960). There were more than 1000 microalgae taxa have been reported as pollution tolerant. The most tolerant microalgae genera towards organic pollutants were *Euglena, Oscillatoria, Chlamydomonas, Scenedesmus, Chlorella, Nitzschia, Naviacula,* and *Stigeoclonium* (Palmer, 1969). Few microalgae genera such as *Chlorella, Ankistrodesmus, Scenedesmum, Euglena, Chlamydomonas, Oscillatoria, Micractinium* and *Golenkinia* were found from

a wide distribution of waste stabilization ponds (also known as oxidation ponds or waste stabilization lagoons) in order of the abundance and occurrence frequency (Palmer, 1974). The use of hyperconcentrated algal cultures in wastewater treatment systems was developed and found to be time effective as the nitrogen and phosphorous were removed in a short periods of times (Lavoie and De la Noüe, 1985). The nutrients such as nitrogen and phosphorous were removed efficiently in such system and it appeared to be more efficiently than conventional activated sewage process (Tam and Wong, 1989; Lau et al., 1995).

Many papers suggested using nutrient-rich wastewater to culture microalgae rather than synthetic fertilizers, as this can improve the sustainability and economic feasibility of microalgae productions (Lundquist et al., 2010; Clarens et al., 2010; Christenson and Sims, 2011; Pittman et al., 2011; Park et al., 2011; Olguín, 2012; Prajapati et al., 2013; Ansari et al., 2017). The microalgae that cultivated in the wastewater are generally used for the production of biofuel and as feed for animals and aquaculture. This integrated process (cultivation of microalgae in wastewater coupled with biofuel production) has several advantages such as (1) less cost and (2) less energy to produce microalgae-based biofuel, and (3) less greenhouse gas emission (Abdel-Raouf et al., 2012).

2.4.1 *Chlorella vulgaris* in Wastewater Treatment

Generally, microalgae are sensitive to the content of wastewater such as nutrients profiles and toxic compounds. There are few microalgae strains such as *Chlorella* sp. and *Scenedesmus* sp. are able to overcome and adapt well with the imbalance in nutrient profile, deficiency of some important trace elements and the presence of toxic compound in wastewater medium. Microalgae are found easier to adapt to the medium which has similar conditions to where they are found. Hence, robust microalgae strains that are more tolerant to specific type of wastewater are preferred in wastewater treatment (Zhou et al., 2014).

In warmer climates, *Chlorella* sp. and *Scenedesmus* sp. are commonly used in treatment tanks as they have high capability to remove nutrient at high rate (Chevalier et al., 2000). Based on the research of Tam and Wong (1994), the *Chlorella pyrenoidosa* which cultivated in primary settled sewage was able to remove 80 % of the inorganic nitrogen and reduce the phosphorus content to 1-2 mg/L in a week of culture. Lau et al. (1995) reported that the *Chlorella vulgaris* has nitrogen removal efficiency of 86 % and phosphorous removal efficiency of 78 %. Jalal et al. (2011) investigated that the *Chlorella vulgaris* removed nitrate from 10.5 mg/L to 2.2 mg/L in 7 days and performed better than *Spiruline platensis* and *Scenedesmus quadricauda* in removing of phosphate from 58.80 mg/L to 24.84 mg/L in 7 days. The *Chlorella vulgaris* has been proven to be excellent candidate in nutrient removal of wastewater. Shriwastav et al. (2014) has summarized the ability of *Chlorella vulgaris* in

nutrient removal from different wastewater which investigated by several researches. The nitrogen and phosphorous removal performed by *Chlorella vulgaris* were 36 % and 61 % respectively in domestic wastewater (Mahapatra et al., 2013), 2.75 % and 8.84 % respectively in pre-treated piggery wastewater (Ji et al., 2013), and 95.9 % and 94.4 % respectively in municipal wastewater (Ryu et al., 2014). Moreover, Wang et al. (2016) cultured *Chlorella vulgaris* with the presence of activated sludge and light for 50 hours in wastewater and found that the removal efficiencies of COD, nitrogen the nitrogen and phosphorous were reach up to 87.3 %, 99.2 % and 83.9 % respectively within 1 day. Mujtaba el at. (2018) reported that *Chlorella vulgaris* can remove nitrogen and phosphorous up to 92 % and 87 % respectively after 2 days of treatment.

Chlorella vulgaris microalgae have great potential in wastewater treatment which mitigates the pollution and aquatic eutrophication problems. Therefore, the application of the *Chlorella vulgaris* as study model on recent research and future development is important.

2.5 Microalgae in Biofuel Production

Fossil fuel plays a vital role in producing energy for transportation and electricity generation. These two sectors are important for improving human living standard and enhancing advance technological development (Lam and Lee, 2012). However, many authorities and researchers have agreed that using

fossil fuel as energy resources is unsustainable due to the depleting supplies in recent year and also the raising of numerous environmental issues caused by greenhouse gases emission which will lead to global warming (Alaswad et al., 2015). Ho et al. (2010b) has predicted that, in year 2100, the emission of carbon dioxide will be more than 26 billion tons per annual. This significant rising in carbon dioxide emission not only will contribute towards global warming but also induce other impacts on environmental and human life. As the energy crisis increases day by day due to the rapid population growth and industrialisation, the researches and developments on biomass based energy have attracted researchers' attention in an attempt to stimulate and seek for alternative sustainable and renewable energy sources (Zhu et al., 2012; Alaswad et al., 2015). A study from International Energy Agency (IEA) (2010) showed that the combustible renewable sources (accounted for 10 % of total primary energy supply) has highest potential in energy production compared to other renewable sources such as hydro (accounted for 2.2 % of total primary energy supply), and solar, wind and geothermal (accounted for 0.7 % of total primary energy supply).

The first and second generation biofuel which derived from food (such as soybean, palm oil, etc.) or non-food crops (such as Jatropha oil, etc) respectively are ultimately unsustainable due to certain drawbacks such as competition for arable lands, high energy consumption, low concentration of free fatty acid, and the fuel versus food feud (Lee and Lavoie, 2013). The requirements to achieve a technically and economically viable biofuel resource are (1) competitive with petroleum fuel, (2) require less land, and (3)

improve air quality (Khosla, 2011). Hence, the third generation biofuel has been developed to convert microalgae biomass into biofuel in an environmentally sustainable and economically effective way. In recent year, microalgae are recognized as promising alternative source for biofuel production due to their high growth rate and their ability to store high amount of lipid and carbohydrate inside their cells. Lipids are generally used for biodiesel production while carbohydrates are used for bioethanol production (Lam and Lee, 2012). The residual biomasses are generally used for biomethane, fuel gas or fuel oil production. The biomass after the production of biofuel can be further used as source to produce other value-added products such as docosahexaenoic acid (DHA), protein supplements, fertilizers, animal feed and aquaculture feed (Tiwari and Kiran, 2018). It is crucial to note that there are other third generation biofuel sources to produce biodiesel (from yeast and fungus) (Nigam and Singh, 2011) and bioethanol (via direct cellulose fermentation) (Carere et al., 2008) other than microalgae. However, microalgae-based biofuel has totally opened up a new dimension in the renewable energy sectors among the third generation biofuel.

In the early 1960s, the first commercial large-scale of microalgae cultivation was started in Japan by Nihon Chlorella. The species used was *Chlorella* sp. In 1970s, due to the oil crisis, the attention in using microalgae as feedstock for renewable energy was increased. In the period of 1978 to 1996, the U.S. Department of Energy (DOE) dedicated a total $25 million to the Aquatic Species Program (ASP) for them to identify lipid yielding strain and develop new technologies for microalgae-based biofuel production. This

program was stopped when they found that the production of biofuel from microalgae is possible yet not economical (Sheehan et al, 1998). From 1987 to 1990, an "Outdoor Test Facility" of 1000 m^2 high rate pond was carried out in Rowell, New Maxico. The low cost production of biodiesel by using microalgae biomass was found viable. However, in 1995, DOE cut the budget allocated to support this program financially and thus the program was stopped before these experiments could be carried out further than the preliminary stages. Recently, again, the interest on microalgae-based biofuel has increased due to the price volatility of crude oil and environmental concern (Liang et al., 2015).

The superior microalgae biomass is used as biofuel feedstock owing to its advantages for biofuel production: (1) microalgae do not require arable land, they need land only utilized as support for cultivation system and hence do not compete with agriculture. (2) the microalgae oil productivity is higher than oil seed crops as it is able to grow throughout the year, (3) microalgae has higher tolerance to high carbon dioxide content and can fix atmosphere carbon through photosynthesis (they consume 1.7 tons carbon dioxide in producing 1 ton of biomass), (4) they need less water than terrestrial plants to grow, the water may be reused (depends on the harvesting process) (5) they have higher growth potential and do not depend on seasons, (6) they can survive under harsh conditions (Behera et al., 2014), (7) they are able to self-purify if coupled with wastewater treatment and obtain nutrient for their growth from wastewater, (8) they reduce or do not trigger with food versus fuel debate (Lam and Lee, 2012), and (9) they carry prodigious amount of lipid, protein

and carbohydrate content for valuable biofuels and other bioproducts production (Zhu et al., 2012). These advantages have proven that the microalga is one of the promising feedstock for biofuel production. However, the cultivation cost of microalgae is higher than the conventional crops. The high energy required during biomass harvesting causes the harvesting steps contribute to 20-30 % of total production cost (Gudin and Thepenier, 1986). Although microalgae-based biofuel production is not cost effective yet in comparison with fossil diesel, researches and experiments in academic and in industry are being carried out in order to meet economically viable one day.

According to the research of Chisti in 2007, at least 0.53 billion m^3/year of biodiesel is required for replacing all transport fuel consumed in U.S. Utilizing of oil crops, waste cooking oil or animal fats as biodiesel feedstock is not practical to satisfy this huge demand in U.S. (Chisti, 2007). The report of U.S. DOE showed that the biodiesel yield produced from microalgae is higher than oilseed crops (Sheehan et al., 1998). Besides that, Chisti (2007) revealed that the microalgae can produce greater oil yields than terrestrial plants. The comparisons between terrestrial plants and microalgae are demonstrated in Table 2.2 in term of oil yield, land area needed for plantation, and percent of existing U.S. cropping area. Obviously, the microalgae need less area for cultivation but has highest oil yield, only 1-3 % of the total cropping area in U.S. is sufficient to produce biodiesel that can meet 50 % of U.S. transport fuel. Also, producing biodiesel from oil crops to replace the fossil diesel completely is infeasible as it requires large cultivation area which is 846 % of total cropping area to achieve 50 % transport fuel

needed. In this regard, microalgae are proven to be the most promising biodiesel feedstock to displace fossil diesel demand completely in U.S. due to its high availability and high oil yield.

Table 2.2: Comparison of Terrestrial Plants and Microalgae (Chisti, 2007)

Terrestrial Plants	Oil Yield (L/ha)	Land Area Needed (M ha)[a]	Percent of Existing U.S. Cropping Area[a]
Corn	172	1540	846
Soybean	446	594	326
Canola	1190	223	122
Jatropha	1892	140	77
Coconut	2689	99	54
Oil Palm	5950	45	24
Microalgae			
Microalgae[b]	136 900	2	1.1
Microalgae[c]	58 700	4.5	2.5

[a] For meeting 50 % of all transport fuel needs of the U.S.

[b] 70 wt % oil in biomass

[c] 30 wt % oil in biomass

Several reports revealed that the lipid content and productivity of microalgae can be increased under various stress condition such as oxidative stress, nitrogen starvation, high light stress and high salt stress conditions (Takagi et al., 2006; Solovchenko et al., 2008; Sharma et al., 2012; Cakmak et al., 2012). The oxidative stress by photocatalyst and high light stress are similar. The microalgae able to accumulate reactive oxygen species (ROS) such as singlet oxygen ($^1O_2$), superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals in huge amount. These may cause the direct decomposition of lipids, proteins and nuclei acids. However, this will trigger the protection

mechanism of microalgae as they have ROS-scavenging ability. Through this mechanism, the antioxidant enzymes (glutathione peroxidase and superoxide dismutase) and antioxidant molecules (ascorbate, glutathione, carotenoids and tocopherols) will increase in response to photo-oxidative stress (Li et al., 2012). Other than that, triacylglycerol (TAG) can be produced efficiently by utilizing the photo-energy in order to reduce damage caused by excess light energy (Solovchenko et al., 2012).

2.5.1 *Chlorella vulgaris* in Biofuel Production

Some of the microalgae species are able to store large quantity of lipid in their cells. The lipid contents of few freshwater microalgae species are shown in Table 2.3. Different species will have different lipid composition. Therefore, the selection of microalgae for biodiesel production is generally based on the fatty acid content, length and branching of fatty acid chain and unsaturation of fatty acid (Sheehan et al., 1998; Li et al., 2008b; Islam et al., 2013). *Chlorella protothecoides* microalgae that grow heterotrophically with the supplement of acetate, glucose, or other organic compounds would have high biomass and high lipid content (Endo et al., 1977). Illman et al. (2000) has suggested that the *Chlorella* sp. might be suitable for replacing diesel after they have studied the calorific values of five different types of *Chlorella* sp. (*Chlorella protothecoides, Chlorella vulgaris, Chlorella emersonii, Chlorella sorokiniana* and *Chlorella minutissima*) that grown in low nitrogen medium. The study of Miao et al. (2004) showed that the lipid content of heterotrophic

Chlorella protothecoides could reach up to 55.2 % by increasing the organic carbon source and decreasing the inorganic nitrogen source in water medium. Based on Table 2.3, Mata et al. (2010) mentioned that the *Chlorella* sp. seems to be a good choice as biodiesel feedstock. Despite the lipid content of *Botryococcus brauniican* can reach up to 75 %, but it has low productivities. The other types of microalgae species including *Chlorella* sp. have lipid contents at around 5-63 % only but have higher productivities. In the study of Ashokkumar et al. (2019), cultivation of *Chlorella sp*. in municipal sewage accumulated 28.5 wt % lipids and 36.2 wt % of carbohydrates. Hence, *Chlorella* sp. is a promising microalgae strain that can reduce nutrient contents for wastewater treatment and produce biomass for the production of renewable biofuel.

Table 2.3: Lipid Content of Various Freshwater Microalgae (Mata, Martins and Caetano, 2010)

Microalgae Species	Lipid Content (% by dry weight)	Lipid Productivity (mg/L/d)	Volumetric Productivity of Biomass (g/L/day)	Areal Productivity of Biomass ($g/m^2/d$)
Ankistrodesmus sp.	24-31	-	-	11.5–17.4
Botryococcus Braunii	25-75	-	0.02	3.0
Chlorella Emersonii	25-63	10.3–50.0	0.036–0.041	0.91–0.97
Chlorella Vulgaris	5-58	11.2–40.0	0.02–0.20	0.57–0.95
Chlorella Sorokiniana	19-22	44.7	0.23–1.47	-
Scenedesmus Obliquus	11-55	-	0.004–0.74	-
Scenedesmus Quadricauda	1.9-18.4	35.1	0.19	-

2.5.2 FAME of Microalgae

Generally, the properties of biodiesel such as cetane number, kinematic viscosity, oxidative stability, cloud point and cold filter plugging point are determined by and well dependent on the composition of FAMEs (Knothe, 2008; Ramírez-Verduzco et al., 2012). Therefore, the FAME composition is necessary to be determined to investigate the feasibility of the particular microalgae species for biodiesel production. FAME is used virtually synonymously as term for biodiesel. In fact, FAME is the chemical composition of biodiesel. Microalgae lipids can be converted to FAME through transesterification with alcohol. Methanol is the most employed alcohol in transesterification and yields methyl ester of fatty acid for biodiesel production. Hence, FAME composed of two building blocks which are fatty acid chain and methanol (Knothe, 2009).

The fatty acid profile of biodiesel is depending on the feedstock and cultivation condition. Knothe (2008) revealed that the most common fatty acids existed in biodiesel are palmitic acid (C16:0), stearic acid (C18:0) and linoleic acid (C18:2n6c). While, the most common fatty acids in microalgae based biodiesel are Palmitic-(hexadecanoic-C16:0), Stearic-(octadecanoic-C18:0), Oleic-(octadecenoic-C18:1), Linoleic-(octadecadienoic-C18:2), Linolenic-(octadecatrienoic-C18:3) acids and small amount of eicosapentaenoic acid (EPA) (C20:5) and DHA (C22:6) (Knotte, 2009). Schenk et al. (2008) suggested that a good quality biodiesel should have fatty acid mass ratio 5:4:1 of palmitoleic (C16:1), C18:1 and C14:0. Jeon et al.

(2017) incubated the silica and methyl-functionalized silica nanoparticles with *Chlorella vulgaris* microalgae. The obtained FAME included C16:1, C16:0, C18:2n6c, γ-linoleic (C18:3n6), oleic (C18:1n9c) and C18:0 acid methyl ester. The acid methyl ester productivity increased in the order of pure *Chlorella vulgaris*, cultivation of *Chlorella vulgaris* with the presence of silica, cultivation of *Chlorella vulgaris* with the presence of methyl-functionalized silica. The FAME productivity of *Chlorella vulgaris* was 0.1409 g/L/d and increased to 0.6204 g/L/d and 1.005 g/L/d with the presence of silica and methyl-functionalized silica respectively due to the environmental stress that increased the cell growth rate during cultivation period of 10 days. The cell growth rate would directly affect the lipid and FAME production. Hence, the cultivation condition is important to affect the FAME productivity.

2.6 Microalgae Separation Methods

In microalgae based biodiesel production, several steps are involved which are selection of microalgae species, cultivation of microalgae, harvesting of microalgae, lipid extraction from microalgae biomass and production of biodiesel (Bligh and Dyer, 1959; Halim et al., 2011; Gamia et al., 2014). The major problem recognized is the harvesting or separation of microalgae from culture medium (James, 1998; Mallick, 2002; Aslan and Kapdan, 2006). When microalgae cultures reach the stationary state, microalgae are required to separate from the growth medium and their biomasses are then recovered at downstream processing. However, harvesting

of microalgae biomass possesses considerable challenges in achieving economically viable. This biomass recovery process which needs high energy consumption accounts for 20-30 % of total production cost (Gudin and Thepenier, 1986). The very small size of microalgae cells and grow in very dilute culture (mass concentration less than 1g/L) cause this process to be not very cost-effective (Barros et al., 2015). Furthermore, the negative charge on the cell surface and the AOM carried by cells cause the cells to be very stable in dispersed state during growth phase (Shelef et al., 1984) which has further increased the difficulty of cell separation process. These are always the major obstacles to a real breakthrough for large scale production. The issue of high energy consumption in harvesting and drying of biomass should be concerned as it may bring adverse effect towards the overall energy balance in producing microalgae biofuel.

The most common existing harvesting methods include centrifugation, filtration, flotation, flocculation, sedimentation and magnetophoretic separation (Uduman et al., 2010; Christenson and Sims, 2011). However, there is no superior harvesting method as different strategies have their advantages and disadvantages. Grima et al. (2003) has suggested that the main criterion in choosing the microalgae harvesting method is on the basis of the final moisture content of microalgae biomass. Barros et al. (2015) stated that, in selecting an adequate harvesting method, there are two criteria that must be taken into consideration, which are (1) characteristics of the microorganism and (2) type and value of the end product.

2.6.1 Flotation

Flotation is used to separate the microalgae cells by the attachment of air bubbles onto the cells. The microalgae cells float on the surface of growth medium are harvested through skimming or filtration (Singh et al., 2013). Some of the microalgae species are able to float on the surface naturally, however, air bubbles are added to enhance the flotation of microalgae cells (Singh et al., 2011). Generally, this process is classified according to the method to produce air bubbles which are (1) dissolved air flotation, (2) electrolytic flotation, and (3) dispersed air flotation (Shelef et al., 1984). The tendency of flotation occurrence is depending on the nature of microalgae cells. Particles with diameter dimension that smaller than 500 μm is easier to be drift up by air bubbles (Matis et al., 1993). Furthermore, the attachment of air bubbles onto microalgae cells is depending on the (1) stability of suspended microalgae cells and (2) air, solid, and aqueous phase contact angle. The greater tendency of air bubble to adhere with the cells is resulting from the (1) lower stability of microalgae cell and (2) larger contact angle (Shelef et al., 1984). Flotation technique may have high investment and operational cost and high energy usage. When the small size air bubbles are required, the cost and energy can be even higher (Brennan and Owende, 2010).

2.6.2 Filtration

Filtration is a process used to harvest the microalgae cells by filter medium. There are many types of filter medium can be used to harvest microalgae cells and this method is suitable for recovery of relatively large microalgae cells especially bigger than 70 µm such as filamentous species or agglomerates (Grima et al., 2003). The membrane filter medium is classified based on the pore size of the membrane which are (1) macrofiltration (>10 µm), (2) microfiltration (0.1-1.0 µm), (3) ultrafiltration (0.02-0.2 µm), and (4) reverse osmosis (<0.001 µm). In order to separate the microalgae cells in more efficient way, the pressure is applied to force the fluid pass through the filter medium. Hence, high operational energy is required especially if the pore size is small. This technique has relatively high cost due to the energy intensive step and frequent changing of the clogged filter membrane. Therefore, this method is temporary not satisfactory in commercial scale recovery of microalgae biomass (Singh et al., 2013).

Polymer membrane has been used for continuous recovery of microalgae biomass. However, several factors have been taken into consideration when using this type of membrane as filter medium such as hydrodynamic condition, concentration, and properties of microalgae (Singh et al., 2013). Moreover, the filtration can be assisted by filter aids and flocculants. These are possible to decrease the equipment operational energy requirement but the cost may be increased due to the additional of materials used. The afterward removal of filter aids or flocculant from the microalgae biomass and

growth medium may increase the operational cost as well (Milledge and Heaven, 2013).

2.6.3 Centrifugation

Centrifugation process utilises the gravitational force and centrifugal force to concentrate the microalgae cells and drive the separation process more efficient. There are two key factors in centrifugal separation process, which are (1) particles size of microalgae cells and (2) density difference between cells and growth medium (Singh et al., 2013). Centrifugal separation can achieve 90-100 % of biomass harvesting efficiency (Heasman et al, 2000). This technique is found to have high effectiveness but the operation cost is high due to its energy intensive. Therefore, this method is only suggested to be applied in production of high value metabolites or utilizing as second stage dewatering technique in order to concentrate the microalgae biomass from 1-5 % solids to about 15 % solids (Singh et al., 2013).

2.6.4 Gravity Sedimentation

Gravity sedimentation is defined as separation of a particle or a fluid from another fluid with different densities by means of only gravitational force without external force. Thus, the energy consumption is relatively low. The sedimentation rate is extremely slow especially when the densities between

two medium is small or the particle size is small (Milledge and Heaven, 2013). This is proven by Stokes' Law which states that sedimentation velocity is proportional to square root of the radius of cells and difference in density between particle and the water medium as shown below (Reynolds, 1984).

$$\text{Setting velocity} = \frac{2}{9} g \frac{r^2}{\eta} (\rho_s - \rho_l) \qquad \text{(Equation 2.1)}$$

Where g is gravitational constant, r is particle radius, η is fluid viscosity, ρ_s is density of particle and ρ_l is density of fluid. This theory is applied in harvesting of microalgae biomass which separates the microalgae cells from growth medium. The sedimentation rate of microalgae cells can be increased by increasing the microalgae cells dimension and the compaction between cells (Shelef et al., 1984). The settling velocity of microalgae can vary between species due to the different in density. Most of the species have density higher than the growth medium such as freshwater (998 kg/m^3) and saltwater (1,025 kg/m^3) (Millero and Lepple, 1973). Hence, the sedimentation of microalgae cells may happen. The example of few microalgae species with different density is shown in Table 2.4. The slow sedimentation process may cause the deterioration of biomass quality. Other than that, it generates dilute microalgae biomass as well.

Table 2.4: Density of Different Microalgae Species

Microalgae Species	Density (kg/m^3)	References
Marine Microalgae	1,030-1,100	Smayda, 1970
Marine Diatom	1,030-1,230	Van Lerland and Peperzak, 1984
Diatom *Cyclotella*	1,114	Edzwald, 1993
Freshwater Green Microalgae	1,040-1,140	Van Lerland and Peperzak, 1984
Freshwater *Chlorella*	1,070	Edzwald, 1993

The slow sedimentation rate is generally assisted by (1) flocculation (Grima et al, 2003) and (2) centrifugation techniques (Singh et al., 2013). In flocculation, the chemical flocculants are added into the medium with microalgae cells for the cells to aggregate into larger bodies (Shelef et al., 1984). The flocs are then collected by gravity sedimentation. The larger bodies of microalgae cells may increase the settling velocity and hence increase the efficiency of this process (Grima et al., 2003). The centrifugation is another approach used to increase the sedimentation rate by means of gravitational force and external centrifugal force. The biomass recovery by this approach is greater than 95 % (Singh et al., 2013). Owing to the high cost of this method, it is temporary not suitable to use in microalgae farms that producing a feedstock for energy production.

2.6.5 Flocculation

Flocculation is one of the techniques to destabilize and aggregate numerous individual microalgae cells into larger flocs. These flocs are then easy to be collected through sedimentation, filtration or centrifugation. Commonly, microalgae are in negative charges, these charges cause an electrostatic repulsion between themselves. Flocculation can be induced by eliminating or overcoming the existing repulsion force (Bux, 2014). Commonly, flocculant is added to enhance the flocculation process that restricted by repulsion between negative charges on the microalgae cells walls.

Using metal salts as flocculants has been intensively studied (Duan and Gregory, 2003). Aluminiun sulphate and iron (III) chloride are metal salts that generally applied for flocculation. When they dissolved in water medium, the aluminium and iron ions hydrolyze to form positively charged hydroxides and start to neutralize the negatively charged microalgae cells (Bratby, 2006). The metal hydroxides attach with microalgae cells and form dense flocs which are settable. However, the use of metal salts flocculant causes the harvested biomass to be contaminated by the high concentration of metals (Şirin et al., 2012). Furthermore, the dosage of metal salts flocculants needed is high and pH adjustment is required for flocculation, which results in significant cost. Hence, autoflocculation and electroflocculation have been developed to harvest the microalgae which have fewer disadvantages.

Autoflocculation is the flocculation of microalgae by increasing the pH of culture medium by pH-dependent chemicals. In some cases, flocculation can occur spontaneously. This approach is usually accomplished by breaking down the carbon dioxide supply during photosynthesis and results in increasing pH level of water medium (Spilling et al., 2011). However, sometimes increase in pH by additional of bases/alkalines such as calcium or magnesium salts or calcium phosphate is necessary (Vandamme et al., 2012). The flocculation at high pH is generally caused by chemical precipitation magnesium, calcium and phosphate salts into hydroxide form in water and these hydroxides with positive charges will interact with negatively charged microalgae and neutralize the microalgae cells to form microalgae flocs (Sathe, 2010). The calcium and magnesium precipitates are less toxic compared to

metal salts which bring less contamination to microalgae biomass. Also, these precipitates can be removed by dissolving them by mild acidification. There are drawbacks for this process as it is not suitable for all kind of microalgae as it will influence marine microalgae more than freshwater microalgae (Barros et al., 2015).

Electroflocculation is induced by small electricity amounts instead of flocculants. Electroflocculation will occur when an electrical current is supplied through the electrodes (aluminium or iron electrodes) in microalgae medium. The anode electrodes produce positive and polyvalent ions and these ions attract the negatively charged cells together to form flocs; thus the flocs formed will accumulate at the bottom (Pearsall et al., 2011; Barros et al., 2015). Nonetheless, depending on the current density and operation time, the electrode may generate toxic towards microalgae biomass. A several studies reported that 1.5 %, 0.56 % and 1.39 % of aluminium was found in biomass after electroflocculation treatment with current density 3 mA/cm (Vandamme et al., 2012), 3.3 mA/cm and 8.3 mA/cm respectively over 10 minutes (Matos, et al., 2013). Furthermore, the depletion of electrode may increase the cost of harvesting.

Bioflocculation of microalgae is induced by the presence of other microorganism such as bacteria (Oh et al., 2001) or filamentous fungi (Zhou et al., 2013). Several types of fungi have positively charged hyphae that can bind with the negatively charged surface of microalgae cells during flocculation (Zhang and Hu, 2012; Zhou et al., 2012; Zhou et al., 2013). Bioflocculation is

constantly employed in high rate microalgae ponds used for wastewater treatment of microalgae due to its effectiveness in harvesting microalgae in facilities (Craggs et al., 2012). The ponds consist of complex communities of microalgae and bacteria, the bioflocculation occurs as a result of the interaction between microalgae cells and between microalgae cells and bacteria. Bioflocculation with fungi or bacteria as flocculating agent could avoid chemical contamination to the biomass but consequently contaminate microbiology which may also disturb with food or feed applications of the microalgae biomass (Vandamme et al., 2013).

Furthermore, flocculation can be induced by polymer flocculants (commonly with positively charged functional groups) by neutralizing the negative charge on microalgae cell surface or forming bridges between microalgae cells. The polymer flocculants are polysaccharides which can be categorized into synthetic or natural. The example of common use cationic natural polymer flocculants for microalgae separation is chitosan, they can induce effective flocculation with freshwater microalgae at low dosages. However, for marine microalgae, they have poor performance in term of flocculation. The high ionic strength of seawater medium causes the coiling of polymer (Muylaert et al, 2017). PDDA is a synthetic cationic polymer which is widely used as primary flocculant in water treatment. Also, PDDA is known as the most chlorine resistant and is functional over a wide range of pH (John et al., 2002). Both synthetic and natural polymers can promote effective flocculation through attractive electrostatic interactions and bridging between cells (Tripathy and De, 2006). Preference of flocculants and the effectiveness

of flocculants are based on the properties of microalgae in terms of the molecular weight or chain length of polymer, ionic strength and pH of culture medium, concentration of biomass, charge density on the polymer and charges on the cell surface (Singh et al., 2013).

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2.6.5.1 Natural Polymer Flocculant

The natural polymer flocculants may be polyelectrolytes (carrying anionic or cationic charge) or uncharged non-ionic polymer. Polyelectrolytes act as an agent to interact with microalgae cells through a combination of charge neutralization and bridging to form flocs (Chatsungnoen, 2019) Polysaccharides cationic chitosan, starch and polypeptide poly-γ-glutamic acid are the example of natural biopolymer that mostly used for flocculation in wastewater treatment (Brostow, 2009). Chitosan can be obtained through deacetylation of chitin (natural polymer derived from arthropod shells constituting *N*-acetylglucosamine, and glucosamine building blocks). Chitosan can be termed as poly-β(1-4)-2-amino-2-deoxy-d-glucopyranose as well (Chen, et al., 2014). The acetamide group on chitin is converted into amino group to give chitosan. This polysaccharides carry positively charged hydroxyl and amino group and very useful to adsorb large number of negatively charged microalgae species. Chitosan is widely used for wastewater treatment as it is non-toxic, non-corrosive, biocompatible, biodegradable, high adhesion to surface, safe to handle, expressed chelating properties and has high flocculation ability (Ahmad et al., 2011; Rashid et al., 2013; Fast et al., 2014).

Chitosan has been proven as an effective bioadsorbent that can adsorb toxic ions, dyes and organic contaminants (Guibal et al., 1998; Chassary et al., 2004; Ma et al., 2009; Mahmoud et al., 2012). It can degrade into products that will not bring negative impacts towards human and environment, which are glucosamine and N-acetylglucosamine. Also, as it has high biocompatibility, it is widely applied in biomedical application (Rinaudo, 2006) as it is effective in dermal regeneration and wound healing (Howling et al., 2001; Azad et al., 2004; Jayakumar et al., 2011). Chitosan is even found has an antimicrobic effect (No et al., 2002; Qi et al., 2004). These properties made chitosan to be used as a surface modifier for various nanometerials (Lemarchand et al., 2004).

Commonly, chitosan is insoluble in water (neutral pH) and organic solvent such as alcohol and pyridine because of the intramolecular and intermolecular hydrogen bonds between polymer chains. It is soluble in some diluted acids such as acetic acid, formic acid and lactic acid (Chen et al., 2014). The protonated amino groups in chitosan may increase in acidic solution (Ahmad et al., 2011). The disadvantage of using chitosan as flocculant is low pH is required for it to function efficiently (Moreno et al., 2015). Hence, it is not very effective in water with high salinity (Grima et al., 2003).

Chitosan is an effective flocculant as it has high positive charge density, long polymer chains, bridging of aggregates and precipitation in neutral or high pH condition (Renault et al., 2009). Adsorption and charge neutralization are most likely to be involved in flocculation of microalgae using chitosan due to its high positively charge density (Chen et al., 2014).

Chitosan is found to be a promising agent to induce flocculation of microalgae in freshwater condition. Ahmad et al. (2011) found that at 10 mg/L of chitosan for *Chlorella* sp. microalgae flocculation, the biomass recovery up to 99 % can be achieved at optimum mixing time (20 minutes), mixing rate (150 rpm) and sedimentation rate (20 minutes). Furthermore, for *Chlorella sorokiniana*, the clarification efficiency by chitosan flocculation could reach up to 99 % at pH value below 7 (Xu et al., 2013). Toh et al. (2018) has proven that with 30 mg/L of chitosan, the *Chlorella* sp. microalgae cell separation efficiency of 96 % can be achieved. In freshwater, only a low dosage of chitosan is required. In the study of Zhu et al. (2018), at 0.25 g/L of chitosan, the *Chlorella vulgaris* microalgae biomass recovery more than 90 % was achieved in 10 minutes sedimentation time. The lipid content of the chitosan harvested biomass reached 32.9 %. The percentage of microalgae cells removed will decline sharply when using overdose of chitosan as the excess amino group will cause restabilization of microalgae and decrease the separation efficiency (Rashid et al., 2013).

2.6.5.2 Synthetic Polymer Flocculant

Synthetic polymer flocculants are generally classified into anionic (eg. sodium polyacrylate, polysodium acrylamido methyl sulphonate and polysodium styrene sulphonate), cationic (eg. polyamine, polyquatenaryester and PDDA) and non-ionic (eg. polyacrylaminde and polyethyleneoxide) categories. These polymer flocculants have the characteristics of fast soluble

in aqueous system and is not affected by the pH of medium. They can achieve high efficiency with only little quantities and the floc is bigger and stronger during flocculation (Razali et al., 2011). PDDA is a synthetic cationic polymer and water soluble polyelectrolyte which is widely used as primary flocculant in water treatment. PDDA is also known as polyquaternium-6 that composed of mainly configurational isomers of pyrrolidinium rings and little pendant allylic double bonds. It is virtually linear in structure with repeating of pyrrolidine rings and completely quaterrnized. Due to its high charge density cation, it is well suited for flocculation. Furthermore, as it possesses pH-independent cationic charge, it is widely used as flocculant agent and composite for biosensor (Jaeger et al., 1996; Dautzenberg et al., 1998).

The PDDA has been applied in microalgae flocculation in order to increase the microalgae harvesting efficiency. In the experiment of Wágner et al (2016), the *Chlorella* sp. and *Scenedesmus* sp. was mixed together and the flocculation was carried out by PDDA. The biomass recovery of the microalgae was 92 % with optimal concentration of 27.3 mg PDDA/g algae. In the study of Toh et al. (2018), at 30 mg/L of PDDA for *Chlorella* sp. microalgae flocculation, the biomass recovery up to 98 % can be achieved.

2.6.5.3 Flocculation Mechanism

Flocculation can be categorized into 4 different mechanisms which are charge neutralization, bridging, electrostatic patch and sweeping. Flocculation can be happened in solely or in combination of the mechanism (Vandamme et al., 2013). Charge neutralization happens when the positively charged flocculants are strongly absorbed onto the negatively charged particles by electrostatic forces (electrostatic attraction force), van der Waals forces and dipole-dipole hydrogen bonding (Singh et al., 2000; Tripathy and D,e 2006). Ultimately, the negative surface charge will be cancelled and the surface charge will be neutralized by flocculants, thus flocculation will occur. There will be no electrostatic repulsion between particles anymore and the particles without charge will attach together to form flocs (Vandamme et al., 2013).

Electrostatic patch mechanism takes place when the positively charged flocculants are bind to the negatively charged particles to give a non-uniform distribution of the surface charge (Yukselen and Gregory, 2004, Bolto and Gregory, 2007). The high charge density flocculants will bind to the particles in flat configuration and locally reverse the charge of the particles surface. Hence, patches of positive charge will be formed on the particle surfaces. Flocculation happens when the particles connect with each other through the positively charged patches on the particle surface to form flocs (Bouyer et al. 2001, Yukselen and Gregory, 2004).

In bridging mechanism, the long chain of positively charged flocculants bind to the two different particle surfaces and link the particles by forming a bridge in between (Li et al., 2006; Bolto and Gregory, 2007). Flocs will form when many particles are connected by the bridge. The particles should have sufficient unoccupied surface for the attachment of segments of polymer chains already adsorbed on other particles (Bolto and Gregory, 2007).

Sweeping flocculation is a phenomenon where the particles are entrapped in a large precipitate formed such as metal hydroxide, calcium hydroxide and magnesium hydroxide and eventually causes flocculation to be happened (Li et al., 2006; Ersoy et al., 2009).

2.6.6 Magnetophoretic Separation

Magnetophoresis is a separation technique that plays an important role in biological and chemical engineering. It is a process that the magnetic particles or magnetized particles are moving through a liquid medium by means of external magnetic field (Zborowski and Chalmers, 2015). The ability and efficiency of cell separation is mainly relied on the degree of cells bind paramagnetic material and the capability of paramagnetic materials imparts a paramagnetic dipole moment to target cells (Chalmers et al, 1998). Most of the biological cells are required to be labelled with magnetic particles. This is to obtain the desired contrast in magnetic susceptibility between biological cells and the liquid medium before a magnetophoresis process can be proceeded

(McCloskey et al., 2003). The magnetic particles with size range of 100 nm to few micrometers are commonly used for magnetophoresis. Smaller magnetic particles sizes have larger surface-to-volume ratios. This may cause the separation efficiency to become low since the magnetic force is proportional to the cube of magnetic particles diameter (Lim et al., 2011; Teste et al., 2011).

Zborowski et al. (2002) stated that this separation method provides several advantages such as (1) the used magnetic nanoparticles may be recycled and reused, (2) the process has high throughput, (3) the operational cost is low, (4) the separation efficiency is high, and (5) the implementation and scalability are flexible. Commonly, the magnetic particles that common be used is iron oxide nanoparticles and the binders that widely used in magnetic separation are chitosan and PDDA. The tagging of polyelectrolyte is commonly achieved through attached-to or immobilized-on strategy (Lim et al., 2012). For attached-to strategy, the microalgae cells are being coated with polymer binder and followed by attaching with magnetic particles. In the immobilized-on strategy, the magnetic particles will be surface functionalized by polyelectrolyte binder and bound to microalgae cells directly. The recovery efficiency achieved by attached-to approach is lower than immobilized-on with an equal dosage of particles. It is because the surface functionalized particles in immobilized-on strategy has enhanced distribution and colloidal stability. The naked magnetic particles in the case of attached-to strategy have poor dispersibility and they agglomerate into large clusters due to magnetostatic and Van der Waals force. The immobilized-on strategy is outperformed compared to attached-to strategy and more preferable in

majority studies (Lim et al., 2012).

Based on Lim et al. (2012) research, the efficiency to remove *Chlorella* sp. in cell separation process can reach up to 99 % within 3 minutes by attaching the PDDA surface functionalized iron oxide nanoparticles onto microalgae cells. Toh et al. (2014a) has surface functionalized the iron oxide nanoparticles by two different cationic polyelectrolye which were chitosan and PDDA. The cell separation efficiency of the *Chlorella* sp. promoted by 300 mg/L chitosan and PDDA surface functionalized iron oxide nanoparticles were 99 % and 98 % respectively. However, according to Toh et al. (2015) review, the iron oxide nanoparticles were toxic to *Chlorella* sp. microalgae when in concentration more than 20 mg/L. The suspending iron oxide nanoparticles created indirect light shading effect which blocked the light from entering the cell medium and absorbed by cells and hence retarted the growth of microalgae.

The environmental exposure of nanoparticles is inevitable when it is applied in large scale or industrial scale. The loss of nanoparticles during the process or disposal is unavoidable. The nanoparticles will leak to aquatic, terrestrial and atmospheric environment directly or indirectly (Nowack and Bucheli, 2007). The nanoparticles tend to bring adverse impact towards the environmental organisms and enzymes through their surface and affect the ecosystems (Navarro et al., 2008). They have potential to affect the environment through (1) direct effect on organisms and enzymes species, (2) changes of biological compounds and nutrients upon contact with pollutants,

and (3) structural alteration of non-living environment. Their presence in environment disturbs the carbon and nitrogen cycle of aquatic ecosystem, affects the photosynthetic organisms, generates ROS and alters the biodiversity (Sarkar et al., 2019). Thus, separation of microalgae cells using magnetic nanoparticles by means of magnetophoresis process in large scale application deserves special concern.

2.7 Properties of Silica

Silica has an International Union of Pure and Applied Chemistry (IUPAC) name which is silicon dioxide (SiO_2). It is a chemical compound that formed by the oxidation of silicon. Silica is in negative charge at neutral pH (Coradin, 2011). It has high chemical, mechanical and thermal stability, resistance to microbial attack and low cost (Budnyak et al., 2015). Silica is a very complex material and is abundantly available in the earth's crust. Generally, it exists naturally as several minerals or can be synthesized manually. Silica mostly occurs in nature as quartz and various living organism and it is the major constituent of most of the sand and glass as well. It can be in amorphous form or crystalline form (Bergna, 1994). Mining or purification of quartz is the most common way to obtain silica in nature. Silica can be in many forms such as fumed silica, silica fume, silica sand, precipitated silica and silica gel. These types of silica are commonly produced from different chemical reagents and processes. However, in laboratory scale, silica is synthesized by Stober process. This process starts with hydrolysis of tetraethyl

orthosilicate (TEOS) via the so-called sol-gel process and followed by condensation of silicic acid in alcoholic solution with the presence of aqueous ammonia (Stober and Fink, 1968).

Nowadays, silica is used in many applications which depend on their characteristics and occupies a prominent position in scientific research. Around 95 % of silica produced is applied in construction industrial. Furthermore, it is used as filter in water filtration, precursor of glass and silicon, main element in sand casting, additive in food and pharmaceutical production, building material and very useful in biomedical science. MacNair et al. (1997) used capillaries packed with 1.5 µm non-porous silica for rapid separation and characterisation of protein and peptide mixtures. The silica nanoparticle is generally acted as an additive for rubber and plastic production, strengthening filler for concrete and other construction composites, and a stable and non-toxic platform for biomedical applications (AzoNano, 2013). With a median lethal dose of 5 g/kg, silica is basically non-toxic if ingested orally. Silica is biodegradable in both crystalline and amorphous form (Liberman et al., 2014).

Jeon et al. (2017) studied the effect of synthesized silica and methyl-functionalized silica nanoparticles (around 200 nm) on *Chlorella vulgaris* microalgae growth and FAME production. They culture the microalgae in fermentation flasks without silica, and with 0.2 wt% of silica and methyl-functionalized silica respectively for 10 days. The dry cell weights were found increased by 177 % and 210 % with the presence of silica and methyl-

functionalized silica nanoparticles. Also, the total FAME productions promoted by silica and methyl-functionalized silica nanoparticles were increased to 340 % and 610 % respectively. However, they concluded that these enhancements might be due to the environmental stress caused by nanoparticles. Kang et al. (2014) found that the accumulation and decomposition of lipid can happen simultaneously by oxidative stress caused by titanium dioxide nanoparticles. After incubated the *Chlorella vulgaris* with 0.1 g/L of titanium dioxide nanoparticles for 2 days, the FAME productivity was highest at 18.2 g/L/d.

The surface functionalization of particles has always been carried out to adjust their physical and chemical properties over a wide range. The interaction between particles and cells is mainly dependent on the surface properties. The particles with positive charge will be more efficiently adsorbed onto negatively charged cells compared to negatively charged particles (Wilhelm et al., 2002). Graf et al. (2012) amino-surface functionalized the negatively charged silica (-43 mV) in ethanol by (3-aminopropyl)trimethoxysilane (APS). The surface charged after surface functionalization become highly positive at 64 mV. This is due to the basicity of primary and secondary amino group in APS. The APS-functionalized silica nanoparticles was found interacting well with negatively charged HeLa cells (immortal human cell line). Chitosan is an attractive agent for modification of oxides such as silica and mixed oxides such as silica/titania and silica/alumina. Silica is found negative at pH higher than 2 (Iler, 1979) and chitosan is positive at pH lower than 6.5 (Anal et al., 2008). The interaction is mainly

driven by the amino groups of chitosan and silanol groups of silica (Podust et al., 2014). Liu et al. (2018) developed galactosylated chitosan (deriavative of chitosan)-functionalized mesoporous silica nanoparticles. The silica before surface functionalization was -3.7 mV and increased to 29.9 mV after surface functionalization. The surface functionalized silica has specific electrostatic affinity towards human colon cancer cells. Che et al. (2014) decorated the negatively charged synthesized silica at -65.3 mV by positively charged PDDA. The charged reversal to positive 58.3 mV happened after surface coating with PDDA on the outmost layer of silica. Fleer et al. (2010) investigated that the orientation of the adsorbed polyelectrolyte might be in trains, loops and tails formed. The positive charge of the PDDA-surface functionalized silica enhanced the attachment with negatively charged iron oxide nanoparticles at -6.6 mV (Che et al., 2014).

In this project, the silica microparticles is used as sedimentation aiding agent in microalgae biomass harvesting. Its biodegradability and non-toxic effects make it possible to be direct disposed to the nature environment after the harvesting and lipid extraction process. The used silica can be returned to environment as sand and the residual biomass can act as fertilizer for plants (Maurya et al., 2016).

CHAPTER 3

RESEARCH METHODOLOGY

3.1 Research Flowchart

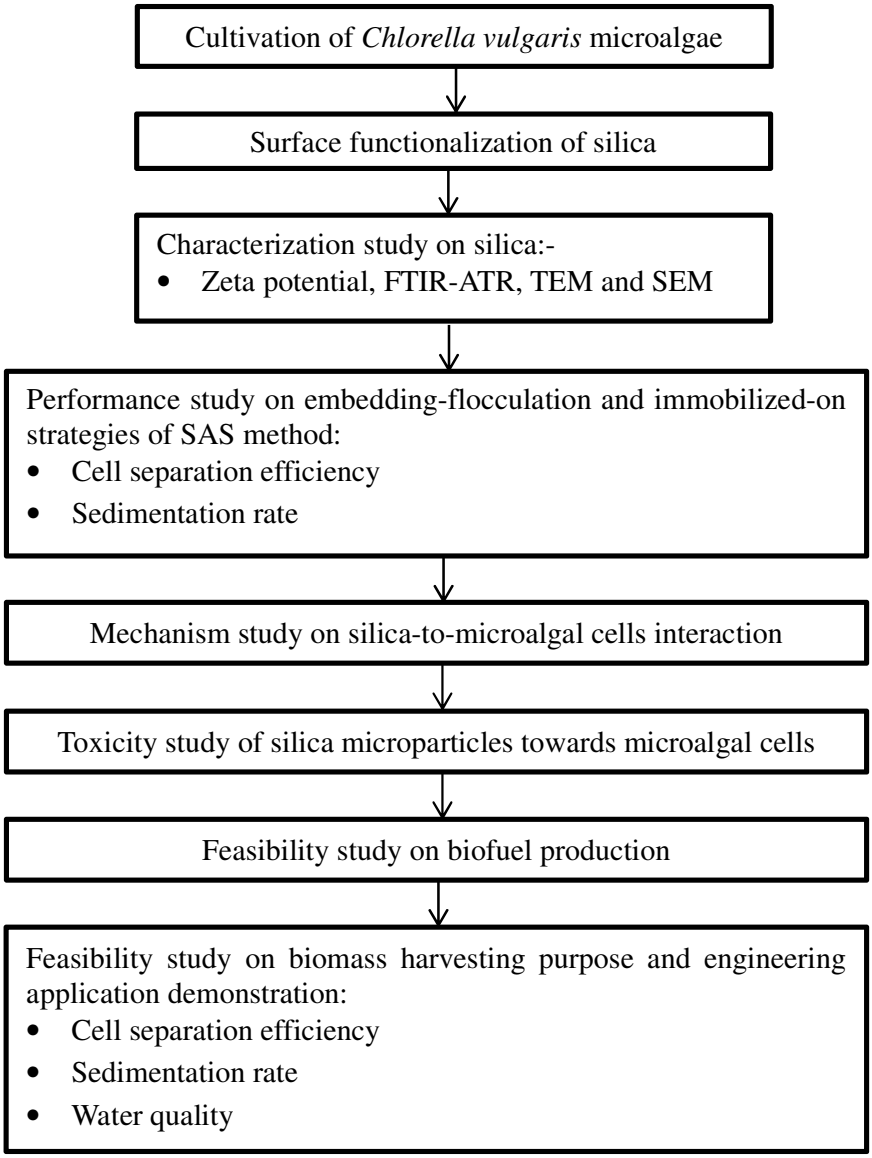

Figure 3.1: Schematic Flowchart of the Experimental Works

3.2 Materials and Chemicals, and Equipment

Table 3.1: List of Materials and Chemicals, and Suppliers/Sources

Materials/Chemicals	Suppliers/Sources
Chlorella vulgaris Microalgae Strain	Culture Collection of Algae and Protozoa (CCAP)
Bold Basal Medium with 3-fold Nitrogen and Vitamins (3N-BBM+V)	Universiti Tunku Abdul Rahman (UTAR)
Chitosan (50,000-190,0000 g/mol)[a]	Sigma-Aldrich
PDDA (100,000-190,000 g/mol)[a]	Sigma-Aldrich
Acetic Acid (99.8 %)[b]	R&M Chemicals
Silica	NovaScientific Resources
Supelco 37 Component FAME Mix	Supelco
Methanol (≥99.9 %)[b]	Sigma-Aldrich
Chloroform (>99-99.4 %)[b]	R&M Chemicals
Hexane (≥99 %)[b]	Sigma-Aldrich
Hydrochloric Acid (37 %)[b]	RCl Labscan
Sulfuric Acid (95-98 %)[b]	Chemiz
Surcose	R&M Chemicals
Protein Assay Bicinchoninate Kit	Nacalai tesque
Albumin Standard	Thermo Scientific
Nitrogen, Ammonia ULR TNTplus Reagent Vial	HACH
Nitrate LR TNTplus Reagent Vial	HACH
Reactive Phosphorus TNT Reagent Vial	HACH
Biochemical Oxygen Demand (BOD) Nutrient Buffer Pillow	HACH
Potassium Hydroxide Pellet (≥85 %)[b]	GENE Chemical
COD TNTplus™ Reagent Set, LR	HACH

[a]average molecular weight

[b]purity

Table 3.2: List of Equipment and Suppliers

Horizontal Laminar Flow Cabinet	ESCO Class II BSC
Autoclave Machine	Hirayama HVE-50
Centrifuge	HERMLE Z326K
Ultrasonic Water Bath	Elma S180H Elmasoic
Ultraviolet-Visible (UV-Vis) spectrophotometer	HACH DR6000
Air pump	Big Boy
Digital Analytical Balance	Sartorius M-pact AX224
Magnetic stirrer plate	2mag MIX15 eco
Magnetic hotplate stirrer	Stuart SB162-3
End-to-end rotator	DLAB MX-RL-E
Zetasizer Nano	Malvern Zetasizer Nano ZS
Fourier Transform Infrared Spectroscopy - Attenuated Total Reflectance (FTIR-ATR)	PerkinElmar UATR – Spectrum Two
Gas Chromatography-Flame Ionization Detector (GC-FID)	SHIMADZU GC-2010 Plus
Transmission Electron Microscopy (TEM)	Phillips CM12
Field Emission Scanning Electron Microscope (FESEM)	JEOL JSM-6701-F
Auto Fine Coater	JEOL JFC-1600
Oven	Memmert UN110
Optical Microscope	Leica ICC50 HD
Microplate reader	BMG LACTECH FLUOstar Omega
BODTrak Incubator	HACH Model 205
BOD Apparatus	HACH BODTrak II
COD Reactor	HACH DRB 200
Portable turbidimeter	HACH 2100Q
Fumehood	IRYAS Laboratory Fumehood

3.3 Cultivation of *Chlorella vulgaris* Microalgae

A total of 250 mL 3N-BBM+V (Appendix A) was added into 500 mL conical flask and covered by cotton and aluminum foil. It was sterilized at 121 ^{o}C for 15 minutes and cooled to room temperature before cultivation. The inoculum size of *Chlorella vulgaris* cell was 1.936×10^5 cells/mL. The cell counting was carried out by using hemocytometer and the cell density was calculated by Equation 3.1 when the grid shown in Figure 3.2 was used. The desired cell density was achieved by dilution using the supernatant of centrifuged medium. The injection of microalgae into 3N-BBM+V was conducted in the horizontal laminar flow cabinet to prevent contamination. The microalgae were cultivated under room temperature with the presence of the continuous light illumination at 2000 lux and the continuous aeration was provided by air pump for the cell medium throughout the cultivation period (14 days). The schematic diagram of microalgae cultivation was shown in Figure 3.3. The wavelength of the *Chlorella vulgaris* microalgae was at 680 nm which was scanned by UV-Vis spectrophotometer.

$$\text{cell density (cells/mL)} = \frac{\text{numbers of cells}}{4 \times 10^{-6}\ \text{mL}} \qquad \text{(Equation 3.1)}$$

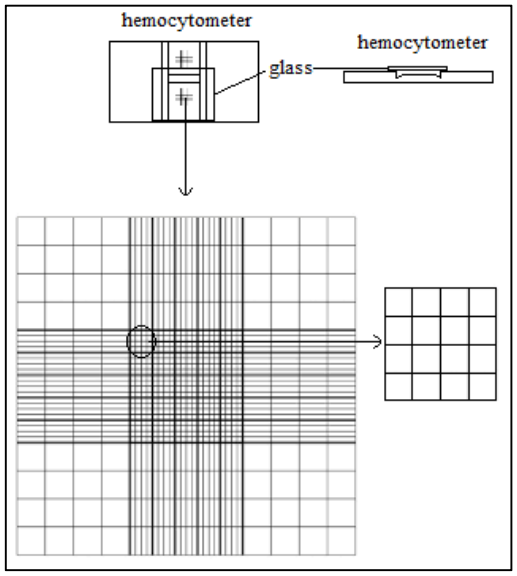

Figure 3.2: Hemocytometer Grid

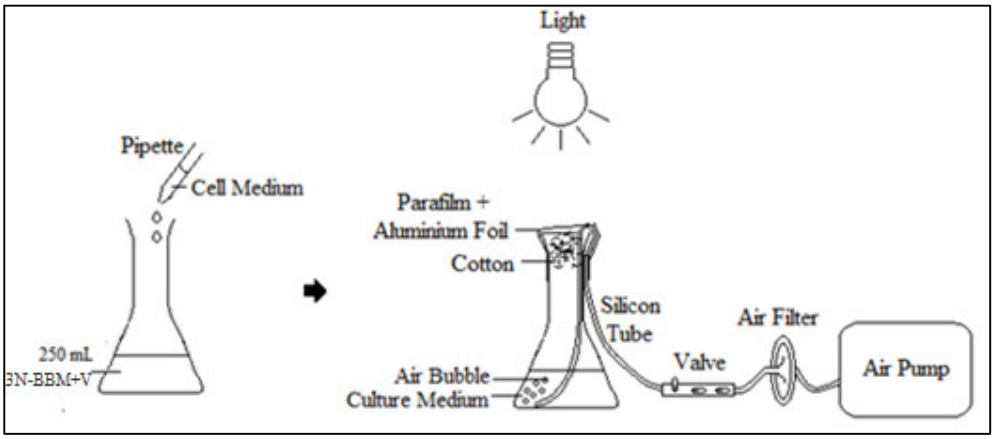

Figure 3.3: Schematic Diagram of Cultivation of *Chlorella vulgaris* Microalgae

3.4 Preparation of Flocculants/Binders

In this project, chitosan and PDDA were being used as flocculants/binders. Both flocculants/binders were prepared in different concentration for embedding-flocculation and immobilized-on strategies.

3.4.1 Preparation of Chitosan Solution

The 99.8 % acetic acid was first diluted to 1 % acetic acid by deionized water. The chitosan solution was prepared by dissolving chitosan powder in 1 % acetic acid. The mixture was stirred overnight to ensure complete dissolution. For embedding-flocculation strategy, the concentration of chitosan solution at 0.96 g/L was prepared and was diluted to desired concentration by deionized water. For immobilized-on strategy, the concentration of chitosan solution at 0.03 g/mL was prepared for surface functionalization of silica.

3.4.2 Preparation of PDDA Solution

The PDDA solution (20 wt % in water, 0.208 g PDDA/mL water) was diluted by deionized water. The diluted PDDA solution was stirred overnight to ensure equilibrium mixing. For embedding-flocculation strategy, the concentration of PDDA solution at 0.96 g/L was prepared and was diluted to desired concentration by deionized water. For immobilized-on strategy the

concentration of PDDA solution at 0.05 g/mL was prepared for surface functionalization of silica.

The concentration of chitosan and PDDA prepared for immobilized-on strategy were 0.03 and 0.05 g/mL respectively. The difference was due to the charge density between chitosan and PDDA. The higher charge chitosan can surface functionalize the silica at lower concentration which was 0.03 g/mL, however, the lower charge PDDA required higher concentration (0.05g/mL) to surface functionalize the silica. At 0.05 g/mL of chitosan, the solution was very viscous which was hard to be stirred due to the higher charge density that increases the viscosity (Bratby, 2016).

3.5 Surface Functionalization of Silica

The silica was surface functionalized by both chitosan solution and PDDA solution prepared respectively. A total of 80 mg silica was dispersed into 5 mL deionized water followed with sonication for 1 hour until a uniform dispersion in concentration of 16 g/L was achieved. A total of 5 mL of 16 g/L silica dispersion was added into 25 mL of 0.03 g/mL chitosan solution and 25 mL of 0.05 g/mL PDDA solution respectively. The mixtures were incubated on an end-to-end rotating mixer overnight to ensure complete surface functionalization. The surface functionalized silica was then separated from the colloidal solution by centrifugation at 4,000 rpm for 5 minutes. The separated surface functionalized silica was washed with deionized water for

three times to remove excessive chitosan solution and PDDA solution. The 80 mg surface functionalized silica was then re-dispersed into 5 mL deionized water and stored at concentration of 16 g/L. The concentration of surface functionalized silica was diluted according to the Table 3.3 in order to achieve respective surface functionalized silica concentration in cell medium after 1 mL surface functionalized silica was added into 15 mL cell medium.

Table 3.3: Concentration of Surface Functionalized Silica

Concentration of Surface Functionalized Silica in Cell Medium (mg/L)	Concentration of Surface Functionalized Silica (g/L)
0	0
200	3.2
400	6.4
600	9.6
800	12.8
1000	16

3.6 Flocculation of *Chlorella vulgaris* Microalgae

For each test, the cell density of the cell medium was set at 3×10^{-7} cells/mL, the desired cell density of the cell medium was achieved with appropriate dilution using the supernatant of centrifuged medium. The cell counting was carried out by using hemocytometer. A total of 15 mL cell medium with desired density was added into a vial followed by adding 1 mL of flocculant prepared with desired concentration (chitosan solution or PDDA solution). The flocculant at 0.96 g/L prepared (in section 3.4.1 and 3.4.2) was

diluted to desired concentration according to Table 3.4 in order to achieve respective flocculant concentration in cell medium after 1 mL flocculant was added into 15 mL cell medium. The sample was stirred at 150 rpm for 20 minutes for flocculation and left for 60 minutes for sedimentation.

Table 3.4: Concentration of Flocculant

Concentration of Flocculant in Cell Medium (mg/L)	Concentration of Flocculant (mg/mL)
0	0
1	0.016
2	0.032
3	0.048
4	0.064
5	0.08
6	0.096
7	0.112
8	0.128
9	0.144
10	0.16
15	0.24
20	0.32
25	0.4
30	0.48

3.7 SAS of *Chlorella vulgaris* Microalgae

In this project, two strategies of SAS were proposed, which were embedding-flocculation and immobilized-on strategies. The main difference of

these two strategies was the silica used. For embedding-flocculation strategy, the bared silica was first added into microalgae cells to ensure complete dispersion and followed by adding flocculant (chitosan solution or PDDA solution) for flocculation. For immobilized-on strategy, the surface functionalized silica was added into microalgae cells directly without adding extra flocculant. For each test, the cell density of the cell medium was set at 3 $\times 10^{-7}$ cell/mL, the desired cell density of the cell medium was achieved with appropriate dilution using the supernatant of centrifuged medium. The cell counting was carried out by hemocytometer.

3.7.1 Embedding-Flocculation Strategy

Embedding-flocculation is a strategy that silica was first added into cell medium for uniform dispersion, once the equilibrium mixture of cell medium and silica was formed, the flocculant (chitosan solution or PDDA solution) was added into the mixture for flocculation. The concentrations of silica and flocculant were prepared according to Table 3.5 and Table 3.6 respectively in order to achieve respective silica and flocculant concentrations in cell medium after 0.5 mL silica and 0.5 mL flocculant were added into 15 mL cell medium. A total of 15 mL of 3 $\times 10^{-7}$ cell/mL cell medium was first added into a vial followed by adding 0.5 mL of 200 mg/L silica and 0.5 mL flocculant with desired concentration according to Table 3.6. The sample was stirred at 150 rpm for 20 minutes for flocculation and left for 60 minutes for sedimentation. The test was repeated for 400, 600, 800 and 1000 mg/L silica.

Table 3.5: Concentration of Silica

Concentration of Silica in Cell Medium (mg/L)	Concentration of Silica (mg/mL)
0	0
200	6.4
400	12.8
600	19.2
800	25.6
1000	32

Table 3.6: Concentration of Flocculant

Concentration of Flocculant in Cell Medium (mg/L)	Concentration of Flocculant (mg/mL)
0	0
1	0.032
2	0.064
3	0.096
4	0.128
5	0.16
6	0.192
7	0.224
8	0.256
9	0.288
10	0.32
15	0.48
20	0.64
25	0.8
30	0.96

3.7.2 Immobilized-on Strategy

For immobilized-on strategy, the chitosan and PDDA surface functionalized silica was added into cell medium directly respectively. A total of 15 mL of 3×10^{-7} cell/mL cell medium was first added into a vial followed by adding 1 mL of surface functionalized silica with desired concentration according to the Table 3.3. The sample was stirred at 150 rpm for 20 minutes for flocculation and left for 60 minutes for sedimentation.

3.8 Determination of Cell Separation Efficiency

Cell separation efficiency is defined as the efficiency of harvesting cells from the sample medium. After 60 minutes of sedimentation, a total of 3.5 mL of sample, that is about 1 mm distance below the liquid surface, was collected and the absorbance intensity (ABS) of the sample collected was measured by UV-Vis spectrophotometer at wavelength of 680 nm. The cell separation efficiency can be calculated by Equation 3.2:

$$\text{cell separation efficiency (\%)} = \frac{\text{ABS}_\text{o} - \text{ABS}}{\text{ABS}_\text{o} - \text{ABS}_\text{centrifuged}} \times 100\ \% \quad \text{(Equation 3.2)}$$

Where ABS_o is absorbance intensity of medium, ABS is absorbance intensity of sample and $\text{ABS}_\text{centrifuged}$ is absorbance intensity of centrifuged medium (clear medium).

3.9 Measurement of Sedimentation Rate

The cell sedimentation rate was determined by measuring the sedimentation distance of the cells in functions of time. The sedimentation distance of the cells was determined quantitatively by measuring the distance between the green cell boundary layer to the water surface as shown in Figure 3.4. The sedimentation duration of cells was recorded by using stopwatch. The sedimentation rate was obtained by dividing the sedimentation distance with sedimentation duration.

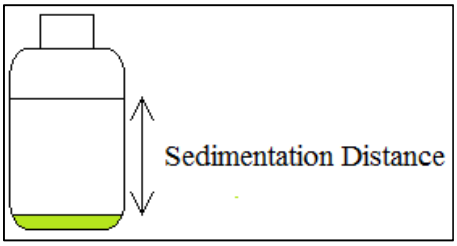

Figure 3.4: Way to Determine the Sedimentation Distance

3.10 Characterization Study

In this project, the bared silica, chitosan, PDDA, surface functionalized silica (by chitosan and PDDA), microalgae cells, flocs formed and environmental lake algae were undergone different characterization studies. The summary of the characterization study was listed in Table 3.7.

Table 3.7: Summary of Characterization Study

Characterization	Silica	Chitosan /PDDA	Surface Functionalized Silica	Microalgae	Cell Flocs	Lake Algae
Zeta Potential Measurement	✓	✓	✓	✓		✓
FTIR-ATR Analysis	✓	✓	✓			
TEM Analysis	✓			✓	✓	
Microscopy Image	✓		✓	✓	✓	
FAME Analysis				✓	✓	

3.10.1 Zeta Potential Measurement

The sample was dispersed in deionized water and injected to Disposable Capillary Cell (DTS1070). The capillary cell with sample was inserted into Malvern Instruments Nanosizer Nano ZS for zeta potential measurement. The zeta potential measurement was conducted in School of Biology Science, Universiti Sains Malaysia (USM).

3.10.2 FTIR-ATR Analysis

The sample was first dried in an oven for 24 hours to ensure moist elimination. The FTIR-ATR spectrum was scanned on a PerkinElmer UATR – Spectrum Two instrument equipped with a diamond crystal iATR reflectance cell with a DTGS detector. The scanning was conducted in the range of 4000 cm^{-1} to 400 cm^{-1} at 4 cm^{-1} spectral resolution to detect the functional groups existed in the sample. The FTIR-ATR analysis was conducted in Faculty of Science (FSc), UTAR.

3.10.3 TEM Analysis

The sample was first dispersed in deionized water and deposited as thin film on a gold mesh grid. The gold mesh grid with sample was then placed in the specimen holder and inserted into Transmission

Electron Microscope Phillips CM12 equipped with Docu Version 3.2 image analysis and operated at 120 kV to observe the particle size. The TEM was carried out in School of Biology Science, USM.

3.10.4 Microscopy Image

The microscopy image was captured under optical microscope equipped with Leica LAS EZ.version 3.4.0 imaging software. The microscopy image was captured in FSc, UTAR.

3.10.5 FAME Analysis

The microalgae cells with density of 9×10^{-7} cell/mL were harvested by centrifugation (physical method) and SAS through embedding-flocculation (chemical method) respectively. For centrifugation method, the microalgae medium was transferred into centrifuge tubes, the tubes were then placed into the centrifuge machine and operated at 2500 rpm for 20 minutes. The harvested microalgae were undergone lipid extraction and transesterification for FAME analysis. The calibration curves of Supelco 37 component FAME mix with different concentration (25, 50 and 100 %) were plotted and used as standard reference (Appendix B). The FAME present in the standard was analyzed by GC-FID equipped with a polar capillary column BPX 70 with dimension of 60 m × 0.25 mm × 0.25 µm. The nitrogen gas with flow rate at 4

cm^2/min was used as carrier gas. Then GC was operated at initial temperature at 155 ^oC. The temperature was elevated at rate of 4 ^oC /min to 220 ^oC on 10 minutes. The injection temperature was 250 ^oC and the split ratio was 80 %. The FAME analysis was conducted in Faculty of Engineering and Green Technology (FEGT), UTAR.

3.10.5.1 Lipid Extraction

The total lipid contents of harvested microalgae biomass by centrifugation and embedding-flocculation were compared. The lipid extraction was conducted to determine the lipid content. The cell biomass was dried and stored to use as seed cells to determine total lipid. The dried biomass was weighed and dispersed in 5.5 mL distilled water in test tube. The dispersion was sonicated for 5 minutes to rupture the cell wall. A total of 6 mL chloroform and 6 mL methanol were added into the same test tube at volume ratio of 5.5:6:6 (distilled water:chloroform:methanol). The sample was maintained at 60 ^oC. and stirred overnight. Then, the sample was centrifuged at 3000 rpm for 2 minutes, two layers of solutions were formed. The bottom layer that contained lipid and chloroform was collected and transferred to a pre-weighed vial and followed by evaporation until dry. The dried lipid was weighed. The lipid yield was calculated by Equation 3.3 (Bligh and Dyer 1959).

$$\text{Lipid yield (wt \%)} = \frac{\text{weight of dry lipid (g)}}{\text{weight of dry biomass (g)}} \times 100\ \% \qquad \text{(Equation 3.3)}$$

3.10.5.2 Transesterification

After the dried lipid was weighed, the transesterification process was performed to obtain the fatty acid profile analysis. A total of 2 mL hexane was added into the dried lipid and the mixture was slightly sonicated to dissolve the lipid. Then a total of 3 mL hexane, 4.25 mL methanol and 215 µL hydrochloric acid (HCl) were added into the dissolved lipid at volume ratio of 5:4.25:0.215 (hexane:methanol:HCl). The mixture was stirred for 2 hours at 85 ^{o}C then cooled to room temperature and followed by centrifugation at speed of 3000 rpm for 5 minutes, two layers of solutions were formed. The upper layer contained FAME and hexane was collected (Toh et al., 2014c). The composition of FAME present in the sample was analyzed by GC-FID equipped with a polar capillary column BPX 70 with dimension of 60 m × 0.25 mm × 0.25 µm, the specification of the process was same as FAME analysis for standard.

3.11 Toxicity Test

A total of 300 mL pre-cultivated cell medium stock at 1.5 × 10^6 cells/mL was added into 500 mL conical flask followed by adding 0.1, 1, 10, 100, 1000 mg/L silica respectively. For control sample, no silica was added. The microalgae were cultivated under room temperature with the presence of

the continuous light illumination at 2000 lux and the continuous aeration was provided by air pump for the cell medium throughout the cultivation period (7 days). Each flask was shaken twice a day during the cultivation period to resuspend settled particles. The toxicity of silica towards the growth and biochemical composition Chl*orella vulgaris* was investigated. At day 7th, microalgae biomass was collected by centrifugation at speed of 2500 rpm for 20 minutes. The microalgae biomass was then dried and stored to use as seed cells. It was then divided into 3 portions for biochemical composition test (total lipid, protein and carbohydrate composition) (Toh et al., 2015).

3.11.1 Growth Test

The cell density of each cell medium was determined every day until day 7th to determine the effect of silica towards the growth of cells. The cell counting was carried out by using hemocytometer. The cell density was calculated by Equation 3.1.

3.11.2 Total Lipid Content

The lipid yield was calculated by Equation 3.3. The lipid extraction was conducted as in section 3.10.6.1 to determine the total lipid.

3.11.3 Preparation of Microalgae Supernatant

The carbohydrate and protein were first extracted from biomass through water extraction method (Barbarino and Lourenco, 2005). The dried biomass was weighed and dispersed in 4 mL distilled water. The dispersion was sonicated for 5 minutes to rupture the cell wall and followed by centrifugation at 2500 rpm for 5 minutes. The cell debris was disposed and the supernatant was collected for the measurement of carbohydrate and protein composition (Toh et al., 2015).

3.11.4 Carbohydrate Content

The carbohydrate content was determined by sulfuric acid-UV method. For sulfuric acid-UV method, a total of 1 mL supernatant was added into test tube and followed by adding 3 mL concentrated sulfuric acid. The temperature of the mixture increased rapidly after the addition of sulfuric acid. The mixture was then stirred for 30 seconds and cooled with ice for 2 minutes to reduce the temperature of the mixture to room temperature. Then, the ABS was measured by UV-Vis spectrophotometer at wavelength of 315 nm. The carbohydrate concentration was determined through the calibration curve with ABS against sucrose concentration constructed (Appendix C). The carbohydrate yield was calculated by Equation 3.4 (Albalasmeh et al., 2013).

$$\text{carbohydrate yield (wt \%)} = \frac{\text{carbohydrates conc. (g/L)}}{\text{biomass conc. (g/L)}} \times 100\ \% \qquad \text{(Equation 3.4)}$$

3.11.5 Protein Content

The protein content was measured by Bicinchoninic Acid (BCA) protein assay with 96 well microplate. The working solution with 10 mL BCA solution and 200 µL copper sulphate solution (ratio 50:1) was prepared. A total of 25 µL supernatant was pipetted into each well and followed by 200 µL working solution and mixed well. The 96 well microplate was sealed and incubated at 37 ^{o}C for 30 minutes. Then, the 96 well microplate was left to room temperature and the ABS of the samples were measured by microplate reader at wavelength of 562 nm. The protein concentration was determined through the calibration curve with ABS against dilute bovine albumin solution concentration constructed (Appendix D). The protein yield was calculated by Equation 3.5.

$$\text{protein yield (wt \%)} = \frac{\text{protein concentration (g/L)}}{\text{biomass concentration (g/L)}} \times 100\ \% \qquad \text{(Equation 3.5)}$$

3.12 Application of SAS through Embedding-Flocculation Strategy on Fishpond Water

The fishpond water sample was collected from a fishpond in Temoh, Perak, Malaysia as shown in Figure 3.5. The wavelength of the water sample was at 300 nm which was scanned by UV-Vis spectrophotometer.

Figure 3.5: Fishpond Water in Temoh, Perak (4.259486, 101.189674)

3.12.1 Determination of Cell Separation Efficiency and Sedimentation Rate

The flocculation of water sample was carried out with and without the presence of silica. For flocculation without adding silica, a total of 15 mL water sample was added into a vial followed by adding 1 mL of chitosan prepared with desired concentration. The chitosan at 0.96 mg/mL prepared (in section 3.4.1) was diluted to desired concentration according to Table 3.4 in order to achieve respective chitosan concentration in cell medium after 1 mL flocculant was added into 15 mL cell medium. The sample was stirred at 150

rpm for 20 minutes for flocculation and left for 60 minutes for sedimentation. For embedding-flocculation strategy, a total of 15 mL water sample was first added into a vial followed by adding 0.5 mL of 1 g/L silica and 0.5 mL chitosan with desired concentration according to Table 3.6 (concentration of chitosan in cell medium from 1 to 10 mg/L). The sample was stirred at 150 rpm for 20 minutes for flocculation and left for 60 minutes for sedimentation. After 60 minutes of sedimentation, a total of 3.5 mL of sample, that is about 1 mm distance below the liquid surface, was collected and the ABS of the sample collected was measured by UV-Vis spectrophotometer at wavelength of 300 nm. The cell separation efficiency can be calculated by the Equation 3.2. The cell sedimentation rate was determined by method in section 3.9.

3.12.2 Water Quality

The fishpond water was treated by centrifugation and SAS through embedding-flocculation respectively. For centrifugation method, the fishpond water was transferred into centrifuge tubes, the tubes were then placed into the centrifuge machine and operated at 2500 rpm for 20 minutes. The water quality of the treated and untreated fishpond water samples in terms of ammoniacal nitrogen level, nitrate level, ortho-phosphate level, turbidity, BOD, COD and total suspended solid (TSS) were measured.

3.12.2.1 Determination of Ammoniacal Nitrogen Level

HACH Method 10205 was used. The content of ammoniacal nitrogen was measured by using Nitrogen, Ammonia ULR TNTplus Reagent Set purchased from HACH. The lid of the DosiCap™ Zip cap was removed and the cap of the test vial that contained Nitrogen, Ammonia ULR TNTplus Reagent was removed. A total of 5 mL sample was added into the test vial and the DosiCap™ Zip cap was turned over immediately and was tightened on the test vial. The test vial was shaken until the reagent in the cap was dissolved and was left for 15 minutes for reaction. Lastly, the test vial was inserted into the UV-Vis spectrophotometer to determine the ammoniacal nitrogen level.

3.12.2.2 Determination of Nitrate Level

HACH Method 10206 was used. The content of nitrate was measured by using Nitrate LR TNTplus Reagent Set purchased from HACH. A total of 1 mL sample was first added into the test vial that contained Nitrate LR TNTplus Reagent and followed by adding 0.2 mL TNTplus Solution A. The cap was tightened on the test vial and the test vial was inverted until completely mixed. Lastly, the test vial was inserted into the UV-Vis spectrophotometer to determine the nitrate level.

3.12.2.3 Determination of Ortho-Phosphate Level

HACH Method 8048 was used. The content of ortho-phosphate was measured by using Reactive Phosphorus TNT Reagent Set purchased from HACH. Program 535 P React. PV TNT was started in the UV-Vis spectrophotometer. Then, 5 mL of the sample was added into the Reactive Phosphorus Test 'N Tube Vial. The cap was tightened on the test vial and the test vial was inverted to mix. The test vial was then inserted into the 16-mm cell holder in UV-Vis spectrophotometer to set zero. After that, the content of PhosVer 3 Phosphate Powder Pillow provided was added into the same test vial. The test vial was shaken for 20 seconds and left for 2 minutes for reaction. Lastly, the vial was inserted back into the 16-mm cell holder in UV-Vis spectrophotometer to determine the ortho-phosphate level.

3.12.2.4 Determination of Turbidity

A total 15 mL of sample was added into the sample bottle provided. Then, the sample bottle was inserted into the portable Turbidimeter and the turbidity was measured.

3.12.2.5 Determination of BOD

Measurement of BOD was done by using BODTrak II apparatus. BOD range from 0 to 70 mg/L was chosen and 355 mL of sample is added into BODTrak II bottle. The content of nutrient buffer pillow was then added into the same BODTrak II bottle. A BODTrak II stir bar was put into the BODTrak II bottle and a seal cup was put into the neck of the bottle. After that, two potassium hydroxide pellets was added into the seal cup. Then, the BODTrak II bottle was put into the BODTrak chassis and the applicable tube was connected. The incubator temperature was set to 20 ^{o}C. Lastly, the test range was set and the test was allowed to run for 7 days before the result was obtained.

3.12.2.6 Determination of COD

HACH Method 8000 was used. The COD level was measured by using COD TNTplus™ Reagent Set, LR purchased from HACH. The DRB200 reactor was on and the temperature was set to 150 ^{o}C. The test vial was inverted several times to mix. A total of 2 mL sample was added to the test vial. The cap was tightened on the test vial and the test vial was inverted to mix. The test vial was inserted in the preheated COD reactor and the lid was closed. The test vial was then kept in the reactor for 2 hours. After 2 hours, the reactor was off to decrease the temperature until 120 ^{o}C. The test vial was inverted gently several times while the test vial was still hot and was left until

the temperature of test vial decreased to room temperature. Lastly, the test vial was inserted into the UV-Vis spectrophotometer to determine the COD level.

3.12.2.7 Determination of TSS

APHA method 3120 (American Public Health Association, Standard Methods for examination of water and wastewater) was used. The samples were tested by KenEp Laboratories (M) Sdn. Bhd, Ipoh, Perak.

3.13 ANOVA Analysis

Statistical analysis by one-way analysis of variance (ANOVA) was carried out to verify the homogeneity of variances among the tested samples. The letters (A, AB, B, BC, C and so on) group the factor levels. Groups that do not share a letter have a mean difference that was statistically significant. Besides that, groups that appear with different letters (AB, BC, CD and so on) were ranged in between the two different letters.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Determination of Optimum Dosage of Flocculants for *Chlorella vulgaris* Microalgae Harvesting

In this study, two flocculants, chitosan and PDDA, were used to promote the cell separation and enhance the sedimentation rate of *Chlorella vulgaris* microalgae respectively by the means of gravity sedimentation. The optimum dosages of flocculants for separation of microalgae cells were studied respectively. A control set (without the presence of flocculants) was performed as a reference. The results in Figure 4.1 showed that the cell separation efficiency of the control sets were low, which around 20 % were obtained. The trends of cell separation efficiencies promoted by both flocculants were similar, which they have lower separation efficiencies with insufficient or surplus of flocculants. In both case, the cell separation efficiencies kept increasing from around 20 % (without the presence of flocculants) to optimum which were 100 % and 99.34 ± 0.61 % at 7 mg/L of chitosan and PDDA dosage respectively. When the concentration of flocculants supplied were further increased to 30 mg/L, the cell separation efficiency started to drop significantly to 41.47 ± 0.58 % in the case of chitosan and decline slightly to 86.58 ± 0.44 % in the case of PDDA.

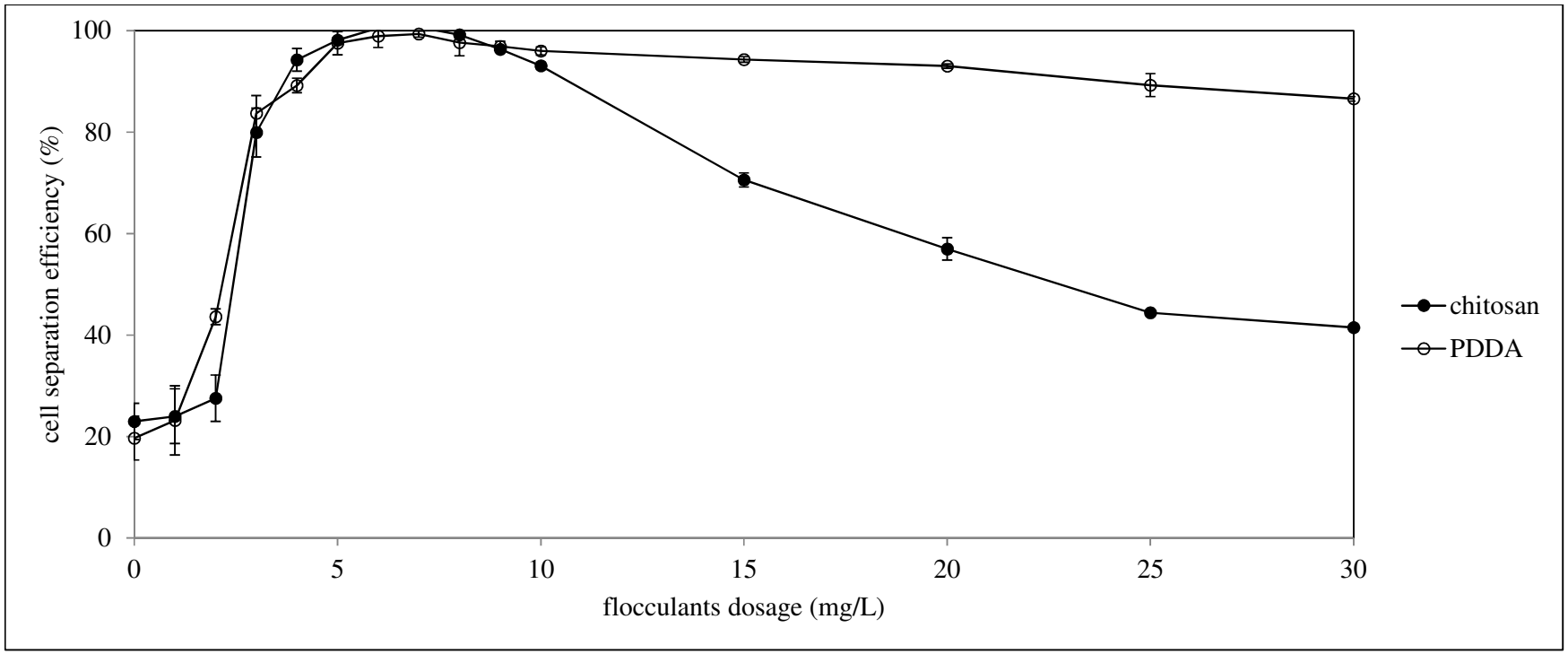

Figure 4.1: The Cell Separation Efficiency of *Chlorella vulgaris* Microalgae Promoted by Chitosan and PDDA at 3×10^{-7} cell/mL Cell Density

The measurement of zeta potential indicated that the *Chlorella vulgaris* microalgae carried a negative charge at -28.73 ± 0.64 mV. Coulumb's Law (Equation 4.1) states that the repulsion force will interact between two surfaces that carry similar charge while attractive force will interact between two surfaces with opposite charge (Coulomb, 1785).

$$F = k_e \frac{q_1 q_2}{d^2} \qquad \text{(Equation 4.1)}$$

Where k_e is Coulomb's constant, q_1 and q_2 are signed magnitude of charges, and d is distance between charges. Hence, the mutual charge between microalgae cells will repel with each other and remain dispersing in water medium. Electrostatic repulsion among the negatively charged cells hindered the formation of cell agglomeration. This in turn brought weak cell separation efficiency at only around 20 %. As shown in Figure 4.2(a), there were no flocs formed when the flocculant was absent.

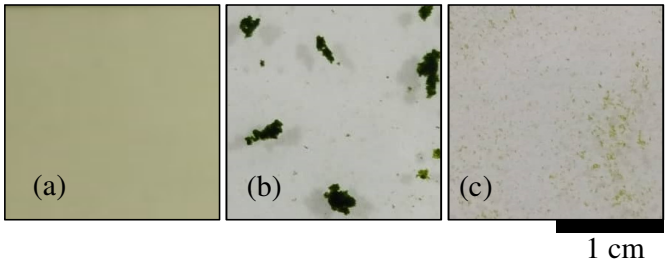

Figure 4.2: The Comparison of (a) size of *Chlorella vulgaris* Microalgae, Floc Size of *Chlorella vulgaris* Microalgae Promoted by (b) Chitosan and (c) PDDA at Optimum Dosage of 7 mg/L Respectively

Both chitosan and PDDA carried a positive charge at 70.20 ± 1.80 mV and 47.67 ± 2.54 mV respectively on their surfaces (-+NH_3 group on each

chitosan monomer and $+N(CH_3)_2$ group on each PDDA monomer) (Kokufuta and Takahashi,1986; Li et al., 2008). According to Coulumb's Law, the attractive force will interact between two surfaces with opposite charges (Coulomb, 1785). Hence, the positively charged flocculants, chitosan and PDDA, were possible to promote an effective flocculation with negatively charged microalgae cells respectively. Based on review, the electrostatic attraction force is proven to be dominant in freshwater compared to van der Waals force and Lewis acid-base interaction (Toh et al., 2014b). The flocculation of microalgae in freshwater condition promotes the effective attachment between flocculants and microalgae cells due to the electrostatic attraction force and achieves high cell separation efficiency up to 99 %.

When the flocculant dosage was insufficient, the flocculant polymer chain was not enough to attach to and fully cover the entire cell for the charge neutralization to be happened as the quantity of free positively charged functional group of polymer was low. The resultant surface charge of the cell flocs below the optimum dosage of flocculant will maintain at net negative charge (Tan et al., 2019). The electrostatic repulsion force between the microalgae cells maintains the colloidal stability of microalgae cells in suspension and inhibits the effective flocculation (Roussy et al, 2005; Ahmad et al, 2011). With increasing flocculant dosage, the cell separation efficiency will increase until reaching maximum. Therefore, with insufficient flocculant dosage (less than 7 mg/L of chitosan and PDDA), the cell separation efficiency will be lower than the optimum cell separation efficiency.

At optimum dosage of chitosan and PDDA (7 mg/L), the cell separation efficiencies up to 100 % and 99.34 ± 0.61 % respectively were achieved. There are two possible bridging flocculation mechanisms as displayed in Figure 4.3. Bridging mechanism A is induced by a long polymer chain that are attached to few cells and connected to another cell in another flocs, whereas bridging mechanism B is induced by multiple polymer chains that are connected to many cells, the bridge formed between two chains is weak and can be destroyed during stirring (Yeap et al., 2012). The study of Toh et al. (2018) showed that the surface charge of microalgae cell flocs formed by chitosan flocculation was nearly neutralized whereas the flocculation with PDDA showed no significant charge neutralization effect at optimum dosage. This indirectly proved that, with optimum dosage of chitosan and PDDA, the flocculations were mainly promoted by bridging mechanism instead of charge neutralization. In this project, for chitosan, the flocculation was mainly driven by bridging mechanism A, and for PDDA, the flocculation was dominantly attributed to bridging mechanism B. This can be proven by the flocs formed by chitosan and PDDA flocculation as shown in Figure 4.2(b) and 4.2(c). Obviously, the flocs size of chitosan-formed flocs was bigger and denser than that of PDDA-formed flocs. Bridging mechanism A is the key mechanism to promote effective flocculation as flocs formed by bridging mechanism B are usually less stable as the bridge formed can be easily broken by shear force (Yeap et al., 2012). Therefore, the bridging mechanism B that prevailed in PDDA flocculation generated flocs smaller than chitosan flocculation which induced by bridging mechanism A.

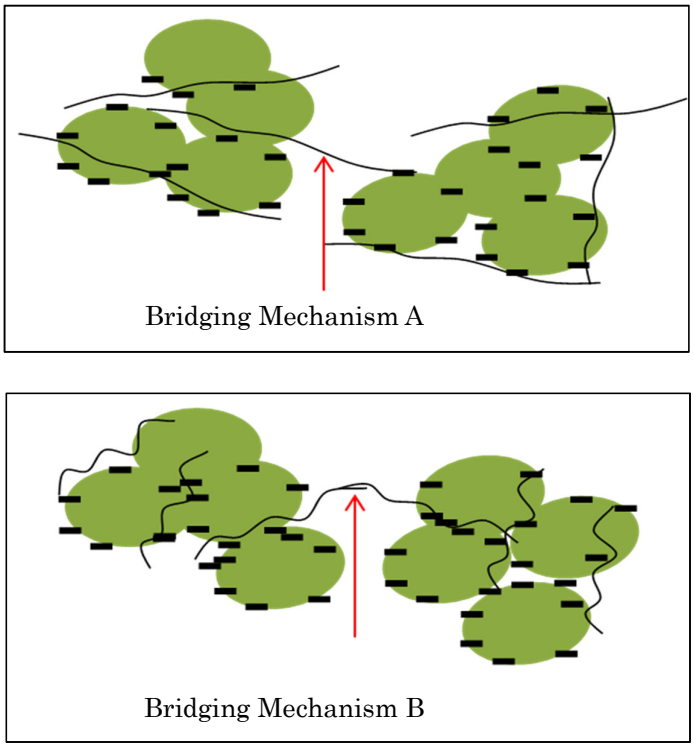

Figure 4.3: Schematic Diagrams Illustrates Bridging Mechanism A and B

When the flocculant dosages were slightly more than the optimum dosage, charge neutralization happened in chitosan flocculation (Tan et al., 2019), while PDDA flocculation was still driven by bridging mechanism B. The cell surface charge was being neutralized after attached with sufficient amount of chitosan and neutralized patches were formed on cell surfaces thus flocs are formed (Low and Lau, 2016). For PDDA flocculation, there was still bridging mechanism B at this dosage of PDDA, as the PDDA has lower charge density compared to chitosan, more positively charged functional group of PDDA was needed for neutralization. However, the cell separation efficiency at this PDDA dosage was lower compared to that of at optimum dosage of PDDA. This was generally due to the hindrance of steric repulsion force between the extended tails and loops protruding away from PDDA-

covered cells (Toh et al., 2018). As a result, lower separation efficiency was observed when excessive dosage of PDDA was applied for flocculation.

Figure 4.1 showed that, when the flocculant dosage was further increased, the cell separation efficiency of microalgae promoted by chitosan dropped significantly while for PDDA the cell separation efficiency was reduced gradually. This means that the chitosan-covered microalgae cells were colloidally more stable than PDDA-covered microalgae cells. When the chitosan dosage supplied was beyond the optimum dosage, the net surface charge goes beyond the neutral point. The microalgae cells tend to be colloidally restabilized in the suspension due to electrostatic repulsion force and steric repulsion, which is known as electrosteric stabilization (Fritz et al., 2002). When the flocculant was oversupplied, the cell flocs will carry a net positive charge on their surface. This proved that the excessive flocculants will fully cover and form a polymer layer on the microalgae cell surface. The extended tails and loops protruding away from the positively charged chitosan-covered cells tended to inhibit the cell flocculation and form a stable suspension. In the case of PDDA, the polymer chains that extended out from the cell surfaces will hinder the attachment with each other due to the electrostatic repulsion force and form a stable colloid in suspension. This increases the difficulty of cell agglomeration to form larger cell flocs. However, the bridging mechanism B still prevailed in PDDA flocculation when the PDDA was oversupplied till 30 mg/L. When microalgae cells were attached with PDDA, another side of chain will extend out from cell surface whereby the extended chains tend to bridge with other to promote flocculation

(Low and Lau, 2016). In short, the dosage of flocculants should be adjusted properly in order to promote the effective flocculation and cell separation.

As shown in Figure 4.1, the performances of both flocculants in term of cell separation efficiencies were above 99 % at optimum flocculant dosages. However, the flocs formed by chitosan flocculation were much bigger compared to that of PDDA flocculation (Figure 4.2). This in turn caused the sedimentation rate promoted by chitosan and PDDA to be extremely different. Figure 4.4 depicted that the sedimentation rate of cell flocs formed by chitosan was 56.54 cm/h, which were 17 and 471 times faster than that of PDDA (3.18 cm/h) and control cell sample (0.12 cm/h) respectively. The sedimentation rate is extremely slow when the densities between two medium is small or the particle size is small (Milledge and Heaven, 2013). This is proven by Stokes' Law which states that settling velocity is proportional to square root of the radius of cells and difference in density between particle and the water medium (Reynolds, 1984). Stokes' Law proves that the sedimentation rate increases with the increasing of particle size. Hence, the flocculation induced by chitosan that formed bigger flocs than PDDA, was more time effective.

In this study, the bridging mechanism exhibited by chitosan in flocculation was found more favourable for colloidal destabilization and cell agglomeration, and enhancement of the performance of flocculation of microalgae cells in terms of cell separation efficiency and sedimentation rate.

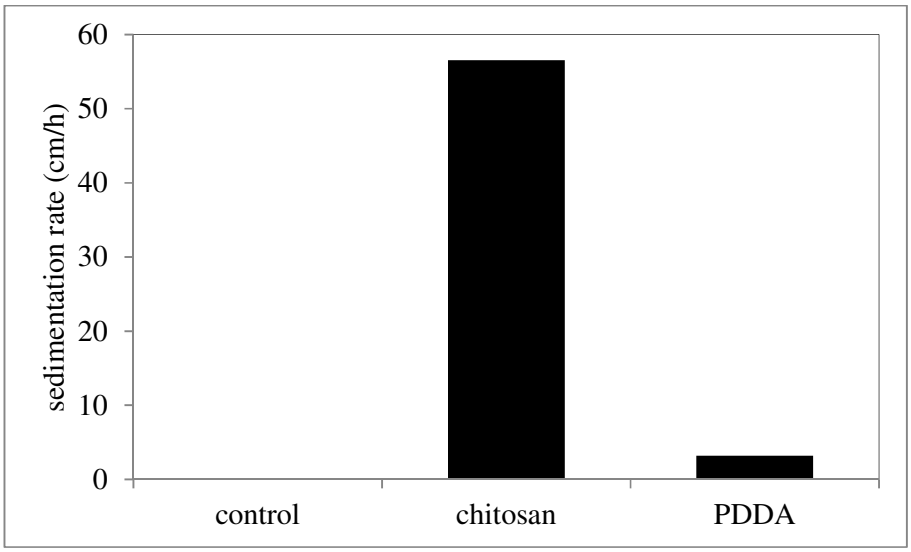

Figure 4.4: Sedimentation Rate of Control Cell and Cell Flocs Promoted by Chitosan and PDDA at Optimum Dosage of 7 mg/L Respectively

4.2 Performance of *Chlorella vulgaris* Microalgae Separation through the Method of SAS

In this study, the method of SAS was carried out in two different strategies including embedding-flocculation and immobilized-on strategies. The cell separation efficiency and sedimentation rate of the SAS method on *Chlorella vulgaris* microalgae were being studied and compared.

4.2.1 Embedding-Flocculation Strategy

The measurement of zeta potential indicated that the surface charge of silica was in negative charge at -33.97 ± 1.91 mV which was same with that of the microalgae cells with negative charge at -28.73 ± 0.64 mV. According to Coulomb's Law, silica and microalgae cells will induce the repulsion force

with each other and the cells cannot attach onto the silica surface effectively (Coulomb, 1785). When positively charged chitosan and PDDA at 70.20 ± 1.80 mV and 47.67 ± 2.54 mV were added into the suspension with negatively charged microalgae cells and silica respectively, the positive charge on chitosan and PDDA surface will promote effective attachment with negatively charged microalgae cells and silica through electrostatic attraction force.

For embedding-flocculation strategy, different concentration of silica at 200, 400, 600, 800 and 1000 mg/L were added into *Chlorella vulgaris* microalgae and followed by flocculants, chitosan and PDDA, respectively and the performances in terms of cell separation efficiency and sedimentation rate were studied. The results in Figure 4.5 and Figure 4.6 showed that the trends of the cell separation efficiencies for different concentration of silica were similar, which they have lower separation efficiencies with insufficient or surplus of flocculants. When the chitosan dosages were beyond the optimum point, the cell separation efficiency has a sharp drop. While for the PDDA, the cell separation efficiency decreased steadily when the PDDA dosages were excessive. This once again proved that the chitosan-covered silica and cells was colloidally more stable than PDDA-covered silica and cells as discussed in Section 4.1.

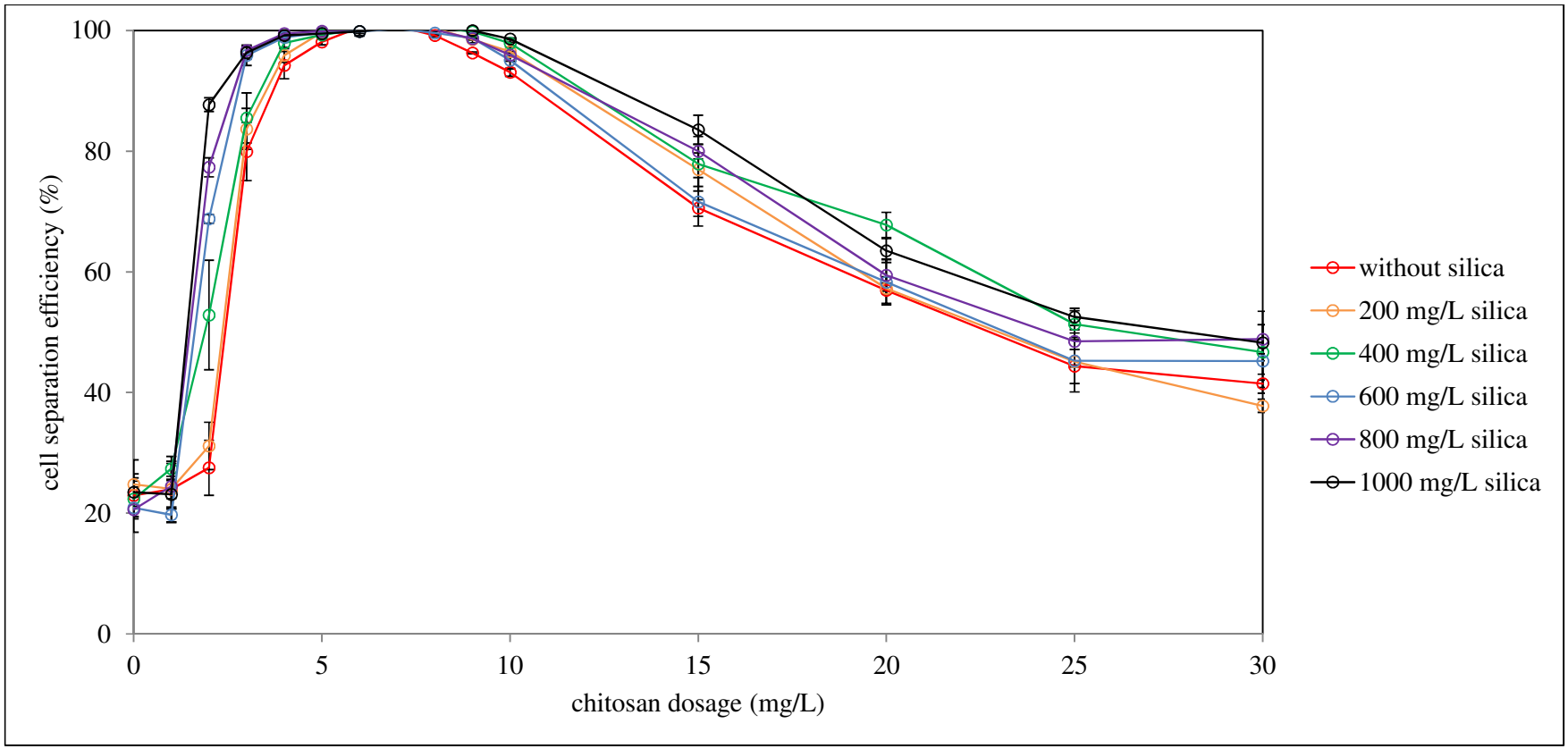

Figure 4.5: The Cell Separation Efficiency of *Chlorella vulgaris* Microalgae Promoted by Chitosan at 3×10^{-7} cell/mL Cell Density through SAS Method

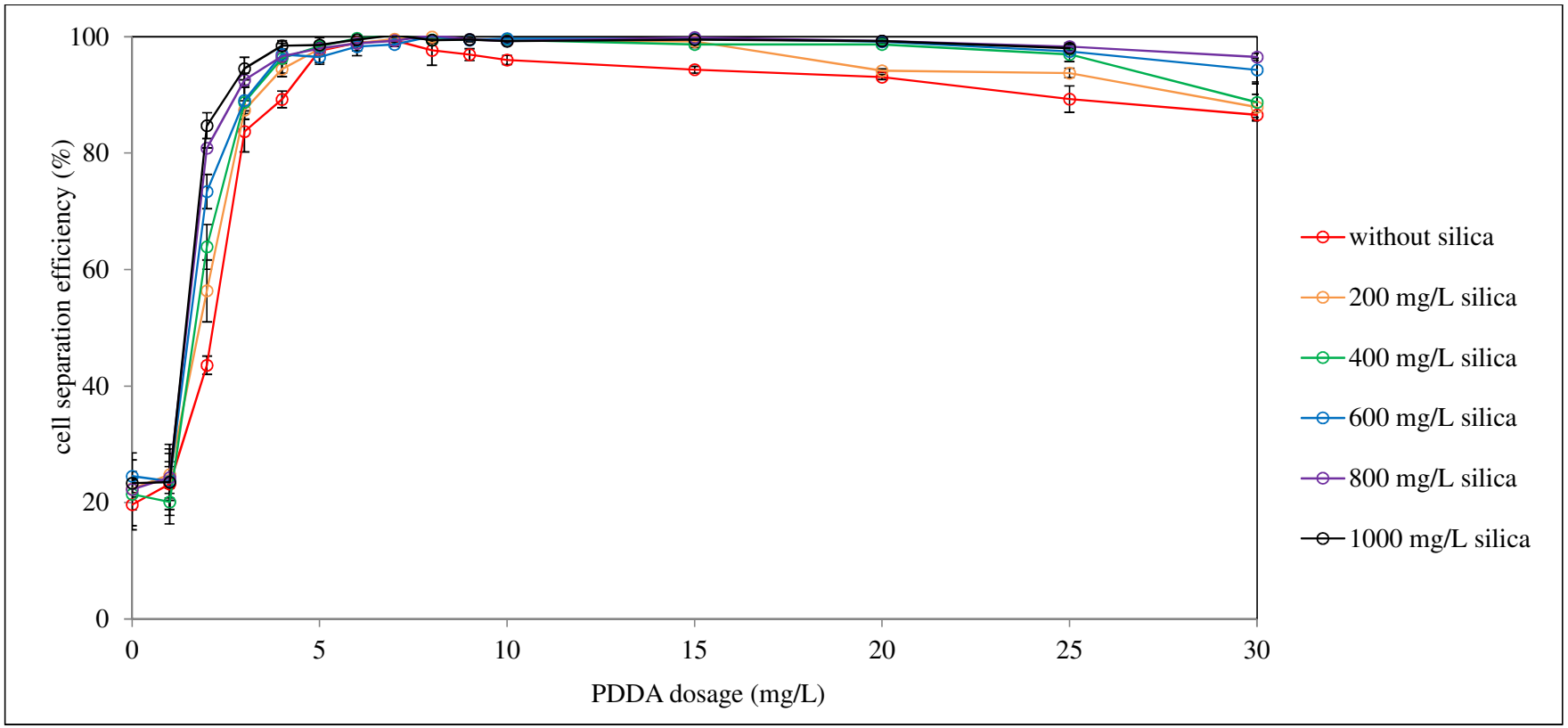

Figure 4.6: The Cell Separation Efficiency of *Chlorella vulgaris* Microalgae Promoted by PDDA at 3×10^{-7} cell/mL Cell Density through SAS Method

The optimum chitosan dosages for 0, 200, 400, 600 and 800 mg silica were same, which were 7 mg/L, while when the silica concentration increased to 1000 mg/L, the optimum chitosan dosage became 8 mg/L. In the case of PDDA, the optimum PDDA dosages range between 7-8 mg/L, which were 7 mg/L for silica concentration at 0, 400, and 1000 mg/L, and 8 mg/L for silica concentration at 200, 600 and 800 mg/L. This verified that the addition of silica particles did not affect much on the optimum flocculant dosage to achieve the highest cell separation efficiency.

At flocculant dosage below 7 or 8 mg/L (optimum dosage), the polymer chains was not enough to attach onto the surface of microalgae cells and silica. At these dosages, the resultant surface charge of the cells and silica maintained at net negative charges (Tan et al., 2019). This means that the quantity of free positively charged functional group of polymer has to be increased. The electrostatic repulsion force between the net negatively charged microalgae cells and silica inhibited the effective flocculation and lead to ineffective electrostatic patch destabilization (Roussy et al., 2005; Ahmad et al., 2011). Therefore, low cell separation efficiencies were promoted due to the failure in producing large and denser flocs (Ahmad et al, 2011).

At optimum dosages of chitosan and PDDA, the cell separation efficiencies above 99 % were achieved. This finding was same with the finding mentioned in Section 4.1 which once again proved that bridging mechanism is dominating at optimum chitosan and PDDA dosage to promote effective flocculation. The chitosan flocculation was mainly driven by

bridging mechanism A and PDDA flocculation was dominantly attributed to bridging mechanism B which was sensitive to shear force (Yeap et al., 2012). This can be proven by the silica-cell flocs formed by chitosan and PDDA flocculation as shown in Figure 4.7 and 4.8 respectively. At particular silica dosaged (0, 200, 400, 600, 800 and 1000 mg/L), the silica-cell flocs formed by chitosan flocculation was much bigger compared to PDDA flocculation.

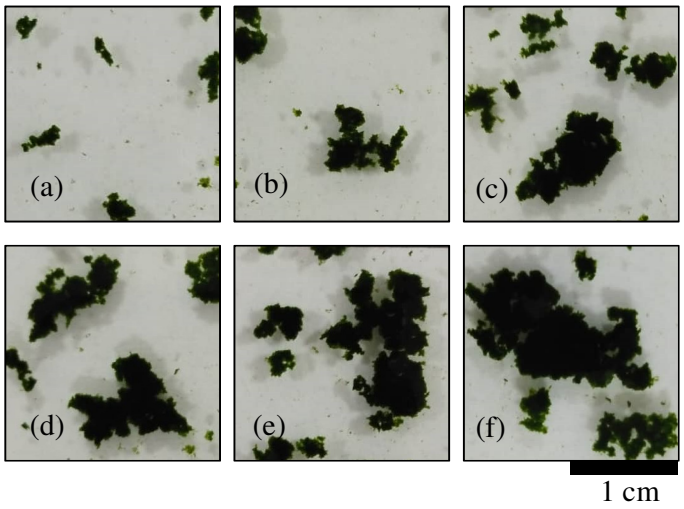

Figure 4.7: The Comparison of (a) Cell Floc Size without Silica, and Silica-Cell Flocs with Silica Dosage at (b) 200 mg/L, (c) 400 mg/L, (d) 600 mg/L, (e) 800 mg/L and (f) 1000 mg/L at Optimum Dosage of Chitosan Respectively

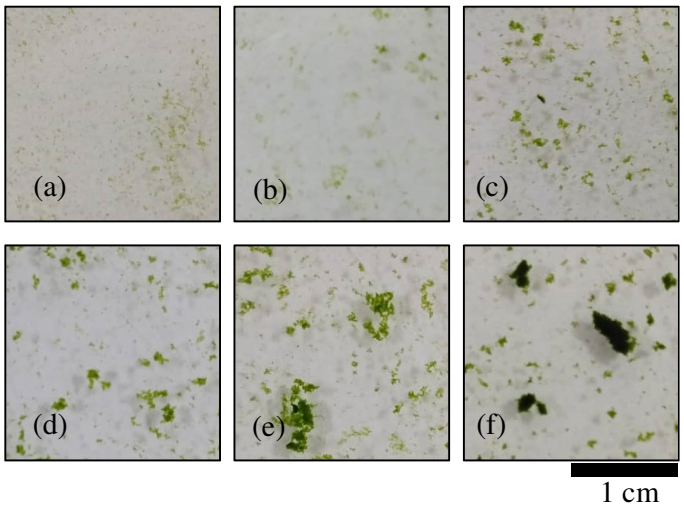

Figure 4.8: The Comparison of (a) Cell Floc Size without Silica, and Silica-Cell Flocs with Silica Dosage at (b) 200 mg/L, (c) 400 mg/L, (d) 600 mg/L, (e) 800 mg/L and (f) 1000 mg/L at Optimum Dosage of PDDA Respectively

As discussed in Section 4.1, a sharp drop was observed when the chitosan dosage was beyond the optimum point and the slight drop happened when the PDDA was oversupplied. When the dosage of chitosan was more than optimum point, the net surface charge of silica and cell turned into positive as there was abundance positively charged chitosan (Low and Lau, 2016; Tan et al., 2019). Hence, the silica and cells tend to be colloidally restabilized due electrosteric stabilization (Fritz et al., 2002). For PDDA, flocculation with excessive PDDA was still prevailed by bridging mechanism B. However, the extended tails and loops on the surface of silica-cell flocs hindered the effective flocculation due to the steric repulsion force (Toh et al., 2018). As a result, lower separation efficiency was observed when excessive dosage of PDDA was supplied. In brief, the failure of appropriate flocculants will decrease the cell separation efficiency.

As shown in Figure 4.5 and 4.6, all the samples were having optimum flocculant dosages of 7-8 mg/L regardless of the silica concentrations. However, Figure 4.9 showed that, for both chitosan and PDDA flocculation, the sedimentation rate was increasing proportionally with the silica concentration. Hewson et al. (2012) revealed that, when the cell concentration increases, the collision frequency increases too and tend to form larger flocs. In this study, the increasing of silica concentration in microalgae cells suspension will lead to higher collision frequency between silica and/or cells, and hence bigger and denser silica-cell flocs was formed, this in turn increased the sedimentation rate. Similar theory as proposed in Section 4.1, the sedimentation rate increases with increasing of flocs size (Reynolds, 1984). Above point of view can be further supported by the images shown in Figure 4.7 and 4.8. The images in Figure 4.7 and 4.8 showed that the size of silica-cell flocs formed increased with the increasing of silica concentrations. Once again, these results proved that the presence of silica did not compete with microalgae cells and affect the flocculant dosages but in turn served as an agent to enhance the cell separation efficiency and promote the sedimentation rate. On the other hand, a sharp increase of the sedimentation rate promoted by the chitosan was noticeable in Figure 4.9. This phenomenon was due to the size of the silica-cell flocs formed which were shown in Figure 4.7 and 4.8. The silica-cell flocs formed by the chitosan that driven by bridging mechanism A was much bigger compared to that of PDDA and in turn led to higher sedimentation rate.

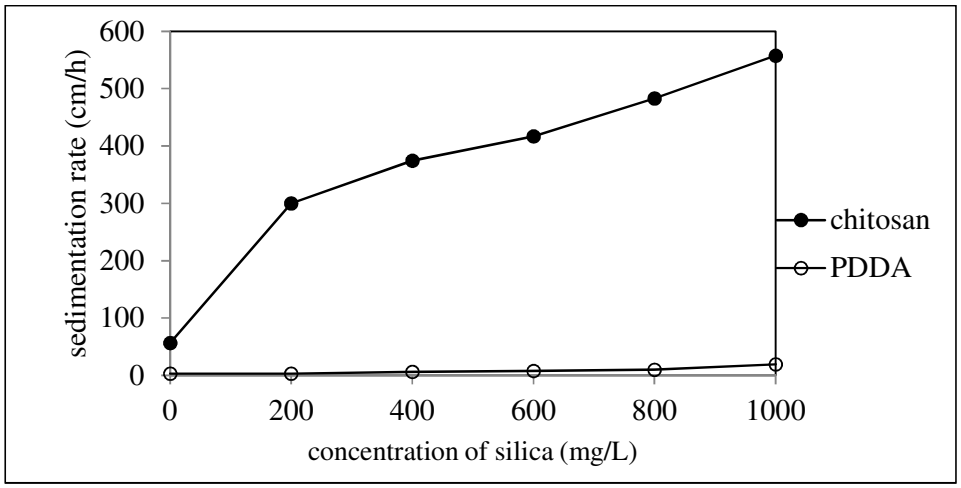

Figure 4.9: Sedimentation Rate of Silica-Cell Flocs Promoted by Chitosan and PDDA at Optimum Dosage

4.2.2 Immobilized-on Strategy

4.2.2.1 Characterization of Surface Functionalized Silica

For immobilized-on strategy, instead of adding silica and flocculants directly into microalgae cells medium as in Section 4.2.1, in this strategy, the silica has to be undergone pre-treatment first for surface functionalization by positively charged flocculants. After surface modification by positively charged chitosan (70.20 ± 1.80 mV) and PDDA (47.67 ± 2.54 mV), the net surface charge on the chitosan-coated silica and PDDA-coated silica become positive charge at 66.57 ± 3.22 mV and 28.87 ± 0.46 mV respectively.

The successful surface functionalization of chitosan and PDDA on the silica surface can also be proven by FTIR-ATR spectral data that demonstrated in Figure 4.10 to Figure 4.14. FTIR-ATR was carried out to identify the functional groups that presented in chitosan-coated silica and

PDDA-coated silica. In Figure 4.10, the characteristic peaks for silica can be observed at 458.84 cm^{-1} (Si-O-Si bending), 796.88 cm^{-1} (Si-O-Si symmetric stretching vibration), and 1075.16 cm^{-1} (Si-O-Si asymmetric stretching vibration). From Figure 4.12, the new additional peaks presented in chitosan-coated silica at 1554.45 cm^{-1} and 3278.98 cm^{-1} were attributed to N-H bending of amide II and N-H stretching of chitosan respectively (Figure 4.11). This proved that the surface of silica was successfully functionalized with chitosan. In Figure 4.14, it can be observed that, an additional peak at 2938.70 cm^{-1} that assigned to C-H symmetric stretching was the characteristic peak for PDDA. Obviously, the PDDA (Figure 4.13) has stronger bands at 1639.42 cm^{-1} and 1468.42 cm^{-1} which correspond to C=C stretching vibrations compared to silica (Figure 4.10). The slightly stronger bands (compared to silica) appeared in PDDA-coated silica at 1633.59 cm^{-1} and 1475.32 cm^{-1} has further confirmed the successfully surface functionalization of silica by PDDA.

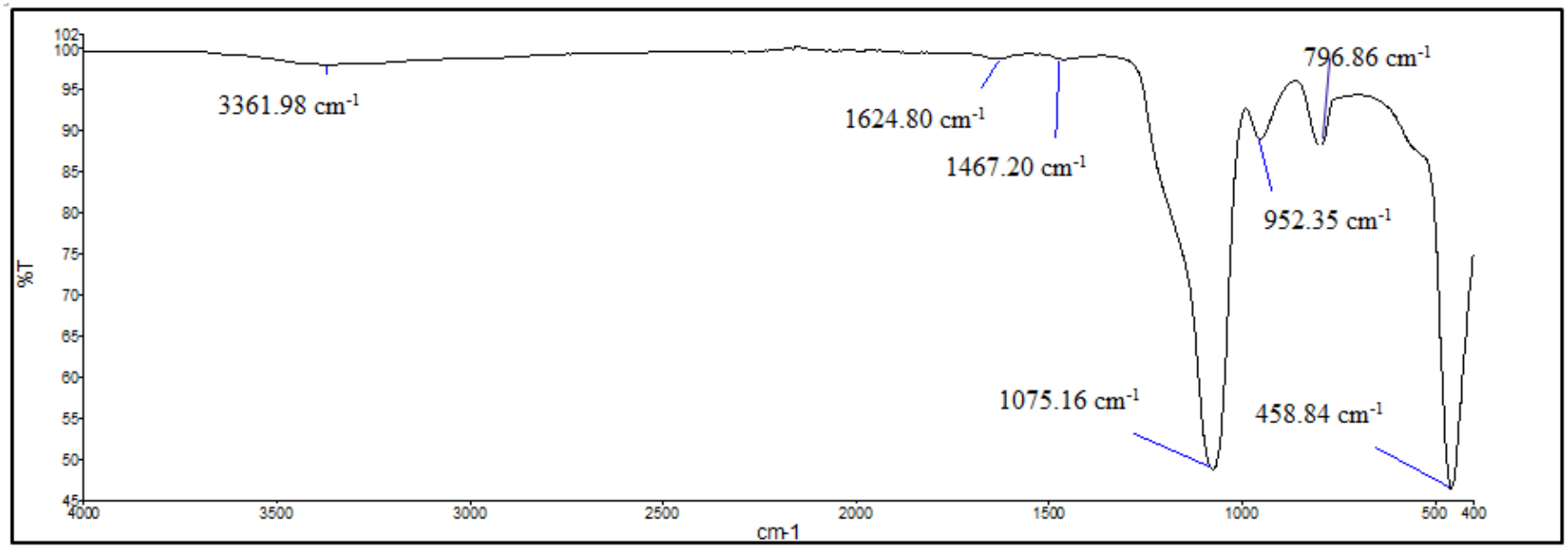

Figure 4.10: FTIR-ATR Spectrum of Silica

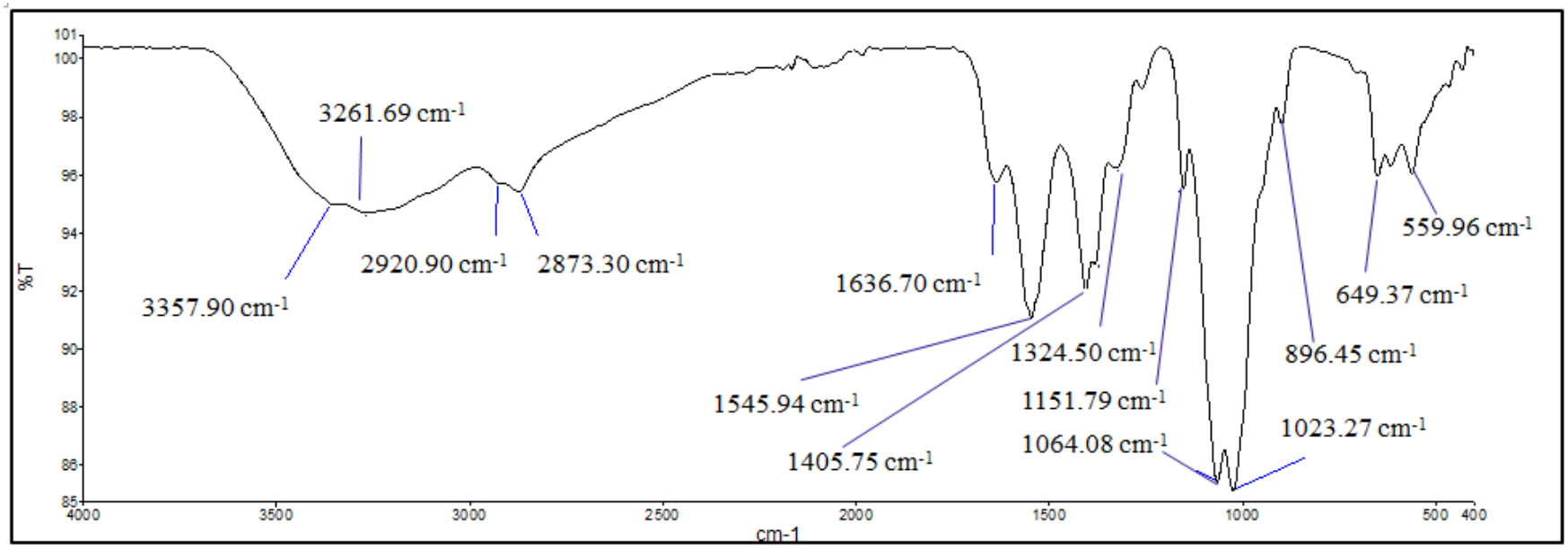

Figure 4.11: FTIR-ATR Spectrum of Chitosan

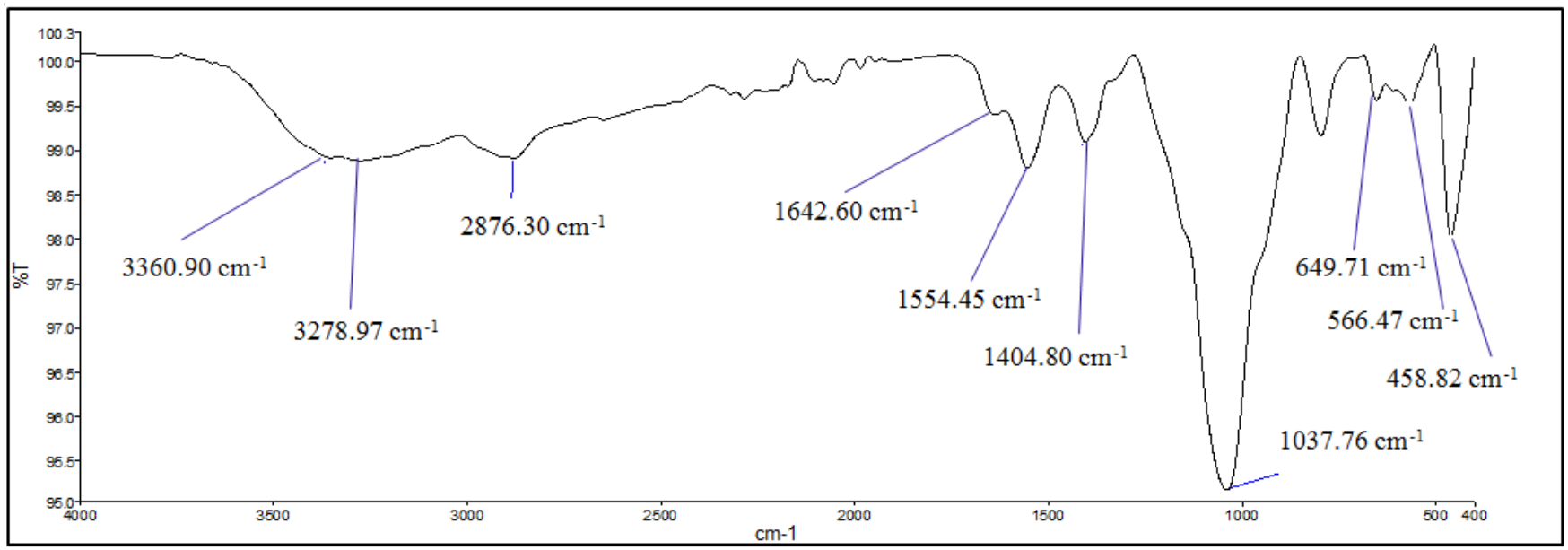

Figure 4.12: FTIR-ATR Spectrum of Chitosan-Coated Silica

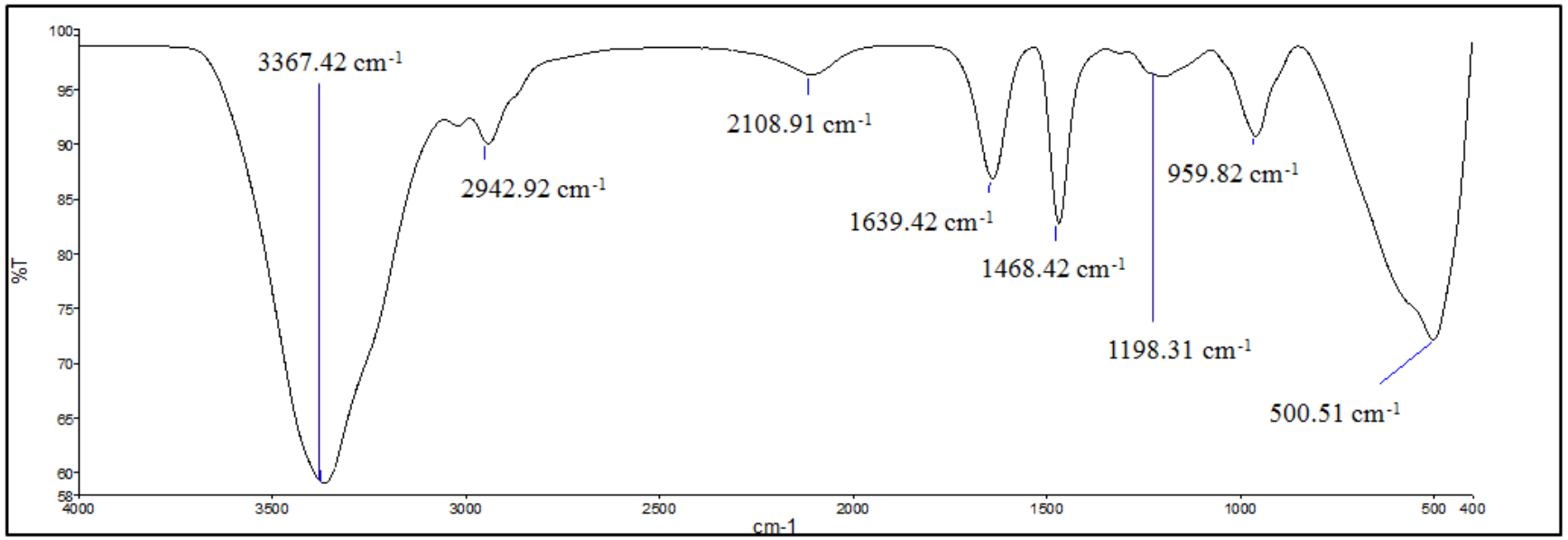

Figure 4.13: FTIR-ATR Spectrum of PDDA

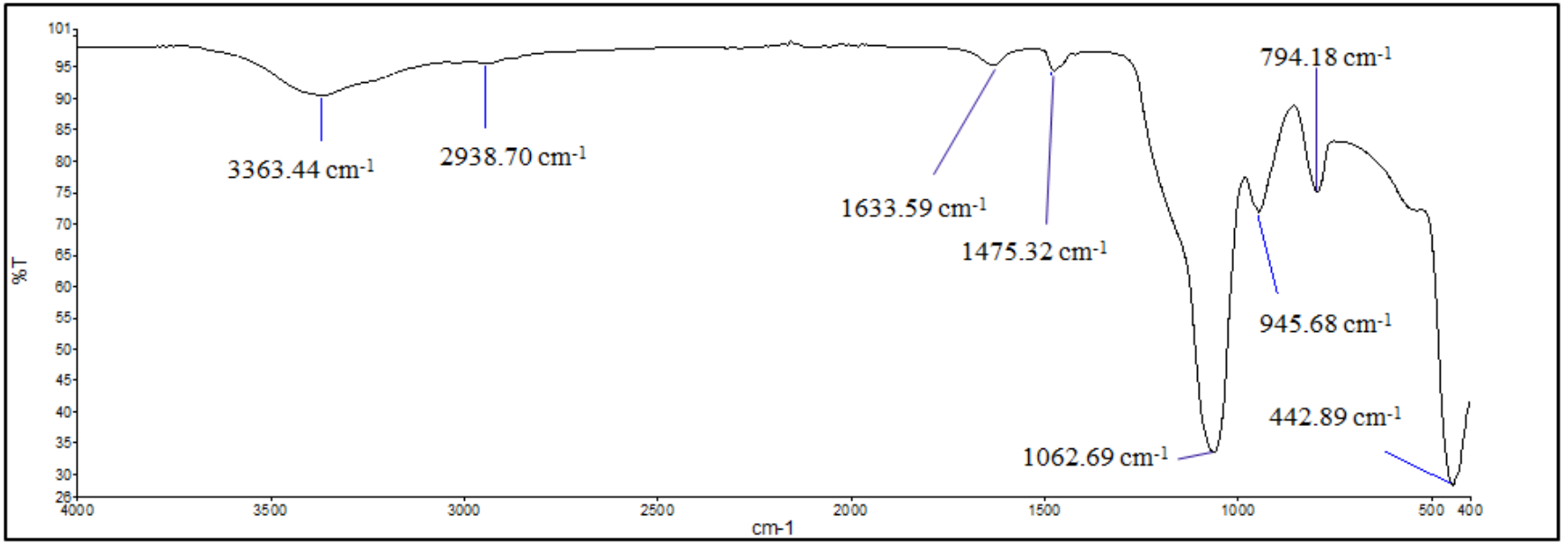

Figure 4.14: FTIR-ATR Spectrum of PDDA-Coated Silica

4.2.2.2 Performance of Immobilized-on Strategy

Results in Figure 4.15 indicated that the surface functionalized silica has successfully attached onto microalgae cell surface and promoted cell separation. By looking at Figure 4.15, the trends of the cell separation efficiencies promoted by chitosan-coated silica and PDDA-coated silica were similar. The increasing of the surface functionalized silica dosage will increase the cell separation efficiency. The cell separation efficiencies up to 99.89 ± 0.22 % and 99.61 ± 0.16 % were achieved by 1000 mg/L of chitosan-coated silica and PDDA-coated silica respectively. When both surface functionalized silica were added into microalgae cells medium respectively, the opposite charges between them will have attractive force according to Coulomb's Law which weaken the electrostatic repulsion and reduce the interparticle repulsion (Coulomb, 1785).

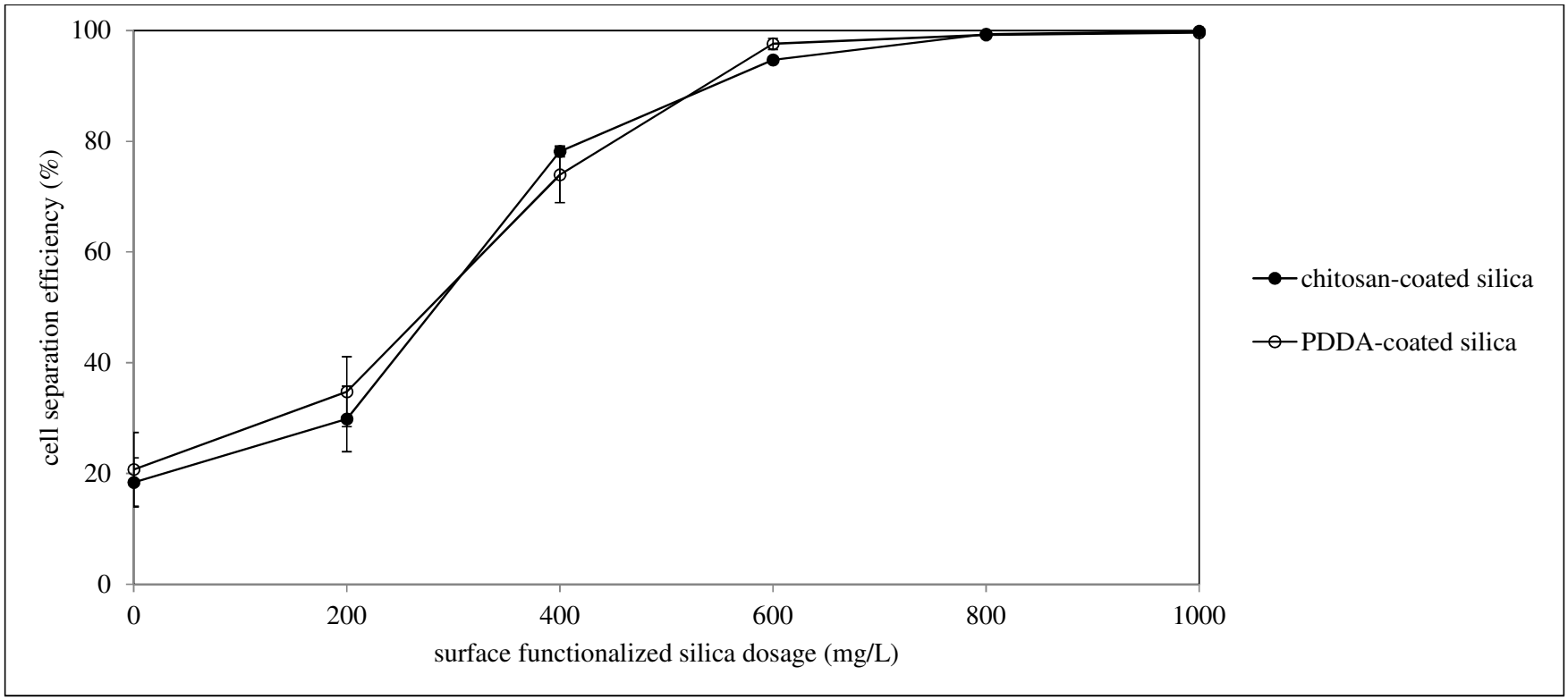

Figure 4.15: The Cell Separation Efficiency of *Chlorella vulgaris* Microalgae Promoted by Chitosan- and PDDA-Coated Silica at 3×10^{-7} cell/mL Cell Density

The cell separation efficiency increases as the dosage of surface functionalized silica increases. A several reports have proven that, in most separation cases, the separation efficiency will increase with the increasing of particles dosage until it reaches a peak separation efficiency value. Generally, after reaching the maximum recovery efficiency point, it will still remain constant even more particle dosage is provided (Fritz et al., 2002; Prochazkova et al., 2013a; Prochazkova et al., 2013b; Hu et al., 2013; Wang et al., 2014; Hu et al., 2014). At low dosage of surface functionalized silica, the microalgae cells cannot adsorb a sufficient amount of the surface functionalized silica and cannot be completely harvested. When the dosage of surface functionalized silica increased, there was sufficient surface functionalized silica to interact with the microalgae cells, hence the collision frequency increased too and led to maximum separation efficiency.

Results in Figure 4.15 showed that, there was no significant increase after 600, 800 and 1000 mg/L of surface functionalized silica was added. For chitosan-coated silica, the cell separation efficiencies can reach up to 94.73 ± 0.43 % at 600 mg/L, 99.35 ± 0.11 % at 800 mg/L and 99.89 ± 0.22 % at 1000 mg/L of chitosan-coated silica. While in the case of PDDA-coated silica, the cell separation efficiencies up to 97.60 ± 1.02 % at 600 mg/L, 99.25 % at 800 mg/L and 99.61 ± 0.16 % at 1000 mg/L of PDDA-coated silica were achieved. The excessive surface functionalized silica will be dispersed in the suspension and have no impact on the cell separation efficiencies even with further increased of the surface functionalized silica.

Although there was no significant different in the cell separation efficiency promoted by both surface functionalized silica, which were chitosan-coated silica and PDDA-coated silica. But the flocs size formed (Figure 4.16) and the sedimentation rate (Figure 4.17) were totally dissimilar. The flocs formed by the chitosan-coated silica were much larger which lead to the faster sedimentation rate (501.48 cm/h) compared to that of PDDA-coated silica (15.38 cm/h), as the sedimentation rate increased with increasing of flocs size (Reynolds, 1984). This might be due to difference between the size of surface functionalized silica and the charge density on the surface functionalized silica.

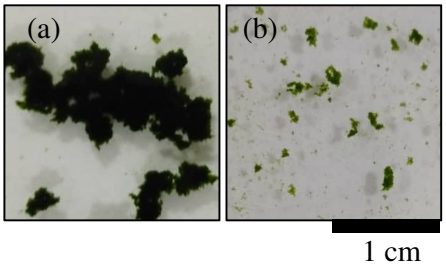

Figure 4.16: The Comparison of Surface Functionalized Silica-Cell Flocs Promoted by 1000 mg/L of (a) Chitosan-Coated Silica and (b) PDDA-Coated Silica

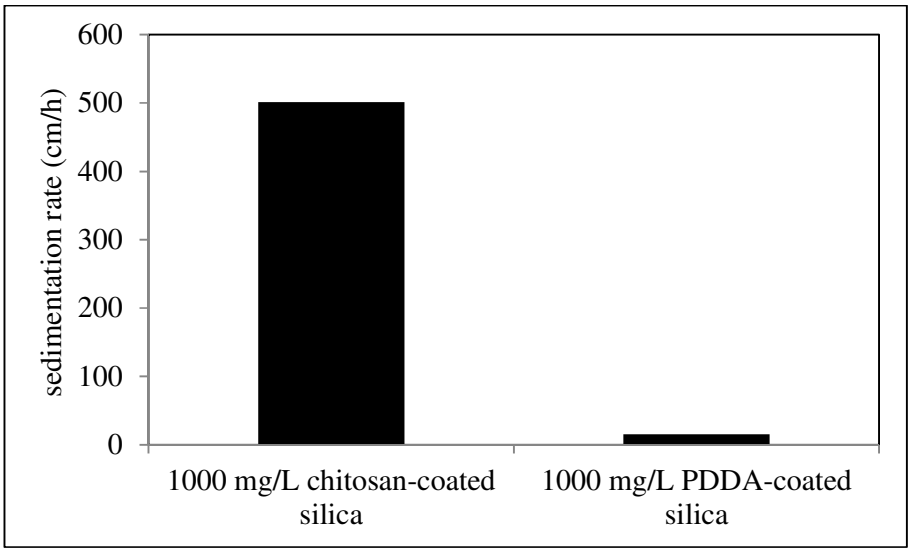

Figure 4.17: Sedimentation Rate of Cell Flocs Promoted by Chitosan-Coated Silica and PDDA-Coated Silica at Dosage of 1000 mg/L Respectively

The size of chitosan-coated silica found larger than that of PDDA-coated silica as shown in Figure 4.18. The larger size of chitosan-coated silica will form larger chitosan-coated silica-cell flocs and undergo gravimetric sedimentation and promote better sedimentation rate. Furthermore, after surface modification, positively charged chitosan-coated silica at 66.57 ± 3.22 mV and PDDA-coated silica at 28.87 ± 0.46 mV were formed. This zeta potential result showed that the chitosan-coated silica has higher surface charge density than PDDA-coated silica. Hence, the chitosan-coated silica might have more remaining positive charges on their surface to attract the negatively charged microalgae cells and showed stronger ability to interact with microalgae cells. While the electrostatic interaction between PDDA-coated silica and microalgae cells was much weaker. Lower surface charge density on PDDA-coated silica made the possibility to interact with microalgae cells became poorer. This in turn caused the size of chitosan-

coated silica-cell flocs to be much bigger compared to PDDA-coated silica-cell flocs as shown in Figure 4.15. Concisely, the higher surface charge and larger size of chitosan-coated silica can form larger chitosan-coated silica-cell flocs to enhance the sedimentation rate.

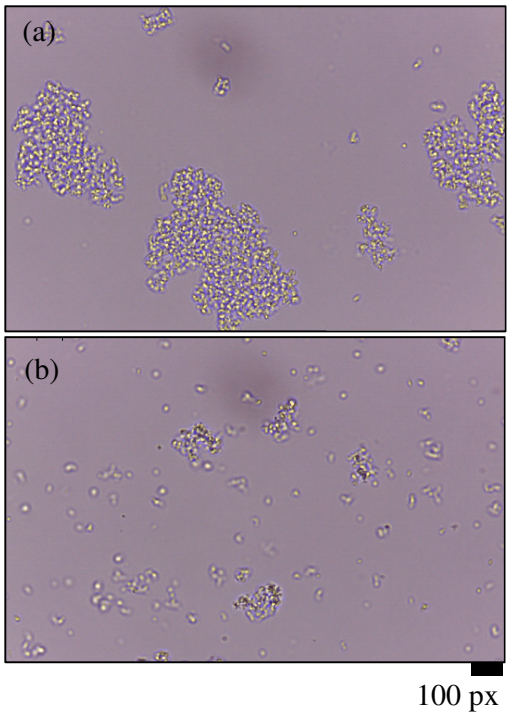

Figure 4.18: Microscopic Images of (a) Chitosan-Coated Silica and (b) PDDA-Coated Silica with Magnification 100X

4.2.3 Comparison between Embedding-Flocculation and Immobilized-on Strategies

A comparison between embedding-flocculation and immobilized on strategies in terms of cell separation efficiency and sedimentation rate was made and shown in Table 4.1. The cell separation efficiencies up to 100 % promoted by embedding-flocculation strategy by using chitosan and PDDA as flocculants with 1000 mg/L silica were achieved. For immobilized-on strategy,

99.89 ± 0.22 % and 99.65 ± 0.16 % of cell separation efficiencies were obtained by chitosan-coated silica and PDDA-coated silica respectively. The cell separation efficiencies were noticeably high for both strategies and flocculants. However, the sedimentation rate promoted by chitosan in both embedding-flocculation (557.21 cm/h) and immobilized-on (501.48 cm/h) strategy as shown in Table 4.1 were higher compared to PDDA (19.35 cm/h in embedding-flocculation strategy and 15.38 cm/h in immobilized-on strategy). Therefore, the one-step embedding-flocculation strategy with chitosan as flocculant which reduce the processing time in surface functionalization of silica was more preferable to apply in microalgae harvesting.

Table 4.1: The Comparison Cell Separation Efficiency and Sedimentation Rate of *Chlorella vulgaris* Microalgae between Embedding-Flocculation Strategy and Immobilized-On Strategy with 1000 mg/L of Silica

Strategies	Flocculants	Cell Separation Efficiency (%)	Sedimentation Rate (cm/h)
Embedding-Flocculation	Chitosan	100	557.21
	PDDA	100	19.35
Immobilized-On	Chitosan	99.89 ± 0.22	501.48
	PDDA	99.65 ± 0.16	15.38

4.3 Study on the Mechanism of Embedding-Flocculation Strategy of SAS Method

The mechanism of silica-to-microalgae cell interaction involved that dictated the successful application of the SAS method for harvesting microalgae cells was studied. In this study, the embedding flocculation

strategy with chitosan as flocculant was proven not only promoted high cell separation efficiency, it was also more time effectiveness compared to immobilized-on strategy. Therefore, the mechanism of the interaction between chitosan, silica and microalgae cells which subjected to embedding-flocculation strategy was being demonstrated as shown in Figure 4.19.

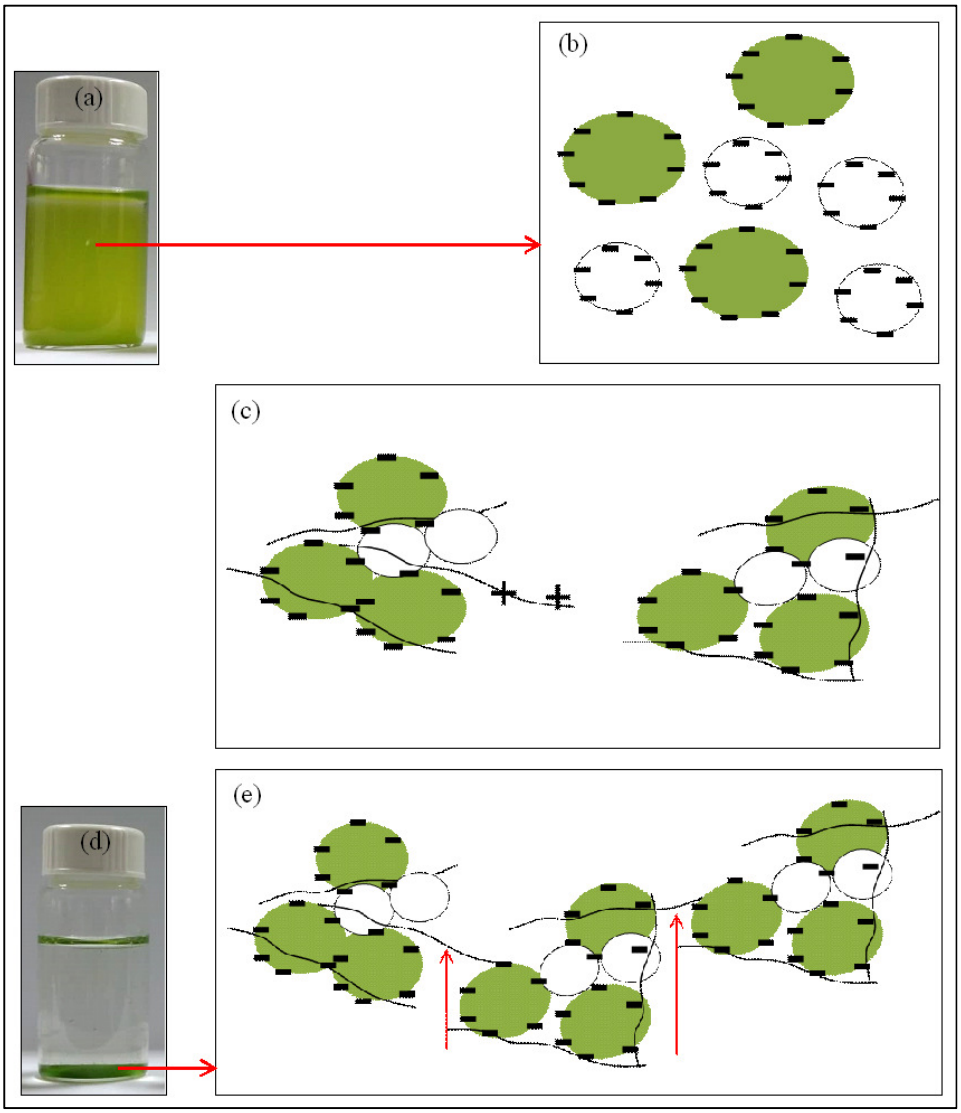

Figure 4.19: (a) The Picture of the Silica and Microalgae Cells Suspension in Vial, (b) The Schematic Diagram of Silica and Microalgae, the Interactions between Chitosan, Silica and Microalgae Cells which Subjected to Embedding-Flocculation Strategy that Formed (c) Microflocs and (e) Macroflocs, and (d) the Sediment of the Macroflocs in Vial

As discussed in Section 4.2.1, the negative charge on silica at -33.97 ± 1.91 mV has similar negative surface charge with microalgae cells at -28.73 ± 0.64 mV. Hence, the repulsion force will be induced between silica and microalgae cells as presented in Figure 4.19(b) (Coulomb, 1785a). During the preparation of chitosan solution, treatment with acetic acid increased the

number of protonated amine group along the chains and formed a soluble cationic polymer (Kurita, 2006; Rinaudo, 2006). The positively charged chitosan at 70.20 ± 1.80 mV was added into the suspension with opposite charged microalgae cells and silica, the positive charge on chitosan surface will promote effective attachment with negatively charged microalgae cells and silica through electrostatic attraction force. The mechanism of flocculation using polymeric flocculant can be the combination of charge neutralization and bridging. The extend of each mechanism is depending on the charge density and chain length of the polymer (Uduman et al., 2010). Charge patch neutralization happens when the silica and microalgae cells, and chitosan, which were in opposite charges, collided and attached together during the stirring step. Hence, silica and cell were being neutralized after attached with sufficient amount of chitosan and neutralized patches were formed on silica and cell surfaces thus microflocs were formed (Low and Lau, 2016). Besides, the bridging mechanisms performed by long chitosan polymer chains are also the main mechanism that uses to promote flocculation process. When silica and microalgae cells were attached with chitosan, the another side of chitosan chain will extend out from surface whereby the extended chains tend to form bridging with others to promote effective flocculation (Low and Lau, 2016). In this study, the resultant surface charge of the silica and microalgae cells at optimum chitosan was in negative, which was not in neutral charge, this depicted that the bridging was the mechanism prevailed in embedding-flocculation strategy (Tan et al., 2019). At optimum chitosan dosage, high electrostatic attraction between chitosan, silica and cells resulted the formation of strong and stable flocs, followed by rapid settling (Rashid et al., 2013). The

chitosan chains that spreaded in the suspension can easily trap onto the silica and microalgae cells by bridging (Liu et al., 2015).

As displayed in Figure 4.19(c), the chitosan chains were attached to the surface of silica and cells, the microflocs were formed and leaving the chitosan tails with available numbers of protonated amine groups. The positively charged extended tails on the microfloc tended to bridge with another microfloc and generated macroflocs and demonstrated in Figure 4.19(e) (Yeap et al., 2012). The chitosan encouraged faster aggregation of silica and cells through the interparticles bridging effect. It enhanced the formation of larger flocs size that promoted rapid settling and attributed to outstanding microalgae cells removal which was shown in Figure 4.19(d) (Ahmad et al., 2011).

4.4 Toxicity of Silica Microparticles towards *Chlorella vulgaris* Microalgae

In order to find out the toxicity effect of silica microparticles towards aquatic life after the disposal of the used silica in harvesting microalgae, the microalgae was employed as study model to investigate the toxicity in terms of growth and biochemical components (total lipid, protein and carbohydrate) of microalgae.

The microalgae was cultured in medium that contained different silica concentrations (0, 0.1, 1, 10, 100 and 1000 mg/L) for 7 days. The growth of

microalgae (cell density) was recorded during 7 days of cultivation as presented in Figure 4.20. Figure 4.20 showed that the growth of microalgae was not affected by the existence of up to 1000 mg/L silica. The cell density of microalgae was 15×10^{-5} cell/mL at day 0 and increased steadily to around 16.69×10^{-5} cell/mL at day 1, 19.14×10^{-5} cell/mL at day 2, 22.5×10^{-5} cell/mL at day 3, 25.75×10^{-5} cell/mL at day 4, 30.11×10^{-5} cell/mL at day 5, 35.36×10^{-5} cell/mL at day 6 and 39.75×10^{-5} cell/mL at day 7. The difference of cell density during 7 days of incubation with different silica concentration was not significant based on the ANOVA analysis ($p < 0.05$). This depicted that the presence of silica did not retard the growth rate of microalgae.

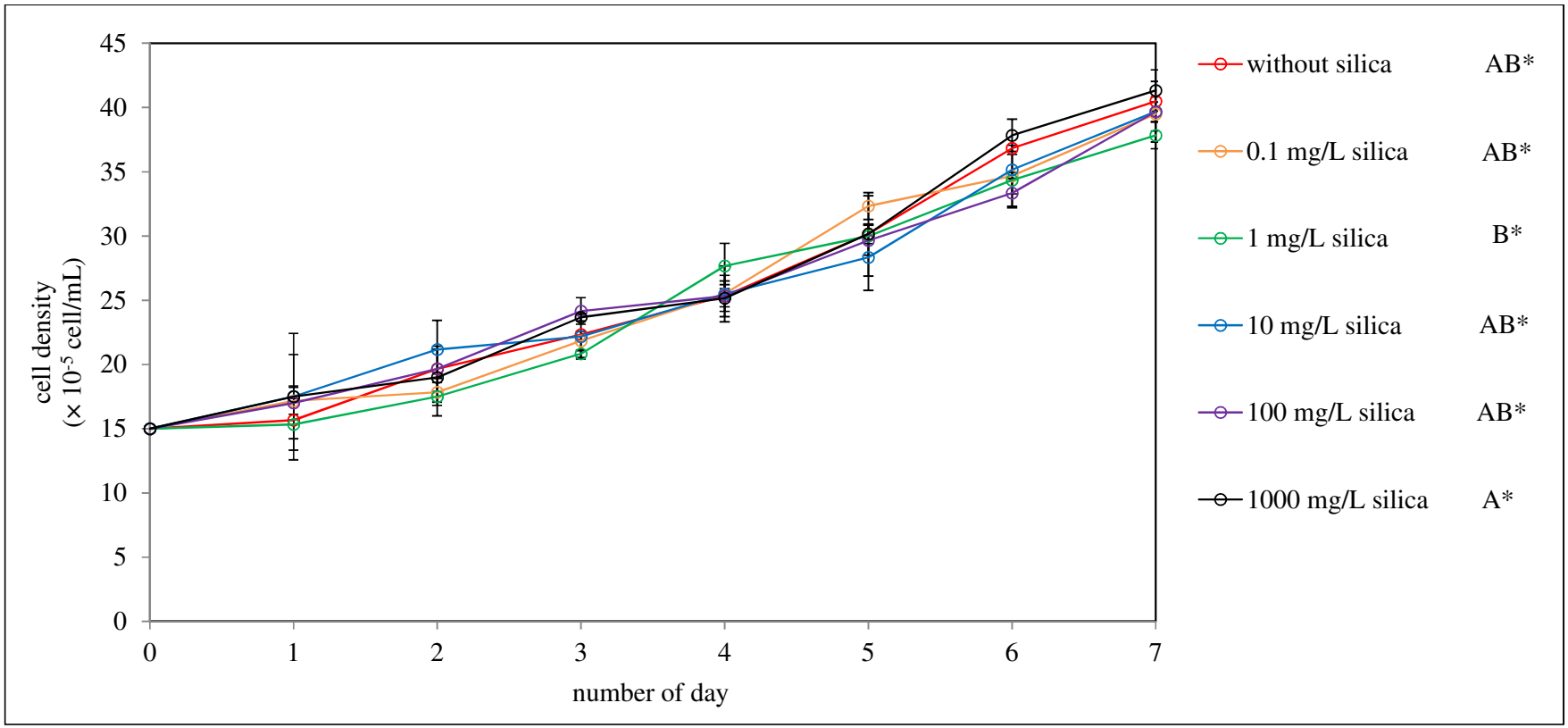

Figure 4.20: Cell Density of *Chlorella vulgaris* in Function of Cultivation Day at Different Concentration of Silica. *Statistically Significant was Evaluated Based on One-Way Analysis of Variance (ANOVA) Followed by LSD All-Pairwise Comparison Test at $p < 0.05$ for Cell Density at Day 7

The biochemical components (total lipid, carbohydrate and protein content) were analyzed after incubation of microalgae cell with different concentration of silica microparticles for 7 days. As shown in Figure 4.21 to 4.23, the reduction and increment of total lipid, carbohydrate and protein were not significant and maintained at about 21.85 %, 1.68 % and 6.82 % respectively regardless the silica concentration. The ANOVA analysis revealed that there was no substantial difference in each of the biochemical component with silica concentration up to 1000 mg/L.

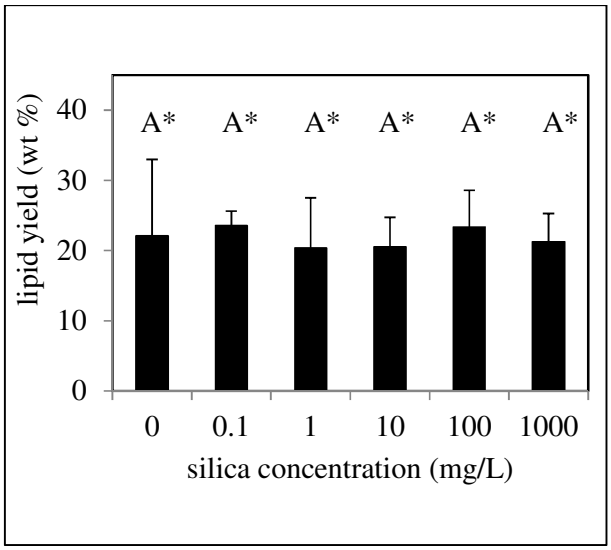

Figure 4.21: The Yield of Total Lipid of *Chlorella vulgaris* Biomass Harvested at Day 7th Cultured in Different Concentration of Silica. *Statistically Significant was Evaluated Based on One-Way Analysis of Variance (ANOVA) Followed by LSD All-Pairwise Comparison Test at $p < 0.05$

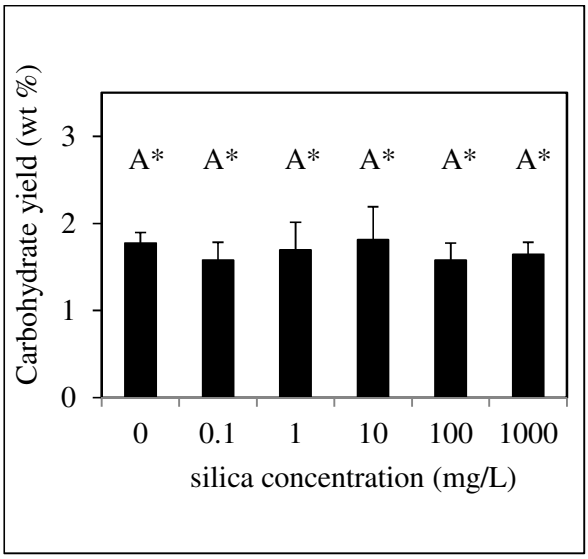

Figure 4.22: The Yield of Carbohydrate of *Chlorella vulgaris* Biomass Harvested at Day 7th Cultured in Different Concentration of Silica. Statistically Significant was Evaluated Based on One-Way Analysis of Variance (ANOVA) Followed by LSD All-Pairwise Comparison Test at $p < 0.05$

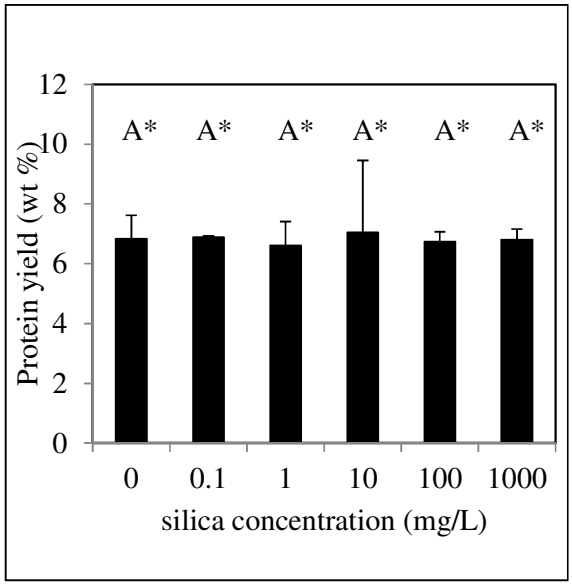

Figure 4.23: The Yield of Protein of *Chlorella vulgaris* Biomass Harvested at Day 7th Cultured in Different Concentration of Silica. Statistically Significant was Evaluated Based on One-Way Analysis of Variance (ANOVA) Followed by LSD All-Pairwise Comparison Test at $p < 0.05$

Most of the common reasons that inhibited the growth and affected the biochemical component of microalgae was oxidative stress (Kang et al., 2014), agglomeration (Sadiq et al., 2011), physical interactions (Al-Awady et al., 2015) or shading effects (Toh et al., 2015). Toh et al. (2015) proved that the bare and surface functionalized iron oxide nanoparticles have tendency to internalize into microalgae cells. This in turned caused the formation of ROS and damaged the proteins, lipids, nucleic acids and pigments of the cells (Barhoumi and Dewez 2013). The silica microparticles which has similar size to *Chlorella vulgaris* microalgae as shown in Figure 4.24 was incapable to internalize into the microalgae cells. As a result, the silica microparticles will not cause toxicity in form of oxidative stress by forming ROS as in the study of Toh et al. (2015). Besides, as silica is having density approximately 2000 kg/m^3, is heavier as compared to microalgae which is around 1000 kg/m^3 (Bangs Laboratories Inc, 1997; Edzwald, 1993). Hence, the colloidal stability of silica will be lower compared to that of microalgae and will settle faster according to Stokes' Law (Reynolds, 1984). Furthermore, the negatively charged silica will never tend to attach on the mutual charged microalgae cells. This declaration was proven by Toh et al. (2015) which the negatively charged cells were well disperse in the culture medium after incubated with iron oxide nanoparticles for 7 days. Due to the sedimentation of silica and the unsuccessful of the formation of silica-cell aggregates in the 7 days of cultivation, there will be no light shading effect that reduces the light reception by cells and inhibits the photosynthesis activity (Sadiq et al. 2011). Therefore, there was no obvious inhibitory effect on the growth of microalgae.

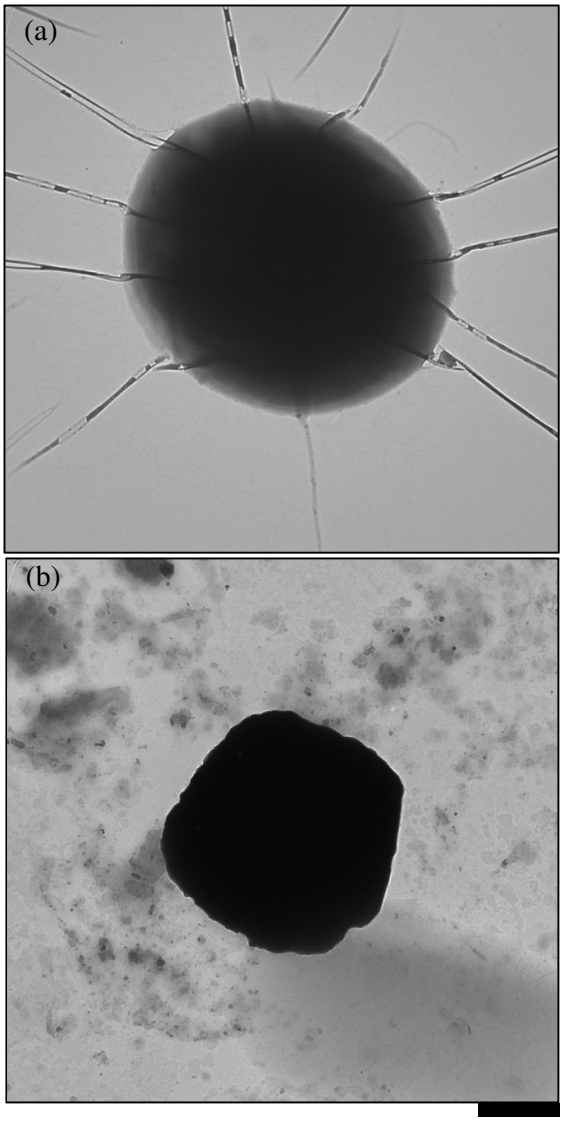

Figure 4.24: TEM Image of (a) *Chlorella vulgaris* Microalgae and (b) Silica Microparticle at the Scale of 1 µm

4.5 Feasibility Study on SAS Method for Fishpond Wastewater Treatment

4.5.1 Performance of Microalgae Separation from Fishpond Wastewater through the SAS Method

From the above finding in this study, the one-step embedding-flocculation strategy of the SAS method with chitosan as flocculant was being proven outperforming in microalgae harvesting in terms of cell separation efficiency and sedimentation rate. A study was being carried out in order to test the feasibility of embedding-flocculation strategy on algae cell separation from real aqueous environment. The sample used for this study was collected from a fishpond which located in Temoh, Perak, Malaysia. The embedding-flocculation strategy was being conducted with and without the presence of silica to compare the performance of cell separation. Furthermore, the water quality was being analyzed after employing of the embedding-flocculation strategy to harvest microalgae from the fishpond wastewater.

The result in Figure 4.25 showed that the relationship of the chitosan dosage and cell separation efficiency was not being affected regardless the presence of silica. Both samples reached optimum cell separation efficiency respectively at 2 mg/L chitosan. At optimum dosage of chitosan, the cell separation efficiency without silica can achieve up to 99.42 ± 0.56 %, when there was 1000 mg/L silica, 99.78 ± 0.76 % of cell separation efficiency was achieved. The optimal cell separation efficiency always achieved when at optimal dosage of chitosan regardless the availability of silica. This finding was same with the aforementioned finding in section 4.2.1, the similar relationship between the cell separation efficiency and chitosan dosage

indicated that the presence of silica did not affect the cell separation efficiency nor the optimum chitosan dosage.

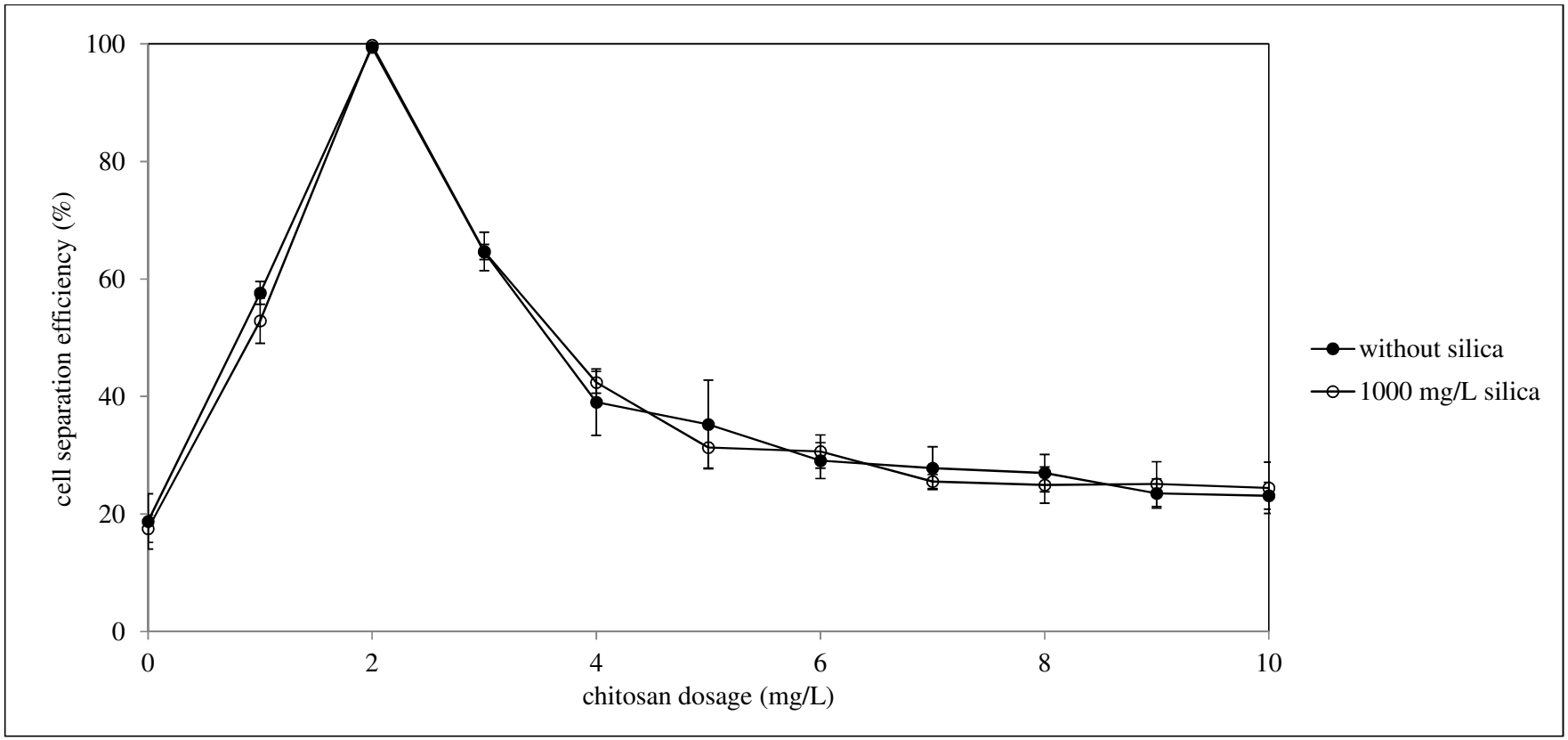

Figure 4.25: The Cell Separation Efficiency of Environmental Lake Algae Promoted by Chitosan through Embedding-Flocculation Strategy

However, the presence of silica will affect the cell sedimentation rate as discussed previously in Section 4.2.1. As displayed in Figure 4.26, the self-sedimentation rate of the environmental lake algae was low, at 0.08 cm/h. After adding the chitosan (without silica), the sedimentation rate was increased to 31.58 cm/h. When the embedding-flocculation strategy of the SAS method was applied, the sedimentation rate further increased to 324.95 cm/h. This study again confirmed the effectiveness of embedding-flocculation strategy able to enhance the performance of cell separation through SAS method effectively and rapidly.

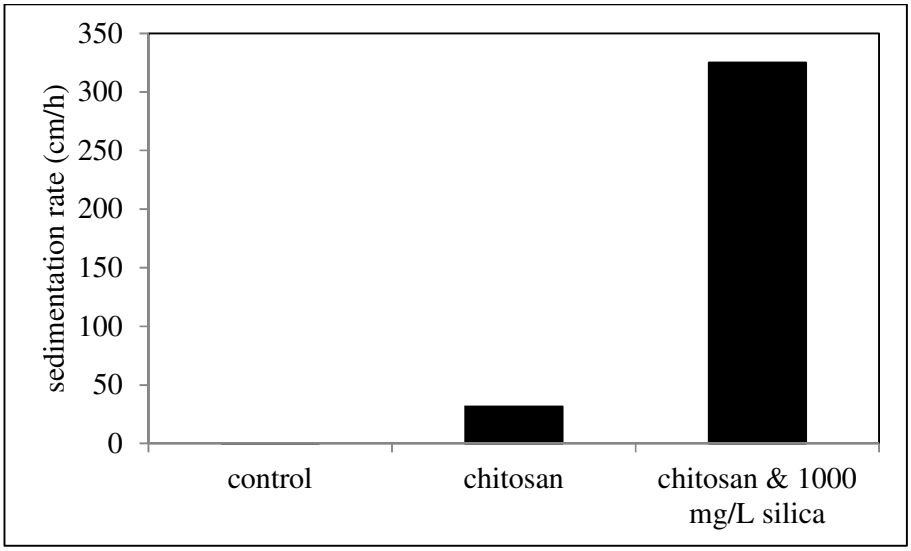

Figure 4.26: Sedimentation Rate of Control Cell (Lake Algae), Cell Flocs Promoted by Chitosan and Chitosan with 1000 mg/L Silica at Optimum Chitosan Dosage of 2 mg/L Respectively

4.5.2 Water Quality Analysis of the Treated Fishpond Wastewater

The water quality of the treated fishpond wastewater was being analyzed. A comparison between chemical harvesting method (SAS through embedding-flocculation strategy) and physical harvesting method (centrifugation) was made. A total 7 different parameters were being measured, which were ammoniacal nitrogen, nitrate, ortho-phosphate, turbidity, TSS, BOD and COD as listed in Table 4.2.

Table 4.2: Comparison of Water Quality before and after Treatment by SAS and Centrifugation Methods

	Untreated sample	Treated sample		Removal efficiency (%)	
		SAS	Centrifugation	SAS	Centrifugation
NH_4^+ (mg/L)	2.20 ± 0.10	0.10	0.70 ± 0.10	95.45	68.18 ± 4.54
NO_3^- (mg/L)	3.03 ± 0.32	0.13 ± 0.06	0.87 ± 0.06	95.60 ± 1.90	71.43 ± 1.90
PO_4^{3-} (mg/L)	29.90 ± 5.83	1.57 ± 0.72	5.47 ± 1.78	94.76 ± 2.42	81.72 ± 5.95
Turbidity (NTU)	18.50 ± 2.57	0.25 ± 0.06	0.26 ± 0.03	98.61 ± 0.33	98.59 ± 0.14
BOD (mg/L)	24.00 ± 2.83	0.30 ± 0.42	0.40 ± 0.14	98.75 ± 1.77	98.33 ± 0.59
COD (mg/L)	128.33 ± 6.11	24.33 ± 1.15	28.00 ± 2.65	81.04 ± 0.90	78.18 ± 2.06
TSS (mg/L)	88.00	56.00	10.00	36.36	88.64

From Table 4.2 above, the removal efficiencies of the ammoniacal nitrogen, nitrate and ortho-phosphate after treatment by SAS method were 95.45 %, 95.60 ± 1.90 % and 94.76 ± 2.42 % respectively, which were higher compared to centrifugation method where the removal efficiencies of ammoniacal nitrogen at 68.18 ± 4.54 %, nitrate at 71.43 ± 1.90 % and ortho-phosphate at 81.72 ± 5.95 %. The results depicted that the removal of ammoniacal nitrogen, nitrate and ortho-phosphate conducted by SAS method was better than that of centrifugation method, the differences were mainly due to the ions presented in the treatment process.

Kim et al. (2014) revealed that the silica particles in aqueous colloid will have an amorphous network of SiO_4^{4-} coordination tetrahedron inside, and leaving silicon and oxygen at particles surface. In aqueous suspensions, the free bonds are neutralized by OH^- or H^+ and hence there will have abundant silanol (Si-OH) groups at the outer surface which showed in Figure 4.27 (Kim et al, 2014; Junior and Baldo, 2014). When the silica is immersed in water, the dissociation of silanol groups may take place and produce a negative surface charge density (Equation 4.2) (Behrens and Grier, 2001). The presence of the negatively charged silica in the treatment process acted as adsorbent for the removal of ammonical nitrogen. The adsorption process comprises various interactions between adsorptive sites and adsorbate ions including electrostatic and chemical interactions, surface adsorption, and intraparticle diffusion (Othman et al., 2010; Darvishi Cheshmeh Soltani et al., 2014; Ayad and Laith, 2019).

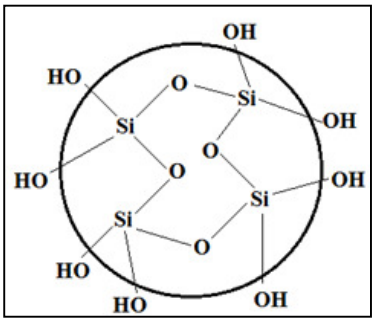

Figure 4.27: Schematic Diagram of Silica

$$SiOH \leftrightarrow SiO^- + H^+ \qquad \text{(Equation 4.2)}$$

Furthermore, the chitosan is a well-known sorbents of nutrients due to its high deacetylation degree and positive charge (Chung et al. 2005). The amine groups in chitosan will be protonated into a polysaccharide structure ($-NH_2 + H^+ \rightarrow -NH_3^+$) (Jozwiak et al., 2018). The sorption capability was mainly owing to the electrostatic attraction between the cationic chitosan and anionic pollutants (nitrate and ortho-phosphate). The positive charge which carried by the amine groups tend to attract NO_3^- and PO_4^{3-} anions electrostatically, which significantly enhance the removal of nutrients from wastewater (Jozwiak et al., 2018). Therefore, the presence of silica and chitosan in the SAS method exhibit better performances in ammoniacal nitrogen, nitrate and ortho-phosphate removal than the physical centrifugation process.

As presented in Table 4.2, after treatment by SAS and centrifugation methods, the removal of turbidity, BOD and COD were similar. The turbidity removal efficiencies were 98.61 ± 0.33 % for SAS method and 98.59 ± 0.14 %

for centrifugation method. As shown in Figure 4.28, the turbidity was mostly caused by the greenish microalgae, hence, the removal of the microalgae regardless the method, the turbidity level will decrease as well. As discussed in Section 4.5.1, the cell separation efficiency was high at 99.78 ± 0.76 %, which means, most of the algae was removed. Besides, centrifugal separation can achieve 90-100 % of biomass harvesting efficiency (Heasman et al, 2000). Therefore, the removal efficiencies of the turbidity in fishpond wastewater were high by both SAS and centrifugation methods.

Figure 4.28: Image of Fishpond Wastewater Sample

The removal efficiencies of BOD promoted by SAS method and centrifugation methods were 98.75 ± 1.77 % and 98.33 ± 0.59 % respectively. While the COD removal efficiencies were 81.04 ± 0.90 % through SAS method and 78.18 ± 2.06 % by centrifugation method. The BOD and COD in the wastewater were mostly due to the presence of organic compound. The decrease in BOD and COD might be correlated to the removal of the

suspended, dissolved organic material, microalgae cells and other microorganisms associated with fishpond wastewater (Cassini et al., 2017). Both SAS and centrifugation (Son et al., 2014) methods can remove organic matter and produced low COD and BOD level.

Overally, the SAS method has better removal efficiencies for all the analyzed parameters except for TSS. The TSS removal efficiency up to 88.64 % was achieved by applying centrifugation which was higher compared to SAS method which has TSS removal efficiency only 36.36 %. According to the study of Toh et al. (2014a), the electrophoretic mobility (zeta potential) of the microalgae was increasing with day. The immature algae with low surface charge has low possibility to be attached and flocculated by chitosan and left as free algae in the suspensions (Toh et al., 2014a). This caused the TSS removal efficiency promoted by the SAS method to be lower compared to centrifugation method which removed the particles through gravitational force and centrifugal force (Singh et al., 2013).

In brief, embedding-flocculation strategy of the SAS method by employing chitosan as flocculant was proven feasible for fishpond wastewater treatment to produce clean water in conjunction with microalgae harvesting.

4.6 Feasibility Study on SAS Method for Biofuel Production

Microalgae, as the source of third generation biofuel, the fatty acid profile of biofuel extracted from microalgae biomass harvested by using SAS method has to be investigated. The results obtained were being compared with the fatty acid profile from centrifugation method as presented in Table 4.3. There were 6 identified fatty acid in both samples, which were C16:0, heptadecanoic acid (C17:0), C18:0, C18:1, C18:2 and C18:3. The ANOVA analysis that showed in Table 4.3 proved that there was no substantial difference in each of the FAME composition in the cells which subjected to both SAS and centrifugation harvesting methods. It statistically showed that the exposure of the microalgae to chitosan and silica during the harvesting has no significant effect to the fatty acids saturation that stored in the cells.

Table 4.3: FAME Content of the Oil Extracted from Microalgae Biomass that Harvested through SAS and Centrifugation Methods. ANOVA Analysis Verified the Homogeneity of Variances on the Fatty Acid Content within Each Group of Fatty Acid

	Concentration of fatty acid (ppm %)		ANOVA	
Fatty acid	SAS	Centrifugation	SAS	Centrifugation
C16:0	22.01 ± 0.27	31.62 ± 8.83	A	A
C17:0	16.70 ± 0.24	9.52 ± 6.80	A	A
C18:0	1.64 ± 0.25	3.32 ± 2.09	A	A
C18:1	4.79 ± 0.24	18.59 ± 17.04	A	A
C18:2	23.02 ± 0.15	17.78 ± 6.85	A	A
C18:3	31.84 ± 0.80	19.16 ± 14.30	A	A

The results also showed that the total unsaturated fatty acids (UFA) which comprising of the fatty acids C18:1, C18:2 and C18:3, were naturally the higher at around 55-60 % as compared to the total saturated fatty acid which were about 40-45 % for both samples harvested by SAS and centrifugation methods. This result was in agreement with the findings of Prommuak et al. (2012), the percentage of unsaturated fatty acids with 65.3 % was higher than the saturated fatty acid which at 34.7 %. Also, Ahmad et al. (2013) showed that the unsaturated fatty acids at 77.85 % was considerably higher than saturated fatty acids which at 21.5 %. As reported by Toh et al. (2014c), the percentage of unsaturated fatty acids with 84.0 ± 7.3 % was higher than the saturated fatty acid which at 16.0 ± 7.3 %. This indirectly proved that the application of embedding-flocculation strategy in harvesting microalgae biomass as the feedstock for biofuel production was reliable.

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

The optimum dosages of the chitosan and PDDA in flocculation were same at 7 mg/L. At optimum flocculant dosages, the cell separation efficiencies at 100 % was achieved by chitosan flocculation and 99.34 ± 0.61 % was obtained through PDDA flocculation .With insufficient and surplus of flocculants, the cell separation efficiencies were very low. These were due to the electrostatic repulsion force between the microalgae cells (flocculant dosages below optimum) and the restabilized microalgae cells (flocculant dosage above optimum). The sedimentation rate of the cell flocs promoted by the chitosan and PDDA flocculation were extremely different. The chitosan-formed flocs has sedimentation rate at 56.54 cm/h, which was 471 times faster than that of PDDA-formed flocs at 3.18 cm/h. The flocs formed by the chitosan flocculation was much bigger (due to the bridging mechanism A) as compared to PDDA flocculation which led to faster sedimentation rate.

Furthermore, this study proved that the presence of silica in SAS method did not affect the flocculation process. The embedding-flocculation

strategy can achieve cell separation efficiencies above 99 % by chitosan and PDDA respectively with 1000 mg/L of silica. However, the sedimentation rate of the silica-cell flocs formed by chitosan flocculation (557.21 cm/h) was faster compared to that of PDDA flocculation (19.35 cm/h) as the chitosan flocculation was driven by bridging mechanism A which can form bigger flocs size and led to higher sedimentation rate. Moreover, in immobilized-on strategy, the cell separation efficiencies promoted by the 1000 mg/L of chitosan-coated silica was at 99.89 ± 0.22 % which was similar to that of PDDA-coated silica which at 99.61 ± 0.16 %. However, the flocs size and the sedimentation rate were substantially different. The chitosan-coated silica-cell flocs (501.48 cm/h) was bigger than the PDDA-coated silica-cell flocs (15.38 cm/h) and subsequently led to higher sedimentation rate. Evidently, the one-step embedding-flocculation strategy with chitosan as flocculant which attributed to bridging mechanism A was outperformed and more preferable in microalgae harvesting.

The toxicity of the silica microparticles was studied by employing microalgae as study model to prevent or minimize the toxic effects caused by the microparticles towards the aquatic life after used and disposal. The similar sizes of silica and microalgae cells caused no toxic effects to the microalgae cells in terms of growth and biochemical compositions of microalgae.

The superior embedding-flocculation strategy with chitosan and 1000 mg/L of silica was feasible to remove the on algal cells from real aqueous environment at efficiency up to 99.78 ± 0.76 % and sedimentation rate at

324.95 cm/h. This strategy also tended to remove 95.45 % of ammoniacal nitrogen, 95.60 ± 1.90 % of nitrate, 94.76 ± 2.42 % of ortho-phosphate, 98.61 ± 0.33 % of turbidity, 98.75 ± 1.77 of BOD, 81.04 ± 0.90 % of COD and 36.36 % of TSS. This strategy was proven to be a potential one pot solution in treating wastewater from fishpond. Besides, the fatty acid profiles extracted from the *Chlorella vulgaris* biomass harvested through embedding-flocculation strategy was comparable as centrifugation method. The exposure of microalgae to the chitosan and silica during harvesting process through embedding-flocculation strategy did not bring any harmful effects on the fatty acid profiles of extracted lipid.

5.2 Recommendations

To improve the quality of this study, some recommendations can be carried out:

i. Perform evaluation of SAS method on microalgae from different type of wastewater source.
ii. Reliability of SAS method toward other bioproducts production.
iii. Further extend the finding for simultaneous water purification by growth of microalgae and the biofuel production from the microalgae biomass.
iv. Conduct the adsorption isotherm for embedding-flocculation strategy so that the exploration on the mechanism between microalgae cells, silica and chitosan can be more detailed and supportive.

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APPENDICES

Appendix A: 3N-BBM+V Media Recipes

stock no.	stocks solution	stock solutions in g/1000 mL distilled water	make up to 1 litre final medium (mL)
(1)	$NaNO_3$	75.0	10
(2)	$CaCl_2.2H_2O$	2.5	10
(3)	$MgSO_4.7H_2O$	7.5	10
(4)	$K_2HPO_4.3H_2O$	7.5	10
(5)	KH_2PO_4	17.5	10
(6)	NaCl	2.5	10
(7)	trace element solution	See below	6
(8)	vitamin B_1	See below	1
(9)	vitamin B_{12}	See below	1

(7) trace element solution

1000 mL of distilled water was added into 0.75 g of Na_2EDTA and the minerals with following sequence:

mineral	mineral (mg)
$FeCl_3.6H_2O$	97.0
$MnCl_2.4H_2O$	41.0
$ZnCl_2$	5.0
$CoCl_2.6H_2O$	2.0
$Na_2MoO_4.2H_2O$	4.0

(8)

vitamin B_1	vitamin in g/100 mL distilled water
thiaminhydrochloride	0.12

(9)

vitamin B_{12}	vitamin in mL/100 mL distilled water
Cyanocobalamin (0.1g/100 mL)	1

Appendix B: FAME Calibration Curve

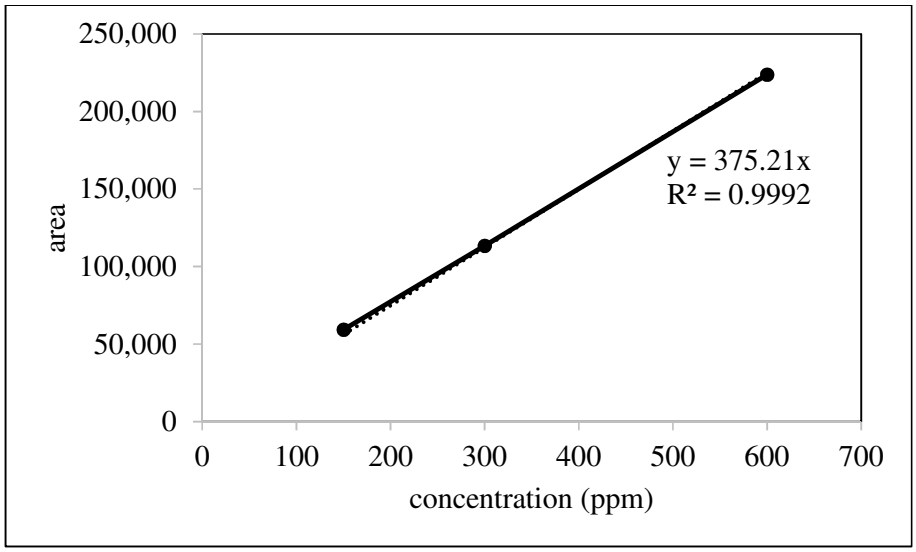


Figure B1: Calibration Curve of C16:0

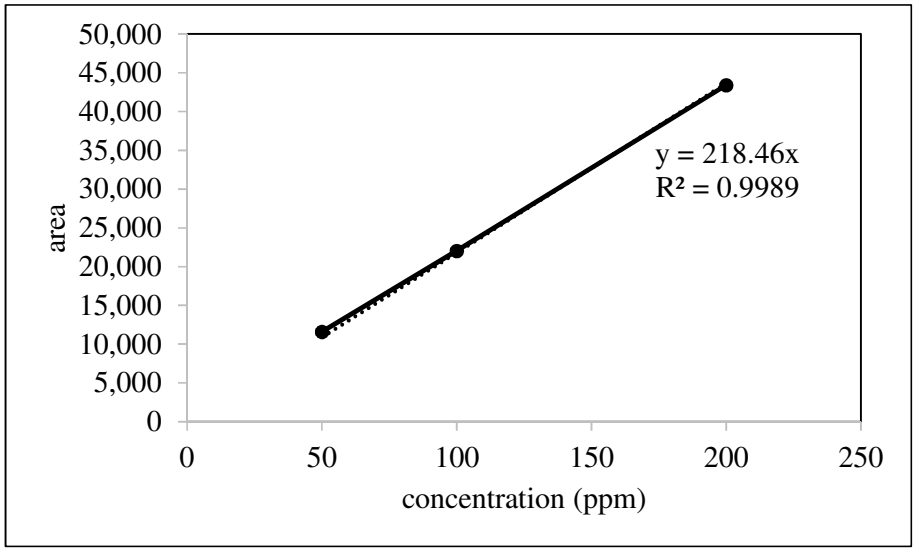


Figure B2: Calibration Curve of C17:0

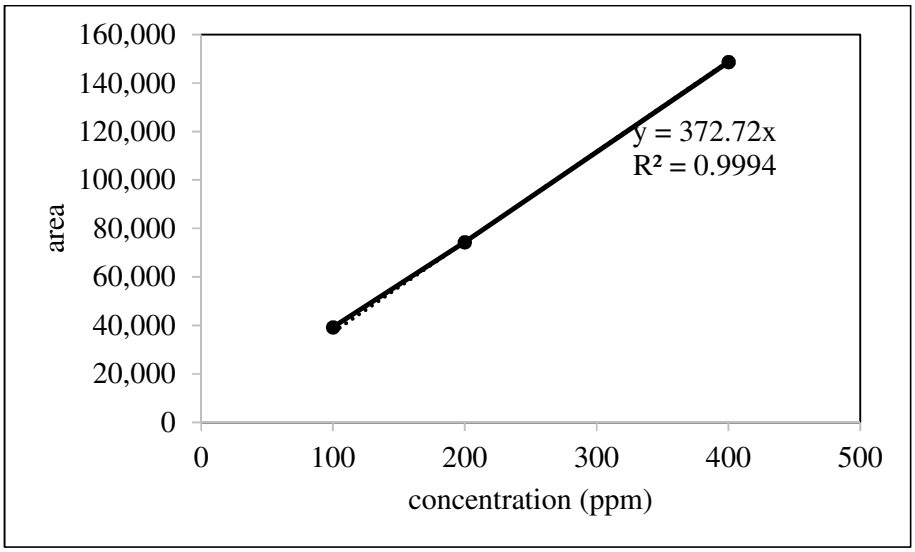


Figure B3: Calibration Curve of C18:0

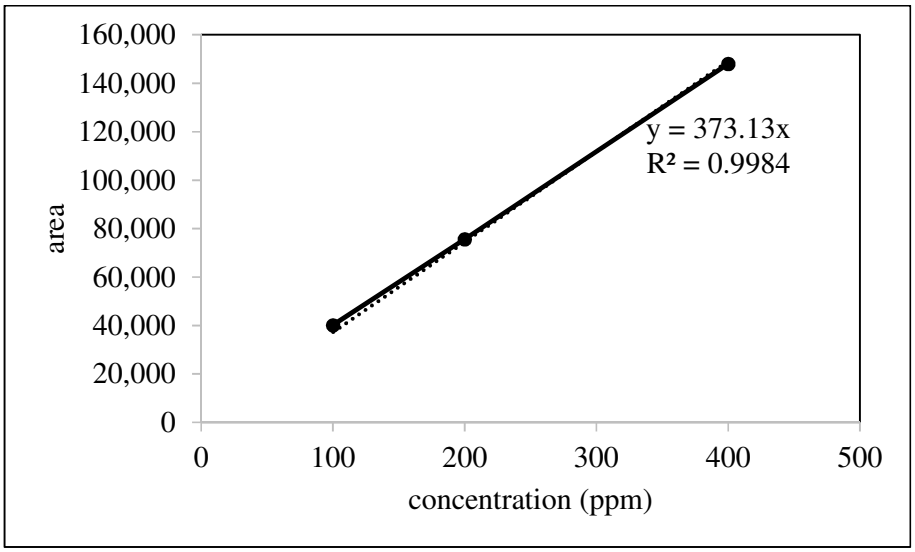


Figure B4: Calibration Curve of C18:0 N9C

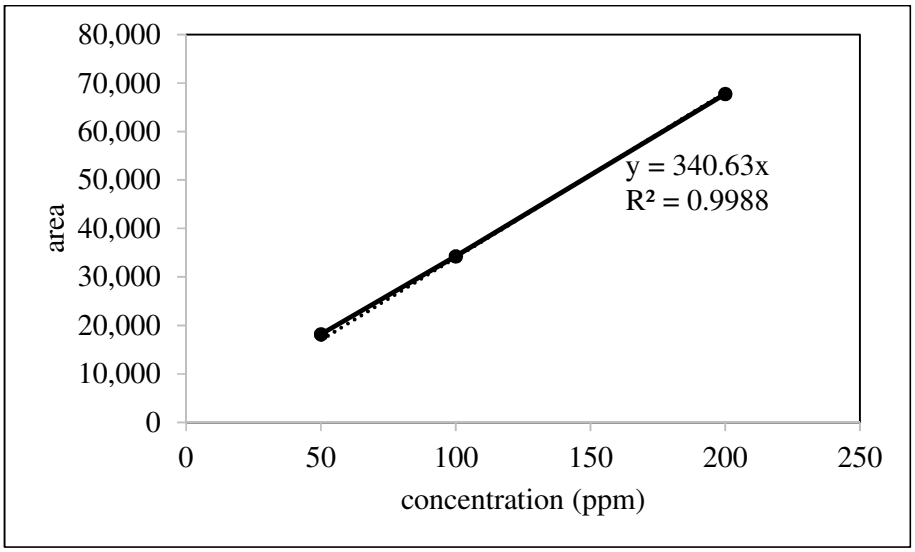


Figure B5: Calibration Curve of C18:2 N6C

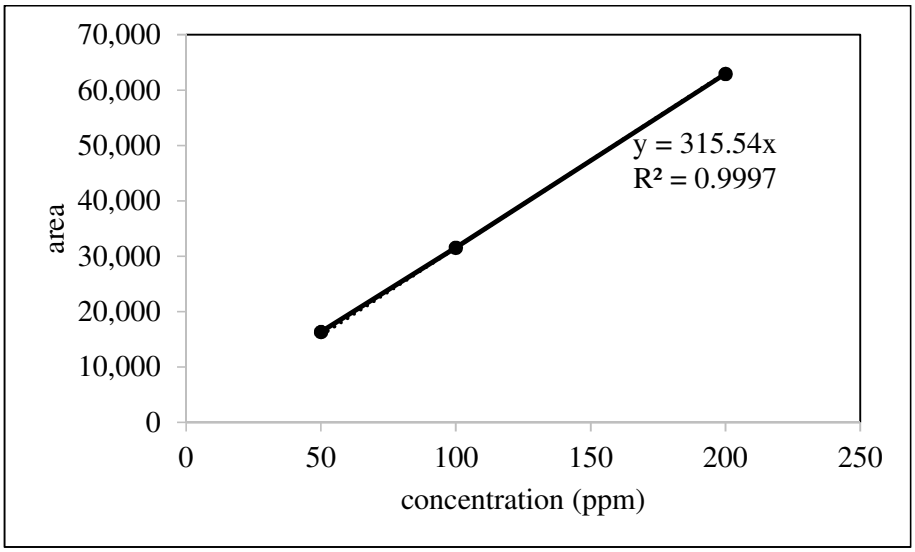
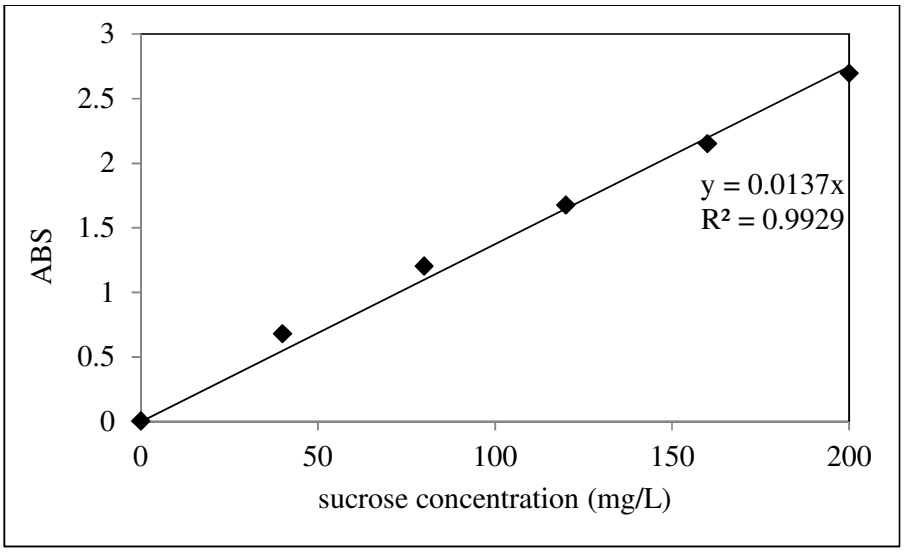
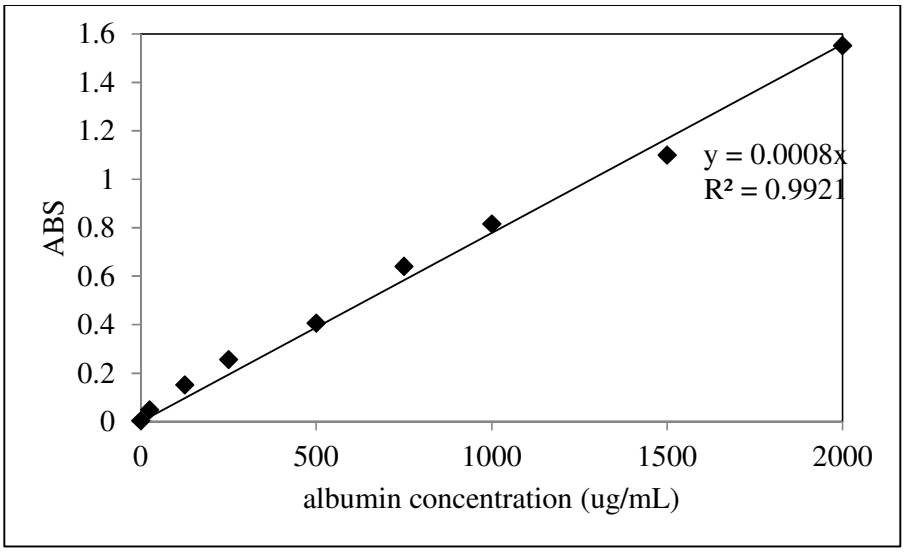


Figure B6: Calibration Curve of C18:3 N3

Appendix C: Carbohydrate Calibration Curve



Appendix D: Protein Calibration Curve



Appendix E: Optimum Flocculant Dosage for Cell Separation

Table E1: Optimum Chitosan Dosage for Cell Separation

ABS_o	0.964							
$ABS_{centrifuged}$	0.022							
Concentration of Chitosan in Cell Medium (mg/L)	**ABS**			**Cell Separation Efficiency (%)**				
	R1	**R2**	**R3**	**R1**	**R2**	**R3**	**Average**	**SD**
0	0.75	0.713	0.78	22.72	26.65	19.53	22.97	3.56
1	0.779	0.681	0.754	19.64	30.04	22.29	23.99	5.41
2	0.743	0.658	0.713	23.46	32.48	26.65	27.53	4.58
3	0.226	0.16	0.247	78.34	85.35	76.11	79.94	4.82
4	0.052	0.092	0.084	96.82	92.57	93.42	94.27	2.25
5	0.037	0.037	0.045	98.41	98.41	97.56	98.12	0.49
6	0.017	0.016	0.018	100.53	100.64	100.42	100.53	0.11
7	0.014	0.013	0.018	100.85	100.96	100.42	100.74	0.28
8	0.028	0.029	0.032	99.36	99.26	98.94	99.19	0.22
9	0.057	0.055	0.058	96.28	96.50	96.18	96.32	0.16
10	0.089	0.08	0.092	92.89	93.84	92.57	93.10	0.66
15	0.313	0.297	0.287	69.11	70.81	71.87	70.59	1.39
20	0.412	0.419	0.451	58.60	57.86	54.46	56.97	2.21
25	0.546	0.544	0.547	44.37	44.59	44.27	44.41	0.16
30	0.568	0.579	0.573	42.04	40.87	41.51	41.47	0.58

Table E2: Optimum PDDA Dosage for Cell Separation

ABS_o	0.922							
$ABS_{centrifuged}$	0.013							
Concentration of PDDA in Cell Medium (mg/L)	**ABS**			**Cell Separation Efficiency (%)**				
	R1	**R2**	**R3**	**R1**	**R2**	**R3**	**Average**	**SD**
0	0.788	0.715	0.727	14.74	22.77	21.45	19.66	4.31
1	0.758	0.641	0.735	18.04	30.91	20.57	23.18	6.82
2	0.516	0.519	0.542	44.66	44.33	41.80	43.60	1.56
3	0.141	0.198	0.144	85.92	79.65	85.59	83.72	3.53
4	0.109	0.125	0.099	89.44	87.68	90.54	89.22	1.44
5	0.02	0.027	0.059	99.23	98.46	94.94	97.54	2.29
6	0.01	0.012	0.046	100.33	100.11	96.37	98.94	2.23
7	0.014	0.025	0.018	99.89	98.68	99.45	99.34	0.61
8	0.014	0.029	0.06	99.89	98.24	94.83	97.65	2.58
9	0.039	0.051	0.033	97.14	95.82	97.80	96.92	1.01
10	0.043	0.048	0.057	96.70	96.15	95.16	96.00	0.78
15	0.066	0.069	0.059	94.17	93.84	94.94	94.32	0.56
20	0.078	0.072	0.079	92.85	93.51	92.74	93.03	0.42
25	0.087	0.121	0.124	91.86	88.12	87.79	89.26	2.26
30	0.135	0.131	0.139	86.58	87.02	86.14	86.58	0.44

Appendix F: Optimum Chitosan Dosage for Cell Separation through SAS Method (Embedding-Flocculation Strategy)

Table F1: Optimum Chitosan Dosage for Cell Separation through SAS Method at 200 mg/L of Silica

ABS_o	0.978							
$ABS_{centrifuged}$	0.015							
Concentration of Silica in Cell Medium (mg/L)	200							
Concentration of Chitosan in Cell Medium (mg/L)	**ABS**			**Cell Separation Efficiency (%)**				
	R1	**R2**	**R3**	**R1**	**R2**	**R3**	**Average**	**SD**
0	0.784	0.713	0.721	20.15	27.52	26.69	24.78	4.04
1	0.731	0.762	0.747	25.65	22.43	23.99	24.02	1.61
2	0.705	0.694	0.635	28.35	29.49	35.62	31.15	3.91
3	0.207	0.167	0.142	80.06	84.22	86.81	83.70	3.40
4	0.062	0.042	0.06	95.12	97.20	95.33	95.88	1.14
5	0.016	0.016	0.021	99.90	99.90	99.38	99.72	0.30
6	0.007	0.01	0.01	100.83	100.52	100.52	100.62	0.18
7	0.009	0.007	0.009	100.62	100.83	100.62	100.69	0.12
8	0.014	0.012	0.009	100.10	100.31	100.62	100.35	0.26
9	0.035	0.026	0.026	97.92	98.86	98.86	98.55	0.54
10	0.066	0.042	0.038	94.70	97.20	97.61	96.50	1.57
15	0.22	0.223	0.268	78.71	78.40	73.73	76.95	2.79
20	0.417	0.429	0.434	58.26	57.01	56.49	57.25	0.91
25	0.561	0.566	0.503	43.30	42.78	49.33	45.14	3.64
30	0.615	0.624	0.603	37.69	36.76	38.94	37.80	1.09

Table F2: Optimum Chitosan Dosage for Cell Separation through SAS Method at 400 mg/L of Silica

ABS_o	0.978							
$ABS_{centrifuged}$	0.015							
Concentration of Silica in Cell Medium (mg/L)	400							
Concentration of Chitosan in Cell Medium (mg/L)	**ABS**			**Cell Separation Efficiency (%)**				
	R1	**R2**	**R3**	**R1**	**R2**	**R3**	**Average**	**SD**
0	0.793	0.738	0.756	19.21	24.92	23.05	22.40	2.91
1	0.714	0.703	0.727	27.41	28.56	26.06	27.35	1.25
2	0.56	0.385	0.462	43.41	61.58	53.58	52.86	9.11
3	0.2	0.137	0.126	80.79	87.33	88.47	85.53	4.15
4	0.04	0.037	0.027	97.40	97.72	98.75	97.96	0.71
5	0.02	0.016	0.03	99.48	99.90	98.44	99.27	0.75
6	0.008	0.012	0.02	100.73	100.31	99.48	100.17	0.63
7	0.007	0.009	0.009	100.83	100.62	100.62	100.69	0.12
8	0.01	0.009	0.006	100.52	100.62	100.93	100.69	0.22
9	0.021	0.019	0.01	99.38	99.58	100.52	99.83	0.61
10	0.039	0.038	0.028	97.51	97.61	98.65	97.92	0.63
15	0.265	0.18	0.238	74.04	82.87	76.84	77.92	4.51
20	0.307	0.322	0.347	69.68	68.12	65.52	67.77	2.10
25	0.498	0.459	0.494	49.84	53.89	50.26	51.33	2.23
30	0.513	0.6	0.472	48.29	39.25	52.54	46.69	6.79

Table F3: Optimum Chitosan Dosage for Cell Separation through SAS Method at 600 mg/L of Silica

ABS_o	0.905							
$ABS_{centrifuged}$	0.011							
Concentration of Silica in Cell Medium (mg/L)	600							
Concentration of Chitosan in Cell Medium (mg/L)	**ABS**			**Cell Separation Efficiency (%)**				
	R1	**R2**	**R3**	**R1**	**R2**	**R3**	**Average**	**SD**
0	0.704	0.736	0.714	22.48	18.90	21.36	20.92	1.83
1	0.734	0.736	0.715	19.13	18.90	21.25	19.76	1.30
2	0.284	0.289	0.297	69.46	68.90	68.01	68.79	0.73
3	0.034	0.045	0.064	97.43	96.20	94.07	95.90	1.70
4	0.019	0.019	0.025	99.11	99.11	98.43	98.88	0.39
5	0.015	0.018	0.011	99.55	99.22	100.00	99.59	0.39
6	0.019	0.009	0.009	99.11	100.22	100.22	99.85	0.65
7	0.008	0.008	0.009	100.34	100.34	100.22	100.30	0.06
8	0.013	0.018	0.013	99.78	99.22	99.78	99.59	0.32
9	0.022	0.025	0.019	98.77	98.43	99.11	98.77	0.34
10	0.057	0.041	0.067	94.85	96.64	93.74	95.08	1.47
15	0.274	0.225	0.295	70.58	76.06	68.23	71.63	4.02
20	0.383	0.351	0.418	58.39	61.97	54.47	58.28	3.75
25	0.448	0.534	0.519	51.12	41.50	43.18	45.26	5.14
30	0.507	0.479	0.516	44.52	47.65	43.51	45.23	2.16

Table F4: Optimum Chitosan Dosage for Cell Separation through SAS Method at 800 mg/L of Silica

ABS_o	0.994							
$ABS_{centrifuged}$	0.012							
Concentration of Silica in Cell Medium (mg/L)	800							
Concentration of Chitosan in Cell Medium (mg/L)	**ABS**			**Cell Separation Efficiency (%)**				
	R1	**R2**	**R3**	**R1**	**R2**	**R3**	**Average**	**SD**
0	0.75	0.819	0.806	24.85	17.82	19.14	20.60	3.73
1	0.751	0.718	0.792	24.75	28.11	20.57	24.47	3.78
2	0.218	0.249	0.236	79.02	75.87	77.19	77.36	1.59
3	0.049	0.038	0.046	96.23	97.35	96.54	96.71	0.58
4	0.016	0.018	0.016	99.59	99.39	99.59	99.52	0.12
5	0.014	0.013	0.012	99.80	99.90	100.00	99.90	0.10
6	0.011	0.009	0.01	100.10	100.31	100.20	100.20	0.10
7	0.007	0.008	0.007	100.51	100.41	100.51	100.48	0.06
8	0.008	0.011	0.013	100.41	100.10	99.90	100.14	0.26
9	0.024	0.026	0.025	98.78	98.57	98.68	98.68	0.10
10	0.054	0.049	0.053	95.72	96.23	95.82	95.93	0.27
15	0.195	0.214	0.217	81.36	79.43	79.12	79.97	1.21
20	0.426	0.38	0.424	57.84	62.53	58.04	59.47	2.65
25	0.506	0.515	0.532	49.69	48.78	47.05	48.51	1.34
30	0.487	0.529	0.527	51.63	47.35	47.56	48.85	2.41

Table F5: Optimum Chitosan Dosage for Cell Separation through SAS Method at 1000 mg/L of Silica

ABS_o	0.994							
$ABS_{centrifuged}$	0.012							
Concentration of Silica in Cell Medium (mg/L)	1000							
Concentration of Chitosan in Cell Medium (mg/L)	**ABS**			**Cell Separation Efficiency (%)**				
	R1	**R2**	**R3**	**R1**	**R2**	**R3**	**Average**	**SD**
0	0.783	0.738	0.769	21.49	26.07	22.91	23.49	2.35
1	0.764	0.791	0.746	23.42	20.67	25.25	23.12	2.31
2	0.141	0.12	0.137	86.86	89.00	87.27	87.71	1.14
3	0.046	0.038	0.059	96.54	97.35	95.21	96.37	1.08
4	0.022	0.018	0.02	98.98	99.39	99.19	99.19	0.20
5	0.01	0.018	0.022	100.20	99.39	98.98	99.52	0.62
6	0.006	0.013	0.021	100.61	99.90	99.08	99.86	0.76
7	0.006	0.006	0.008	100.61	100.61	100.41	100.54	0.12
8	0.004	0.007	0.008	100.81	100.51	100.41	100.58	0.21
9	0.01	0.015	0.011	100.20	99.69	100.10	100.00	0.27
10	0.024	0.025	0.027	98.78	98.68	98.47	98.64	0.16
15	0.146	0.186	0.189	86.35	82.28	81.98	83.54	2.44
20	0.348	0.384	0.379	65.78	62.12	62.63	63.51	1.99
25	0.489	0.483	0.462	51.43	52.04	54.18	52.55	1.44
30	0.486	0.538	0.536	51.73	46.44	46.64	48.27	3.00

Appendix G: Optimum PDDA Dosage for Cell Separation through SAS Method (Embedding-Flocculation Strategy)

Table G1: Optimum PDDA Dosage for Cell Separation through SAS Method at 200 mg/L of Silica

ABS_o	0.984							
$ABS_{centrifuged}$	0.007							
Concentration of Silica in Cell Medium (mg/L)	200							
Concentration of PDDA in Cell Medium (mg/L)	**ABS**				**Cell Separation Efficiency (%)**			
	R1	**R2**	**R1**	**R2**	**R1**	**R2**	**R1**	**R2**
0	0.781	0.754	0.781	0.754	0.781	0.754	0.781	0.754
1	0.743	0.698	0.743	0.698	0.743	0.698	0.743	0.698
2	0.489	0.425	0.489	0.425	0.489	0.425	0.489	0.425
3	0.116	0.128	0.116	0.128	0.116	0.128	0.116	0.128
4	0.062	0.049	0.062	0.049	0.062	0.049	0.062	0.049
5	0.029	0.018	0.029	0.018	0.029	0.018	0.029	0.018
6	0.018	0.018	0.018	0.018	0.018	0.018	0.018	0.018
7	0.014	0.011	0.014	0.011	0.014	0.011	0.014	0.011
8	0.01	0.007	0.01	0.007	0.01	0.007	0.01	0.007
9	0.011	0.009	0.011	0.009	0.011	0.009	0.011	0.009
10	0.01	0.017	0.01	0.017	0.01	0.017	0.01	0.017
15	0.019	0.013	0.019	0.013	0.019	0.013	0.019	0.013
20	0.064	0.067	0.064	0.067	0.064	0.067	0.064	0.067
25	0.071	0.074	0.071	0.074	0.071	0.074	0.071	0.074
30	0.127	0.146	0.127	0.146	0.127	0.146	0.127	0.146

Table G2: Optimum PDDA Dosage for Cell Separation through SAS Method at 400 mg/L of Silica

ABS_o	0.984							
$ABS_{centrifuged}$	0.007							
Concentration of Silica in Cell Medium (mg/L)	400							
Concentration of PDDA in Cell Medium (mg/L)	**ABS**				**Cell Separation Efficiency (%)**			
	R1	**R2**	**R1**	**R2**	**R1**	**R2**	**R1**	**R2**
0	0.795	0.784	0.795	0.784	0.795	0.784	0.795	0.784
1	0.811	0.766	0.811	0.766	0.811	0.766	0.811	0.766
2	0.374	0.317	0.374	0.317	0.374	0.317	0.374	0.317
3	0.107	0.095	0.107	0.095	0.107	0.095	0.107	0.095
4	0.023	0.036	0.023	0.036	0.023	0.036	0.023	0.036
5	0.02	0.021	0.02	0.021	0.02	0.021	0.02	0.021
6	0.008	0.015	0.008	0.015	0.008	0.015	0.008	0.015
7	0.005	0.003	0.005	0.003	0.005	0.003	0.005	0.003
8	0.009	0.016	0.009	0.016	0.009	0.016	0.009	0.016
9	0.014	0.012	0.014	0.012	0.014	0.012	0.014	0.012
10	0.009	0.015	0.009	0.015	0.009	0.015	0.009	0.015
15	0.019	0.016	0.019	0.016	0.019	0.016	0.019	0.016
20	0.019	0.021	0.019	0.021	0.019	0.021	0.019	0.021
25	0.046	0.023	0.046	0.023	0.046	0.023	0.046	0.023
30	0.149	0.116	0.149	0.116	0.149	0.116	0.149	0.116

Table G3: Optimum PDDA Dosage for Cell Separation through SAS Method at 600 mg/L of Silica

ABS_o	0.973							
$ABS_{centrifuged}$	0.007							
Concentration of Silica in Cell Medium (mg/L)	600							
Concentration of PDDA in Cell Medium (mg/L)	**ABS**			**Cell Separation Efficiency (%)**				
	R1	**R2**	**R3**	**R1**	**R2**	**R3**	**Average**	**SD**
0	0.765	0.732	0.711	21.53	24.95	27.12	24.53	2.82
1	0.785	0.694	0.755	19.46	28.88	22.57	23.64	4.80
2	0.232	0.286	0.274	76.71	71.12	72.36	73.40	2.94
3	0.098	0.103	0.138	90.58	90.06	86.44	89.03	2.26
4	0.029	0.037	0.043	97.72	96.89	96.27	96.96	0.73
5	0.045	0.047	0.031	96.07	95.86	97.52	96.48	0.90
6	0.022	0.017	0.031	98.45	98.96	97.52	98.31	0.73
7	0.024	0.017	0.019	98.24	98.96	98.76	98.65	0.37
8	0.004	0.007	0.009	100.31	100.00	99.79	100.03	0.26
9	0.014	0.015	0.01	99.28	99.17	99.69	99.38	0.27
10	0.012	0.01	0.009	99.48	99.69	99.79	99.65	0.16
15	0.011	0.007	0.013	99.59	100.00	99.38	99.65	0.32
20	0.009	0.017	0.021	99.79	98.96	98.55	99.10	0.63
25	0.029	0.037	0.028	97.72	96.89	97.83	97.48	0.51
30	0.045	0.058	0.084	96.07	94.72	92.03	94.27	2.06

Table G4: Optimum PDDA Dosage for Cell Separation through SAS Method at 800 mg/L of Silica

ABS_o	0.973							
$ABS_{centrifuged}$	0.007							
Concentration of Silica in Cell Medium (mg/L)	800							
Concentration of PDDA in Cell Medium (mg/L)	**ABS**			**Cell Separation Efficiency (%)**				
	R1	**R2**	**R3**	**R1**	**R2**	**R3**	**Average**	**SD**
0	0.819	0.756	0.698	15.94	22.46	28.47	22.29	6.26
1	0.741	0.763	0.711	24.02	21.74	27.12	24.29	2.70
2	0.192	0.192	0.192	80.85	80.85	80.85	80.85	0.00
3	0.078	0.068	0.091	92.65	93.69	91.30	92.55	1.19
4	0.04	0.051	0.027	96.58	95.45	97.93	96.65	1.24
5	0.038	0.024	0.018	96.79	98.24	98.86	97.96	1.06
6	0.022	0.017	0.016	98.45	98.96	99.07	98.83	0.33
7	0.014	0.016	0.011	99.28	99.07	99.59	99.31	0.26
8	0.003	0.004	0.007	100.41	100.31	100.00	100.24	0.22
9	0.014	0.012	0.009	99.28	99.48	99.79	99.52	0.26
10	0.017	0.011	0.015	98.96	99.59	99.17	99.24	0.32
15	0.01	0.007	0.008	99.69	100.00	99.90	99.86	0.16
20	0.013	0.014	0.016	99.38	99.28	99.07	99.24	0.16
25	0.03	0.019	0.021	97.62	98.76	98.55	98.31	0.61
30	0.047	0.037	0.038	95.86	96.89	96.79	96.51	0.57

Table G5: Optimum PDDA Dosage for Cell Separation through SAS Method at 1000 mg/L of Silica

ABS_o	0.973							
$ABS_{centrifuged}$	0.007							
Concentration of Silica in Cell Medium (mg/L)	1000							
Concentration of PDDA in Cell Medium (mg/L)	**ABS**			**Cell Separation Efficiency (%)**				
	R1	**R2**	**R3**	**R1**	**R2**	**R3**	**Average**	**SD**
0	0.728	0.749	0.766	25.36	23.19	21.43	23.33	1.97
1	0.763	0.759	0.716	21.74	22.15	26.60	23.50	2.70
2	0.146	0.139	0.179	85.61	86.34	82.19	84.71	2.21
3	0.053	0.081	0.046	95.24	92.34	95.96	94.51	1.92
4	0.022	0.031	0.014	98.45	97.52	99.28	98.41	0.88
5	0.016	0.027	0.019	99.07	97.93	98.76	98.59	0.59
6	0.013	0.014	0.009	99.38	99.28	99.79	99.48	0.27
7	0.003	0.006	0.004	100.41	100.10	100.31	100.28	0.16
8	0.008	0.018	0.012	99.90	98.86	99.48	99.41	0.52
9	0.011	0.016	0.009	99.59	99.07	99.79	99.48	0.37
10	0.011	0.014	0.019	99.59	99.28	98.76	99.21	0.42
15	0.017	0.011	0.008	98.96	99.59	99.90	99.48	0.47
20	0.017	0.008	0.017	98.96	99.90	98.96	99.28	0.54
25	0.028	0.031	0.019	97.83	97.52	98.76	98.03	0.65
30	0.054	0.041	0.033	95.13	96.48	97.31	96.31	1.10

Appendix H: Zeta Potential of Samples

Samples	Zeta Potential (mV)				
	R1	**R2**	**R3**	**Average**	**SD**
***Chlorella vulgaris* microalgae**	-28	-29	-29.2	-28.73	0.64
Chitosan	71.7	70.7	68.2	70.20	1.80
PDDA	50.6	46.1	46.3	47.67	2.54
Silica	-33.4	-32.4	-36.1	-33.97	1.91
Chitosan-coated silica	70.1	65.8	63.8	66.57	3.22
PDDA-coated silica	29.4	28.6	28.6	28.87	0.46
Lake algae	-15	-18.2	-16.2	-16.47	1.62

Appendix I: Toxicity Test

Table I1: Growth Test at 0 mg/L of Silica

Silica Concentration (mg/L)	0				
Day	**Cell Density (number of cell/mL)**				
	R1	**R2**	**R3**	**Average**	**SD**
0	1500000	1500000	1500000	1500000	0
1	1650000	1550000	1500000	1566666.67	76376.26
2	1900000	1950000	2050000	1966666.67	76376.26
3	2200000	2300000	2200000	2233333.33	57735.03
4	2600000	2450000	2550000	2533333.33	76376.26
5	2950000	3050000	3050000	3016666.67	57735.023
6	3450000	3900000	3700000	3683333.33	225462.49
7	3750000	4300000	4100000	4050000	278388.22

Table I2: Growth Test at 0.1 mg/L of Silica

Silica Concentration (mg/L)	0.1				
Day	**Cell Density (number of cell/mL)**				
	R1	**R2**	**R3**	**Average**	**SD**
0	1500000	1500000	1500000	1500000	0
1	1600000	1800000	1750000	1716666.67	104083.30
2	1700000	1850000	1800000	1783333.33	76376.26
3	2200000	2250000	2100000	2183333.33	76376.26
4	2550000	2650000	2450000	2550000	100000
5	3350000	3150000	3200000	3233333.33	104083.30
6	3500000	3450000	3450000	3466666.67	28867.51
7	4100000	3850000	3900000	3950000	132287.57

Table I3: Growth Test at 1 mg/L of Silica

Silica Concentration (mg/L)	1				
Day	Cell Density (number of cell/mL)				
	R1	R2	R3	Average	SD
0	1500000	1500000	1500000	1500000	0
1	1650000	1650000	1300000	1533333.33	202072.59
2	1900000	1750000	1600000	1750000	150000
3	2100000	2100000	2050000	2083333.33	28867.51
4	2950000	2750000	2600000	2766666.67	175594.23
5	3100000	3250000	2650000	3000000	312249.90
6	3400000	3650000	3250000	3433333.33	202072.59
7	3700000	3900000	3750000	3783333.33	104083.30

Table I4: Growth Test at 10 mg/L of Silica

Silica Concentration (mg/L)	10				
Day	**Cell Density (number of cell/mL)**				
	R1	**R2**	**R3**	**Average**	**SD**
0	1500000	1500000	1500000	1500000	0
1	1900000	1200000	2150000	1750000	492442.89
2	2100000	1900000	2350000	2116666.67	225462.49
3	2200000	2050000	2400000	2216666.67	175594.23
4	2400000	2450000	2800000	2550000	217944.95
5	2550000	2900000	3050000	2833333.33	256580.07
6	3300000	3650000	3600000	3516666.67	189296.94
7	3700000	4050000	4150000	3966666.67	236290.78

Table I5: Growth Test at 100 mg/L of Silica

Silica Concentration (mg/L)	100				
Day	**Cell Density (number of cell/mL)**				
	R1	**R2**	**R3**	**Average**	**SD**
0	1500000	1500000	1500000	1500000	0
1	1650000	1850000	1600000	1700000	132287.56
2	1800000	2150000	1950000	1966666.67	175594.23
3	2300000	2500000	2450000	2416666.67	104083.30
4	2350000	2650000	2600000	2533333.33	160727.51
5	2900000	3100000	2900000	2966666.67	115470.05
6	3400000	3400000	3200000	3333333.33	115470.05
7	4050000	3900000	3950000	3966666.67	76376.26

Table I6: Growth Test at 1000 mg/L of Silica

Silica Concentration (mg/L)	1000				
Day	Cell Density (number of cell/mL)				
	R1	R2	R3	Average	SD
0	1500000	1500000	1500000	1500000	0
1	2050000	1400000	1800000	1750000	327871.93
2	2050000	1650000	2000000	1900000	217944.95
3	2350000	2400000	2350000	2366666.67	28867.51
4	2400000	2600000	2550000	2516666.67	104083.30
5	3000000	3100000	2950000	3016666.67	76376.26
6	3900000	3800000	3650000	3783333.33	125830.57
7	4250000	4200000	3950000	4133333.33	160727.51

Table I7: Total Lipid Yield

Silica Concentration (mg/L)	Weight of Dry Biomass (g)			Weight of Dry Lipid (g)			Total Lipid Yield (%)	
	R1	R2	R3	R1	R2	R3	Average	SD
0	0.0207	0.0315	0.0235	0.0037	0.0044	0.0081	22.104	10.885
0.1	0.0251	0.0277	0.0232	0.0065	0.0063	0.0051	23.556	2.0767
1	0.0300	0.0292	0.0399	0.0081	0.0062	0.0051	20.338	7.1518
10	0.0222	0.0417	0.0277	0.0036	0.0103	0.0057	20.508	4.2312
100	0.0239	0.0222	0.0228	0.0066	0.0039	0.0057	23.374	5.2156
1000	0.0233	0.0268	0.0261	0.0040	0.0057	0.0066	21.247	4.0445

Table I8: Carbohydrate Yield

Silica Concentration (mg/L)	Carbohydrate Concentration (g/L)			Biomass Concentration (g/L)			Carbohydrate Yield (%)	
	R1	R2	R3	R1	R2	R3	Average	SD
0	101.97	110.07	92.63	6150	5800	5250	1.77	0.12
0.1	77.81	85.47	101.75	5521.49	4721.96	6720.97	1.58	0.21
1	58.25	73.80	62.12	3003.55	5517.73	3430.80	1.70	0.32
10	98.03	40.29	83.14	7006.30	2123.12	3880.52	1.81	0.38
100	92.34	103.58	98.39	5117.34	7015.69	6776.16	1.58	0.20
1000	95.91	88.76	101.82	5635.60	5995.21	5825.07	1.64	0.14

Table I9: Protein Yield

Silica Concentration (mg/L)	Protein Concentration (g/L)			Biomass Concentration (g/L)			Protein Yield (%)	
	R1	R2	R3	R1	R2	R3	Average	SD
0	447.50	423.75	311.25	6150	5800	5250	6.84	0.79
0.1	381.25	326.25	460	5521.49	4721.96	6720.97	6.89	0.04
1	210	313.75	245	3003.55	5517.73	3430.80	6.61	0.80
10	306.25	191.25	301.25	7006.30	2123.12	3880.52	7.05	2.40
100	325	486.25	468.75	5117.34	7015.69	6776.16	6.73	0.33
1000	402.50	386.25	398.75	5635.60	5995.21	5825.07	6.81	0.35

Appendix J: Optimum Chitosan Dosage for Lake Algae Separation

Table J1: Optimum Chitosan Dosage for Lake Algae Separation

ABS_o	0.935							
$ABS_{centrifuged}$	0.013							
Concentration of Chitosan in Cell Medium (mg/L)	**ABS**			**Cell Separation Efficiency (%)**				
	R1	**R2**	**R3**	**R1**	**R2**	**R3**	**Average**	**SD**
0	0.794	0.713	0.78	15.29	24.08	16.81	18.73	4.70
1	0.415	0.413	0.383	56.40	56.62	59.87	57.63	1.94
2	0.017	0.024	0.014	99.57	98.81	99.89	99.42	0.56
3	0.326	0.348	0.344	66.05	63.67	64.10	64.61	1.27
4	0.518	0.62	0.588	45.23	34.16	37.64	39.01	5.66
5	0.687	0.552	0.591	26.90	41.54	37.31	35.25	7.54
6	0.663	0.697	0.641	29.50	25.81	31.89	29.07	3.06
7	0.689	0.706	0.641	26.68	24.84	31.89	27.80	3.66
8	0.654	0.711	0.694	30.48	24.30	26.14	26.97	3.17
9	0.698	0.743	0.714	25.70	20.82	23.97	23.50	2.47
10	0.746	0.713	0.707	20.50	24.08	24.73	23.10	2.28

Table J2: Optimum Chitosan Dosage for Lake Algae Separation through SAS Method at 1000 mg/L of Silica

ABS_o	0.935							
$ABS_{centrifuged}$	0.013							
Concentration of Silica in Cell Medium (mg/L)	1000							
Concentration of Chitosan in Cell Medium (mg/L)	**ABS**			**Cell Separation Efficiency (%)**				
	R1	**R2**	**R3**	**R1**	**R2**	**R3**	**Average**	**SD**
0	0.749	0.784	0.788	20.17	16.38	15.94	17.50	2.33
1	0.422	0.488	0.432	55.64	48.48	54.56	52.89	3.86
2	0.022	0.008	0.015	99.02	100.54	99.78	99.78	0.76
3	0.307	0.367	0.342	68.11	61.61	64.32	64.68	3.27
4	0.525	0.558	0.549	44.47	40.89	41.87	42.41	1.85
5	0.679	0.614	0.646	27.77	34.82	31.34	31.31	3.53
6	0.678	0.654	0.626	27.87	30.48	33.51	30.62	2.82
7	0.705	0.707	0.687	24.95	24.73	26.90	25.52	1.19
8	0.691	0.687	0.738	26.46	26.90	21.37	24.91	3.08
9	0.705	0.668	0.738	24.95	28.96	21.37	25.09	3.80
10	0.689	0.756	0.684	26.68	19.41	27.22	24.44	4.36

Appendix K: Water Quality Analysis

Table K1: Water Quality Analysis of Untreated Fishpond Wastewater

Parameters	R1	R2	R3	Average	SD
NH_4^+ (mg/L)	2.1	2.2	2.3	2.20	0.10
NO_3^- (mg/L)	3.4	2.9	2.8	3.03	0.32
PO_4^{3-} (mg/L)	23.2	32.7	33.8	29.90	5.83
Turbidity (NTU)	18.41	21.11	15.97	18.50	2.57
BOD (mg/L)	26	22	-	24.00	2.83
COD (mg/L)	123	127	135	128.33	6.11
TSS (mg/L)	-	-	-	88	-

Table K2: Water Quality Analysis of Treated Fishpond Wastewater

Parameters	SAS					Centrifugation				
	Readings			Removal Efficiency (%)		Readings			Removal Efficiency (%)	
	R1	R2	R3	Average	SD	R1	R2	R3	Average	SD
NH_4^+ (mg/L)	0.1	0.1	0.1	95.45	0	0.6	0.7	0.8	68.18	4.55
NO_3^- (mg/L)	0.1	0.2	0.1	95.60	1.90	0.8	0.9	0.9	71.437	1.90
PO_4^{3-} (mg/L)	1.2	1.1	2.4	94.76	2.42	4.7	4.2	7.5	81.72	5.95
Turbidity (NTU)	0.19	0.27	0.31	98.61	0.33	0.24	0.25	0.29	98.60	0.14
BOD (mg/L)	0	0.6	-	98.75	1.77	0.3	0.5	-	98.33	0.59
COD (mg/L)	25	23	25	81.04	0.90	26	27	31	78.18	2.06
TSS (mg/L)	-	-	-	56	-	-	-	-	10	-

Appendix L: Fatty Acid Profile

Table L1: Fatty Acid Profile of Microalgae Biomass Harvested through SAS Method (Embedding-Flocculation Strategy)

Compound	Area			Concentration (%)				
	R1	R2	R3	R1	R2	R3	Average	SD
C16:0	43527	45459	55000	22.32	21.82	21.89	22.01	0.27
C17:0	19254	20217	24108	16.96	16.66	16.48	16.70	0.24
C18:0	2845	3979	3824	1.47	1.92	1.53	1.64	0.25
C18:1 N9C	9837	9686	11565	5.07	4.67	4.63	4.79	0.24
C18:2 N6C	41049	43451	52240	23.19	22.97	22.90	23.02	0.15
C18:3 N3	50815	55995	68822	30.99	31.95	32.57	31.84	0.80

Table L2: Fatty Acid Profile of Microalgae Biomass Harvested through Centrifugation Method

Compound	Area			Concentration (%)				
	R1	R2	R3	R1	R2	R3	Average	SD
C16:0	91972	41771	224248	28.21	25.00	41.64	31.62	8.83
C17:0	23569	14007	5516	12.42	14.40	1.76	9.52	6.80
C18:0	7323	3275	30658	2.26	1.97	5.73	3.32	2.09
C18:1 N9C	38674	9788	203246	11.93	5.89	37.95	18.59	17.04
C18:2 N6C	60874	34590	48786	20.57	22.80	9.98	17.78	6.85
C18:3 N3	67454	42076	13301	24.61	29.94	2.94	19.16	14.30