Towards the development of an energy efficient microalgae biodiesel process

Qingyu Tian

A thesis submitted in total fulfilment of the requirements of the Degree of Master of Philosophy

Department of Biochemical Engineering
University College London

August 2020

Declaration

This is to certify that:

i) The thesis comprises only my original work towards the MPhil;

ii) The thesis is fewer than 30000 words in length, exclusive of contents, tables, figures and appendix.

Signed:

Nomenclature

% v/v	Volume percentage
% w/w	Weight percentage
mol: mol	Molar ratio
DW	Dry weight
FAME	Fatty acid methyl esters
FFA	Free fatty acids
DoE	Design of experiment
Chlorella S.	*Chlorella Sorokiniana*
Chlorella sp.	*Chlorella species*
NL	Neutral lipid
PL	Phospholipid
TAG	Triacylglycerides
C15	Pentadecanoic acid
C16	Hexadecanoic acid
C18	Oleic acid

Contents

Abstract

Biofuel is a powerful energy source to replace fossil fuels and has received much attention in recent years. This project used microalgae *Chlorella Sorokiniana* UTEX-1230 to produce the biodiesel. The process included cell cultivation, harvesting, lipid extraction, and transesterification.

There are two different processes that have been evaluated in this project: the base line and the modified process. These two processes have the same biofuel production steps including cultivation, harvesting, lipid extraction, and transesterification. In this project, different conditions were used in cultivation and the highest lipid content obtained was ~50% (w/w) after eight cultivation days. After cultivation, the biomass was harvested for the lipid extraction and the transesterification. The base line process, which was the benchmark, was established using centrifugation followed by ultrasonication, and acid catalysed transesterification. This process can reach high lipid extraction yield (~50%) and conversion performance (90%), but the energy requirement was relatively high. Thus, a modified processes were established. A flocculation step was added before centrifugation to reduce the sample volume, which can reduce the energy requirement. Enzymes or ethanol were used in cell breaking stage and lipid conversion stage to decrease the energy input. Moreover, chloroform was replaced by hexane in the modified process during lipid extraction due to the low cost, easy of recovery, and low toxicity. Then, enzyme transesterification was used to replace the acid transesterification. The modified process aimed to have the same process performance and lower energy input than the base line.

Two modified process where established in this project to reduce the energy input with wet biomass and have a similar FAME produce performance. The base line performance was 48.26%. The modified process 1 performance was 17.57% and modified process 2 performance was 17.26%. The performances of modified processes were still lower than that of base line. However, the energy requirements of modified process 1 (11.40% of base line) and modified process 2 (12.08% of base line) were much lower than base line. Besides, when not considering about the waste of cultivation material and with the same energy input, the power output ratio of modified process 1 to baseline was 325%. The power output ratio of modified process 2 to baseline was 275%. The power output ratio of modified process 2 was lower than modified process 1 and both modified processes were higher than the baseline. These results indicated that in this project, both modified processes can have higher energy output than the baseline when under same energy input.

Impact statement

Biofuel is a powerful energy source to replace fossil fuels and has received much attention in recent years. Using microalgae to produce the biofuel is a highly efficient way because the microalgae can be cultivated in large-scale with high lipid content in a short cultivation cycle. However, most biofuel production processes were established on dry biomass, which required much energy input for drying the microalgae cells. This project aimed to establish lipid extraction processes on wet microalgae cells to reduce the energy requirement. Moreover, the enzymes were used to replace the acid used in transesterification, which will reduce the chemical input and energy input because the enzyme transesterification required lower temperature.

This project can help the biofuel production process establishment with less complex steps and lower energy input. Moreover, the chemical used was more environmentally friendly when comparing to the traditional biofuel production process. This project also suggested that the wet biomass can be used in the biofuel production instead of dry biomass.

Acknowledgement

Here I would like to extend my utmost gratitude to both my supervisors, Dr. Frank Baganz and Dr. Brenda Parker. Throughout my MPhil years, Frank has always made himself available for support, while giving me the advices to complete my research. Through working with Frank (with considerable time investment and patience on his part), I have learned what were required to perform the biofuel production research. Brenda has also been an extremely supportive mentor throughout my research. She is a great listener and problem solver. With her breadth of expertise, she often provided wise nuggets that helped to overcome the obstacles I had during the project. This journey would have been impossible without the constant supports from either of them.

I would like to thank all my team members in Algal Group, past or present, particularly Dr. Max CF and Dr. Zhongdi Song, for the lab guidance when I started my degree; Milena Rivera, and Ricaardo Heredia, for many helping during the research; Victor Sanchez Tarre for sharing his knowledge and passion about microalgae. I would also like to thank Anabela for being a great company in the university. To Yiheng Yang and Chris Wayne, it has been my great pleasure to meet and to work with all of you and thank you all for the opportunities to lead a research.

Last but not the least, I would also like to thank my parents back in China. For without their supports, I would not be able to undertake this journey in United Kingdom without any reservation!

List of tables

Table 17. Particle size distribution of microalgae of all the samples.

Table 18. The particle distribution of initial microalgae and the particle distribution of microalgae after sonication. Dx (10) means 10% of the particles were below this size. Dx (50) means 50% of the particles were below this size. Dx (90) means 90% of the particles were below this size.

Table 19. particle distribution of microalgae of all the measurements. In this experiment, the microalgae were treated by enzymes. Dx (10) means 10% of the particles were under this size. Dx (50) means 50% of the particles were under this size. Dx (90) means 90% of the particles were under this size. For the other groups, the Dx (10), Dx (50), and Dx (90) had the same meaning.

Table 20. Particle distribution of microalgae of all the samples. In this experiment, the microalgae were aggregated by flocculation and then enzymes were added to break the cell walls. Dx (10) means 10% of the particles were under this size. Dx (50) means 50% of the particles were under this size. Dx (90) means 90% of the particles were under this size. For the other samples, the Dx (10), Dx (50), and Dx (90) had the same meaning.

Table 21. The clarification level for each group. In the first group, the microalgae were firstly aggregated by chitosan and then the enzymes were added to break the cells. In experiment 2, the microalgae cells were treated by enzymes at first and then, the chitosan was added to aggregate the cells.

Table 22. Saccharification yield after enzyme treatment and acid hydrolysis. In the first experiment, the microalgae were firstly aggregated by chitosan and then the enzymes were added to disrupt the cells. In experiment 2, the microalgae cells were treated by enzymes at first and then, the chitosan was added to aggregate the cells. In control experiment 3, the sugars were released by acid hydrolysis.

Table 23. The analysis of variance between each group. The saccharification yields from table 22 were used for the analysis of variance. The “a” value is the significance level.

Table 24. The comparison of the extraction yield between the two groups. For the first group, the microalgae cells were concentrated by flocculation first. Then, the microalgae cells were treated by the enzymes. For the second group, the microalgae cells were treated by the enzymes firstly and then concentrated by the flocculation.

Table 25. The FDE (factorial design experiment) groups and the results. The three factors and levels were: pH (3 & 5.5), reaction time (4 hours & 8 hours), and the enzyme concentration (2 & 6 ug/g DCW). The results were analysed by GC-FID and compared with the Nile red results.

Table 26. the ANOVA of selected factorial model.

Table 27. The optimal conditions for enzymatic treatment.

Table 28. The comparison of extraction yield when different extraction solvents were used. In the three experiments, the flocculation was used to aggregate the cells and enzymes were used to break the cells.

Table 29. The amount of lipid (g) after using different ethanol volume (3ml, 5ml, and 10ml) in first extraction, second extraction, and third extraction.

Table 30. The extraction yields when using ethanol in cell wall treatment and hexane in lipid extraction. 1.1 represented the extraction yield after the first-time cell wall treatment and the first-time lipid extraction. 1.2 represented the extraction yield after the first-time cell wall treatment and the second time lipid extraction. 2.1 represented the extraction yield after the second time cell wall treatment and the first-time lipid extraction. 2.2 represented the extraction yield after the second time extraction and second cell wall treatment.

Table 31. The transesterification yield of acid transesterification. C15, C16, and C18 were added in samples and concentrations determined by GC-FID.

Table 32. The results for enzymatic esterification after 6 hours using factorial design experiment. C15, C16, C18, C18:1 and C18:2 was used in these samples. The three parameters were: the methanol: FFA ratio (mol: mol), the water content, and the lipase: FFA amount (w/w%).

Table 33. The results for enzymatic esterification after 24 hours using a factorial design experiment. C15, C16, C18, C18:1 and C18:2 was used in these samples. The three parameters were the methanol: FFA ratio (mol: mol), the water content, and the lipase: FFA amount (w/w%).

Table 34. the ANOVA analysis for factorial design experiment model after 6 hours' reaction.

Table 35. The optimal conditions for 6 hours reaction.

Table 36. the ANOVA analysis for factorial design experiment model after 6 hours' reaction.

Table 37. The optimal conditions for 24 hours reaction.

Table 38. The results of the response surface methodology experiment after the reaction time of 6 hours. C16 and C18 were used in these samples. The three factors were: the FA/ solvent concentration (mg/mL), the methanol: FFA ratio (mol: mol) and the lipase: FFA amount (w/w%). The responses were the esterification yields of MC16, MC 18, and the total esterification yield from GC-FID.

Table 39. The results of the response surface methodology experiment after the reaction time of 24 hours. C16 and C18 FFAs were used in this experiment. The three parameters were: the FA/ solvent concentration (mg/mL), the methanol: FFA ratio (mol: mol) and the

lipase: FFA amount (w/w%). The responses were the esterification yields of MC16, MC 18, and the total esterification yield from GC-FID.

List of figures

Figure 16. Lipid and cell concentration of C. Sorokiniana cultivated in TAP medium with glucose (11g/L) under heterotrophic conditions. The error bars represented standard deviations of the mean (n=3).

Figure 17. Particle distribution of microalgae before and after flocculation. The microalgae were treated by chitosan.

Figure 18. Particle distribution of microalgae of all the samples. In this experiment, the microalgae were treated by enzymes firstly to disrupt the cell walls and then chitosan was added to aggregate the cells.

Figure 19. The particle distribution of microalgae before and after sonication.

Figure 20. Particle distribution of microalgae of all the measurements. In this experiment, the microalgae were treated by enzymes.

Figure 21. Particle distribution of microalgae of all samples. The microalgae were treated by flocculation firstly and then by enzymatic treatment.

Figure 22. FAMEs analysis by GC-FID after conversion. In the first experiment, the centrifuge was used to harvest the microalgae cells. Then, sonication was used to break the cells. The chloroform-methanol (2:1v/v) mixture was used as extraction solvents, which was the same solvents that were used in experiment 2 and experiment 3. In experiment 2, the flocculation was used to concentrate the cells and enzymes were used to break the cell walls. In experiment 3, the flocculation was used to aggregate the cells and there was no cell breaking method.

Figure 23. the normal plot of selected factors from Design Expert 10 analysis.

Figure 24. the normal plot of residuals.

Figure 25. The 3D surface of factor A(pH), factor C (enzyme concentration; μl/g DCW), and the extraction yield after enzymatic treatment when factor B (time; hours) was at 4 hours.

Figure 26. Comparison of lipid extraction performance after using different cell treatment methods, reaction time, and extraction solvents. For these experiments, the flocculation was used to aggregate the cells. For the cell treatment, the enzymes were used in the experiment: 1 and 3. In experiment 2 and 4, the enzymes were not added, but the samples were kept at the same conditions. In experiment 1 and 2, the reaction time was 6 hours and in experiment 3 and 4, the reaction time was 24 hours. The extraction solvents used were hexane and methanol.

Figure 27. The lipid concentration in bottle 1 (3ml ethanol as treatment solvent) during cell wall treatment. ■ represented the first 24 hours treatment. ● represented the second 24 hours treatment. ▲ represented the third 24 hours treatment. The error bars represented the triplicates of the experiment.

Figure 28. The lipid concentration in bottle 2 (5ml ethanol as treatment solvent) during cell wall treatment. ■ represented the first 24 hours treatment. ● represented the second 24 hours treatment. ▲ represented the third 24 hours treatment. The error bars represented the triplicates of the experiment.

Figure 29. The lipid concentration in bottle 3 (10ml ethanol as treatment solvent) during cell wall treatment. ■ represented the first 24 hours treatment. ● represented the second 24 hours treatment. ▲ represented the third 24 hours treatment. The error bars represented the triplicates of the experiment.

Figure 30. The methyl esters composition after chloroform -methanol (2:1, v/v) extraction

Figure 31. The methyl esters composition after extraction when using hexane as extraction solvent.

Figure 32. The methyl esters composition after transesterification when using the ethanol-hexane (1:1 v/v) as extraction solvents

Figure 33. The 3D surface when the methanol: FFA molar ratio (parameter B) and the FA/ solvent (mg/ml) (parameter A) were parameters for the first 6 hours. The lipase: FFA ratios were at 10%, 30%, and 50%.

Figure 34. The 3D surface when the lipase: FFA amount (parameter C) and the FA/ solvent (mg/ml) (parameter A) were as parameters. The methanol: FFA molar ratio was at 9:1, 12:1, and 15:1 w/w.

Figure 35. The 3D surface when the lipase: FFA amount (parameter C) and the methanol: FFA molar ratio (parameter B) were the variables. The FA/solvent (mg/ml) (parameter A) were at 19.23mg/ml, 57.42mg/ml, and 76.9mg/ml.

Figure 36. The 3D surface when the methanol: FFA molar ratio (parameter B) and the FA/ solvent (mg/ml) (parameter A) were parameters after 24 hours. The lipase: FFA amounts were at 10%, 30%, and 50%.

Figure 37. The 3D surface when the lipase: FFA amount (parameter C) and the FA/ solvent (mg/ml) (parameter A) were parameters for 24 hours reaction. The methanol: FFA molar ratio was at 9:1, 12:1, and 15:1.

Figure 38. The 3D surface when the lipase: FFA amount (parameter C) and the methanol: FFA molar ratio (parameter B) were parameters. The FA/solvent (mg/ml) (parameter A) were at 19.23mg/ml, 54mg/ml, and 76.9mg/ml.

Figure 39. The transesterification yield change after extraction over 6 hours with two different solvents: hexane and t-butanol. The ● represented the yield when using hexane as a solvent and ● represented the yield when using t-Butanol as solvent.

Figure 40. The energy input for base line process (per 5mL).

Figure 41. The energy input of the modified process 1 (per 5 mL sample). The energy requirement presented for each step was based on 5mL sample.

Figure 42. The energy input of the modified process 2 (per 5 mL sample). The energy requirement presented for each step was based on 5mL sample.

Figure 43. OD-dry mass curve by dilution. The optical density and the dry mass were measured by dilution. The day 7th culture was diluted and the concentration after dilution were: 10%, 20%, 25%, 50%, 75%, and 100% (raw material) to the raw material. Then, the linear ship between these two parameters was: Y=0.2031X, where Y was dry mass (g/l) and X was optical density. The R2 for this curve was 0.9849.

Figure 44. OD-dry mass curve by everyday measuring. The optical density and the dry mass were measured every day during cultivation. Then, the linear ship between these two parameters was: Y=0.2009X, where Y was dry mass (g/l) and X was optical density. The R2 for this curve was 0.9604.

Figure 45. the peaks that has been detected by GC-FID. There were 5 main peaks and some small peaks. The main peaks were: C15 (internal standard), C16, C18, C18:1, and C18:2.

Figure 46. The interaction between the acid concentration and the esters yield when the methanol: FFA molar ratio was at 9:1 and 15:1 and the lipase amounts were at 10%,30%, and 50% w/w.

Figure 47. The interaction between the acid concentration and the esters yield when the methanol: FFA molar ratio was at 9:1 and 15:1 and the lipase amounts were at 10%,30%, and 50% w/w.

1. Introduction

1.1 Background

The demand for energy has sharply increased in recent years. Fossil fuels, formed from prehistoric plants and animals that died hundreds of millions of years ago, provided over 88% (J/J) of the energy demanded in 2010 (Brennan L. and Owende P., 2010). However, fossil fuels are not a renewable energy source and have a limited availability. It was reported that the fossil fuels, including petroleum, coal and natural gas, will last less than another 50 years at current energy using rate. Thus, it is necessary to develop renewable energy sources to meet the growing demand (Demirbas, A., 2017). In addition, the combustion of fossil fuels will release carbon dioxide and other pollutants, which has a significant influence on the environment. Thus, many efforts have been made to develop renewable fuels. It was reported that wind, solar, geothermal, and marine energy sources are sustainable and can be the most likely energy source in future because these sources are more environmentally friendly compared to fossil fuels. Among all the energy sources, the biofuel energy source is very important as the biofuel can replace the fossil fuels. Moreover, the biofuel will produce no net carbon dioxide into the atmosphere as all the carbon released was from the environment (Demirbas A., 2017).

Biofuels can be divided into three groups: the solid fuels such as biochar, the liquid fuels such as ethanol and gaseous energy such as biogas (Mubarak et al., 2017). The first generation of bio-liquid fuels was produced by terrestrial crops such as the soybean, jatropha, and corn. However, because the demand of food source and the requirement of the arable land for cultivation and living, those biofuels were not sustainable (Daroch, M. et al., 2017). Thus, using microalgae to produce biofuels received much attention. Microalgae have been living on earth for over 450 million years. They have mechanisms for energy conversion, and they can survive even in an unsuitable environment (Falkowski, P. and Raven, J, 2007). Microalgae are primitive plants and mainly aquatic and grow well in saline, brackish and wastewater that are unsuitable for food crops. These make it possible to reduce the arable land used and it is possible to develop a method for large-scale algae cultivation. Also, the microalgae can grow faster than plants and have only simple growth requirements such as lights, sugars, carbon dioxides, and nitrogen source. Besides, using microalgae to produce biofuel in wastewater can remove the nutrients, which can help the wastewater treatment (Chisti Y., 2007). Moreover, the microalgae have a faster growth rate than food crops and they can grow all year round with nearly no influence by the seasons, which will maximize the whole period of production and have a higher economic effectiveness. It is reported that the green algae have a doubling time of less than 24h and even as short as 3.5 hours and can often have a lipids content of more than 50% (w/w) (Chisti Y., 2007). Furthermore, microalgae can have a high biomass production with a high content of lipids, which often is higher than 60% (w/w) of the dry biomass with a high efficient carbon dioxide fixing (Sheehan et al., 1998). Table 1 shows the

oil content per hectare of different crops and from which, the oil content of algae is the highest by several orders of magnitude. (Demirbas A. and Fatih Demirbas M., 2017).

Table 1. Yield of various plant oil content (Demirbas A .and Fatih Demirbas M., 2017; Katsumi Y., and Hiroshi N.,1987; Parsons, S., and Raikova, S.,2020.).

Crop	Oil in Litres per hectare	Lipid compositions (number of carbon atoms in the fatty acid chain: number of double bonds in the chain)				
		Medium chain esters (8:0-14:0)	Palmitic (16:0)	Stearic (18:0)	Mono-unsaturated (16:1, 18:1)	Poly-unsaturated (18:2, 18:3)
Algae	100000	1.7	4.2	1.6	85	2.1
Coconut	2689	82	-	3	6	2
Palm	5950	-	45	5	38	11
Soy	446	0	11	4	22	62
Sunflower	952	0	6	5	20	69
Corn	172	0	13	3	31	53

The algal organisms, they can be divided into 2 groups, the macro algae and the microalgae, which can both grow in salt or fresh water. Because of different pigmentation, the microalgae can also be divided into 3 groups: brown seaweed (*Phaeophyceae*), red seaweed (*Rhodophyceae*), and green seaweed (*Chlorophyceae*). The carbon dioxide will be fixed inside of the algal cells by photosynthesis (John P., 1986). For the natural microalgae, the lipids and fatty acids content can be as high as 55% (w/w) and can be almost everywhere inside the microalgae, from the membrane components, storage products, to the sources of energy (Gong Y., 2020). Table 2 shows the lipids content of samples from different microalgae (Demirbas A. and Fatih Demirbas M., 2017). The highest lipid content was found in Prymnesium parvum and then Chlorella vulgaris. The lowest lipid content was found in Chlorella pyrenoidosa species and the lipid content was around 2%, which was not considered in this project. In addition, there are some additional products. Some microalgae will produce the polyunsaturated fatty acids (omega-3 and omega-6). These are often found in fish oils as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). These can make the marketability larger for the algae as the additional products can be used as feed or fertilizer after the oil extraction (Vazhappilly R. and Chen F., 1998).

Table 2. Lipids contents of different algae on a dry matter basis (% w/w) (Demirbas A. and Fatih Demirbas M., 2017).

Species	Lipids content (%, w/w)
Chlorella vulgaris	14-22
Chlorella pyrenoidosa	2
Prymnesium parvum	22-38
Spirulina platensis	4-9
Spirulina maxima	6-7
Anabaena cylindrica	4-7

However, although the microalgae have numerous advantages, their use to produce biofuels still has some barriers. Firstly, the species of the microalgae should be chosen very carefully because different species will have different lipid content, which will make a difference for biofuel production. Then, the growth conditions for the microalgae should be considered carefully. The way of growing microalgae will influence the final lipid content and the economic efficiency of the biofuel process because the energy required are not the same for heterotrophic and autotrophic conditions (Gong Y., 2020). Moreover, there is not a lot of data for large scale microalgae culturing because using the microalgae to produce biofuels is still a relative new approach and there are still a lot of details to decide. Also, the whole process still needs to be developed into a more economically efficient process. For example, the growing of the microalgae might be influenced by available sunlight, which makes it a limiting factor for the outdoor cultivation process, thus the artificial lighting should be considered as an additional energy source. However, the artificial lighting is too expensive for low value products like biodiesel, which requires the balance of the benefit and the total input.

The typical process of biofuel production was the base line of this process. The steps of this process were: cultivation, harvesting, cell breakage (cell wall treatment), extraction and transesterification. The different cultivation conditions can provide different lipid contents of microalgae, and the condition that can provide the best lipid content will be chosen for the next steps. This base line had high power input but high performance. The productivity of it can provide a base performance of the biofuel production.

1.2 Cultivation

Microalgae can have two modes of cultivation, the autotrophic mode and the heterotrophic mode. The autotrophic mode requires light as energy source but only needs inorganic compounds, such as carbon dioxide and salts. The heterotrophic mode requires organic compounds as nutrients to provide the energy for growth. However, light energy is not required. Also, some microalgae can grow both autotrophically and heterotrophically and require organic nutrients and lights. This mode is called the mixotrophic cultivation (Lee R., 2009).

In many cases, the lipids can be accumulated inside microalgal cells. The growth temperature, pH, nutrient (carbon source, phosphorous source, nitrogen), light, and the microalgae ages can affect the accumulated lipids (Wen Z. and Chen F., 2003; Chisti Y., 2007). It was reported that for *Chlorella Sorokiniana*, the accumulation of lipids can be affected

by the sugar feeding during cultivation, which represented that if the rate of sugars consumption was higher than the rate of cell generation, the microalgal cell would convert the excess sugar into lipids (Chen et al., 1991;). Also, there was a survival response when microalgal cells under a nitrogen source limitation or silicate source limitation. The lipids accumulation occurred under this survival response (Wilhelm et al., 2006). In the nitrogen limitation condition, the lipid accumulation was directly dependent on the remaining sugars. The more sugars remained the more lipid accumulated (Wen Z. and Chen F., 2003). Besides, the microalgae under heterotrophic condition can accumulate more lipids than under autotrophic condition. This was because the energy from triglycerides oxidation under heterotrophic growth was much higher than that from polyunsaturated fatty acids. Besides, the autotrophic growth would produce more unsaturated fatty acids. It was reported that more unsaturated acid inside biodiesel would have a lower cetane number and heating value respectively (Day M. et al., 1991; Gladue K. and Maxey M., 1994).

1.2.1 Photoautotrophic production

1.2.1.1 Mechanisms

The lipids or oil bodies (triacylglycerol) are produced inside the cells with autotrophic growth as shown in figure 1 and most of them are stored in the cytoplasm while some of them are in chloroplasts. The Calvin cycle happening inside the chloroplast can fix the carbon from carbon dioxide and release oxygen. The fixed carbon can provide the carbon source to form triacylglycerol or sugars inside the chloroplast.

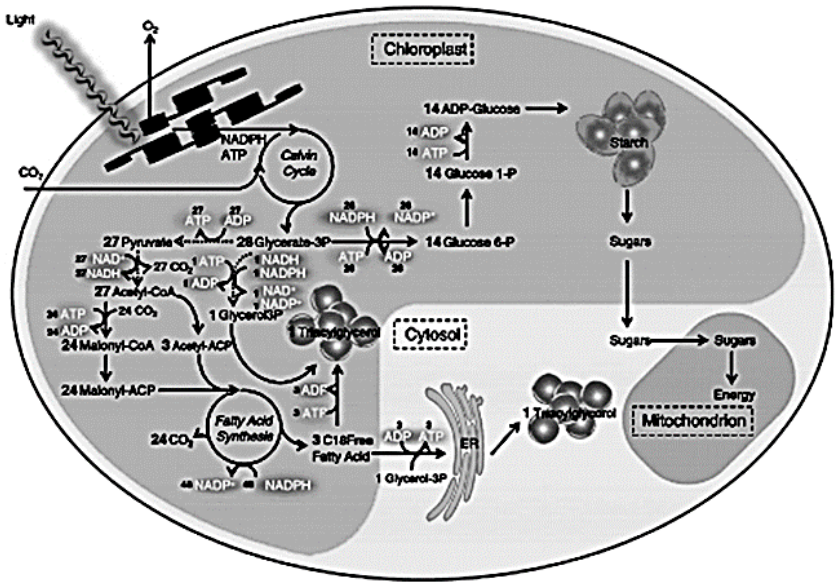

Figure 1. Simplified triacylglycerol and starch metabolism in microalgae with autotrophic growth (Jaeger, L. et al., 2014).

Most of the lipids produced by microalgae are neutral lipids and they are surrounded by a monolayer of phospholipids and some proteins. The proteins can play a distinct role and can transport the lipids across the cells. The lipids can be used as a secondary energy source within the cell cycle. Besides, the lipids not only can be used as energy and carbon source but also help the microalgae cell to adapt to different environmental conditions. The long-chain fatty acid can help the rearrangement of cellular membranes and the lipophilic carotenoids which was made from the lipids can create an optical screen that the microalgae can be protected from photo-damage when under a high light intensity environment. (Shibata et al., 2009; Shibata et al., 2010; Yang et al., 2012)

1.2.1.2 Photoautotrophic production systems

For the photoautotrophic production, the open pond production system and photobioreactor system have been deployed to produce the microalgae biomass. For the open pond production system, the raceway ponds are the most typically used system for microalgae growing (Jiménez et al., 2003). It is a closed loop system, which will be used by the natural waters (lakes, lagoons, and ponds) and containers as shown in figure 2. In a continuous production system, the medium which provides the nutrients is pumped into the system with the algae broth in front of the paddlewheel and will spend time travelling through the loop and will be harvested at the end. The natural sunlight is used to provide the energy for the microalgae biomass formation and although a carbon dioxide system exists on the surface of the loop, the aerators might still be needed to increase the carbon dioxide usage rate (Terry and Raymond, 1985).

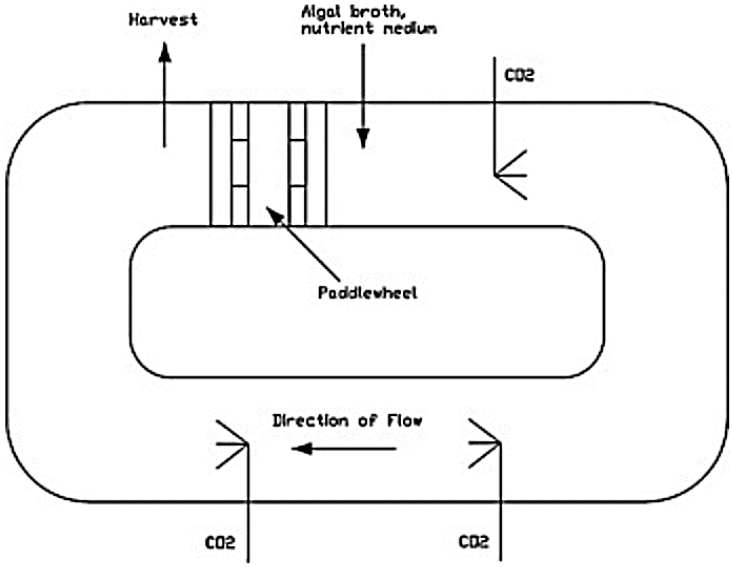

Figure 2. The open pond system (Terry and Raymond, 1985)

An open pond system is cheap and can be cleaned very easily. Also, it does not require a high energy input because the sunlight is used. This is both convenient and energy effective during large-scale culturing (Ugwu, Aoyagi and Uchiyama, 2008). But when using the open pond system, the operators should take care of the contamination from the other algae species and protozoa, which makes the environment of the systems very strict. Despite the strict environment, the strains of the microalgae should be selected very

carefully because many microalgae strains are not suitable for monoculture cultivation. For example, the species Chlorella is adaptable in media with a high level of nutrients in monoculture cultivation, as the species Spirulina can survive when cultured in high alkalinity media in monoculture cultivation (Borowitzka, 1999). Microalgae cultivation of open pond systems is less efficient when compared with closed photobioreactors, this is because of the evaporation losses, the temperature fluctuation, the carbon dioxide deficiencies, and the light limitation during cultivation (Chisti Y., 2007).

Closed photobioreactor systems can be divided into tubular photobioreactor system, flat plate photobioreactor system, and column photobioreactor system. A closed photobioreactor can have less risk of getting contaminated by the other species of microalgae and the protozoa, which makes the environment for culturing much easier to achieve. The closed photobioreactor systems often have a higher biomass content compared to open pond systems, which makes the cost during harvesting lower (Carvalho, Meireles and Malcata, 2006). Because of these advantages, the closed culturing systems were considered as a highly efficient system and table 3 shows the advantages and the limitations of the open pond systems and closed photobioreactor systems (Brennan and Owende, 2010).

Table 3: The advantages and limitations of different photobioreactor systems (Brennan and Owende, 2010).

Production system	Advantages	Disadvantages
Tubular photobioreactor	Relatively cheap	Fouling
	Good for outdoor cultivation	Requires large space
	Large surface area	pH, dissolved oxygen and co_2 are not the same along inside the photobioreactor
	Good biomass productivities	
Flat plate photobioreactor	Good biomass productivities	Difficult to scale-up
	Large surface area	Difficult temperature control
	Good for outdoor cultivation	Small degree of hydrodynamic stress
Column photobioreactor	High mass transfer	Small surface area
	Easy to sterilize	Expensive compared to open pools
	Compact	Shear stress

A hybrid production system was developed to improve the microalgae biomass, which combined the open pond systems and closed photobioreactor systems. The first step of the hybrid production system was the photobioreactor step, which can minimize contamination. When the microalgae biomass reached a certain level, the second production stage, which was the open pond system, begins. The second production stage can stimulate the microalgae to have a higher lipid content because the second step environment was more suitable for the microalgae to produce lipids. Thus, the

hybrid production systems had a higher lipid content when compared to the open pond systems and closed photobioreactor systems respectively (Huntley and Redalje, 2006).

1.2.2 Heterotrophic production

1.2.2.1 Mechanisms

Some microalgae can grow under the heterotrophic conditions, the required characteristics have been listed below (Chen G. and Chen F., 2006):

1. The microalgae cell division and metabolisms can be active without lights
2. Can easily adapt to the environment change
3. Can use the organic source as an energy source in the absence of photosynthesis.
4. Strong enough to survive from the hydromechanical stress during cultivation

Glucose was the most used organic source in microalgae heterotrophic growth because glucose produced more energy (w2.8kJ/mol) compared to other organic acids such as acetate (w0.8kJ/mol). Many other substrates such as sugars, sugar alcohols, and organic acids were used in heterotrophic growth although these substrates give lower cell growth rates (Boyle and Morgan, 2009). It was reported that heterotrophic culture of *C. pyrenoidosa* generated more ATP from the energy supplied as glucose than the autotrophic and mixotrophic cultures with energy supplied as light (Yang F. et al., 2000).

For aerobic dark conditions, glucose is mainly metabolised through the Pentose Phosphate Pathway (PPP) while the EMP pathway is the main glycolytic process of cells in light conditions (Yang F. et al., 2000). Figure 3 shows the heterotrophic metabolism inside the microalgae and only the important pathways for heterotrophic growth are shown. The first step of oxidative assimilation of glucose is the phosphorylation of hexose. Glucose-6-phosphate is produced which can support cell synthesis and respiration. During this step, one mole of phosphate bond is required for one mole glucose assimilating to glucose-6-phosphate. Then the glucose-6-phosphate will be converted to pyruvate in the cytoplasm through Pentose Phosphate Pathway (PPP) and release NADPH and ATP.

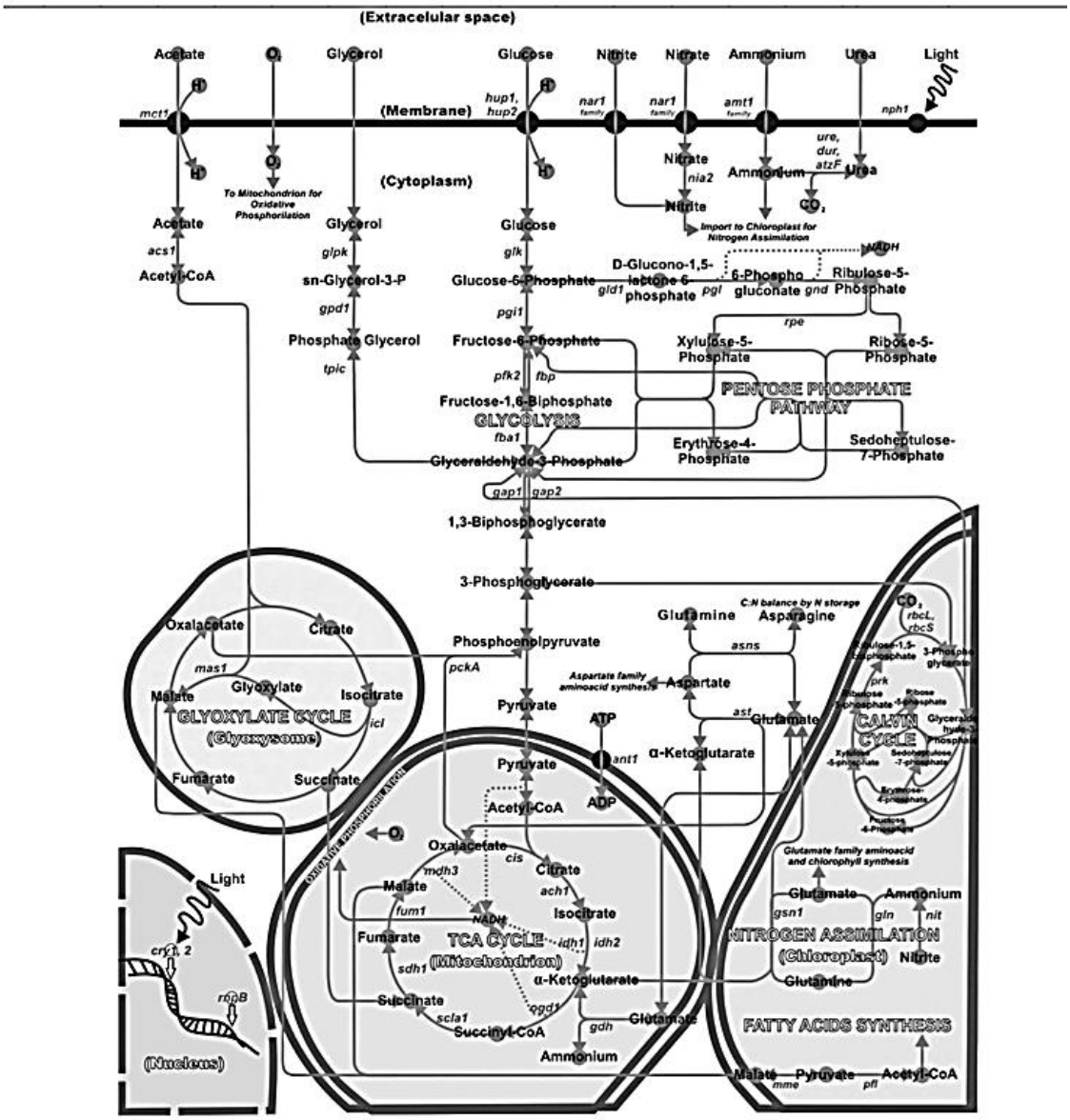

Figure 3. The heterotrophic metabolism in the microalgae. Only the important pathways for heterotrophic growth are shown (Perez-Garcia, O., et al., 2011). The glycolysis, and the assimilation of acetate, nitrite, ammonium, and urea via the glyoxylate cycle, the TCA cycle, fatty acids synthesis, and the Calvin cycle are shown.

It was reported by Yang et al., (2000) that for *Chlorella Sorokiniana*, the PPP pathway (Pentose Phosphate Pathway) via glucose-6-phosphate dehydrogenase (EC:1.1.1.49) accounts for 90% (w/w) of glucose assimilation while the EMP pathway, which is catalyzed by glucose-6-phosphate isomerase (EC:5.3.1.9) is totally turned off under darkness condition. Because *Chlorella Sorokiniana* can survive under both phototrophic cultivation and heterotrophic cultivation, and it can have relatively high lipid content, this species was used in this project. (Yang et al., 2000). The activity of Tricarboxylic Acid Cycle (TCA cycle) under darkness condition is at the same level of the light condition. This shows that the light has much less effect on this pathway (Yang et al., 2000). Moreover, the two trans-membrane amino acid transport systems can be induced by the glucose in the

medium and the uptake rate of neutral amino acids and basic amino acids is about 5exp10 times faster than the rates of other plant cells or algae. This can give a higher growth rate when comparing to the other plant cells or algae.

1.2.2.2 Assimilation of acetate

In heterotrophic condition, acetate can be used as carbon source as well. The monocarboxylic transporter protein can help to transport the monocarboxylic molecules across the membrane (Yangli Y., 2018). The acetylation occurs in the cytoplasm by acetyl-CoA synthetase (EC.6.2.1.1) and acetyl coenzyme A (acetyl-CoA) formed by consuming an ATP molecule (Boyle and Morgan, 2009). Then, there are two pathways for acetate, which carried by coenzyme A to be oxidized: the glyoxylate cycle and the tricarboxylic acid cycle (TCA cycle). The first pathway can form malate in glyoxysome by isocitrate lyase (EC.4.1.3.1) and malate synthetase (EC.2.3.3.9) and the second pathway can form citrate in mitochondria. The citrate can provide carbon skeletons and energy in further metabolism (Boyle and Morgan, 2009).

1.2.2.3 Heterotrophic production system

During heterotrophic production, the microalgae use the organic carbon source in the medium to produce the lipids. A conventional bioreactor can be used in a heterotrophic production because sunlight is not required. Thus, during the scale-up, the bioreactor surface to volume ratio is not a necessary parameter as there is no requirement of using sunlight energy, which makes the scale-up much easier (Eriksen N., 2008). Also, when utilizing heterotrophic production, the harvesting cost is much lower when compared to phototrophic production because the final cell density of heterotrophic production is much higher (Chen G. and Chen F., 2006). However, the cost of energy input during heterotrophic production is much higher than during phototrophic production (Chisti Y., 2007). According to the report of Miao and Wu (2006), the lipid content of the microalgae under heterotrophic production can be as high as 55%. When under autotrophic production, the lipid content is only around 15% (Miao and Wu, 2006). In this project, the heterotrophic production was the best choice of lipid production because the lipid content was higher than the autotrophic production.

1.2.3 Mixotrophic production

The mixotrophic production was also developed to produce lipids but this method was only for the microalgae that can use both metabolic processes (autotrophic and heterotrophic) for growth such as the *Spirulina platensis* and *Chlamydomonas reinhardtii* (Chen F., et al.,1996). During the mixotrophic production, the light energy and the organic carbon substrate were the parameters affecting the growth rate, which means the

microalgae were less sensitive to light variation or the kind of organic carbon substrate (Zhang X., et al., 1999). The microalgae can use the light to grow during the daytime and when they were in the darkness, the organic carbon substrate will be used. Thus, the biomass loss will be less during the dark phase and the final cell density will be higher than both the autotrophic and heterotrophic culture, which can make the harvesting more efficient (Chojnacka K. and Noworyta A., 2004).

1.2.4 Metabolism of nitrogen sources

Microalgae use ammonium as the most preferred nitrogen source during cultivation because less energy is required during uptake and the absent of light will not affect the uptake rates (Wilhelm et al., 2006). The ammonium assimilation forms amino acids such as glutamate, glutamine, and aspartate, and TCA cycle can provide the carbon skeletons (keto-acids) and energy (ATP and NADPH) for this step (Lea and Miflin, 2003). The glutamate synthase (GOGAT; EC1.4.1.14) and glutamine synthetase (GS; EC 6.3.1.2) can catalyse ammonium to glutamate and glutamine respectively under both autotrophic and heterotrophic conditions (Vanoni and Curti, 2005). Also, the glutamate dehydrogenase (GDH, EC 1.1.1.2) can catalyse ammonium into glutamate by the amination of ketoglutarate (Lea and Miflin, 2003).

1.3 Harvesting

When the cell density was high and the lipid content was at the required level, harvesting was the next step to separate the cells from the medium. The harvesting could include more than one step, such as flocculation, flotation, centrifugation, and filtration. It was reported that the harvesting step will cost 20-30% of the whole biofuel process (Olaizola, 2003). Thus, if reasonable harvesting steps were chosen, the entire cost for the biofuel production will decrease. Several parameters should be considered for choosing reasonable harvesting steps. For example, the size of the microalgae and the cell density of the final medium would influence the method used for harvesting (Olaizola, 2003). Moreover, the chosen of microalgae species should be considered because if the size of the microalgae was small, the harvesting would be difficult.

1.3.1 Centrifugation

The centrifugal force could separate the microalgae and the medium. The centrifugation technique was normally used for recovery of high-quality microalgae with a short time, and it required no chemical input (Mata Martins and Caetano, 2010). Thus, centrifugation was used in the base line to harvest the microalgae cells to have a high harvesting level. However, the centrifugation requires a high-energy input and some newly designed centrifuges for microalgae harvesting need large capital investment. Thus, some pre-

concentration methods were used to reduce the energy input. The micro flocculation or combining filtration and centrifugation will sharply reduce the energy needed and the cost of harvesting (Salim, Vermuë and Wijffels, 2012).

1.3.2 Flotation

Flotation is a technique that uses rising gas bubbles in the medium to capture the microalgae cells. The microalgae cells were harvested at the surface of media as the cells were carried up by the gas bubbles. The gas bubbles can be divided into three parts due to their size: nanobubbles (b1 μm), microbubbles (1–999 μm), and fine bubbles (1–2 mm) and the smaller size (Zimmerman, Tesař and Bandulasena, 2011). As the surface area to volume ratio increases, the bubble would last longer, rise slower, and have a larger carrying capacity. The diameter from 10μm to 500μm would be suitable for the flotation (Hanotu, Bandulasena, and Zimmerman, 2012).

Because the gas bubbles are hydrophobic negatively charged and the microalgae are hydrophilic negatively charged, how to change the microalgae cells' hydrophobicity or how to change the gas bubbles' hydrophobicity becomes the most important question. Using ozonizing can negatively charge the microalgae surface but can enhance the strength of the gas bubbles. Also, it would make the gas bubbles more hydrophilic to capture the microalgae (Cheng et al., 2010). The presence of cationic surfactant can make microalgae more hydrophobic, which would increase the efficiency of flotation.

1.3.3 Filtration

The filtration has several advantages, such as high efficiency of separation, simple operation, and it can be run as a continuous process. Therefore, filtration has become a widely used separation method in biofuel process. In addition, there are no coagulants during the filtration (Toh Y., et al., 2012). The constraint of membrane filtration is the membrane fouling. The membrane fouling can reduce the permeate of the membrane, and it was caused by the algal cake layer and the attachment of allogeneic organic matter (AOM) (Frappart et al., 2011). Hence, the cross- flow filtration was widely used as it can reduce the fouling. Moreover, the cross- flow filtration can also reduce the energy requirement and increase the efficiency of harvesting. For a system which includes cultivation, harvesting, and extraction together, membrane filtration is widely used because the water after harvesting can be recycled (Ríos et al., 2012).

1.3.4 Flocculation

Flocculation can be used as a pre-concentration step to increase the microalgae cell density to decrease the energy input (Mata Martins and Caetano, 2010). During flocculation,

the microalgae will form algal flocs and the flocs can be separated by gravity. As the surface of microalgae was negatively charged, the positively charged flocculants will be added during the flocculation. After the flocculation, the concentration of the material would increase. Thus, the energy requirement would be less if the centrifuge was used in the next step.

The choice of flocculants was important. Although the positively charged flocculants will help the microalgae flocs generating, the remaining of the flocculants after concentration should be considered (Zhang X. et al., 1999). The flocculants such as the aluminium salts and iron-based metal salts would not only require a high dosage but impacts the next steps (Estevez et al., 2001). The organic polymer flocculants such as chitosan, anionic polyacrylamide and cationic polyacrylamide required lower dosage and had less impact. Thus, these flocculants can be used in microalgae flocculation. This method can be used for marine microalgae, as the high ionic strength of seawater has a low impact on flocculants (Zheng et al., 2012). Chitosan was used in this project because it had low price and the harvesting performance of it was high. Besides, it can be easily found in nature. Supatchalee S. and Robert E. found that the settlement rate increased until the levels of chitosan were at 200mg/L, 5-200rpm and when the levels increased from 250rpm, the settlement rate decreased (Supatchalee S. and Robert E., 2015). It was also reported that when increasing the pH of flocculation culture, the flocculation efficiency will increase until pH equalled to 7, and this pH was used in this project as well. Also when increasing the chitosan concentration, the settling time was decreased（Ravi Divakaran and Sivasankara Pillai, 2002）.

Although there are several techniques of harvesting, there are still some barriers. The key bottlenecks of microalgae harvesting are the similar microalgae density to water density and the small average size of microalgae. Thus, different harvesting techniques could be combined to cover the shortage of individual techniques and table 4 shows the different combination of the separation techniques.

Table 4. The combination of different separation techniques.

Combination	Performance	Reference
Bio-flocculation and centrifugation	90% of centrifuge energy input has been reduced using S. obliquus of bio-flocculation before centrifugation	Salim et al. (2012)
Filtration and centrifugation	90% of centrifuge energy input has been reduced using filtration before centrifugation	Salim et al. (2012)
Inorganic flocculation and flotation	99.2% of flotation energy input has been reduced by using metallic coagulant before	Hanotu et al. (2012)

1.4 Cell breakage

The microalgae cell breakage required constant pressure and temperature condition, which would need energy input. The main energy input was to break microalgae's rigid and thick cell wall. When doing the lipid extraction of dry biomass, a mixture of a non-

polar solvent and a polar solvent can be used but the energy needed to dry the biomass is high and a way that does not need to dry the microalgae should be developed (Bligh and Dyer, 1959). However, the wet biomass has a low yield of lipid extraction when the same method is used. This is due to the microalgae remaining in the water phase due to their surface charges, which prohibits contact with the organic phase and therefore inhibits lipid extraction. Thus, the cell breakage step needed.

The methods for cell disruption contain the mechanical methods, the chemical methods, and the biological methods. Currently, there is no efficient microalgae cell disruption method that has been developed for wet biomass, especially for a large-scale process. To compare the different ways of microalgae cell disruption, the standard of material, energy and time used should be established.

1.4.1 Mechanical methods

The mechanical method is the way to break the microalgae directly using physical force such as ultrasonication, bead beating, high-pressure homogenization (HPH), and electroporation (Harrison, 1991).

When using a mechanical method to rupture the microalgae, the species of the microalgae was not important and the risk of degradation or degeneration can be ignored. Some mechanical methods, such as grinding, cannot be used with wet biomass (both microalgae paste and the microalgae suspension with a water ratio over 60%). Some methods can be used for cell breakage using wet microalgae cells. For example, the ultrasonication technique can be very effective for wet microalgae cell disruption. Therefore, the choice of disruption method is very important in the process.

1.4.1.1 Ultrasonication

Ultrasonication uses the ultrasound to radiate to liquid media and small "cavitation" will be generated. The cell envelopes will be damaged by the shockwaves, heat, sonic luminescence, and free radicals. These are generated when the ultrasound is strong enough which makes the microbubbles become smaller and implode (Miller, Miller and Brayman, 1996).

Ultrasonication can be the best cell disruption method for some algal species because it was rapid and efficient. However, during the ultrasonication rupturing process, the temperature of the media would increase very quickly so that a continuous cooling system was necessary to keep the media temperature at a constant level. Moreover, the power requirement during ultrasonication rupturing was high. Therefore, it is not suitable for a large-scale process (Jiang, Y. et al., 2006). Another reason that the ultrasonication cannot be used in a large-scale process is the cavitation, which causes breakage to be restricted to small regions near ultrasonic probes. Thus, ultrasonication was used in the base line in lab scale to have a high cell breaking performance with a high energy input.

1.4.1.2 Bead beating

Bead beating uses the quartz or metal beads to destroy the microalgae cells. The collision or friction is the force that makes the cells break. The factors that will affect the bead beating include the container shape, the shaking rate, the bead size, the number of beads used, and the types of beads (Cheng et al., 2010). The equipment is simple and can be provided to cells without any preparation, which made the bead beating a very useful method in microalgae rupturing. Moreover, the bead beating method can result in complete cell wall rupture in a short amount of time (Cheng et al., 2010). However, the bead beating method is hard to scale up and a cooling system is needed to prevent thermal degradation of the target product, which will cause a high cost in the lipid extraction process.

1.4.1.3 High-pressure homogenization

High-pressure homogenization is a rupturing method using the hydraulic shear force, which occurs when the medium is under high pressure and is sprayed through a narrow tube. During the rupturing process, the temperature in the high-pressure homogenizer will not increase sharply, which means there is little risk for degradation of the microalgae, and it will need low cost for cooling (Sheng, V. et al., 2012). Moreover, it is easy to scale up a homogenizer. For the microalgae cell rupturing using a high-pressure homogenizer, higher pressure and higher microalgae cell concentration can cause a higher efficiency. However, high-pressure homogenizer also has disadvantages. The time of cell rupture is long, and the energy input is large as a high hydraulic shear force will be provided. For this method, increasing the efficiency and decreasing the power input are the most important targets to develop a better downstream process for biodiesel production (Zheng et al., 2011).

1.4.2 Chemical methods

Chemical methods can disconnect the chemical linkages on the cell envelope or break the osmotic pressure. The chemical treatments often used acids, alkalis, and surfactants. The energy input for chemical methods was low because there was no need for heat input. However, it requires the chemical input. In this project, ethanol was chosen as the cell lysis solvent for it had low price and can act as the extraction co-solvent in the extraction step.

Ethanol was approved as a safe solvent and was used as cell wall treatment and extraction solvent in Fajardo et al.'s report on dry cell mass (Fajardo.A.R.et al, 2007). In Gonzalez et al.'s report, the ethanol was used to extract lipids from wet biomass (Gonzalez lez M.J., et al, 1998). However, their experiment required high temperature. In Yang's study

(Yang F. et al, 2014), the ethanol was used at room temperature and required no dewatering step, which means this process can require less energy input than the other studies.

Ethanol is a cheap solvent and has an affinity with lipids. Thus, ethanol can bind the membrane-associated lipids, which are strongly linked with the protein in the membrane (Halim, R. et al, 2012). Besides, ethanol can easily pass through the cell membrane into the cytoplasm when it was used during cell wall treatment. Then, the cellular lipids can diffuse and be extracted quickly through the cell wall. During the cell wall treatment, the cell lysis happens. The cellular lipids can be released directly through the disrupted cell walls. However, these cell lysis efficiencies were relatively low (19-25% performance) (Halim, R. et al, 2012).

During the cell wall treatment, Yang et al. found that there were some gaps and pits on the microalgae cell walls, which indicated that the ethanol can weaken or disrupt the cell wall in the treatment. Thus, the following extraction solvent can benefit from the broken cell wall structure. Yang also found that using 5mL ethanol on 1g wet microalgae can have the highest lipid extraction performance (Yang f., and Wenzhou X., 2014). Thus, the operating condition of this project was 3ml, 5ml, and 10ml of ethanol to 1g wet microalgae.

Furthermore, the cell wall structure may influence the cell wall treatment efficiency and the extraction efficiency. Yang et al. found that, under same conditions, the pits and holes can be observed on *Chlorella sp.*'s cell wall. However, the cell wall of *Picochlorum sp.* was relatively smooth. This might be because the *Picochlorum sp*. has a more rigid cell wall structure than Chlorella sp. (Yang F., and Changhong C., 2015). It was reported that during the cell wall treatment, most cells were deformed with pits and gaps and around 17%-25% of cells were broken into pieces (Phukan, M. M.2011).

Acid hydrolysis was used in this project to have a base sugar content of the microalgae cell wall. From Naoko (2010), the cell wall of Chlorella species may contribute up to 22% of the dry weight of the cell (Naoko A., 2010). Thus, the detected sugar content after acid hydrolysis would be compared with this data and would be used as a base line when comparing with the enzyme hydrolysis.

1.4.3 Biological methods

Biological methods using different biological ways to degrade the cell envelope, such as the enzymes, phage, and microalgae autolysis. (Geciova, Bury, and Jelen, 2002) Mostly, biological methods use enzymes to degrade the cell wall because they are easy to control and commercially available. The choice of the enzyme when doing the microalgae disruption should be very carefully considered because the enzymes can select a specific chemical linkage to break, which makes this enzymatic biological method purposeful (Braun and Aach, 1975). In addition, the condition of the enzyme reaction is mild. Thus, the energy requirement is small when comparing to the acid cell breakage. Different enzymes can be mixed during the microalgae rupturing, which can increase the effectiveness of the lipid extraction process. To increase the efficiency of the biological

method, the enzyme process can be developed. For example, the immobilization of enzymes can recycle the enzymes easily. The performance of cell breakage can be increased, and the cost of the extraction process can be decreased.

The cell wall of Chlorella sp. was shown to be species-specific. The cellulase, hemicellulases and pectin were shown in the two- layer cell wall structure (Cristina G, Bruno S. and Nicolas B, 2011). The out layer contains algaenan, which was a nonhydrolyzable aliphatic biopolymer and consisting of long-chain saturated hydrocarbons with ether and ester cross-linkages. The algaenan can provide the resistance for the algal cell wall (B. Allard and J. Templier, 2000). Moreover, the glucose, rhamnose, arabinose, mannose, xylose, fucose and galactose were hydrolysed from cell wall in the report of Edwin Kapaun and Eckhard Loos (Edwin K. and Eckhard L. 1992). The cell wall of Chlorella sp. can be divided into two kinds of cell walls: the present of glucosamine or not. The *Chlorella Sorokiniana* cell wall are the glucosamine-rigid wall, and the glucosamine was in form of the chitin-like glycan (Henri G. and Bryon D., 2013). Thus, the enzymes such as cellulase (CEL), hemicellulose, xylanase (AXC), and esterase (AGL) can be used to hydrolyse the microalgae cell wall (Henri G. and Bryon D., 2013).

Viscozyme has been chosen to hydrolyse the cell wall in this project. Viscozyme is a multi-enzyme complex and contains a wide range of carbohydrase, including arabanase, cellulase, β-glucanase, hemicellulose, and xylanase. The complex of enzymes can improve the cell lysis efficient. For the hydrolysis reaction, the 1,4-beta-D-glycosidic linkages were broken in cellulose, hemicellulose, glycan, lichenin, and beta-D-glucans and can produce glucose, galactose, and fructose. Thus, the hydrolysis performance can be determined when comparing the released sugars (saccharification yield) after acid hydrolysis and enzyme hydrolysis. It is difficult to hydrolyse the cell wall because the linkages between each unit are strong. Thus, exploring the best condition during hydrolysis would be important in this project. The experiment conditions were determined by the Viscozyme operating condition (pH: 3-5.5, 37℃). J.M. Romero et al. found that when the reaction time increasing from 4 hours to 8 hours, the hydrolysis increased from 42% to 59% when pH was 7 (J.M. Romero et al., 2012). Thus, the time condition for this experiment was from 4 hours to 8 hours.

Because chitosan was chosen as a flocculant in this project and it can be hydrolysed by the cellulase, which is contained in Viscozyme. Thus, the chitooligomer will be degraded from chitosan. This would not affect the project results much because the chitosan concentration used was relatively low.

1.5 Transesterification

The lipid extracted from microalgae cells cannot be used as fuel directly because its viscosity is high. So, the engines will fail quickly due to the generated oil sludge. Because of this, the lipid transesterification will be used to decrease the viscosity to make the biofuel suitable for the engine. The transesterification is a chemical reaction, which can transfer the oil into FAME (fatty acid methyl ester), in which the C14-C24 methyl esters

can be used as biodiesel. The acyl acceptor was used to accept the acyl from the extracted lipid (triglyceride). The fatty esters and the glycerol will be produced after the reaction. Figure 4 shows the reaction scheme of the transesterification (Ivana, Lukić et. al., 2017).

Catalysts such as the enzymes, acids, and bases were added to accelerate the reaction. However, the liquid catalysts would increase the difficulty of biodiesel recovery, which will lower the efficiency of the microalgae biodiesel process. The glycerol will be produced as a by-product during this reaction. For the critical factors, the moisture and free fatty acid content are the most influential factors during the transesterification process.

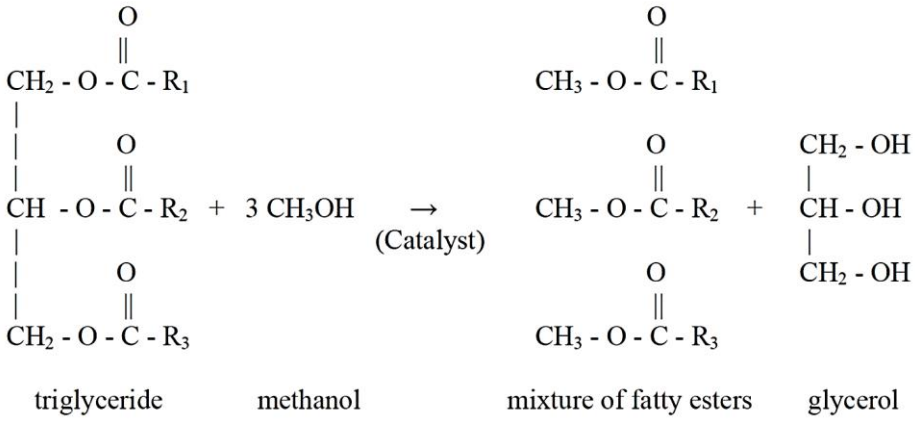

Figure 4. The transesterification reaction of triglycerides with methanol (Ivana, Lukić et.al., 2017).

1.5.1 Catalyst

1.5.1.1 Alkali catalyst

The alkaline catalysis can achieve a high conversion yield with low temperature and atmospheric pressure. Besides, the time required to complete the reaction is short. Thus, the alkaline catalysis has been mostly used in biodiesel production. However, the microalgal lipids have a high content of free fatty acids and when using alkaline catalysts, the soap will be produced. Thus, alkaline catalysts are not suitable for microalgal biodiesel production (Azean and Yilmaz,2012).

Figure 5 shows the mechanism of the alkali-catalyzed transesterification. When using alkali as a catalyst in transesterification, the alkoxide ion will attack the carbonyl carbon of the triglyceride molecule and a tetrahedral intermediate will be formed in the first step. The intermediate will react with alcohol and produce the alkoxide ion in the second step. Then the tetrahedral intermediate will rearrange, and a diglyceride and an ester will form (Chen Y., 2017).

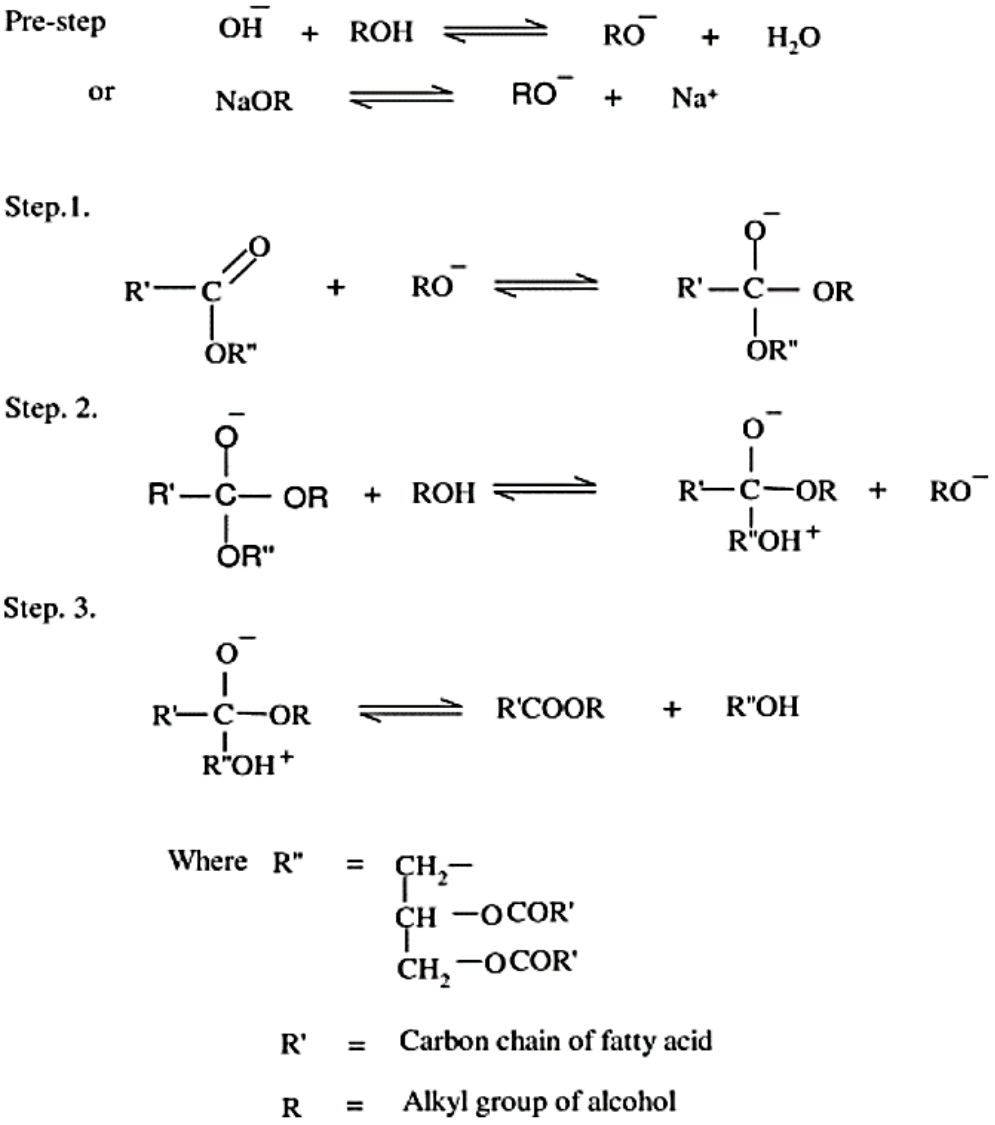

Figure 5. The mechanism of base catalyzed transesterification (Chen Y., 2017)

1.5.1.2 Acid catalyst

Acids can be used in the transesterification as catalyst, but the reaction time is longer than alkaline transesterification. Thus, corrosion of the equipment might happen. Moreover, the temperature required for acidic transesterification is high (typically above 100° C) and the yield for this type of conversion is high as well. Sulfuric acid (H_2SO_4) and hydrochloric acid (HCl) are the most used acid catalysts (Thanh et al. 2012).

The mechanism of acid catalyzed transesterification is shown in figure 6. Firstly, a protonation happens, and a carbocation is formed. Then, the carbocation will attack the alcohol and a tetrahedral intermediate will be formed. Finally, the intermediate will eliminate glycerol and form a new ester (Vidya. N. Naik, 2004).

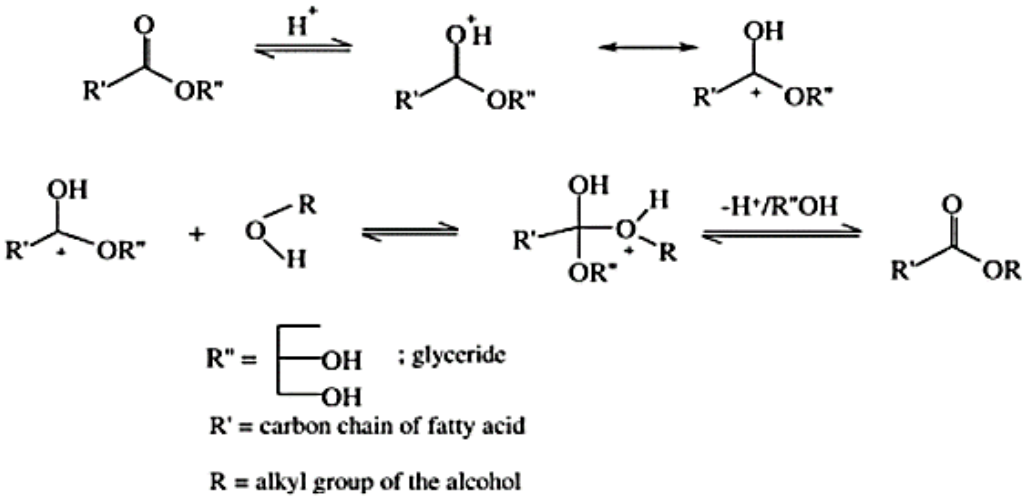

Figure 6. Mechanism of acid catalyzed transesterification (Vidya. N. Naik, 2004)

1.5.1.3 Enzyme catalyst

Enzymes can also be used in transesterification as catalyst (Azean and Yilmaz, 2012). Table 5 shows the comparison between the enzymatic transesterification and the chemical transesterification. It is shown that for the enzymatic transesterification, there would not be soap formation and the glycerol recovery is easy. The catalyst cost is high for the enzymatic transesterification, but the reusability of the catalyst has been shown. Moreover, there would not be a wastewater treatment when using enzymes while the chemical process needs this treatment.

Table 5. Comparison of enzymatic catalysis versus chemical (alkaline and acid) catalysis for biodiesel production (Gao M., 2013; Vidya. N. Naik, 2004, Chen Y., 2017)

Parameter	Enzymatic process	chemical process	
		alkaline process	acid process
FFA content in the raw material	FFA are converted to biodiesel	Soaps formation	FFA are converted to biodiesel
Water content in the raw material	It is not deleterious for lipase	Soaps formation. Oil hydrolysis resulting more soaps	Catalyst deactivation
Biodiesel yield	High, usually around 90%	High, usually>96%	High, usually>90%. However, only for high alcohol to oil molar ratio, high catalyst concentration and high temperature
Reaction rate	Low	High	High
Catalyst recovery and reuse	Easy	Difficult	Difficult
Energy costs	Low	Medium	High
Environmental impact	Low	High, wastewater treatment needed	High: wastewater treatment needed

Figure 7 shows the mechanism of enzymatic transesterification when using methanol. The glycerol and methyl esters will be produced in this reaction.

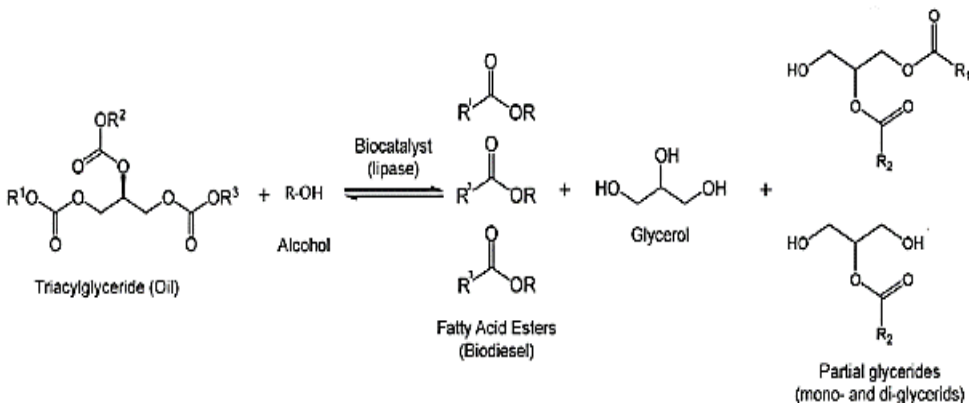

Figure 7. The metabolism of enzymatic transesterification when using methanol（Amini, Z. et al., 2016).

The enzyme used in this project was Novozyme 435, which was from Candia Antarctica and immobilized on acrylic resin. Novozyme 435 was chosen because it was immobilized on acrylic resin and had a higher resistance to inactivation from methanol (Noraini et al, 2014). Thus, it can be recycled after the transesterification. Besides, it was the most broadly used yeast lipase and have been used to convert the plant oil (Gog et al, 2012 and Noraini et al, 2014). It was the most effective enzyme in microalgae lipid transesterification according to Wu's research (Wu et al, 2017) The immobilized lipase was added in plate with oil and alcohol, and the lipase can be recovered by filtration after transesterification. Furthermore, the enzyme transesterification required low temperature during reaction, which means that the energy requirement would be less than the acid transesterification when the time cost was similar.

According to Pu's research, the lipase transesterification performance can reach the stationary phase after 20 hours (Wang Pu and Yang li-rong, 2001). Thus, in this project, the lipase transesterification time was settled as 6 hours and 24 hours.

1.5.2 Lipid source

The biocatalysts are competitive catalysts when comparing to acids and bases because a wild variety of triglyceride substrates can be used during transesterification. Table 6 shows the triglyceride substrates that have been used in biocatalytic transesterification. For this project, the oil used was microalgal lipids and fatty acids.

Table 6: Triglyceride substrates that have been used in biocatalyst transesterification (Amini, Z. et al., 2016 and Bajaj, A et al., 2010).

Triglyceride substrate	Enzyme	Acyl acceptor	Yield (%)
Cottonseed oil	Novozyme 435	Methanol	91.5
Jatropha oil	Pseudomonas cepacian	Ethanol	98
Microalgal oil	Candida sp. (immobilized)	Methanol	98
Soybean oil	Novozyme 435	Ethanol	82
Olive oil pitch	Thermomyces lanuginosa (immobilized)	Ethanol	90
Waste activated	Candida cylindracea	Methanol	97
Tallow	Lysozyme IM-60	Primary alcohols	94.8-98.5

Pure fatty acids were chosen in this project during lipase transesterification because it was difficult to determine the component of the microalgae lipid after extraction. Using pure fatty acids can easily determine the fatty acid content before and after the transesterification. Thus, the transfer rate can be easily calculated.

The lipid composition of *C. Sorokiniana* has been found to contain mainly C16, C18:0, C18:1, and C18:2, and the C16 and C18:0 had the highest lipid content after cultivation (Kumar et al. 2014). Thus, for the lipase transesterification, the fatty acids used were mainly C16 and C18:0.

1.5.3 Acyl acceptor

In enzymatic transesterification, many different alcohols and esters can be used as acyl acceptor. Table 7 shows the different acyl acceptors that can be used in enzymatic transesterification.

Table 7: Different acyl acceptors that can be used in enzymatic transesterification (Christopher, P. 2014).

Acyl acceptor	Oil/fat	Lipase	System	Yield (%)
Methanol	Sunflower oil	Pseudomonas fluorescents	Hexane	94
			Heptane	70
			Petroleum ether	80
			Isooctane	80
			Acetone	20
	Soybean oil	Novozyme 435	Solvent-free	93.8
	Cotton oil	Novozyme 435	Tert-Butanol	90
Ethanol	Sunflower oil	Pseudomonas fluorescents	Solvent-free	82
	Fish oil	Novozyme 435	Solvent-free	100
Isopropanol	Palm oil	Pseudomonas fluorescents	Solvent-free	24
	Tallow	Mucor miehei IM60	Solvent-free	90.3
2-Butanol	Tallow	Candida Antarctica SP435	Solvent-free	96.4
Methyl acetate	Soybean oil	Novozyme 435	Solvent-free	92

Various types of alcohols can be used in the enzymatic catalysis as acyl acceptors. Methanol, ethanol, and isopropanol are the most used alcohols in biodiesel production due to their low price and availability. According to the transesterification response equation, three moles of alcohols to one mole of triglycerides were required to produce one mole of glycerol and three moles of FAME (Guldhe et al, 2015 and Meher etal, 2006). However, when more alcohol was added, the reaction would be pushed to the product side. Meher found that when the molar ratio of alcohol to triglycerides arrived 6:1, the transesterification was found to be the most effective (Meher et al, 2006). In this project, to

detect the optimal molar ratio of methanol to lipid, the range of the molar ratio between 9:1 and 15:1 was tested.

1.5.4 Organic solvent

The transesterification can be performed without an organic solvent, but the conversion rate will be increased when organic solvent is present. Using organic solvent in transesterification can improve the solubility of the triglycerides and alcohols to help the conversion. Various types of organic solvent have been used in transesterification and it was found that using the hydrophobic organic solvent such as n-heptane, n-hexane, and isooctane will have the best performance. However, the produced glycerol is insoluble in the hydrophobic organic solvent and the glycerol will adsorb onto the enzymes if it remains in the reactor. Thus, the conversion performance will be influenced.

The hydrophilic organic solvents are much less used in enzymatic transesterification because there will be a water layer around the enzymes when using them. However, using tert-butanol can solubilize the glycerol and methanol because of its moderate polarity. Thus, the negative influence of glycerol and methanol will be avoided (Sevil Y. and Pinar T., 2013).

Using organic solvent will improve the conversion rate, and the addition of alcohols can be finished in one step. There will be a purification step after conversion to extract the lipids.

1.5.5 Temperature

To protect the lipase activity, the temperature of enzymatic transesterification was usually lower than the chemical transesterification. Another parameter to determine the conversion temperature was the transesterification rate. It was reported that the transesterification rate catalyzed by lipase increases when increasing the temperature from 30°C to 50 °C. However, the transesterification rate will decrease when the temperature is higher than 50°C (Gog et al, 2012). This was because the enzyme stability was decreasing. Thus, the operating conditions in this project was 30℃.

1.5.6 Water content

The existence of water can increase the enzymatic transesterification yield. The water-oil interface can make a conformational change, and the lipase activity and stability can be influenced. Therefore, the enzymatic transesterification yield can be increased by adding a certain amount of water to form the water-oil interface. Besides, the water content has an influence on the enzyme lifetime. Researches on *C. Antarctica* lipase (Novozyme 435)

showed that the low water amount will have a high transesterification yield because a high-water content will decrease the enzyme lifetime. However, the excess of water will stimulate the competing hydrolysis reaction of ester and the transesterification yield will decrease. Shah and Gupta reported that a 0.5% water content will increase the transesterification yield to 98% while the non-water transesterification yield is 70%. Thus, in this project, the water content was 0.1-1 (volume %, v/v). In conclusion, the water content can have an influence on the enzyme lifetime, the ester hydrolysis reaction, and the availability of the water-oil interface (Shah and Gupta, 2007). A suitable water content will have an optimal transesterification yield.

1.5.7 Direct transesterification

Direct transesterification combined the lipid extraction and biodiesel conversion into one step. Recently, the single step transesterification method has received more attention because this process was simple and can have a high yield. The acid catalyst and pure methanol were added to dried microalgae biomass and the transesterification can happen in this one step process. The methanol can extract the lipid from the biomass and the acid can transfer the lipid to the mono-alkyl ester of fatty acids (El-shimi et al. 2013).
When using the direct transesterification, the oleaginous biomass, alcohol, and catalyst are mixed at a temperature, during which the transesterification will occur. This method can be used with both dry and wet biomass. However, when using wet biomass, harsher conditions will be needed or a cell disruption pre-treatment should be undertaken (Ji-Yeon P. and Min P., 2015).
Direct transesterification still has other disadvantages. For example, due to the high temperature during the process, many side reactions can happen between cell materials and alcohol, which will make the biodiesel separation more difficult (Young et al., 2011). Thus, the in situ-transesterification was not used as base line in this project.

1.6 Energy estimation

This project was aimed to develop lower energy input process by replacing the process steps. Thus, the energy estimation was required in this project.
The lab scale was used in this project. Thus, the energy requirement was calculated as lab scale machine. The energy requirement calculation would be different when during an industry calculation.
For the centrifuge energy estimation, Eppendorf 5804R Refrigerated Centrifuge with A-4-44 Rotor was used in this project and the max capacity of it was: 4 × 250 mL/2 × 5 MTP. The power consumption was: 1650W. Thus, the total capacity was:

$$4x250ml = 1000ml = 1L$$

The total weight that can be centrifuged was

$$\frac{5g}{L} x\ 1L = 5g$$

For one gram of raw material, the power input was:

$$1650W/5g = 330W/g$$

For the samples, the centrifuge time was 10min. Thus, the energy input can be calculated as:

$$1650w/l * 10min * 60s = 990000J/L$$

For 5mL sample, the energy input was:

$$990000J/L * 0.005L = 4950J$$

For space reasons, the rest of the energy input calculation was in the references.
However, these calculations were developed on lab scale. When transfer these steps to large scales, different units would be used. Thus, different calculations should be developed. The filtration, sedimentation, and flotation also were used in industry to separate the microalgae. When focused on the centrifuge, the disc stack centrifuge and the decanter centrifuge were mostly used to separate the cells (Mariam Al hattab and Abdel Ghaly, 2015). The disc stack centrifuge required the concentration of the samples, and the solid content was required in the range of 2-25%. Thus, the pre-concentration step such as flocculation can be added before the centrifuge step (Yang J et al., 2011). The operating of dis stack centrifuge speed and gravitational force would be changed when using different microalgae strains. Sharma et al. found when using Chlorella sp., 5.5KWh/m^3 power would be consumed by using disc stack centrifuge (Yang J et al., 2011). For the decanter centrifuge, the flow rate would influence the microalgae recovery. Dassey and Theegala had a harvesting efficiency as 28.5% when the flow rate was 18L/min of continuous flow decanter centrifuge (Dassey AJ. et al., 2013). A pre-concentration step (air flotation) can be used to improve the cell density of the samples for the decanter centrifuge. The microalgae concentration was from 0.02-0.05 (weight, %) to 2-3 (weight, %) (Vasudevan V. et al,.2012). It was reported that when using a decanter centrifuge, the cell density can be concentrated to 22% (w/v), and the power consumption was 8k Wh/m^3 (Molina G. E. et al., 2003).

1.7 Conclusion

Microalgae were potential lipid provider and using microalgae lipid to produce biofuel attracted much attention in the world. The microalgae cultivation, the harvesting of the biomass, the lipid extraction from the cells, and the transesterification were the typical steps of the process. To complete a powerful biofuel production process, the energy input for each step was very important. Thus, the choice of methods used for each step should be considered very carefully. According to the literature, the centrifuge required a high energy input. Thus, in this project, a pre-concentration step (flocculation) was chosen. An enzyme treatment step or chemical step was used to disrupt the cells instead of the sonication. Moreover, lipase was used as the catalyst in transesterification to replace the acid to decrease the energy input. The modified processes were aimed to have the similar performance as the base process but lower energy input.

2. Hypothesis and project objectives

This project was trying to establish a modified process using wet microalgae cells (*Chlorella Sorokiniana*, UTEX-1230). The modified process used flocculation as a concentration method, enzymatic treatment or ethanol treatment as a cell wall treatment, and lipase as the transesterification catalyst. This project aimed to develop a DSP using wet algae biomass. The hypothesis was the modified process can obtain similar product yields with less energy input compared to the established (baseline) process.

There were four steps in the established process: the harvesting, cell breakage, extraction, and transesterification. For the harvesting, centrifugation was used in the baseline process. This step was followed by sonication to disrupt the microalgae cells. Chloroform-methanol (2:1 v/v) mixture was used as the solvent in the lipid extraction step. In the final transesterification step, the sulfuric acid was used as the catalyst.

To reduce the energy input, flocculation was used before the centrifugation to decrease the sample volume in the centrifuge. The enzymatic treatment was used to replace the sonication step and the ethanol was used as an alternative cell wall treatment. Hexane was used to replace the chloroform due to its toxicity. Finally, the lipase was used in transesterification to reduce the energy input in the modified process.

1. Evaluate the flocculation as a pre-step to centrifugation.
2. Evaluate enzyme treatment and addition of ethanol as alternatives to mechanical cell breakage for lipid extraction.
3. Evaluate hexane as an alternative extraction solvent to chloroform-methanol (2:1 v/v) mixture.
4. Evaluate the lipase transesterification as an alternative to acid transesterification.
5. Determine and compare the energy balance of the baseline and modified process.

3. Flow chart for the project

3.1 Process base line (lab scale)

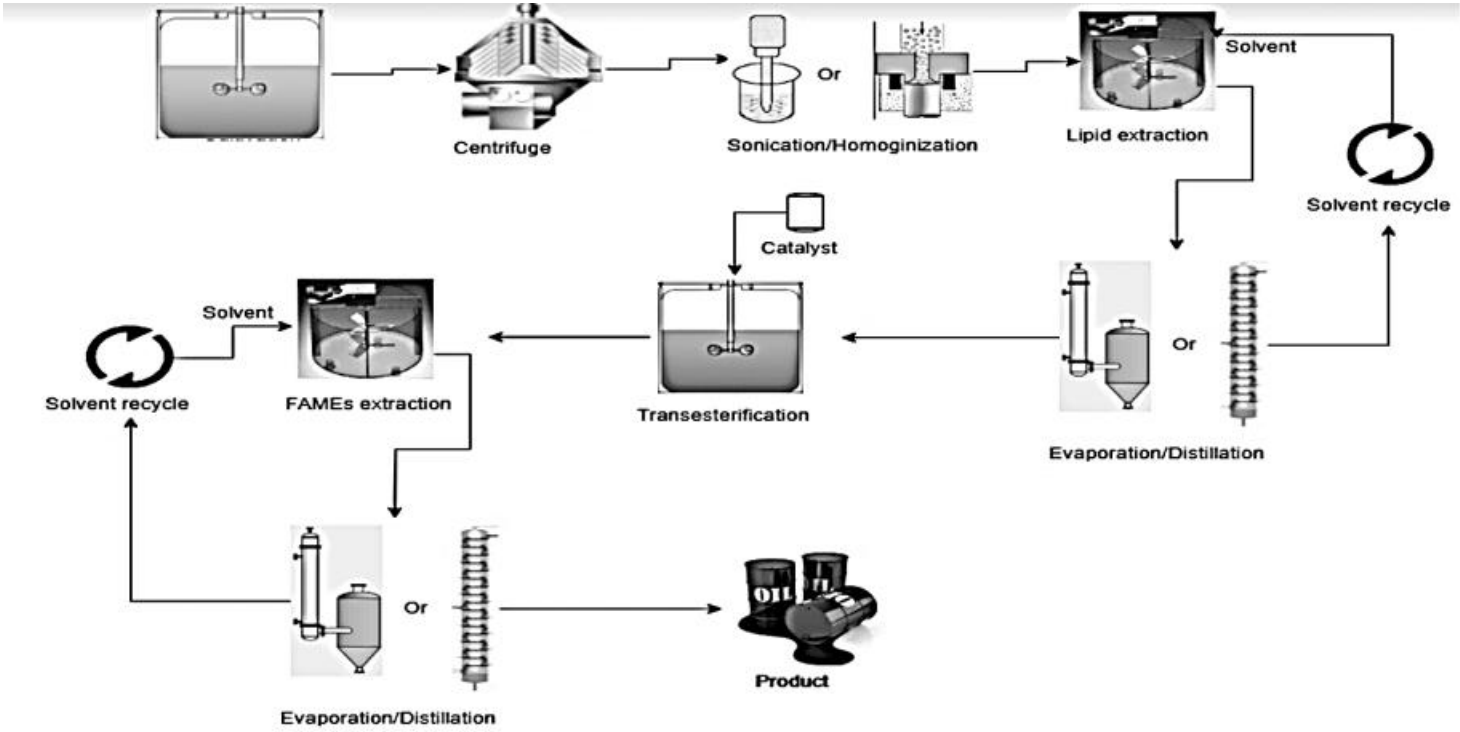

Figure 8. The flow chart of process base line (lab scale)

The base process consisted of five different steps to produce the biofuels from wet microalgae. These steps were chosen because they can have the highest process performance in the shortest time. The cultivation step aimed to increase the lipid content of the microalgae cells. The harvesting step used a centrifuge to remove the supernatant. Sonication was used to break up the cells. A chloroform-methanol mixture was then used to extract the lipids. Finally, sulfuric acid was used in the transesterification step as the catalyst. After transesterification, the FAMEs (fatty acid methyl esters) were in the reaction mixture. Thus, the hexane was used to extract the FAMEs from the mixture. This process had a high biofuel product yield but required high energy input.

3.2 Modified process 1(lab scale)

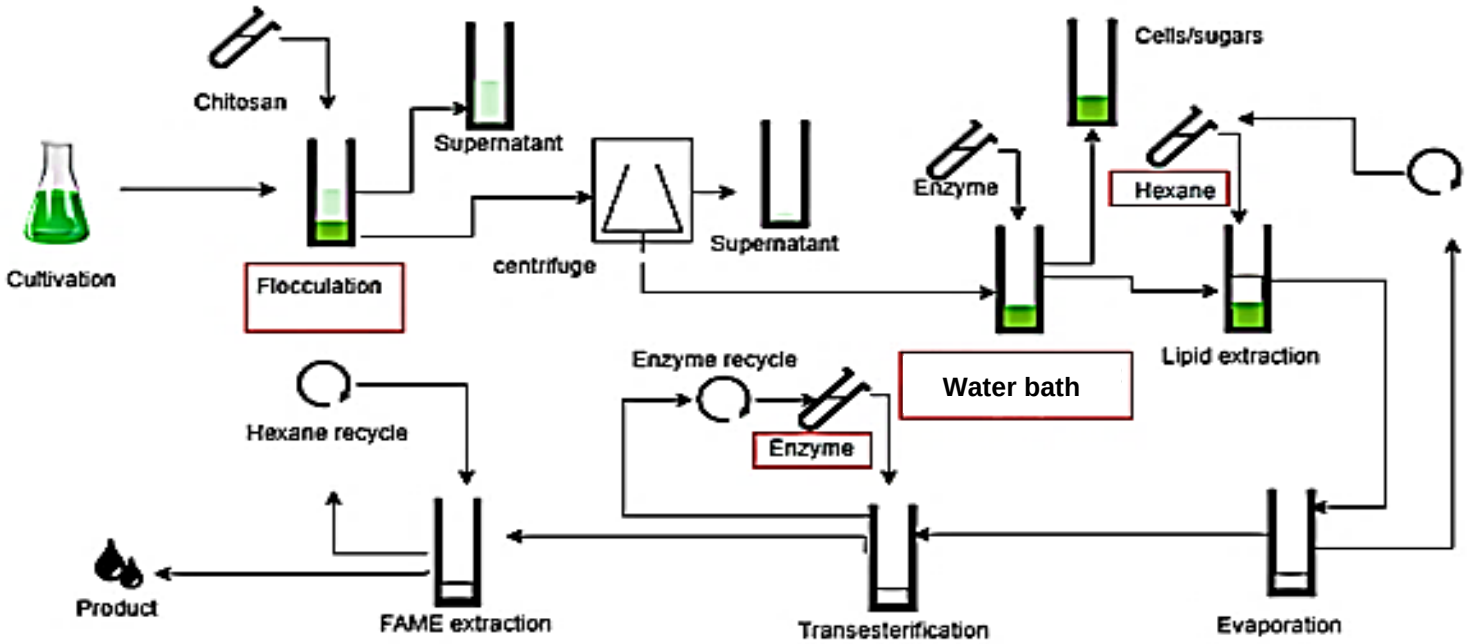

Figure 9. The flow char of modified process 1.

This project aimed to establish a modified algal biodiesel process. In the cultivation step, different media and cultivation methods were used to increase the lipid content of the microalgae cells, which was the same as the base line process to keep the raw material same in the next steps. Then, flocculation with chitosan followed by centrifugation was used to harvest the microalgae cells. For cell disruption, it was proposed to use enzyme or ethanol treatment. There was not a cell debris remove step because after the enzymatic treatment, the microalgae cells were not whole broken. The enzymes can hydrolyse the microalgae cell wall and leave some holes on it rather than hydrolyse the whole cells. Thus, some of the lipids were still in the cell envelope and a cell debris remove step will remove these lipids and decrease the performance. Hexane was used to extract the lipids. Lipase was used in the transesterification step as an alternative catalyst. After transesterification, the FAME (fatty acid methyl ester) was in the reaction mixture. Thus, the hexane was used to extract the FAME from the mixture. It was expected that the modified process 1 has a lower energy input assuming similar product yield.

3.3 Modified process 2 (lab scale)

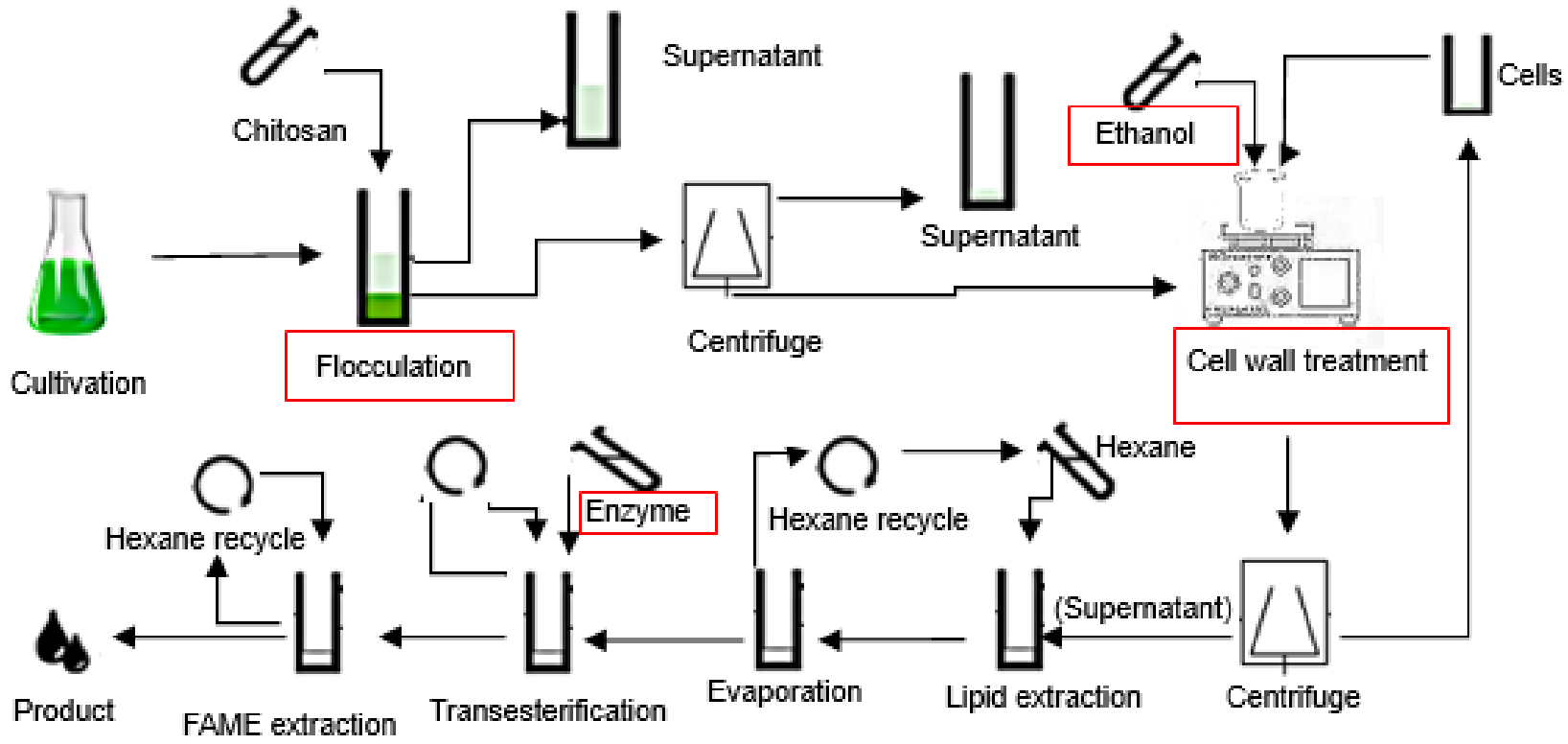

Figure 10. The flow chart of modified process 2.

The modified process 2 was aimed to using the ethanol as a co-extraction solvent and cell wall treatment when using hexane as extraction solvents. Firstly, the microalgae were cultivated under different conditions. The condition that can provide the highest lipid content was chosen and the microalgae cultivated under this condition were used in the next steps. After centrifuge, the chitosan was used for supernatant. Then, the ethanol and hexane were used as cell wall treatment and extraction solvent. After the hexane was evaporated, enzyme was used in transesterification to transfer the lipids into FAMEs (fatty acids methyl esters). After transesterification, the FAMEs (fatty acid methyl esters) were in the reaction mixture. Thus, the hexane was used to extract the FAMEs from the mixture. It was expected that the modified process 2 would have a lower energy input and a similar biofuel yield than the base line.

4. Materials and Methods

4.1 Cultivation

The microalgae species used is *Chlorella Sorokiniana* (UTEX1230) from Austin, Texas, USA. The microalgae were incubated in 1L flasks with 300mL working volume in TAP medium, 3N-BBM+V medium, low nitrogen source TAP medium, TAP medium (heterotrophically), and TAP medium with glucose (1 g/l, 3.5 g/l, 6 g/l, 8.5 g/l, 11g/l) (heterotrophically) separately. Table 8 showed the media used in this project.

Table 8: the media used in this project. The conditions of all media were the same (30° C, 200rpm)

Media	Conditions	Cultivation	Carbon source	Nitrogen source	C/N (mol/mol)
TAP medium	30℃ /200RPM	Mixotrophic	Acetate	NH_4Cl	~7.0
3N-BBM+V		Phototrophic	----	$NaNO_3$	~0
Low-N TAP media		Mixotrophic	Acetate	NH_4Cl	~70.0
TAP medium		Heterotrophic	Acetate	NH_4Cl	7.0
TAP with glucose(1g/l)		Heterotrophic	Acetate/Glucose	NH_4Cl	11.67
TAP with glucose (3.5g/l)					23.33
TAP with glucose (6g/l)					35.00
TAP with glucose (8.5g/l)					46.67
TAP with glucose(11g/l)					58.33

The initial optical density was ~0.1 for the cultivation (~0.02g/l for the initial biomass). 30ml microalgae material and 270ml media were added in 300ml working volume flask to make the optical density equal to 0.1 and then, the flask was put in shaker for cultivation. The conditions of the cultivation were 30°C, 200rpm, and pH 7.

The lipid concentration and the dry cell weight were measured every day during cultivation.

4.2 Harvest

The microalgae were harvested by either centrifugation or flocculation and centrifugation.

4.2.1 Centrifugation

5 mL of culture was added to a 15 mL plastic Falcon tube and the tube was centrifuged at 4 °C and 600 g. The centrifuge used in this experiment was Eppendorf 5804R Refrigerated Centrifuge with A-4-44 Rotor. After centrifugation, the supernatant was discarded, and the cell pellets were frozen in -20°C freezer until analysis.

4.2.2 Flocculation

100 mg of chitosan (Across 100-300kDa) was dissolved in 10 mL of HCl (0.1 M) and agitated for 30 – 60 min or until full dissolution. Then 90 mL of MilliQ water was added. After cultivation, the pH of the culture was 7.8. Thus, 0.1M HCl was used to adjust the pH to 6. Chitosan stock was added in the microalgae culture to a final concentration of 22 mg of chitosan per g of DCW (dry cell weight) and 0.1M HCL were used to adjust the pH to 7. Then, the mixture was agitated in vortex for 10min and left for settling for 30min in room temperature. After the recovery of the supernatant, the cell aggregates were used for enzymatic treatment. For the supernatant, the absorbance was measured at 750nm and the clarification levels were calculated.
The clarification levels:

$$\text{Clarification (\%)} = 100 - \left[\frac{final\ biomass}{initial\ biomass} X100\%\right]$$

The biomass concentration (dry cell weight) can be calculated from the optical density using the optical density-dry mass calibration curve.

4.2.3 Particle size distribution

The Master sizer 2000 (Malvern Analytical, United Kingdom) was used to measure the particle size distribution. The particle type was non-spherical particles. The refractive index was 1.03 and the density of microalgae was 1.05 (g/cm^3). The dispersant was water and the refractive index was 1.33. The background measurement duration was 10s and the sample measurement duration was 10s. The number of measurements was 5 and the delay between measurements was 1s. The obscuration lower limit was set at 5% and the higher lipid limit was 25%.

4.3 Cell wall treatment

The microalgae were disrupted by either sonication or enzymatic treatment.

4.3.1 Sonication

For the sonication, 5 mL of microalgae cells were added in 30mL Falcon tube. The type of bath sonicator was Bath-sonicator-Bransonic-R-5510. The ultrasonic frequency was 40kHz. The ice-water mixture was added to the bath sonicator to keep the temperature (~0°C). Then, the samples were put in the bath sonicator. The sonication time was 30min (continuously) to make sure the microalgae cells were broken.

4.3.2 Acid hydrolysis

2mL of fresh algae culture were centrifuged at 10000rpm at 4° C for 10min. Then the supernatant was discarded. 4mL of MilliQ water was added to wash the pellet and centrifuged again with the same condition. Then, the supernatant was discarded. 2 mL of MilliQ water was added to resuspend the pellet and the samples were sonicated in ice bath sonicator (ultrasonic cleaner, Branson, W.W. Grainger Inc.) with 10s on, 15s off for 25cycles at 10 A amplitude. Then, the samples were centrifuged, and the supernatant was discarded. 2 mL of MilliQ water was added and 175μL of sulfuric acid 72% (v/v) was added. The samples were autoclaved at 121 ° C for 1 h. Then, the samples were centrifuged at 15000rpm for 10 min. The supernatant was recovered and diluted to 500 times for the analysis.

4.3.3 Enzymatic treatment

The enzymatic treatment was used as a cell wall treatment. The enzymes used in this project was Viscozyme.

For the particle size distribution measurement, the pH of microalgae culture was adjusted from 7.8 to 5.5 by 0.1M HCl. Then, 5ml of microalgae culture was added to a 15ml Falcon tube. 2μL/g (DCW) of Viscozyme was added to the same tube. The water bath was used to keep the temperature at 37° C for 4 hours. Then, the Mastersizer 3000 was used to measure the particle size distribution (see section 4.2.3 for details).

1. For the FDE (factorial design experiment), firstly, the groups were designed by using the DOE (design of experiment). The table below shows the groups of the FDE. Before centrifuging for 10min at 600g, the pH of microalgae culture was adjusted to 3-5.5 by 0.1M HCl. 5ml of microalgae culture was added to a 15mL Falcon tube. Then, 2-6 μL/g (DCW) Viscozyme was added in the tube. The water bath was used

to keep the temperature at 37°C for 4 - 8hours. Finally, the samples after enzymatic treatment were extracted by Chloroform-methanol (2:1 v/v).

Table 9. The FDE (factorial design experiment) groups for the conditions of enzymatic treatment.

Run	Factor 1 A: pH	Factor 2 B: Time (hour)	Factor 3 C: Enzyme concentration (μg DCW)
2	3.0	8	6
4	3.0	4	6
6	3.0	8	2
8	3.0	4	2
1	5.5	4	6
3	5.5	4	2
5	5.5	8	2
7	5.5	8	6

2. For the positive and negative control of enzymatic treatment, the treatment time was 6 hours and 24 hours. 5ml of culture was added in 30mL Kimble tube. The temperature was 37°C and the pH was 3. For the positive control 6 μL/g (DCW) Viscozyme were added. The negative control samples were kept in the same conditions but without enzymes. After the treatment time, 5 mL of the hexane or hexane-methanol (3:1 v/v) solvents was added for the extraction. All the groups were treated by double extraction.

4.3.4 Saccharification yield

The saccharification yield (ratio of sugar released from the cell walls to the dry mass) can determine the cell wall hydrolysis level. The cell wall lysis levels of different method can be compared by comparing the saccharification yields. The saccharification yield can be calculated as below:

$$\text{saccharification yield } (\%\frac{w}{w}) = \frac{sugar\ released\ (g)}{dry\ cell\ weight\ (g)}$$

4.3.5 Ethanol as treatment solvent

1. The treatment using different ethanol volume (3ml, 5ml, and 10ml)
   The microalgae were harvested by centrifugation (centrifuge conditions: 4℃,3000rpm, 5min). 1g of wet microalgae was put in 20ml Duran bottle and 3ml, 5ml, and 10ml of ethanol was added respectively. A cross stirrer was used for mixing and the stirrer rate was 1000rpm. After 24 hours treatment, the ethanol and the biomass residual biomass were separated by centrifugation (centrifuge conditions: 4℃,3000rpm, 5min). The ethanol phase (containing lipids) was transferred to a Kimble tube for the extraction and the ethanol (3ml, 5ml, and 10ml) was added to the

residual biomass for the second cell wall treatment. After 24 hours treatment, the ethanol and the residual biomass was separated by centrifugation again. The ethanol was transferred to another Kimble tube and the residual biomass was added 3ml, 5ml, and 10ml of ethanol for the third cell wall treatment. The third treatment had the same conditions as before. The ethanol was evaporated after separation by Genevac (Sp Scientific, U.S.) and the Nile red assay (see section 4.7 was used to quantify the lipids.

2. The treatment using 5ml of ethanol
   After harvesting, the microalgae were treated with 99.0% ethanol. 1.01g wet biomass was added in 25ml Duran Bottle. 5ml of ethanol was added for treatment. A cross stirrer was used during the cell wall treatment (treatment time: 2 hours; stirrer speed: 1000rpm). Then, a centrifuge was used to separate the ethanol (~4.5ml) and the residual biomass (centrifuge conditions: 4℃,3000rpm, 5min). After separating the residues and the upper phase, 5ml of ethanol was used for the second treatment.

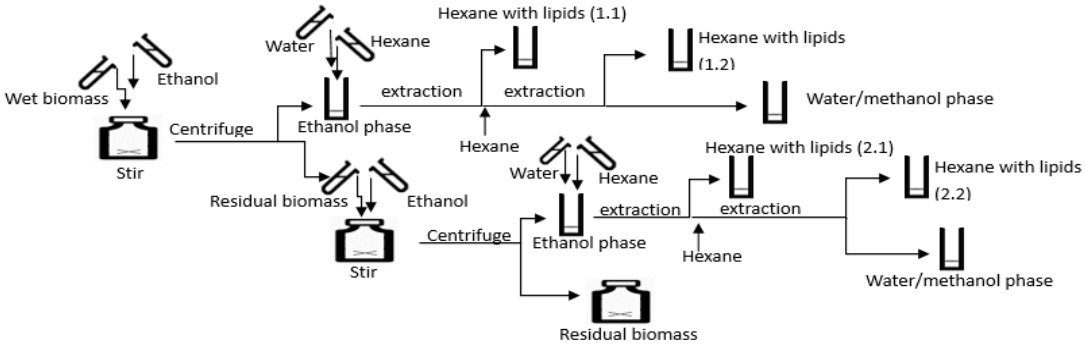

Figure 11. The flow chart of ethanol treatment (5ml ethanol)

4.4 Lipid extraction

Different solvents were used during extraction. For each method, the procedures are described below.

4.4.1 Hexane as the solvent

1. Using hexane as extraction solvent after cell wall treatment (sonication, and enzymatic treatment)

Before the extraction, 200μg of C15 (pentadecylic acid) was added into the material after cell broken step as an internal standard of the extraction. Then, the lipids were extracted with hexane and the volume ratio of hexane to microalgae cell volume was 1:1. (In hexane-methanol extraction, the microalgae culture volume, methanol, and hexane volume ratio were: 1:1:1). After agitating for 2-10 minutes by vortexing, centrifugation was used to separate the hexane and water. The Vortex used in this experiment was a

Chemical Fixed Speed Vortex Mixer (Scilogex, U.S.). The upper phase, which was the hexane containing lipids was transferred to another Kimble tube. Then, hexane was used for a double extraction (volume ratio of hexane to microalgae cell volume 1:1).
Then, the Genevac machine was used to evaporate the hexane. The Genevac machine used in this experiment was: Genevac EZ-2.3 Plus Mk3 Personal Evaporator (Sp Scientific, U.S.). After evaporation, 200 µL of heptane was used to suspend the lipids and the samples were kept at -20℃ for the next step.

2. Using hexane as extraction solvent after ethanol cell wall treatment

Hexane was used to extract the lipids after ethanol treatment. 3ml of water was added in the ethanol-lipid part to form the water-ethanol layer. Then, 7.5ml of hexane was added to extract the lipids from the water-ethanol layer. A cross stirrer was used during extraction (extraction time: 20 hours, 1000rpm). Then, centrifugation was used to separate the two layers (centrifuge conditions: 4° C,3000rpm, 5min). The hexane with lipids (the top layer) was taken out to a Kimble tube for transesterification. Then, 7.5ml of hexane was added to the water-ethanol layer to re-extract the lipids. The same cross stirrer was used during extraction (extraction time: 4 hours; 1000rpm). Then, centrifugation was used again to separate the two layers as before. The hexane with lipids was taken out to a Kimble tube for the next experiments. The ethanol of first cell wall treatment and second cell wall treatment were separately determined. Similarly, the first time lipid extraction and second time lipid extraction were separately determined to estimate the necessity of second time cell wall treatment and extraction.

4.4.2 Chloroform: methanol (2:1 v/v) as solvent

The chloroform: methanol (2:1 v/v) solvent was used in the project as a standard method. The chloroform: methanol (2:1 v/v) mixture was added into the samples after enzymatic treatment with a volume ratio of 1:1 and 200µg of C15 were added into the mixture to have an internal standard for the extraction. After agitating for 2 minutes, the same volume with the chloroform-methanol mixture of water was added into the mixture and agitated again. Then, centrifugation was used to separate the chloroform (containing the lipids) and the water phase (containing the methanol and the cells) for 10 minutes, 10000rpm, and room temperature. The lower phase, which contained the chloroform and the lipids was transferred into another Kimble tube and the Genevac machine was used to evaporate the chloroform. Finally, the lipid was suspended into 200 µL of hexane for the next step.

4.4.3 Sonication and Chloroform: methanol (2:1 v/v) as the solvent in lipid extraction.

Sonication was used to disrupt the microalgae cells before the lipid extraction. The

microalgae were centrifuged at first and the supernatant was discarded. Then, the pellets were suspended in 5 ml chloroform: methanol (2:1 v/v) mixture. The internal standard C15:0 free fatty acid was added before the sonication. The microalgae cells were sonicated for 30min in a bath sonicator. Then, 5ml of the water was added and the tubes were shaken for 2 min. After centrifugation, the solvent was separated into two phases. The upper phase contained water and methanol and the lower phase contained chloroform and lipids. The lower phase was transferred to another Kimble tube and the chloroform was evaporated in Genevac. Finally, the lipids were suspended in 120μL heptane and frozen for the transesterification.

4.4.4 Lipid composition after extraction.

The lipid samples were from chapter 4.4.1, 4.4.2, and 4.4.3 in this project. After acid transesterification, the compositions of methyl-esters were detected by GC-FID. The acid transesterification conditions were shown in chapter 4.5.1. The GC-FID conditions were shown in chapter 4.8. The groups were:

Table 10. The groups of lipid composition.

No.	Steps	Extraction solvents
1	Flocculation-enzymatic treatment	Chloroform
2		Hexane
3		Ethanol-hexane

4.5 Transesterification

4.5.1 Acid-catalysed transesterification

The methanol was used in the transesterification and sulfuric acid was used as the catalyst.

15μL of the sample from the lipid extraction was added to a Kimble tube and 3mL of 5. Methanol with 2.5% (v/v) H_2SO_4 mixture was added. After mixing, the Kimble tubes were put into a 60°C water bath and heated for four hours to convert the lipids into the esters. Then, after the Kimble tubes were cooled down to the room temperature, 3mL of hexane and 3mL of water were added into the Kimble tubes and agitated for extracting the FAMEs. Centrifugation was used to separate the hexane and the water phase. The hexane phase (containing the FAMEs) was transferred into another Kimble tube and this extraction step was repeated. Then, the Genevac machine was used to evaporate the hexane and 120 μL heptane was used to resuspend the FAMEs prior to the GC-FID analysis.

4.5.2 Chemical Transesterification performance

Pure fatty acids were used to determine the conversion performance of acid transesterification. 200 mg pentadecanoic acid, 200 mg palmitic acid, and 200 mg stearic acid were dissolved in 9 ml of hexane in a Kimble tube. Then, 15 μL of this sample was taken out and added to 3 mL H_2SO_4 (2.5% v/v) in methanol mixture. After mixing, the mixture was put in a 60°C pre-heated water bath for four hours. Then, the sample was taken out and cooled to room temperature. 3 mL of deionized water was added in the tube to quench the reaction. Then, 3 mL of hexane was added. After 2 minutes' vortexing, the sample was put in a centrifuge at 3500 rpm for 10 minutes. After the phase separation was achieved, the upper phase was transferred to another Kimble tube and 3 mL of hexane was added to the lower phase to repeat the extraction. The hexane was evaporated by Genevac and the esters were dissolved in 120 μL n-heptane and stored at -20°C for further analysis.

4.5.3 Enzymatic Transesterification

Novozyme 435 (Sigma-Aldrich, Candida Antarctica lipase B immobilized on acrylic resin) was used as catalysts in this project for transesterification.
Design-Expert 10 Stat-Ease, USA) was used in this stage to analyse results.
From the literature, 5 parameters (the water content, lipase amount, time, methanol: fatty acid ratio and molar ratio) were involved in this stage and the Factorial Design Experiment (FDE) and Response Surface Methodology (RSM) were used to design and analyse the experiments.

4.5.3.1 Factorial Design Experiment

Three parameters were chosen (Methanol: Fatty acid ratio, water content, and lipase amount) in this experiment and each parameter had two levels. There were no centre points because for factorial design experiment, the trends of the parameters were tested. For the next step: the response surface experiment, the optimal condition would be found out. Thus, a 2^3 fractional factorial design was used, which resulted in 8 runs. Then, the response data was analysed by using the factorial coding. Then, the RSM was used to analyse the relationship between the parameters and the transesterification performance. Table 11 shows the values chosen in this project using FDE.

Table 11. The design of the FDE experiment showing the factors and levels.

Run	Methanol: FFA ratio (mol: mol)	Water content (volume % v/v)	Lipase: FFA amount (% w/w)	Methanol volume (µL)	Total fatty acid weight (mg)	Hexane volume (mL)	MilliQ water volume (µL)	Lipase weight (mg)
1	3:1	1	50	45.07	100	1.3	13.45	50
2	3:1	0.1	10	45.07	100	1.3	1.35	10
3	3:1	0.1	50	45.07	100	1.3	1.35	50
4	3:1	1	10	45.07	100	1.3	13.45	10
5	9:1	1	50	135	100	1.3	14.36	50
6	9:1	1	10	135	100	1.3	14.36	10
7	9:1	0.1	50	135	100	1.3	1.44	50
8	9:1	0.1	10	135	100	1.3	1.44	10

Two Polytetrafluoroethylene (PTFE) plates (Innovative laboratory products, U.S.) were used in this experiment. Each of the PTFE plate had 18 wells with a maximum volume of 5mL and each plate had a sealing film, foam, and screw-on top for air-tight sealing. One plate had 6 hours reaction time and another plate had 24 hours reaction time.

200mg C15, 200mg C16, and 600mg C18 (total weight: 1g) were dissolved in hexane with 1:13w/v ratio of free fatty acids and then methanol was added. For one tube, the molar ratio of methanol to fatty acids was 3:1 and for the other tube it was 9:1. Then, 0.1%v/v milliQ water was added. After mixing, the samples were added to PTFE plate wells and for the wells that were designed to contain 1%v/v milliQ water, 0.9%v/v milliQ water was added. Then, the amount of lipase was added in each well and after tightening the cover (from top to bottom: support, foam, and sealing film) by the screw, the plate was put in the shaking incubator (Innova, Large-capacity, Eppendorf, U.S.) at 30°C and 250rpm. After 6 hours, the first plate was taken out and put on ice to stop the transesterification. Then, the samples in the wells were transferred to a Kimble tube and 1mL water and 1mL hexane were added. After mixing, the two phases were separated by centrifuge at 3500rpm for 10min. Then, the upper phase, which was hexane with the esters was transferred to a GC vial to store at -20°C until analysis. For the second PTFE plate, which was designed for 24 hours reaction time, the same steps were taken after incubating for 24 hours.

Then, another experiment was carried out and the fatty acids were composed of 200 mg Palmatic Acid, 200 mg Pentadecanoic Acid, 200 mg Steric Acid, 200 mg Oleic Acid, and 200 mg of Lineloic Acid. This mixture was to mimic the composition of fatty acids in the microalgae. Then other conditions were the same as shown in table 11.

4.5.3.2 Response Surface Methodology Experiment

The response surface methodology experiment was used to determine the optimal conditions of the lipase transesterification. Thus, the centre points were chosen in these experiments. A total of 17 runs, including 5 centre points were designed for the RSM experiment. The parameters in this design were the methanol ratio, lipase amount, and fatty acid to solvent ratio. To determine the transesterification productivity of each lipids,

the mixture of fatty acids was used. 80mg C16 and 20mg C18 FFAs were used. This composition was chosen because after hexane-ethanol extraction, the ratio of C16:C18 was around 4:1. Table 12 shows the variation of parameters. In the DOE, a multiple linear regression was used to model the relationship between the factor and response data. Analysis of variance (ANOVA) was used to assess the fitness of the model.

Table 12. Experimental design for the RSM experiment showing the input factors and levels. In the DOE, a multiple linear regression was used to model the relationship between the factors and response data.

Methanol: FFA ratio (mol: mol)	FFA: solvent (mg/mL)	Lipase: FFA amount (% w/w)	Methanol volume (μL）	MilliQ water volume (μL)	Lipase weight (mg)
9.1	25mL/1.3 mL	30	39	1.5	8.66
12:1	25mL/1.3 mL	50	52	1.55	14.43
12:1	25mL/1.3 mL	10	52	1.55	2.89
15:1	25mL/1.3 mL	30	65	1.57	8.66
9:1	50mL/1.3 mL	10	78	1.58	5.77
9:1	50mL/1.3 mL	50	78	1.58	28.85
12:1	50mL/1.3 mL	30	104	1.6	17.31
12:1	50mL/1.3 mL	30	104	1.6	17.31
12:1	50mL/1.3 mL	30	104	1.6	17.31
12:1	50mL/1.3 mL	30	104	1.6	17.31
12:1	50mL/1.3 mL	30	104	1.6	17.31
15:1	50mL/1.3 mL	10	131	1.63	5.77
15:1	50mL/1.3 mL	50	131	1.63	28.85
9:1	100mL/1.3 mL	30	156	1.66	34.6
12:1	100mL/1.3 mL	10	208	1.7	11.54
12:1	100mL/1.3 mL	50	208	1.7	54.7
15:1	100mL/1.3 mL	30	260	1.76	34.6

The fatty acid compounds (800mg C16 and 200mg C18) were dissolved in three volume levels of hexane and then transferred into each well. After this, the designed lipase, methanol, and milliQ water were added in each well. Two PTFE plates were prepared, and the reaction time was 6 hours and 24 hours respectively. Then, the PTFE plates

were put into the incubator with 250rpm and 30°C. After 6 hours, the first plate was taken out and put on ice to stop the transesterification. Then, the samples in wells were transferred to Kimble tubes and 1mL water and 1mL hexane were added. After mixing, the two phases were separated by centrifugation at 3500rpm for 10min. Then, the upper phase, which was hexane with the esters was transferred to a GC vial and stored at -20°C until analysis. For the second PTFE plate which was designed for 24 hours reaction time, the same steps were taken after incubating for 24 hours.

4.5.4 Comparison of Scale and Types of Solvent

Duran bottles were used for the scale up and tert-butanol was used as an alternative solvent to hexane.

Table 13. The preparation of samples for scale-up experiment.

	Solvent mixture volume (mL)	C15 weight (mg)	C16 weight (mg)	Lipase weight (mg)
Duran bottle hexane *	20	242.2	769.2	40.5
Duran bottle t-butanol*	20	242.2	769.2	40.5
PTFE well hexane*	1.5	18.8	57.7	0.1
PTFE well t-butanol*	1.5	18.8	57.7	0.1
PTFE well algae	1.5	Sample	Sample	0.04

*Duplicate samples

200mL hexane with 5μL methanol and 200mL t-butanol and 5μL methanol were mixed respectively. Then, 20mL of the mixture was added to 4 Duran bottles respectively (two with hexane/methanol and two with t-butanol/methanol). Then, fatty acids, i.e. 0.05M C15 and 0.15M C16, were added with a 3:1 methanol to fatty acid molar ratio. Then, the lipase was added to the bottles and the weight ratio was 4% w/w of fatty acids. The same conditions were designed in PTFE plates for comparison. Besides, the lipids extracted from microalgae were added to the wells using the same conditions. Table 13 shows the conditions for the experiment. Then, the PTFE plates and the Duran bottles were put in the incubator at 30°C and 250rpm. The reaction time for bottles and PTFE plates were 6 hours and 24 hours and for the Duran bottles, a sample (0.5 mL) was taken out every hour to show the trend of the reaction. Then, the same procedures were taken to extract the esters from the samples, and the products were kept at -20°C until analysis.

4.6 Dry weight measurement

For the dry cell weight, a gravimetric method was used. The cell suspension was filtered using pre-weighted filter paper (Whatman, 47mm circle, 100 pcs, South Miami, U.S.). Then, the oven was used to dry the filter paper at 90°C for 24 hours. Then the filter paper with dry cells was taken out and put in a desiccator to cool to room temperature. An analytical balance (Semi Micro Analytical Balances, GH-202, GPS Instrumentation Ltd.) was used to weight the filter paper with dry cell biomass. The dry cell weight was calculated as below:

$$\text{Filter paperweight and dry cell mass} - \text{Filter paperweight} = \text{Dry cell mass}$$

The cell concentration can be calculated as below:

$$\frac{Dry\ cell\ mass\ (g)}{Sample\ volume\ (mL)} = Cell\ concentration\ (g/mL)$$

4.7 Nile red assay

For determination of the lipid concentration, the Nile red method was used.
10mg of Nile red (Sigma-Aldrich, USA) was dissolved in 40mL acetone to make the Nile red stock (250µg/ml). The Nile red stock was kept at -20°C and in darkness when it was at room temperature. 20mL of pure DMSO (Dimethyl sulfoxide, Fisher Scientific, U.S.) was added in 75mL of MilliQ water to make 25% (v/v). 20µLof Nile red stock (250µg/ml) was added in 10ml of DMSO 25% to make the Nile red reagent (0.5µg/mL). The canola oil was used in this project because 94.1% to 99.1% of the canola oil were triglycerides (Chen W. et al., 2009). Thus, the canola oil and microalgae oil had similar fluorescence intensity values under same concentration. 110µL canola oil (Insight Biotechnology, UK) was added in 10mL of isopropanol final volume to make the canola oil stock (10mg/mL). Then, 30µL of canola oil (10mg/ml) was added in 2970µL of DMSO 25% to make the canola oil standard (100µg/ml). Then 750µl, 500µl, 250µl, 100µl, 50µl of canola oil standard (100µg/ml) was added in 250µl, 500µl, 750µl, 900µl, and 950µl DMSO 25% separately to make the canola oil standard (75µg/ml, 50µg/ml, 25µg/ml, 10µg/ml, and 5µg/ml), which was for the calibration curve. 2ml of microalgae culture were centrifuged at 10000rpm for 10min in centrifuge (C3100 Centrifuge, Benchmark Scientific, USA). Then, 1-10ml of DMSO 25% was added until the fluorescence intensity values were between calibration curve values.
96 microplate (Polystyrene, Universal medical, USA) was used for the measurement. 10µL of canola oil standard or microalgae sample was added in each well and 310µL of Nile red reagent (0.5µg/mL) was added after the canola oil or microalgae sample. For blank, the DMSO 25% was added to replace the canola oil or microalgae sample. For the microalgae sample blank, the DMSO 25% was used to replace the Nile red. Then, the plate was incubated at 40°C for 15 min at 500 rpm in Thermomixer (Thermomixer R Mixer, Marshall scientific, USA) in darkness. The measurements were treated in triplicate. Plate reader (Tecan, Infinite M200Pro, Switzerland) was used to measure the fluorescence intensity. The settings were: Emission / Excitation - 530 / 580, shaking: 30s,

number of flashes:25, multiple read per well: 2x2. According to the canola oil concentration and fluorescent intensity, the calibration curve equation can be calculated. Then, the microalgae oil concentration can be calculated by the calibration curve and the microalgae fluorescence intensity. Finally, the initial microalgae oil concentration can be calculated by the dilution rate.

Then, the lipid content was calculated as below:

$$\frac{Lipid\ concentration\ x\ Sample\ volume\ (g)}{Dry\ cell\ mass\ (g)}\ x\ 100\% = Lipid\ content\ (\frac{g}{g}\ \%)$$

4.8 GC-FID analysis:

In this project, the GC-FID analysis was used to quantification the FAME after transesterification of lipids extracted from the microalgae cells by GC-FID (Agilent, United States). The column used was Rxi 5Sil MS (fused silica, RESTEK, USA). The length of it was 30m and the diameter was 0.25mm. The injection volume was 1µl. The column temperature was at 60 °C for 2 minutes and then increased to 150°C for 1min. Then, the temperature was increased to 230°C and the hold time was 2min. The detector temperature was at 250°C with a hydrogen flow of 35ml/min and airflow of 350ml/min.

Before the analysis, the external standards were prepared for the calibration curve. The C15, C16:0, C18; C18:1, and C18:2 free fatty acids were used to make different concentration standards and the concentrations were: 2mg/mL, 1 mg/mL, 0.5 mg/mL, 0.25 mg/mL, 0.125 mg/mL, and 0.0625 mg/ml. These standards were used in the GC-FID analysis to generate the standard curves. The standard curves were used to calculate the FAMEs obtained from the process.

4.9 ICS (ion chromatography system)

Before the analysis, the calibration curve was made. The galactose, glucose, and the fructose were used to make the different concentration standards. The concentrations were: 0.02g/L, 0.01g/L, 0.005g/L, 0.0025g/L, and 0.00125g/L. These standards were used for the standard curves.

The ion chromatography system (ICS-6000, Thermo Fisher, U.S.) was used in this project. 5mM KOH was used as the mobile phase with a pumping rate of 0.25 mL/min and the duration was 5min. The column temperature was 30°C. The injection volume was 50µl using an autoinjector. The wash volume was 250µL and wash speed was 19.2µL/s. The draw speed was 5.0µL/s and the dispense speed was 5.0µL/s.

4.10 In-situ transesterification

1. Acid-catalysed in-situ transesterification

Firstly, the microalgae cells were concentrated by centrifugation for 10 minutes at 600g and 4°C. Then, after removing the supernatant, the bath sonicator was used to disrupt the cells. Then, 3 mL of methanol: H_2SO_4 (H_2SO_4 2.5% in methanol) mixture was added

to the samples without an extraction step. The samples were kept at 60°C for 4 hours in a pre-heated water bath. Then, the samples were extracted with 3 mL of hexane and 3 mL of water. The Vortex was used to mix the solvents for 2 minutes. Then, the centrifuge was used to separate the two phases for 10 minutes at 600g and 4°C. The upper phase, which contains the hexane and the FAMEs were transferred to another Kimble tube. The lower phase was added to 3 ml of hexane for a re-extraction. Then, after vortexing and centrifugation under the same conditions as before, the upper phase was combined with the previous upper phase. Then, the Genevac was used to evaporate the hexane. Finally, the samples were suspended in 120 µL heptane and analysed by the GC-FID.

2. Lipase-catalysed in-situ transesterification

Firstly, the microalgae cells were concentrated by centrifugation for 10 minutes at 600g and 4°C. Then, after removing the supernatant, the lipase (Novozyme 435) were added, and the same amount of solvent and methanol were added to create the transesterification system. The lipase amount was 50% w/w of FFA. FA/solvent ratio was: 50mg/1.3ml, and Methanol: FFA ratio was 9:1. The internal standard 200 mg of C15 was added before the transesterification. The samples were kept in the water bath at 37°C for 24 hours during the in-situ transesterification. Then, the samples were extracted with 3 mL of hexane and 3mL of water. The Vortex was used to mix the solvents for 2 minutes. Then, the centrifuge was used to separate the two phases for 10 minutes at 600g and 4°C. The upper phase, which contains the hexane and the FAMEs, was transferred to another Kimble tube. The lower phase was added in 3 ml of hexane for re-extraction. Then, after vortexing and centrifugation (the same conditions as the first mixing and separation), the upper phase was combined with the previous upper phase. Then, the Genevac was used to evaporate the hexane. Finally, the samples were suspended in 120µL heptane and analysed by the GC-FID.

3. Control of lipase transesterification

Firstly, 5 ml of microalgae cells culture were concentrated by centrifuge for 10 minutes at 600g, 4°C in Kimble tube. Then, the bath sonicator was used for 30 minutes. After sonication, the hexane (5ml) and methanol (5mL) were used as extraction solvent. Vortexing was used for mixing the solvents for 2 minutes. The centrifuge was used to separate the two phases at 600g and 4°C for 10 minutes. Then, after transferring the upper phase to another Kimble tube, 5 ml of hexane was added to have a re-extraction step. For the transesterification step, the conditions were the same as described before (lipase amount: 50% w/w, FA/solvent: 50mg/1.3ml, and Methanol: FFA ratio: 9:1), and there was no water in the system because the water required was too small to be measured. The transesterification time was 24 hours, and the temperature was 37°C in bath water. Then, the samples were extracted by 3mL of hexane with 3mL of water. The Vortex was used to mix the solvents for 2 minutes. Then, the centrifuge was used to separate the two phases. The separation time was 10 minutes at 600g and 4°C. The upper phase, which contains the hexane and the FAMEs were transferred to another Kimble tube. The lower phase was added in 3ml of hexane to have the re-extraction, and the same procedures were taken to extract and separate the phases. Then, the Genevac was used to evaporate the hexane. Finally, the samples were suspended in 120µL

heptane and analysed by the GC-FID.

4.11 Determination of water content

Dry 15 ml Falcon tubes were weighted on the balance (Weight A). Then, 5 ml of microalgae culture was added to the tubes. After centrifuge, the supernatant was discarded and the tubes with the wet cells were weighted on the balance (Weight B). Then, the tubes were put in an oven for 48 hours (until the weight was constant) at 90°C. After drying, the tubes with the dry cell mass were weighted again (Weight C).
The water content was calculated as below:

$$\frac{weight\ (water)}{weight\ (water + dry\ cells)} x100\% = \frac{B - C}{B - A} x\ 100\% = water\ content\ (\frac{g}{g}\%)$$

In which:
A: the tube's weight
B: the tube and the weight of the wet cells
C: the tube and the weight of the dry cells

5. Results:

5.1 Chlorella Sorokiniana cultivation

The aim of the cultivation was to determine the cultivation conditions to maximize lipids accumulation.

C. Sorokiniana was cultivated in different media to characterize the growth curve. The medium used included TAP medium, 3N-BBM+V medium, TAP medium with low nitrogen (10% nitrogen source of TAP medium), and TAP with glucose (11g/l). For the TAP medium, the microalgae cells were cultivated under mixotrophic and heterotrophic conditions. For TAP medium with low nitrogen, the cells were cultivated under mixotrophic conditions with light, and for the 3N-BBM+V medium, the cells were cultivated under phototrophic conditions. For the TAP with glucose medium, the cells were cultivated under heterotrophic conditions.

Figure 12 shows the growth curve of *C. Sorokiniana* growing in different media. In this graph, it was shown that the TAP with glucose medium had the highest yield of biomass and in 3N-BBM+V medium, the yield of microalgae was the lowest.

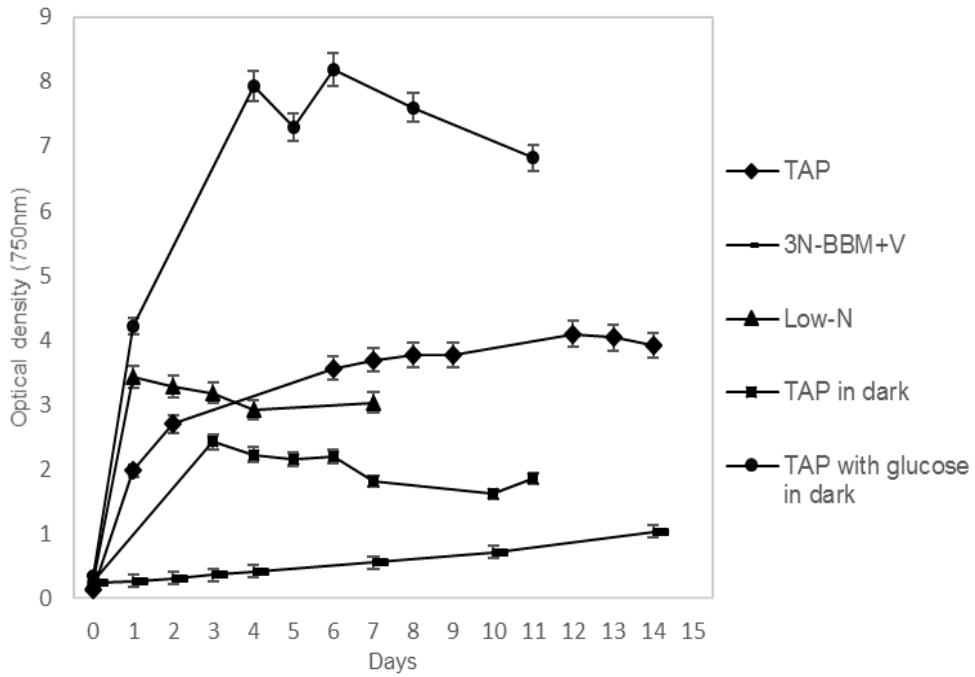

Figure 12. The microalgae cultivation in the different media. The microalgae were cultivated in TAP, 3N-BBM+V, Low Nitrogen source TAP medium, Heterotrophic growing in TAP and TAP with glucose medium. The error bars are the standard deviation of replica experiments (n=3).

From figure 12, the microalgae were cultivated in TAP media with glucose under heterotrophic growth can have the highest optical density. This was because this media contained extra glucose, which can provide the energy and the carbon source for the carbon skeleton during cell growth. The stationary phase started after day 5. Besides, the

3N-BBM+V media had the lowest optical density. This might because there was no carbon source in this media and the energy that can help microalgae growth was low.

5.1.1 OD-dry mass curve for the microalgae cultivated in different media

Figure 13 shows *Chlorella Sorokiniana* OD-dry mass curve when using different media for cultivation. The cell concentration can be calculated when the OD of the culture was known.

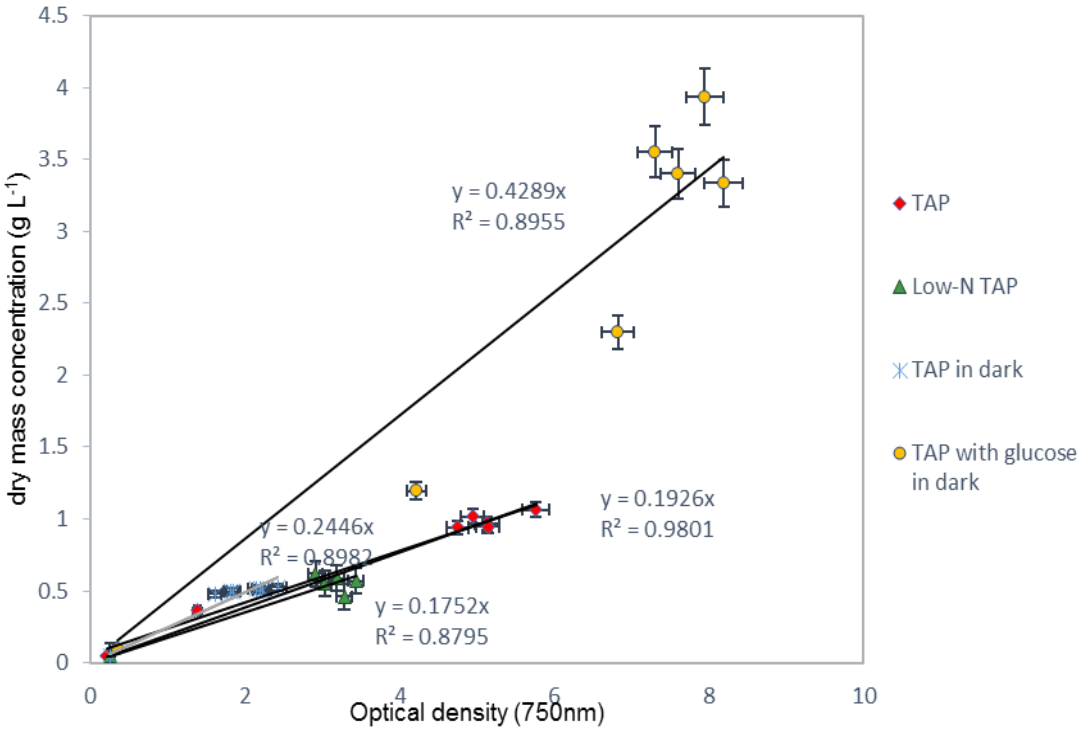

Figure 13. Optical density-dry mass curve when using different media. The optical density was measured every day during cultivation. The error bars represent triplicates for each sample.

Table 14. Optical density-dry mass curve in different media.

Media name	Linear relationship		R^2
TAP media	Y=0.1926X	Y was dry mass (g/l) and X was optical density	0.9801
Low nitrogen source media	Y=0.1752X		0.8795
TAP media and heterotrophic growth	Y=0.2446X		0.8982
TAP with glucose	Y=0.4289X		0.8955

According to table 14, the relationship of the optical density and the dry biomass was a linear relationship. The dry biomass can be calculated in the next cultivation by detecting

the optical density of the cell culture.
Because different conditions were used for cultivation, the lipid content of the microalgae cells were different, and the lipid content might influence the absorbance. Besides, when at the same OD, the TAP with glucose condition had the highest dry mass. In this condition, the lipid amount in the cells was the highest, which means the dry mass of this condition was the highest of all the other conditions. Thus, although the same strain of the microalgae was used (the *Chlorella Sorokiniana*), the correlation between the OD (optical density) and the dry mass concentration would be different.

5.1.2 Lipid content in different media and different conditions

Table 15 and figure 14 showed the maximum lipid content in different media and different conditions.
From table 15, it was observed that when using TAP medium with 11g/L glucose in heterotrophic condition, the lipid content reached as high as 52.59% and the second highest lipid content was seen when using low nitrogen source TAP medium in the phototrophic condition. The lipid content was 12.11%

Table 15. The maximum lipid content in different media and different C/N ratio.

Medium and condition	Condition	Day	C/N (mol/mol)	Cell concentration (g/l)	Lipid content (mg lipid/mg DCW)	Productivity (mg/L/day)
TAP	Mixotrophic	5	~7.0	0.61±0.09	1.70%± 0.51%	2.00±1.00
3N-BBM+V	Phototrophic	14	~0	0.23±0.006	5.14%± 0.82%	0.86±0.36
Low-N source TAP medium	Mixotrophic	7	~70.0	0.55±0.026	12.13%± 0.63%	9.57±0.43
TAP	Heterotrophic	7	7	0.50±0.027	3.52%± 0.23%	2.43±0.14
TAP with glucose (1g/l)	Heterotrophic	2	11.67	0.92±0.04	7.23%± 0.44%	35.00±2.00
TAP with glucose (3.5g/l)	Heterotrophic	8	23.33	2.02±0.10	15.34%± 0.82%	38.75±3.50
TAP with glucose (6g/l)	Heterotrophic	8	35	2.9±0.20	13.86%± 0.91%	50.00±5.00
TAP with glucose (8.5g/l)	Heterotrophic	8	46.67	5.12±0.26	45.97%± 2.40%	293.75± 11.25
TAP with glucose (11g/l)	Heterotrophic	8	58.33	5.6±0.45	52.59%± 3.26%	368.75± 15.00

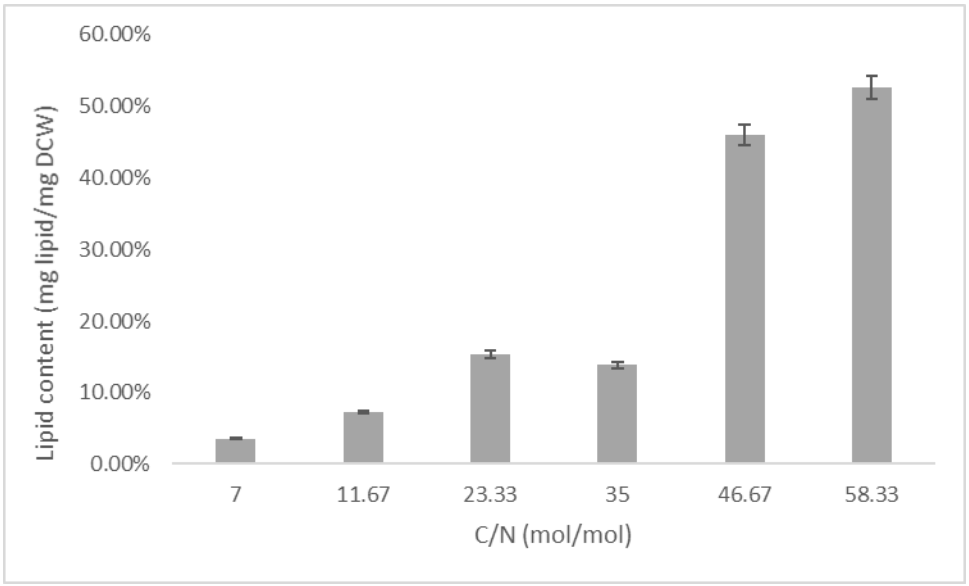

Figure 14. The maximum lipid content in different media and different C/N ratio.

In table 15, when the microalgae were cultivated in TAP media, the maximum lipid content was around 1.7% on the 5th day, which was the lowest lipid content. When the

microalgae were cultivated in 3N-BBM+V media, the maximum lipid content was around 5.1% on the 14th day, which took the longest time to reach the highest lipid content. The optimal condition to cultivate the microalgae was using TAP media with 11g/L glucose and keeping the microalgae in dark, which had the lipid content of around 52.59% and only required 8 days. Besides, the lipid productivity was increasing when the C/N ratio increasing under the heterotrophic condition. This was because the carbon source can provide the energy and the carbon atom to form the lipid.

A time course of the heterotrophic cultivation in TAP medium with glucose (11g/l) was carried out because, in this medium and condition, the highest lipid content was obtained. According to Xiao-Fei Shen's report, the productivity of heterotrophic cultivation was 51 mg L^{-1} day^{-1}. However, in this experiment, acetate was the only carbon source (Xiao-Fei Shen, 2019). Manoranjan Nayak found that the microalgae lipid productivity was 65.17mgL mg L^{-1} d^{-1} when using urea as nitrogen source (Manoranjan N., 2016). In this experiment, the highest lipid productivity was ~369 mg L^{-1} d^{-1}. This productivity was higher than both experiments and can be used in the next steps.

As shown in Figure 15 acetate was consumed firstly after day 1 and glucose was consumed during the whole cultivation. The lipids will be produced inside the microalgae cell when the glucose concentration was decreasing because the microalgae were storing the lipids. The glucose did not run out after day 8 and during day 6 and day 8, the glucose concentration remained at a similar level, which indicates that in this experiment, the glucose was in excess.

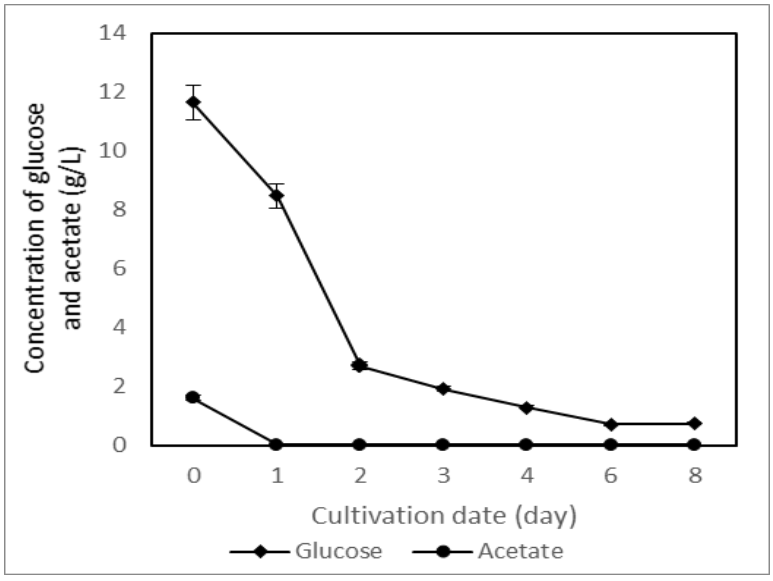

Figure 15. Glucose and acetate concentration in the culture. C. Sorokiniana were cultured in TAP medium with 11 g/L glucose under heterotrophic conditions. The error bars represented standard deviations of the mean (n=3).

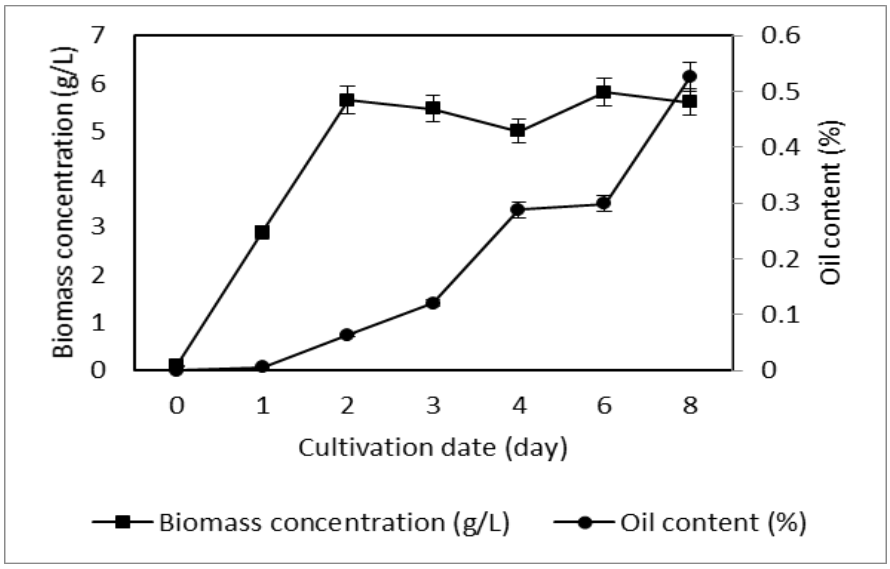

Figure 16. Lipid and cell concentration of *C. Sorokiniana* cultivated in TAP medium with glucose (11g/L) under heterotrophic conditions. The error bars represented standard deviations of the mean (n=3).

*The other figures of growth curve and consumptions for other media were in the appendix.

Intracellular lipid concentration was increasing for the first 3 days because the microalgae were consuming the acetate and glucose and the lipids were accumulated as energy storage. At the same time, the cell concentration was increasing because there were sufficient nutrients in the medium. And then, the cell mass remained, and the lipid concentration increased due to the storage of the energy.

It was reported that for biodiesel production, the microalgae lipid content should be higher than 10% (Kumar R., 2015). In this project, the microalgae that were cultivated in low-nitrogen source TAP medium with light reached a lipid content of 12.1%±0.6%. The microalgae that were cultivated in TAP with glucose (11g/L) under heterotrophic conditions reached a lipid content of 52.6%. Under these conditions the microalgae reached a cell concentration of around 5.6g/L DCW after 8 days of cultivation. According to Han and Xiaoling, the lipid content of heterotrophically cultivation of Chlorella sp. can reach 54.7% with 10g/l glucose, which was very similar to this project's results (Han X. and Xiaoling M. 2006). Because the sugar (glucose) can provide the energy and the carbon atom to form the lipids, the media with glucose can provide more lipid and had higher microalgae biomass. Thus, the microalgae that were cultivated in TAP with glucose (11g/L) with heterotrophic growth was used in the downstream process as it had the highest cell concentration and the highest lipid content.

5.2 Flocculation and Enzymatic treatment

5.2.1 Flocculation

Flocculation was used as a pre-harvesting method in this process. The cell size after flocculation was detected in this part to identify if the flocculation will change the particle size.

5.2.1.1 Effect of flocculation on algal cell size

In this experiment, the microalgae were directly treated by flocculation. The size distribution was measured before the flocculation and after the flocculation. After flocculation, the supernatant and lower phase (the aggregated cells) were measured separately. Figure 17 and table 16 showed the particle size distribution of the particles before and after flocculation.

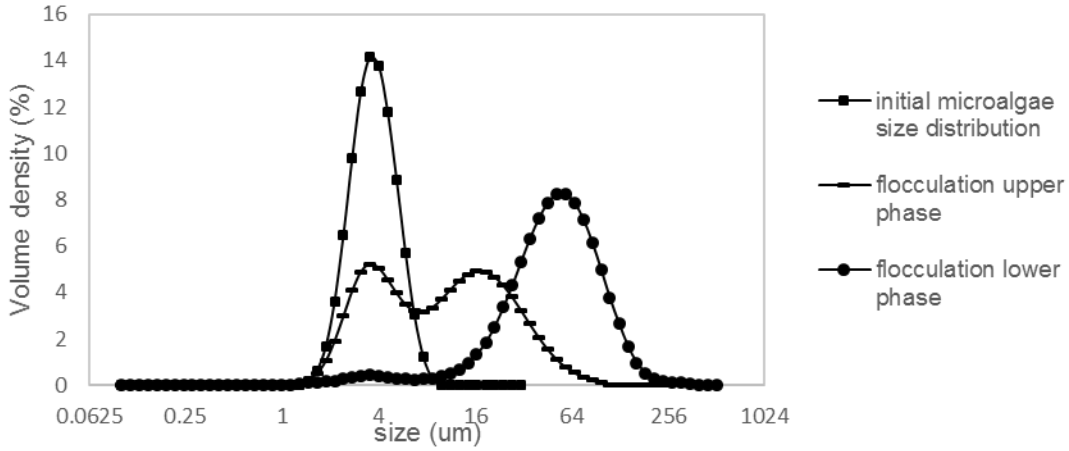

Figure 17. Particle distribution of microalgae before and after flocculation. The microalgae were treated by chitosan.

Table 16. Particle distribution of microalgae of all the measurements. In this experiment, the microalgae were aggregated by flocculation. Dx (n) means n% of the particles were under this size (n=10, 50, and 90). For initial microalgae particles, 10% of the particle were under 2.61μm.

Name of treatment	Sample name	Dx (10) (μm)	Dx (50) (μm)	Dx (90) (μm)
Initial microalgae	Average of "microalgae"	2.61±0.016	4.06±0.036	6.92±0.034
After flocculation data upper phase	Average of "microalgae"	3.05±0.026	10.7±0.092	34.6±0.32
After flocculation data lower phase	Average of "microalgae"	21.2±0.28	54.3±0.58	113±1.60

For the initial microalgae size, table 16 showed that 90% of the particles were under 6.92 μm and 80% of the particles were between 2.61 μm and 6.92 μm. After flocculation, for the upper phase, 80% of the particles were between 3.05 μm and 34.6 μm. The sizes of these particles were higher than the initial microalgae cells, which means that some of the particles remain in upper phase and some of these particles were flocculated by chitosan. However, the size of these particles was not huge enough for settling. In the lower phase after the flocculation, 80% of the particles were between 21.2 μm and 113 μm, which was as high as 10-20 times before the flocculation. This result showed that after the flocculation, the cells were flocculated, and the particle sizes increased.

5.2.2 Size distribution of flocculation (after enzymatic treatment)

In this experiment, the microalgae were treated with enzymes firstly. Then the flocculation was used for harvesting.

The size of microalgae cells was measured before the enzymatic treatment and after enzymatic treatment. Then, the flocculation was used. After the flocculation, the size of microalgae cells in the upper phase (supernatant) and the lower phase were both measured. Figure 18 and table 17 showed the particle size distribution of all the samples.

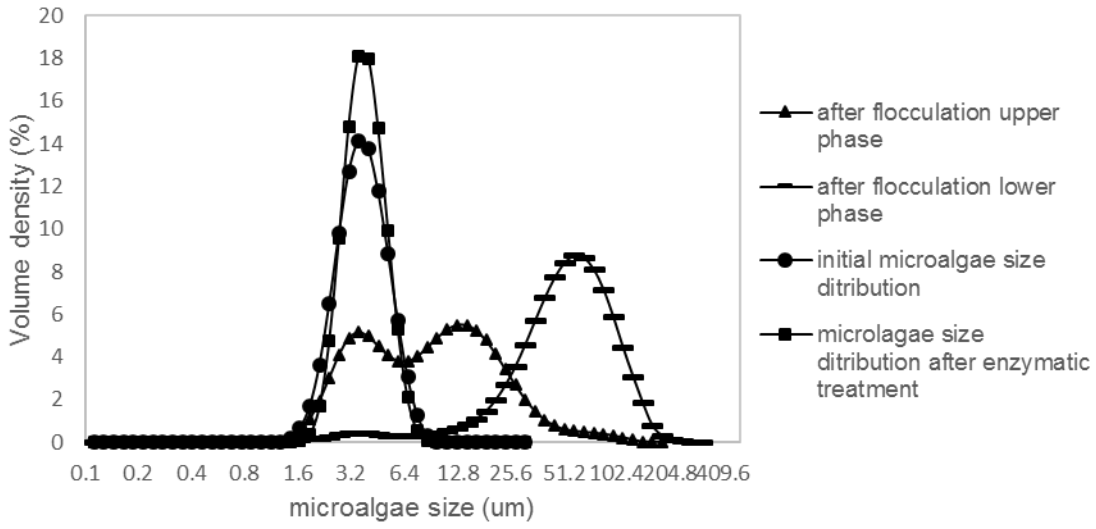

Figure 18. Particle distribution of microalgae of all the samples. In this experiment, the microalgae were treated by enzymes firstly to disrupt the cell walls and then chitosan was added to aggregate the cells.

Table 17. Particle size distribution of microalgae of all the samples.

Name of Treatment	Dx (10) (μm) *	Dx (50) (μm)	Dx (90) (μm)
Initial microalgae	2.61±0.014	4.06±0.045	6.92±0.075
After enzymatic treatment	2.87±0.020	4.05±0.045	5.77±0.053
After flocculation (upper phase)	3.05±0.028	9.75±0.083	28.6±0.62
After flocculation (lower phase)	22.1±0.68	58.1±0.88	113±1.65

- DX (10) means 10% of the detected particle sizes were smaller than this size.

In this experiment, the microalgae were treated by enzymes to break the cell walls and then chitosan was added to aggregate the cells. For initial microalgae particles, 10% of the particle were under 2.61μm (Dx 10), 50% of the particle were under 4.06μm (Dx 50) and 90% of the particles were under 6.92μm.

For the initial microalgae size, table 17 shows that 90% of the particles were under 6.92um and 80% of the particles were between 2.61μm and 6.92μm. After enzymatic treatment, 80% of the particles were between 2.87μm and 5.77μm. Then, the samples were flocculated by chitosan and the upper phase data shows that the particle size of the microalgae was between 3.05μm and 28.6μm. The first wave indicated that some cells were not flocculated and existed as single cells. The second wave indicated that although some of the cells were flocculated, the sizes of the particles were not large enough. Thus, the particles were not settled and remained in the supernatant. This data showed that there were still some microalgae cells not being flocculated and the clarification levels can be calculated by using the optical density of the upper phase. For the lower phase data, 80% of the particles were between 22.1μm and 113μm. This data was very similar to the data of the last experiments (direct flocculation), which showed the size of microalgae aggregates after flocculation was between 22μm and 113 μm.

Moreno (2015) reported that, after the flocculation, the microalgae aggregated size was between 2-4μm to 70-80μm. In this experiment, the particle size was between 21μm-113μm. The particle size of this experiment was larger than that reported in the literature. This was because, in this experiment, the pH was adjusted to 6 and in the work by Moreno (2015), the pH used was 7. When the pH decreased, larger size of the particles will be formed due to a more positive charge (Moreno, 2015).

5.3 Enzymatic treatment

Aim:

To test the microalgae size changing after enzymatic treatment.

To test the amount of released sugar levels, which represented the hydrolysed cell walls, after different treatments.

5.3.1 Cell size distribution

5.3.1.1 Cell size distribution after sonication

The microalgae were treated by sonication in this experiment. A sonication probe was used to break the cells for 5 minutes. Then, Mastersizer 2000 was used for the cell size distribution. Figure 19 and Table 18 showed the results.

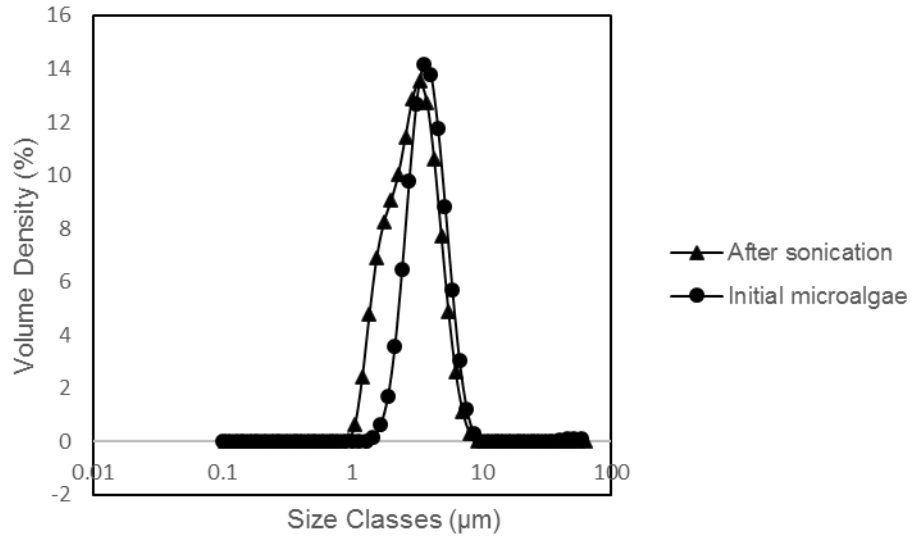

Figure 19. The particle distribution of microalgae before and after sonication.

Table 18. The particle distribution of initial microalgae and the particle distribution of microalgae after sonication. Dx (10) means 10% of the particles were below this size. Dx (50) means 50% of the particles were below this size. Dx (90) means 90% of the particles were below this size.

Name of Treatment	sample name	Dx (10) (µm)	Dx (50) (µm)	Dx (90) (µm)
Initial microalgae	Average of "microalgae"	2.61±0.014	4.06±0.045	6.92±0.075
After sonication	Average of "microalgae"	1.56±0.008	2.89±0.029	4.85±0.065

Figure 19 and Table 18 presented the cell size distribution of initial microalgae and the microalgae after sonication. For initial microalgae particles, 10% of the particle were under 2.61µm. For the microalgae after sonication, 10% of the particle were under 1.56µm. From table 18, 50% of the initial microalgae size was under 4.06µm. For microalgae after sonication, 50% of the particle were under 2.89µm. For initial microalgae particles, 90% of the particles were under 6.92µm. For the microalgae after sonication, 90% of the particle were under 4.85µm. It should be noticed that there were some small fines (~3µm) after the sonication, this would not influent the next steps because the cells

and the fines were mixed with the extraction solvents together during extraction.

5.3.1.2 Cell size distribution after enzymatic treatment

In this experiment, the microalgae were treated by enzymatic treatment.

The size of microalgae cells was measured before the enzymatic treatment and after enzymatic treatment. Figure 20 and table 19 showed the particle size distribution of all the samples.

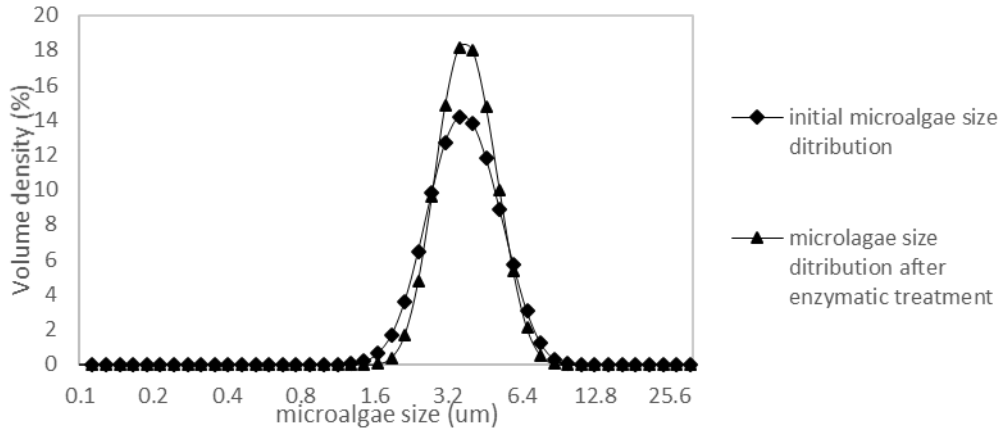

Figure 20. Particle distribution of microalgae of all the measurements. In this experiment, the microalgae were treated by enzymes.

Table 19. particle distribution of microalgae of all the measurements. In this experiment, the microalgae were treated by enzymes. Dx (10) means 10% of the particles were under this size. Dx (50) means 50% of the particles were under this size. Dx (90) means 90% of the particles were under this size. For the other groups, the Dx (10), Dx (50), and Dx (90) had the same meaning.

Name of Treatment	sample name	Dx (10) (μm)	Dx (50) (μm)	Dx (90) (μm)
Initial microalgae	Average of "microalgae"	2.61± 0.014	4.06± 0.045	6.92± 0.075
After enzymatic treatment	Average of "microalgae"	2.87± 0.020	4.05± 0.045	5.77± 0.053

For the initial microalgae size, table 19 showed that 90% of the particles were under 6.92um and 80% of the particles were between 2.61μm and 6.92μm. After enzymatic treatment, 80% of the particles were between 2.87μm and 5.77μm, which showed that after enzymatic treatment, the particle size was similar to the initial microalgae size. This result suggested that the enzymes would not change the microalgae size a lot.

5.3.1.3 Cell size distribution of enzymatic treatment following the flocculation

In this experiment, the microalgae were harvested by flocculation firstly. Then, the enzymes were used to disrupt the cell wall.

The cell size distribution was measured before the flocculation and after the flocculation

(lower phase). Then, after the enzymatic treatment, the size distribution was measured again. Figure 21 and table 20 shows the particle size distribution of all the samples.

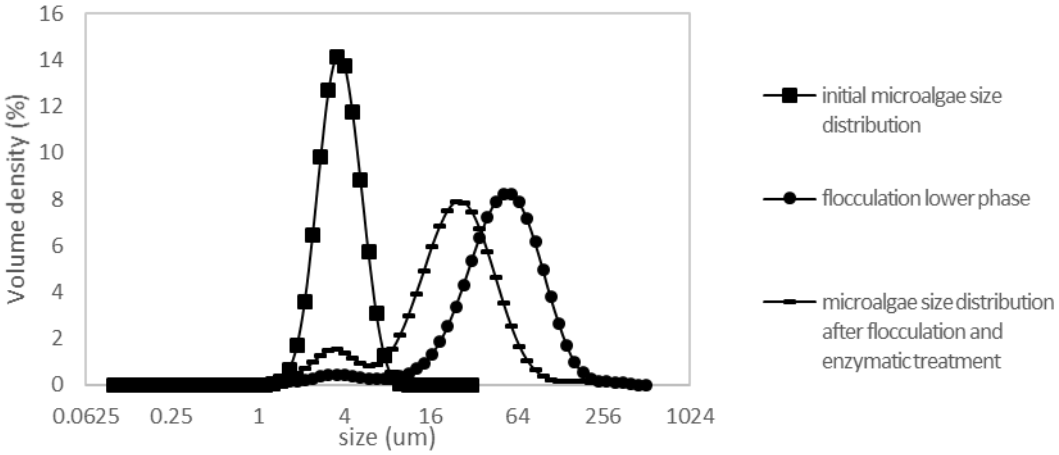

Figure 21. Particle distribution of microalgae of all samples. The microalgae were treated by flocculation firstly and then by enzymatic treatment.

Table 20. Particle distribution of microalgae of all the samples. In this experiment, the microalgae were aggregated by flocculation and then enzymes were added to break the cell walls. Dx (10) means 10% of the particles were under this size. Dx (50) means 50% of the particles were under this size. Dx (90) means 90% of the particles were under this size. For the other samples, the Dx (10), Dx (50), and Dx (90) had the same meaning.

Name of Treatment	sample name	Dx (10) (μm)	Dx (50) (μm)	Dx (90) (μm)
Initial microalgae	Average of "microalgae"	2.61±0.016	4.06±0.036	6.92±0.034
After flocculation (lower phase)	Average of "microalgae"	21.2±0.28	54.3±0.58	113±1.60
After enzymatic treatment	Average of "microalgae"	5.55±0.023	24.5±0.25	53.3±0.65

For the initial microalgae size, table 20 showed that 90% of the particles were under 6.92μm and 80% of the particles were between 2.61μm and 6.92μm. In the lower phase after the flocculation, 80% of the particles were between 21.2μm and 113μm, which was as high as 10-20 times before the flocculation. After the enzymatic treatment, the particles were smaller. Because the chitosan is a polysaccharide and the Viscozyme enzymes can hydrolyse it. It is likely that some of the cell aggregates were broken up. Thus, the particle sizes after the enzymatic treatment were smaller than the particle sizes after flocculation.

In conclusion, the results suggest that the Viscozyme could break the microalgae aggregates after flocculation.

5.3.2 Comparison between two groups: group 1: flocculation before enzymatic treatment, group 2: enzymatic treatment before flocculation

5.3.2.1 Clarification level calculation

Aim:

The aim of this part was to compare different flocculation clarification level under different steps.

Table 21. The clarification level for each group. In the first group, the microalgae were firstly aggregated by chitosan and then the enzymes were added to break the cells. In experiment 2, the microalgae cells were treated by enzymes at first and then, the chitosan was added to aggregate the cells.

No.	Group name	OD of the supernatant	Clarification level	P-value	F	F crit	a
1	Flocculation followed by enzymatic treatment	0.86±0.03	91.4%±0.34%	0.003	41.59	21.20	0.05
2	Enzymatic treatment before flocculation	1.07±0.05	89.3%±0.45%				

From table 21, the clarification level of flocculation before enzymatic treatment group was around 91% and the clarification level of enzymatic treatment before flocculation group was 89%. The clarification level of the first group was slightly higher than that of the second group. This was because after the enzymatic treatment, some of the cells were broken and those cells were more difficult to flocculate. According to the analysis of variance, the P value (0.003) was less than significant level (a=0.05), and the F was bigger than F crit, which means that the difference of these two clarification levels was statistically significant.

It was reported that, for Chlorella species, the flocculation using chitosan as flocculant had a separation efficiency of 94%-99% (Scenedesmus) and 52%-75% (Chlorella sp.) (Moreno, 2015). In this experiment, the recovery efficiency was around 90%, which was higher than that in the literature. .

In conclusion, the clarification levels of two groups (the flocculation followed by enzymatic treatment and the enzymatic treatment followed by flocculation) had statistically significant difference and the average clarification level was around 90%.

5.3.2.2 The saccharification yield after enzymatic treatment and acid hydrolysis

Aims:
To compare released sugar concentration after different steps (enzymatic hydrolysis and acid hydrolysis).

The Viscozyme was used during the experiment to hydrolyse the cell walls. The saccharification yield (ratio of sugar released from the cell walls to the dry mass) can determine the cell wall hydrolysis level. Besides, the sugars could be recovered and might can be reused for the cultivation. For the three groups, the enzymatic treatment before or after the flocculation groups and the acid hydrolysis group, the supernatant was transferred and detected by ion chromatography system (ICS). Table 22 showed the sugars detected by ICS.

Table 22. Saccharification yield after enzyme treatment and acid hydrolysis. In the first experiment, the microalgae were firstly aggregated by chitosan and then the enzymes were added to disrupt the cells. In experiment 2, the microalgae cells were treated by enzymes at first and then, the chitosan was added to aggregate the cells. In control experiment 3, the sugars were released by acid hydrolysis.

No.	Description	galactose (g/l)	glucose (g/l)	fructose (g/l)	Total concentration of all sugars (g/l)	Saccharification yield (% w/w) *
1	Flocculation before enzymatic treatment	0.047±0.006	0.638±0.059	0.516±0.115	1.201±0.115	3.641%±1.03%
2	Enzymatic treatment before flocculation	n.d.	0.057±0.010	0.1206±0.012	0.177±0.002	5.382%±0.74%
3	Sulfuric acid hydrolysis	0.045±0.041	1.05±0.473	0.011±0.001	1.111±0.261	20.160%±4.74%

*the saccharification yield was based on the dry cell biomass (w/w %).

Table 22 indicated that the flocculation before enzymatic treatment group had a glucose concentration of 0.64 g/l and total sugar concentration of 1.201g/l. For the enzymatic treatment before flocculation group, the glucose concentration was 0.057g/l and the amount of all sugars was 0.177g/l, which was quite low. This was because, in this group, the volume of the sample was much higher than the first group during enzymatic treatment. Thus, the sugars concentration after flocculation was around 0.177g/l, which was lower than the first group. However, when comparing the enzymatic saccharification yield, group two had a slightly higher yield (5.38%±0.79%) than the first group (3.64%±

1.06%). The microalgae aggregation might influence the sugar release because the superficial area decreased.

For the acid hydrolysis of algae cell wall group, the enzymatic saccharification yield was around 20% of the dry cell biomass, which can release most of the sugar from the cell wall. This yield was much higher than that of other groups.

Table 23. The analysis of variance between each group. The saccharification yields from table 22 were used for the analysis of variance. The “a” value is the significance level.

Groups	P-value	F	F crit	a
1 & 2	0.076	5.65	7.71	0.05
2 & 3	0.006	28.35752	7.71	
1 & 3	0.004	34.65764	7.71	

*group 1, 2, and 3 were from table 22.

According to table 23, the P-value of group 1 and 2 was 0.076, which was bigger than the significant level (a=0.05). The P-value of group 1 and 3 was 0.004 and the P-value of group 2 and 3 was 0.005, which were less than significant level (a=0.05). The F value of group 1 and was less than F crit. The F values of group 1 and 3; group 2 and 3 were bigger than F crit. Thus, saccharification yields of the group 1 and group 2 had no statistically significant difference and the saccharification yields of group 3 and group 2; group 3 and group 1 were statistically significant.

In conclusion, the enzymes had an influence on the microalgae cell wall after the reaction and the concentration of sugars after enzymatic treatment had a large difference to each other due to the volume changes. The acid hydrolysis had the highest saccharification yield and the other two groups had lower yield, which indicated that the enzymes can have some influence on the microalgae cell wall but not hydrolysed all the cell wall. For the enzymatic saccharification yield, group 2 has a higher yield than group 1 because the microalgae aggregation might influence the sugar release because the superficial area decreased. According to the results from the enzymatic treatment, some sugars were hydrolysed, which showed that the Viscozyme had some impact on the microalgae cell wall. To determine the total sugars that can be released, the sulfuric acid hydrolysis was used. In the acid hydrolysis group, the saccharification yield was around 20%. From Naoko (2010), the cell wall of Chlorella species may contribute up to 22% of the dry weight of the cell. Thus, the yield of acid hydrolysis can be considered as the base line (Naoko A., 2010). The enzymatic saccharification yield of the two groups were 3.64%±1.03% and 5.38%±0.74%. These two groups had much lower saccharification yields than that of the acid hydrolysis group. From Lee (2013), the enzymatic saccharification yield was around 10.7% when the pH was at 5.5, and the temperature was 35℃ (Lee et al., 2013). The saccharification yield of both groups was lower than that yield.

5.3.2.3 The lipid extraction yield of two groups

Aim:

The extraction yield comparison between the two groups.

Table 24. The comparison of the extraction yield between the two groups. For the first group, the microalgae cells were concentrated by flocculation first. Then, the microalgae cells were treated by the enzymes. For the second group, the microalgae cells were treated by the enzymes firstly and then concentrated by the flocculation.

No.	Steps	Lipid (GC-FID) concentration (mg/ml)	Total extracted lipid (mg)	Yield (%)	F	P-value	F crit	a
1	Flocculation before enzymatic treatment	0.021±0.004	0.033± 0.006	1.11%± 0.19%	2.13	0.22	7.71	0.05
2	Enzymatic treatment before flocculation	0.018±0.002	0.029±0.003	0.95%± 0.001%				

The GC-FID data showed the lipid concentration from the FC-FID analysis. The total extracted lipid showed the lipid amount after the extraction. The extraction ratio was compared to the Nile red assay results.

From the table above, the extraction ratio of group 1 was higher than the extraction ratio of group 2 although the difference was insignificant. From the flocculation results, fewer microalgae cells were harvested in group 2. This might cause the extraction yield of group 2 lower than that of group 1 because the total initial amount of cellular lipids was less. According to the analysis of variance, the P value was larger than the significant level (a=0.05) and the F was less than F crit, which meant the two groups' extraction yields were similar to each other.

In conclusion, the flocculation clarification level and the extraction yield of these two groups were very similar. The sugar concentration of the samples from the flocculation after enzymatic treatment group was higher than another group. Besides, when considering the energy input, after flocculation, the volume of the sample was reduced by 10 times. Thus, the energy required to keep the temperature constant during enzymatic treatment decreased. Thus, the samples treated by flocculation before enzymatic treatment were used for the next experiments.

5.3.3 Evaluation of the conditions for the process

Aims:

To compare extraction yield after different harvesting/ cell wall treatment/ extraction methods when using the same culture.

5.3.3.1 The comparison of extraction performance using different pre-treatment methods (same extraction solvents)

There were three groups in this experiment. In group 1, the sonication was used to break up the cells after centrifugation. In group 2, enzyme treatment was used to disrupt the cells after flocculation. In group 3, samples were only treated by flocculation and all these three groups used chloroform-methanol (2:1 v/v) as extraction solvent. All three groups had the same acid transesterification conditions.
The extraction yields were shown in figure 22 below.

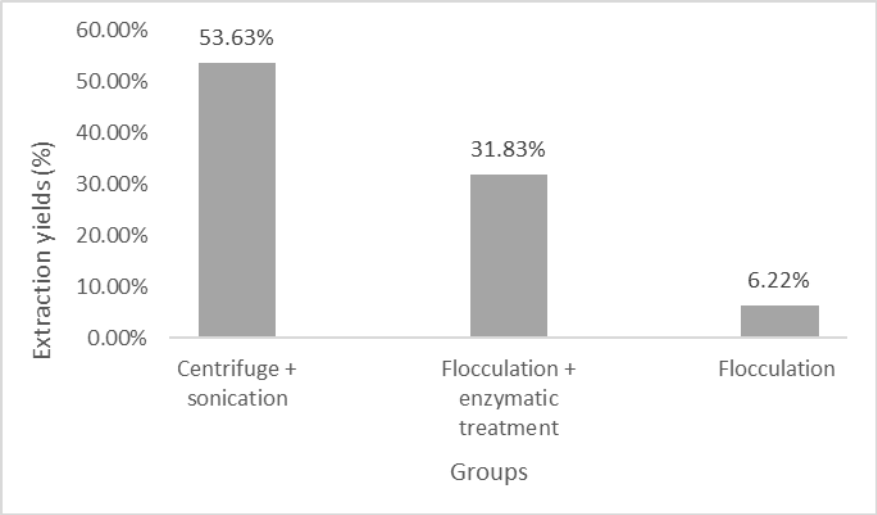

Figure 22: FAMEs analysis by GC-FID after conversion. In the first experiment, the centrifuge was used to harvest the microalgae cells. Then, sonication was used to break the cells. The chloroform-methanol (2:1v/v) mixture was used as extraction solvents, which was the same solvents that were used in experiment 2 and experiment 3. In experiment 2, the flocculation was used to concentrate the cells and enzymes were used to break the cell walls. In experiment 3, the flocculation was used to aggregate the cells and there was no cell breaking method.

Figure 22 showed that using sonication to break up microalgae cells and using chloroform-methanol (2:1 v/v) mixture to extract the lipids had the highest product yield, which was 53.63%. Using flocculation to harvest and using enzymatic treatment to break up cells gave an extraction yield of 31.83%. The extraction yield was quite low when there was no cell breakup step and the performance was only 6.22%. Thus, a cell wall treatment step can improve the extraction yield.

5.3.3.2 The exploration of enzymatic treatment conditions

To determine the optimal conditions of the enzymatic treatment, the FDE (factorial design experiment) of DOE (design of experiment) was used in this experiment. The pH range

(3-5.5) was chosen to be suitable for Viscozyme. The time range and the enzyme concentration were taken from previous work (personal communication Max Cardenas Fernandez).The factors and levels in this experiment were: pH (3-5.5), reaction time (4 hours - 8 hours), and the enzyme concentration (2-6 ug/g DCW). The groups and the results are shown in table 25.

Table 25. The FDE (factorial design experiment) groups and the results. The three factors and levels were: pH (3 & 5.5), reaction time (4 hours & 8 hours), and the enzyme concentration (2 & 6 ug/g DCW). The results were analysed by GC-FID and compared with the Nile red results.

Run	Factor 1 A: pH	Factor 2 B: Time (hour)	Factor 3 C: Enzyme concentration (μl/g DCW)	Response 1 Yield %
1	5.50	4.00	6.00	2.09
2	3.00	8.00	6.00	5.90
3	5.50	4.00	2.00	2.69
4	3.00	4.00	6.00	7.27
5	5.50	8.00	2.00	2.30
6	3.00	8.00	2.00	2.22
7	5.50	8.00	6.00	5.22
8	3.00	4.00	2.00	2.15

Then, Design Expert 10 was used to analyse the data.
The analysis used coded data and from the normal plot (showed below), the selected factors were: A: pH, C: enzyme concentration (μl/g DCW), and AC: the interaction of pH and enzyme concentration (μl/g DCW).

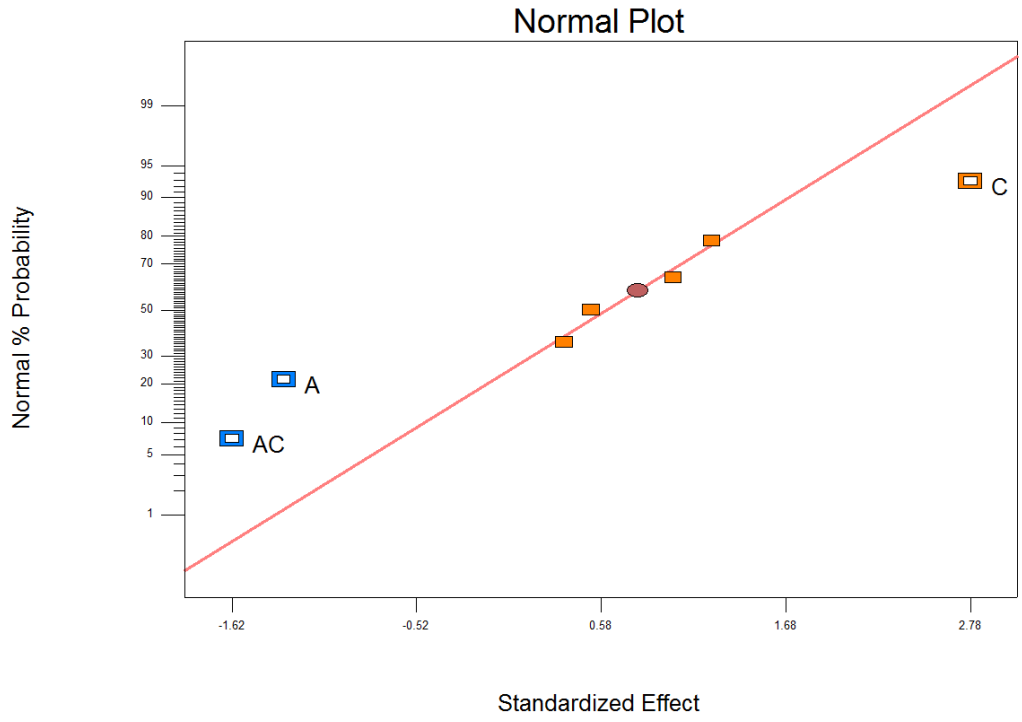

Figure 23. the normal plot of selected factors from Design Expert 10 analysis.

The ANOVA of selected factors were showed below:

Table 26. the ANOVA of selected factorial model.

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	24.1378	3	8.045933	5.440669	0.0677	
A-pH	3.4322	1	3.4322	2.320857	0.2023	
C-Enzyme concentration	15.4568	1	15.4568	10.45191	0.0319	Significant
AC	5.2488	1	5.2488	3.549244	0.1327	
Residual	5.9154	4	1.47885			
Cor Total	30.0532	7				
Adeq Precision			5.116884			

From table 26, the factor C: enzyme concentration (μl/g DCW) Prob>F was 0.0319, which was less than 0.05 and this factor was significant. The other factors were insignificant. The Adeq precision measured the signal to noise ratio, which was 5.12 and was greater than 4. This indicated that this model can be used to navigate the design space.

The final equation was showed below (coded factors):

$$\text{Yield} = 3.73 - 0.66 * A + 1.39 * C - 0.81 * A * C$$

From the equation, the coefficient of factor A was negative which indicated that the interaction between factor A and yield had negative relationship. The coefficient of factor C was positive, which indicated that the interaction between factor C and yield was positively relevant.

Figure below showed the diagnostics of the model.

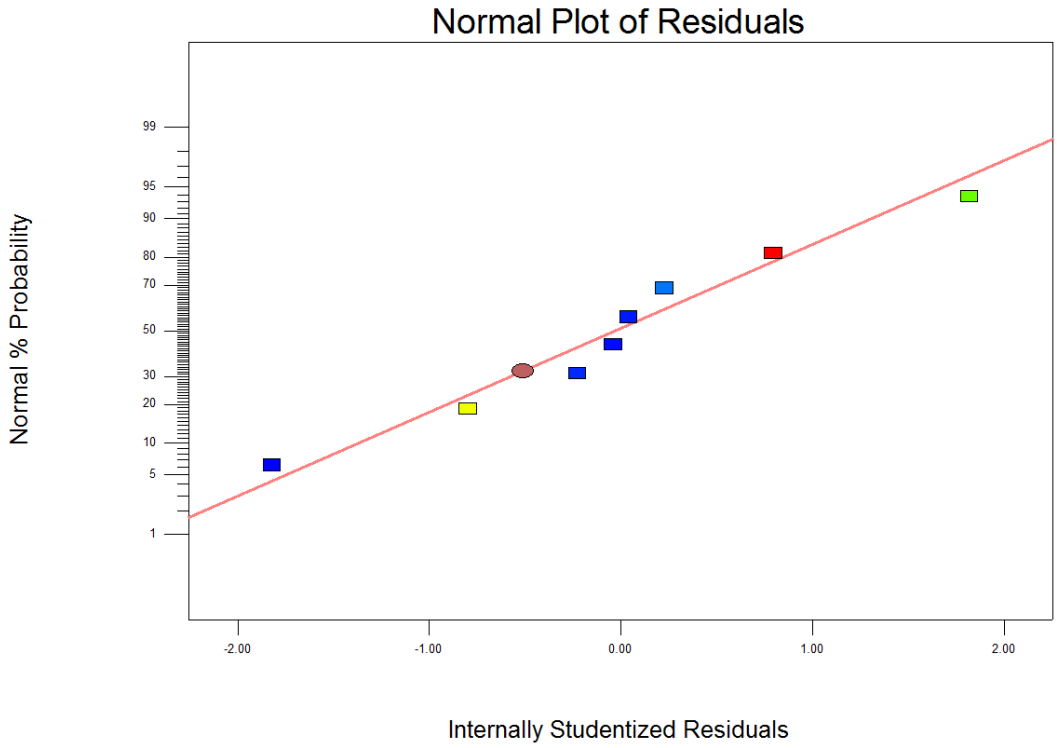

Figure 24. the normal plot of residuals.

*The figure was from Design Expert 10.

Figure above showed the normal plot of the residuals. The line indicated that this regression model fitted well.

Figure below indicated the 3D surface of factor A(pH), factor C (enzyme concentration; μL/g DCW), and the extraction yield after enzymatic treatment when factor B (time; hours) was at 4 hours.

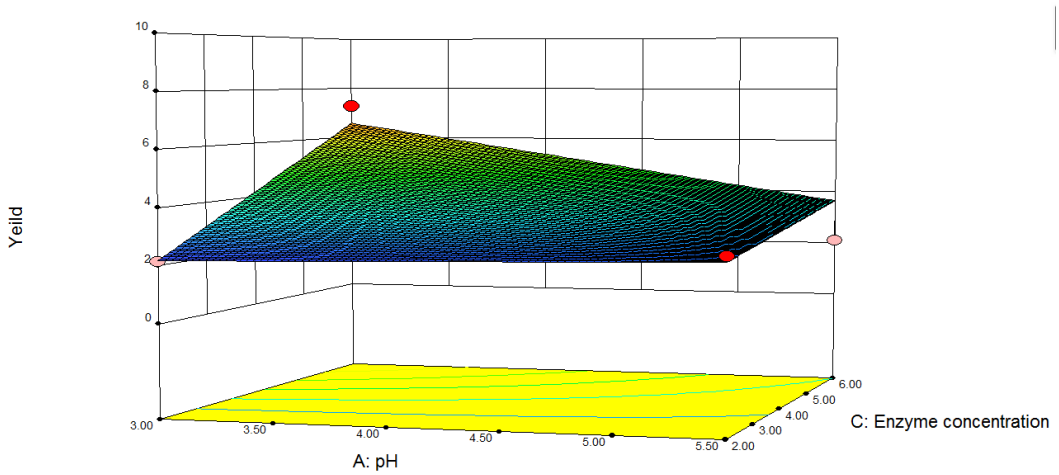

Figure 25. The 3D surface of factor A(pH), factor C (enzyme concentration; μl/g DCW), and the extraction yield after enzymatic treatment when factor B (time; hours) was at 4 hours.

From figure above, the highest point was reached when the pH was at 3 and enzyme concentration was at 6(μl/g DCW) when the time was 4 hours. Thus, for this experiment, the best conditions for enzymatic treatment were as follows:

Table 27. The optimal conditions for enzymatic treatment.

pH	Time	Enzyme concentration
3	4 hours	6 μg/g DCW

However, the factorial design experiment was used in this experiment and the results showed just a trend for each factor. The optimal conditions for enzymatic treatment might lie outside of the range tested in this experiment. Thus, a response surface design experiment should be run to determine the optimal conditions.

5.3.3.3 The exploration of different extraction solvents.

Aim:

To compare the extraction yields after different extraction solvents when the culture was treated by the same procedures.

There were three groups in this experiment. The concentration method in these experiments was flocculation and the cell disruption method was enzymatic treatment. The only changes were in experiment 1 (No. 1), the extraction solvent was chloroform-methanol (2:1v/v) mixture. The raw material (microalgae culture) used was 5mL and the solvent mixture volume used was 5mL. For experiment 2 (No.2), the extraction solvent was hexane. In this experiment, the raw material (microalgae culture) used was 5mL and the hexane volume used was 5mL. For experiment 3 (No.3), the extraction solvent was hexane-methanol. In this experiment, the raw material (microalgae culture) used was 5mL and the hexane used was 5mL and the methanol used was 1mL.

Table 28. The comparison of extraction yield when different extraction solvents were used. In the three experiments, the flocculation was used to aggregate the cells and enzymes were used to break the cells.

No.	Concentration	Cell breakage	Extraction	Extraction yield*
1	Flocculation	Enzymatic treatment	Chloroform -methanol	31.83%± 2.31%
2	Flocculation	Enzymatic treatment	Hexane	1.20%± 0.03%
3	Flocculation	Enzymatic treatment	Hexane -methanol	18.38%± 0.39%

*The extraction yield was calculated based on the Nile red results.

In experiment 1, the extraction solvents used were chloroform and methanol (2:1 v/v) and the extraction yield was around 32%. In experiment 2, the hexane was used as an extraction solvent and the yield was around 1%. In experiment 3, the hexane and methanol were used as extraction solvents and the yield was around 18%

For the baseline, the extraction was performed using chloroform and methanol mixture (2:1 v/v) to extract the lipids and the yield was 31.83%. Due to the toxicity of the chloroform, the industry cannot use it in large-scale production. Thus, the hexane was used instead. The hexane is non-polarity molecule and can extract the non-polar lipids. However, after using chitosan to flocculate the microalgae cells and after the enzymatic treatment, the microalgae were still wet, and the hexane cannot have a good mixing with

microalgae to extract the lipids under these conditions. Thus, the extraction yield was very low in this experiment (Experiment No. 2). The yield was around 1%. Then, the methanol was added to help extraction. The methanol can change the polarity of the water to help the mixing and according to experiment 3 (No. 3), the extraction yield increased from about 1% to 18%.

From the experiments above, adding methanol as co-extraction solvent can increase the extraction yield. From Chen-His and Tz-Bang's report, the lipid extraction yield can reach 44.7% (w/w) by using hexane with methanol after sonication (Chen-His C. and Tz-Bang D.,2011). However, the extraction yield was still lower than using the chloroform-methanol mixture. Thus, the volume ratio of the hexane to methanol should be tested to find the optimal volume ratio to maximise the extraction yield.

5.3.3.4 Effect of cell wall treatment time (with or without enzymes) on lipid extraction

To determine how much the enzymatic treatment can help to improve the lipid extraction, these experiments were carried out. For experiment 1 (No. 1), the concentration method was flocculation and enzymes were added to break the cells. The temperature for the enzymatic treatment was 37°C for 6 hours. The extraction method was hexane and methanol. For the experiment 2 (No.2), the harvesting method was flocculation without enzyme treatment. Instead, the samples were kept in the same conditions (37°C) for 6 hours to make a direct comparison with experiment 1. Then, a mixture of hexane–methanol was used for the extraction.

To determine how much the enzymatic treatment time can influence the extraction, experiments 3 and experiment 4 were carried out. In experiment 3, the cell concentration method was flocculation and the enzymes were added afterwards. The temperature for enzymatic treatment was 37°C and the treatment time was 24 hours. The hexane and methanol mixture were used as extraction solvents. In experiment 4, the chitosan was used for flocculation and then the samples were kept at 37°C for 24 hours without enzymes. The extraction solvents again were hexane and methanol.

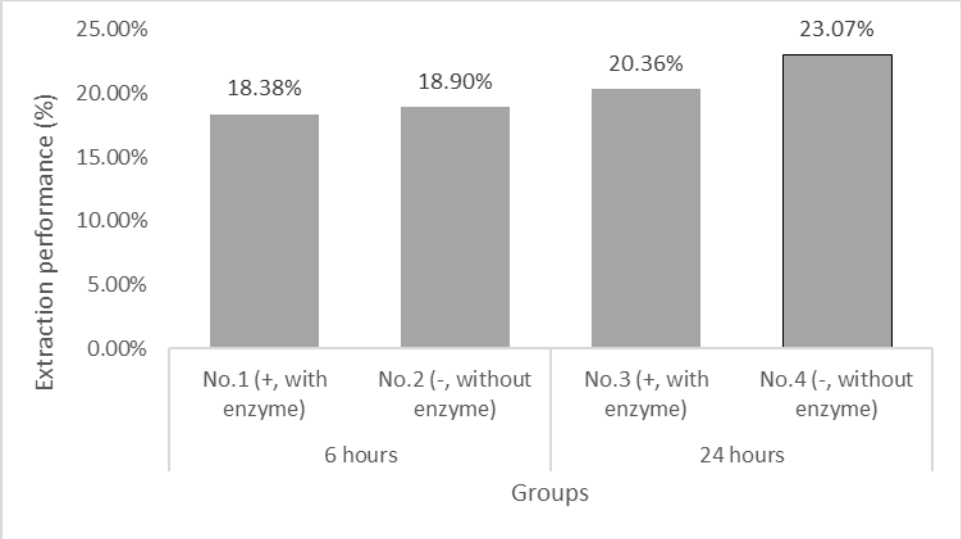

Figure 26. Comparison of lipid extraction performance after using different cell treatment methods, reaction time, and extraction solvents. For these experiments, the flocculation was used to aggregate the cells. For the cell treatment, the enzymes were used in the experiment: 1 and 3. In experiment 2 and 4, the enzymes were not added, but the samples were kept at the same conditions. In experiment 1 and 2, the reaction time was 6 hours and in experiment 3 and 4, the reaction time was 24 hours. The extraction solvents used were hexane and methanol.

*The extraction yield was calculated by the Nile red results.

The results showed that there was no significant difference between experiment 1 (No.1) and experiment 2 (No.2). Experiment 1 (No. 1) showed that when the enzymes were added for cell breaking, the extraction yield was around 18.38%. Experiment 2 (No.2) showed that when the enzymes were not added for cell breaking, the extraction yield was around 18.90% and this yield was very similar to the experiment 1.

Similarly, the results showed that there was no significant difference between experiment 3 (No.3) and experiment 4 (No.4). Experiment 3 (No. 3) showed that when the enzymes were added for cell breaking, the extraction yield was around 20%. Experiment 4 (No.4) showed that when the enzymes were not added for cell breaking, the extraction yield was around 23%, and this yield was very similar to the experiment 3.

These experiments showed that, although the enzymes can help to hydrolyse part of the cell walls (outer layer), the extraction yield was not improved when using the enzymes. This result was similar to Ahmed and Lara's results and the microalgae used was *Chlorella reinhardtii* (Ahmed M. and Lara, 2014).

However, when comparing experiment 1 and experiment 3, experiment 2 and experiment 4, the extraction yield increased by around 2%-5%. The only difference between these two groups was the cell wall treatment time. In experiment 1 and experiment 2, the time was 6 hours. In experiment 3 and experiment 4, the time increased to 24 hours. One possible explanation is that when the samples were kept for a long time in the water bath, the cell walls became weaker. Thus, the extraction performance can be more efficient.

The optimal conditions of these experiments were harvesting: flocculation; cell wall treatment (without enzyme): 24hours; extraction solvents: hexane with methanol.

5.4 Ethanol cell wall treatment

5.4.1 The water content after centrifuge

The water content after centrifuge was required because the wet microalgae were used after centrifuge in the ethanol cell wall treatment.
The weight of tube was 5.30±0.03g (weight A). The weight of tube with wet cells (water +dry microalgae) was 5.34±0.06g (weight B). The weight of tube with dry cells was 5.31±0.03g (weight C). Thus, the water content was calculated below.

$$\frac{weight\ (water)}{weight\ (water + dry\ cells)} x100\% = \frac{B - C}{B - A} x\ 100\% = 67.36\% \pm 12.30\%$$

Thus, the water content after centrifuge was 67.36%±12.30%.

5.4.2 The treatment using different ethanol volume (3ml, 5ml, and 10ml)

Figure 27, 28, and 29 showed the lipid concentration in ethanol measured by Nile read assay after cell wall treatment when the ethanol volume was 3mL, 5mL, and 10mL. The microalgae were harvested by centrifugation. 1g of wet microalgae was put in 20ml Duran bottle for the cell wall treatment.

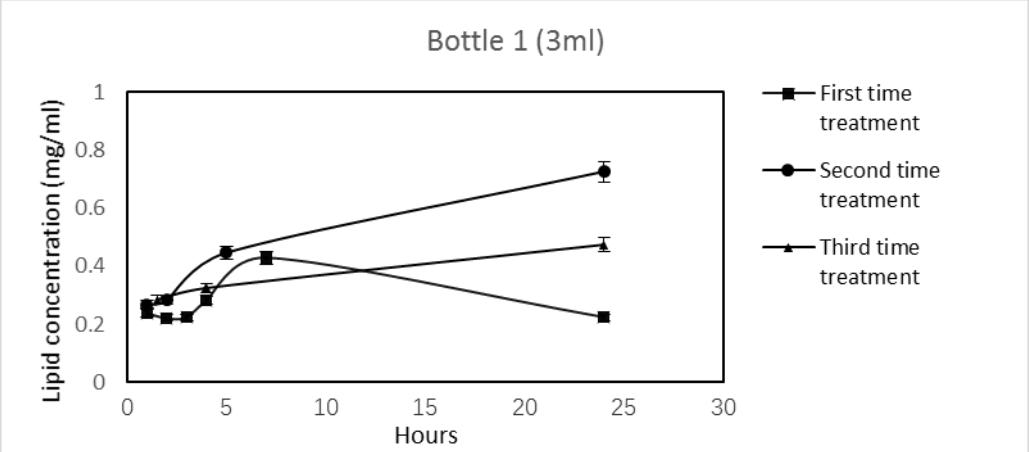

Figure 27. The lipid concentration in bottle 1 (3ml ethanol as treatment solvent) during cell wall treatment. ■ represented the first 24 hours treatment. ● represented the second 24 hours treatment. ▲ represented the third 24 hours treatment. The error bars represented the triplicates of the experiment.

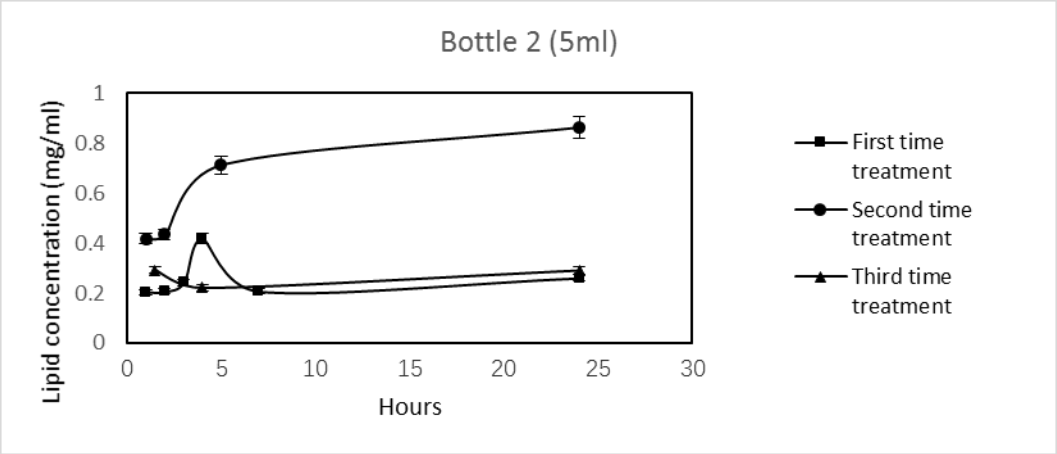

Figure 28. The lipid concentration in bottle 2 (5ml ethanol as treatment solvent) during cell wall treatment. ■ represented the first 24 hours treatment. ● represented the second 24 hours treatment. ▲ represented the third 24 hours treatment. The error bars represented the triplicates of the experiment.

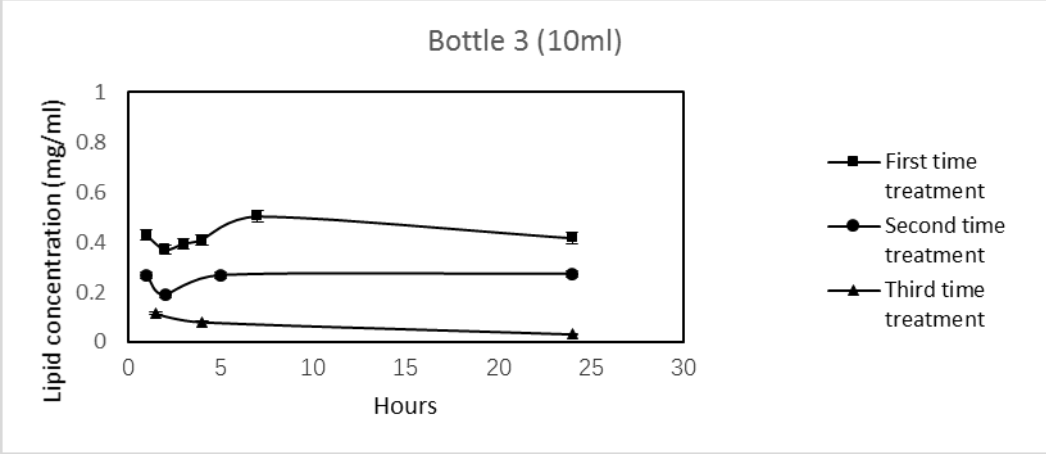

Figure 29. The lipid concentration in bottle 3 (10ml ethanol as treatment solvent) during cell wall treatment. ■ represented the first 24 hours treatment. ● represented the second 24 hours treatment. ▲ represented the third 24 hours treatment. The error bars represented the triplicates of the experiment.

Figure 27 showed the lipid concentration measured by Nile red assay after cell wall treatment when the ethanol volume was 3ml. The lipid concentration increased during the first 7 hours and then decreased to 0.2mg/ml after 24 hours. In the second treatment experiment, the lipid concentration increased from 0.25mg/ml to 0.7mg/ml and in the third experiment, it increased from 0.25mg/ml to 0.4mg/ml. This indicated that during the cell wall treatment, the lipid concentration in the ethanol increased over time. During the second treatment, the concentration of lipid increased around four-fold, which indicated that the second treatment was necessary in this experiment. During the third treatment, the lipid concentration in ethanol doubled after 24 hours, which indicated that the third time treatment can help to increase the final extraction yield.

Figure 28 showed the lipid concentration (measured by Nile red assay) in ethanol after cell wall treatment when the added ethanol volume was 5ml. The lipid concentration increased during the first 4 hours to 0.4mg/ml and then decreased to 0.2mg/ml after 24 hours. The falling of the bottle might cause the decrease between 7 hours and 24 hours. After each extraction, the lipid concentration was tested. In the second cell wall treatment, the lipid concentration increased from 0.4mg/ml to 0.85mg/ml and in the third

treatment, the change was insignificant. This indicated that during the cell wall treatment, the lipid concentration in the ethanol increased over time and the third time cell wall treatment had no huge influence on extraction yield because the third time extraction was insignificantly increased. During the second treatment, the concentration of lipid increased around two times, which indicated that the second treatment was necessary in this experiment. During the third treatment, the lipid concentration had insignificant changes, which indicated that the third time treatment had less influence when using 5ml of ethanol as cell wall treatment.

Figure 29 showed the lipid concentration (measured by Nile red assay) in ethanol after cell wall treatment when the added ethanol volume was 10ml. The lipid concentration increased during the first 7 hours to 0.5mg/ml and then decreased to 0.2mg/m. The falling of the bottle might cause the decreasing during 7 hours to 24 hours. In the second cell wall treatment, the lipid concentration did not have a huge change and the same situation happened in the third time cell wall treatment. This indicated that during the cell wall treatment, the lipid concentration in the ethanol increased over time, and the second and the third time cell wall treatment had no huge influence on extraction yield.

Table 29. The amount of lipid (g) after using different ethanol volume (3ml, 5ml, and 10ml) in first extraction, second extraction, and third extraction.

	Amount of lipid (mg)		
Extraction No.	bottle 1 (3ml)	bottle 2 (5ml)	bottle 3 (10ml)
First treatment	0.78±0.10	1.38±0.11	4.38±0.25
Second treatment	2.39±0.22	4.41±0.16	2.72±0.02
Third treatment	1.60±0.26	1.63±0.33	0.36±0.08
Summary	4.77±0.58	7.42±0.59	7.46±0.36

Table 29 represented the amount of lipid extracted after using ethanol as cell wall treatment solvent. The amounts of lipid were 4.77mg, 7.42mg, and 7.46mg from bottle 1, bottle 2, and bottle 3 respectively. Thus, when using 3ml as treatment solvent, the amount of lipid was the lowest. Besides, there was no huge difference when using 5ml and 10ml of ethanol as extraction solvent. In conclusion, 5ml of ethanol when using 1g of wet cell mass (~67% water content) was the optimal condition in this experiment.

5.4.3 The extraction yield when using 5ml of ethanol

According from the last experiments, the optimal ethanol volume was 5ml. Thus, this volume was used in this experiment, and different steps' extraction yields were analysed. The extraction yields were shown in table 30.

Table 30. The extraction yields when using ethanol in cell wall treatment and hexane in lipid extraction. 1.1 represented the extraction yield after the first-time cell wall treatment and the first-time lipid extraction. 1.2 represented the extraction yield after the first-time cell wall treatment and the second time lipid extraction. 2.1 represented the extraction yield after the second time cell wall treatment and the

first-time lipid extraction. 2.2 represented the extraction yield after the second time extraction and second cell wall treatment.

Step No.	Total extraction weight	Extraction yield	Extraction percentage	Sum	Extraction yield	Extraction percentage of 1st and 2nd step extraction percentage
Unit	mg	% w/w	%	mg	% w/w	%
1.1	12.43± 0.78	32.71%± 1.40%	60.81%	20.43 ±0.35	12.18%± 0.21%	53.76%
1.2	8.01± 0.43	21.1%± 1.56%	39.19%			
2.1	13.70± 1.46	36.02%± 3.09%	77.87%	17.56 ±0.46	10.47%± 0.28%	46.24%
2.2	3.87± 1.00	10.17%± 2.93%	22.13%			
Sum	37.995± 0.81	100.00%	/	38.00 ±0.81	22.66%± 0.49%	100.00%

According to table 30, the total extraction yield was around 22.7%, which was the summation of all extraction yields and still less than the Chloroform-methanol extraction yield after sonication (53.6% w/w). For the first cell wall treatment, the first extraction yield was around 61% and the second extraction yield was around 39%. The second time extraction yield was around 58.7% of the first-time extraction yield, which indicated the second time extraction was necessary in this experiment. For the second cell wall treatment, the first extraction yield was around 77.9% and the second time extraction yield was around 22.1%. The second time extraction yield was around 21.5% to the first-time extraction yield, which indicated the second time extraction was necessary in this experiment. When comparing the total extraction between the first time ethanol treatment and the second time ethanol treatment, the extraction yields were 53.8% and 46.2% respectively, which indicated that the second time cell wall treatment was necessary in this experiment.

In conclusion, when comparing different volume of ethanol, the 5ml of ethanol with 1g of wet biomass (water content: ~67%) was the optimal condition and the extraction yield was around 23%. When comparing the total extraction between the first time ethanol treatment and the second time ethanol treatment, the extraction yields were 53.60% and 46.4% respectively, which indicated that the second time cell wall treatment was necessary in this experiment.

5.4.4 The composition of the extracted lipids

Different extractions solvents were used in this project. It would be necessary to detect the lipid composition after extraction. Figure 30 showed the lipid composition when using chloroform-methanol as extraction solvents.

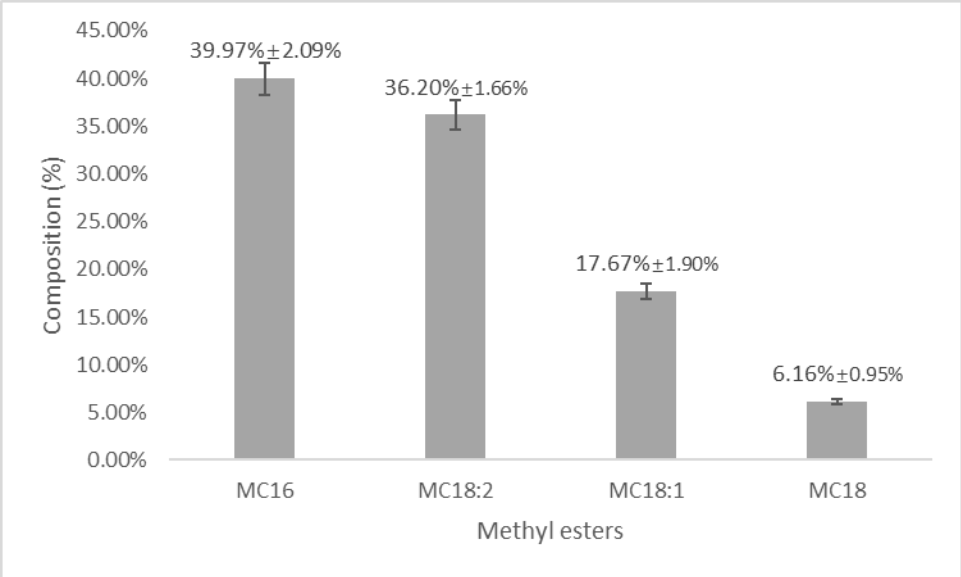

Figure 30. The methyl esters composition after chloroform -methanol (2:1, v/v) extraction

Figure 30 showed that after the chloroform-methanol (2:1, v/v) extraction, 39.97% of the extracted lipids were methyl ester 16 (16 carbons on the TAG carbon chain before transesterification). The MC 18:2 was 36.20% of the methyl mixture and the MC 18:1 was around 17.67% of the mixture. These two methyl esters were unsaturated methyl esters. The lowest proportion was MC18, which was around 5.16%.

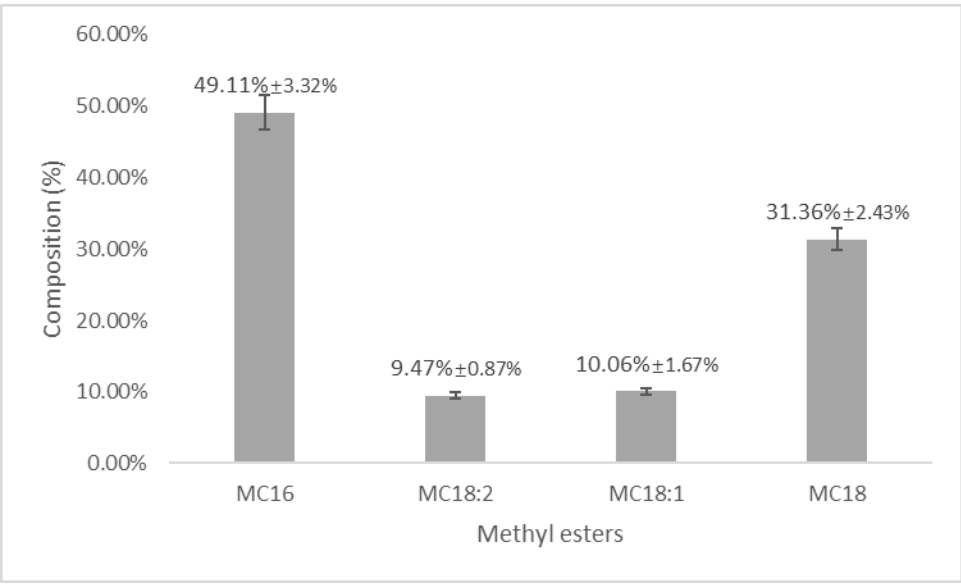

Figure 31. The methyl esters composition after extraction when using hexane as extraction solvent.

Figure 31 showed the composition of methyl esters when using hexane as extraction solvent. The saturated methyl esters (MC16 and MC18) had high proportions. 49.11% of the methyl esters after transesterification were MC 16, and 31.36% of them were MC18. The unsaturated methyl esters (MC 18:1 and MC 18:2) had low proportions. 9.47% of the methyl esters after transesterification were MC 18:2. 10.06% of the methyl esters after transesterification were MC 18:1.

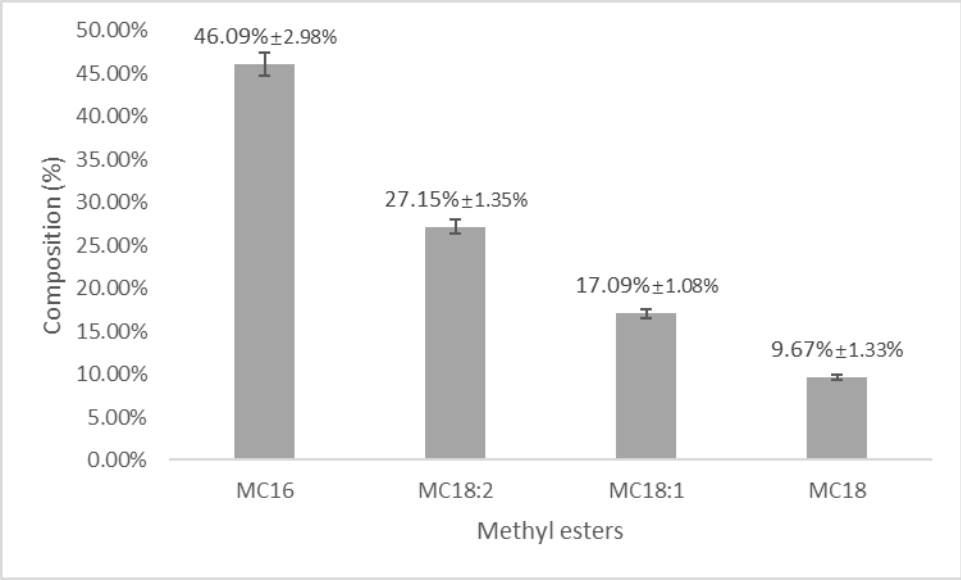

Figure 32 The methyl esters composition after transesterification when using the ethanol-hexane (1:1 v/v) as extraction solvents

When using ethanol-hexane (1:1, v/v) as extraction solvents, MC 16 had the highest proportion. 46.09% of the methyl esters were MC16. 27.15% of the methyl esters were MC 18:1, and 17.09% of the methyl esters were MC 18:1. MC 18 had the lowest proportion, which was 9.67%.

These experiments can detect the methyl esters after transesterification when using different extracted samples. The compositions of methyl esters can represent the compositions of the carbon atom numbers on the TAG's carbon chains. Thus, this composition can be used in the transesterification to mimic the microalgae lipids.

5.5 Acid transesterification yield

To determine the acid transesterification yield, the C15, C16, and C18 FAs were used and the initial input concentration was 66.67mg/ml. The FFAs were used because it would be helpful to determine the transesterification yield of each fatty acids. Then, 15μL of samples were added in the Kimble tube. After the acid transesterification, the sample was stored at 120μL of heptane. So, the total fatty acid input concentration was:

$$15\mu L = 0.015mL;\ 120\mu L = 0.12mL$$

$$\frac{0.015mL\ x\frac{66.67mg}{mL}}{0.12mL} = 8.33mg/mL$$

According to the data from GC-FID, the mean acid transesterification yield was shown in table 31.

Table 31. The transesterification yield of acid transesterification. C15, C16, and C18 were added in samples and concentrations determined by GC-FID.

	Concentration (mg/mL)				Yield (%)
Sample	MC15	MC16	MC18	Sum of FFA	Total FAME conc./Input FFA conc.
1	2.1718	2.5348	2.5346	7.2411	86.92
2	2.0859	2.3855	2.3021	6.7735	81.31
3	2.6237	2.9895	2.8611	8.4742	101.73
	Mean transesterification yield:				89.99± 10.56

From table 31, the third sample had a highest yield and the second sample had the lowest yield. Besides, the three samples were in a reliable range and the mean acid transesterification yield was 89.99%±10.56%.

This acid transesterification yield will be used when calculating the extraction yield and as a comparison of enzyme transesterification yield.

5.6 Enzymatic Esterification

Aim:

To find the optimal conditions of enzymatic esterification and the highest enzymatic esterification yield.

5.6.1 Factorial Design Experiment （lipase esterification）

In this experiment, C15, C16, C18, C18:1, and C18:2 (20mg each) FFAs were added in the esterification system for 6 hours and 24 hours. This composition was chosen because it would be easy to compare the yield of each fatty acids. Table 32 showed the results of 6-hours reaction.

Table 32. The results for enzymatic esterification after 6 hours using factorial design experiment. C15, C16, C18, C18:1 and C18:2 was used in these samples. The three parameters were: the methanol: FFA ratio (mol: mol), the water content, and the lipase: FFA amount (w/w%).

Run	A: Methanol: FFA ratio (mol: mol)	B: Water content (V/V)	Factor 3 C: Lipase: FFA amount (% w/w)	Response yield (%)					
				MC15	MC16	MC18 %	MC18:1	MC18:2	ME Sum
1	3.00	1.00	10.00	63.73	29.51	40.58	48.53	42.30	45.94
2	3.00	1.00	50.00	86.90	37.76	51.60	62.74	54.19	58.64
5	3.00	0.10	50.00	68.29	33.21	39.30	45.28	43.84	48.97
6	3.00	0.10	10.00	64.99	38.06	49.69	60.41	52.31	53.09
3	9.00	1.00	50.00	85.61	38.03	41.05	61.05	55.46	56.24
4	9.00	0.10	10.00	101.80	39.45	51.00	66.49	55.58	50.79
7	9.00	0.10	50.00	99.34	36.94	53.87	68.33	60.43	60.85
8	9.00	1.00	10.00	113.40	22.13	28.28	37.61	29.74	29.44

Table 33. The results for enzymatic esterification after 24 hours using a factorial design experiment. C15, C16, C18, C18:1 and C18:2 was used in these samples. The three parameters were the methanol: FFA ratio (mol: mol), the water content, and the lipase: FFA amount (w/w%).

Run	A: Methanol: FFA ratio (mol: mol)	B: Water content (V/V)	C: Lipase: FFA amount (% w/w)	Response yield (%)					
				MC15	MC16	MC18	MC 18:1	MC 18:2	ME Sum
2	3.00	1.00	50.00	102.10	36.19	51.60	62.74	53.70	50.99
5	3.00	0.10	50.00	85.41	39.13	48.51	55.71	53.83	59.52
1	3.00	1.00	10.00	95.36	43.28	58.09	70.88	61.54	65.83
6	3.00	0.10	10.00	87.86	42.15	57.98	70.16	61.05	63.84
3	9.00	1.00	50.00	118.70	41.06	57.08	74.89	58.61	57.91
7	9.00	0.10	50.00	115.20	38.91	62.55	79.65	71.49	54.63
4	9.00	0.10	10.00	89.66	47.78	64.61	85.88	67.73	71.13
8	9.00	1.00	10.00	123.00	45.34	59.18	80.10	65.44	62.52

From table 32 and table 33, the yield of 24 hours reaction time was higher than that of 6 hours except for sample 1 and sample 7. Also, C15 had the highest transfer rate of all

fatty acids, some of the yields were higher than 100%. This was because in the Factorial design, the conditions were designed without triplicates and the results was not accurate.

5.6.1.1 The results of Factorial Design Experiment （6 hours reaction）

Table 34. the ANOVA analysis for factorial design experiment model after 6 hours' reaction.

Source	Sum of Squares	df	Mean Square	F-value	p-value Prob > F
Model	679.779	6	113.2965	141620.6	0.002034
A-methanol: FFA ratio	10.8578	1	10.8578	13572.25	0.005464
B-water content	68.6792	1	68.6792	85849	0.002173
C-Lipase: FFA amount	258.0992	1	258.0992	322624	0.001121
AB	101.3888	1	101.3888	126736	0.001788
AC	99.9698	1	99.9698	124962.2	0.001801
BC	140.7842	1	140.7842	175980.2	0.001518
Adeq Precision			1187.18		

The Model F-value of 141620.6 implied the model was significant. There was only a 0.20% chance that an F-value this large could occur due to noise. "Values of ""Prob > F"" less than 0.0500 indicated model terms were significant. "In this case A, B, C, AB, AC, BC were significant model terms. "Adeq Precision" measured the signal to noise ratio. A ratio greater than 4 was desirable. The ratio of 1187.186 indicated an adequate signal. Thus, this model can be used to navigate the design space.

The final equation of sum yield was showed as below (coded factor):

Sum Yield=+50.50+1.16*A -2.93*B+5.68*C -3.56* A* B +3.54*A*C +4.20* B*C

* The equations of the other responses were shown in the appendix.

The equation indicated that the factor B had the negative relationship with the yield, and the factor A and factor C had the positive relationship with the yield. Besides, the equation in terms of coded factors can be used to make predictions about the response for given levels of each factor. The coded equation was useful for identifying the relative impact of the factors by comparing the factor coefficients. From this equation, the impact of the factors was: C>BC>AB>AC>B>A.

Besides, the results showed that the optimal conditions for this experiment was:

Table 35. The optimal conditions for 6 hours reaction.

methanol: lipid molar ratio	water content (v/v)	lipase amount (w/w %)	yield (%)
9:1	0.1	50	60.85

5.6.1.2 The results of Factorial Design Experiment after 24 hours reaction

Table 36. the ANOVA analysis for factorial design experiment model after 6 hours' reaction.

Source	Sum of Squares	df	Mean Square	F-value	p-value Prob > F
Model	287.7953	5	57.55905	162.1553	0.00614
A-Methanol: FFA ratio	4.515013	1	4.515013	12.71969	0.070415
B-Water content	17.61211	1	17.61211	49.61683	0.019565
C-Lipase: FFA amount	202.7091	1	202.7091	571.0719	0.001747
AB	0.183013	1	0.183013	0.515583	0.54728
ABC	62.77601	1	62.77601	176.8525	0.005607
Adeq Precision			39.03		

The Model F-value of 162.16 implied the model was significant. There was only a 0.61% chance that an F-value this large could occur due to noise. Values of Prob > F less than 0.0500 indicated model terms were significant. In this case B, C, ABC were significant model terms. "Adeq Precision" measured the signal to noise ratio. A ratio greater than 4 was desirable. In this project, the ratio of 39.034 indicated an adequate signal. Thus, this model can be used to navigate the design space.

The final equation was showed as below (coded factor):

Sum Yield =+60.80+0.75* A-1.48*B-5.03*C+0.15*A*B +2.80*A*B*C

* The equations of the other responses were shown in the appendix.

The equation indicated that the factor B and factor C had the negative relationship with the yield and the factor A had the positive relationship with the yield. Besides, the equation in terms of coded factors can be used to make predictions about the response for given levels of each factor. The coded equation was useful for identifying the relative impact of the factors by comparing the factor coefficients. From this equation, the impact of the factors was: C>ABC>B>A>AB.

The results also showed the optimal conditions for the enzymatic esterification after 24 hours:

Table 37. The optimal conditions for 24 hours reaction.

methanol: lipid molar ratio	water content (v/v)	lipase amount (w/w %)	yield (%)
9:1	0.1	10	71.13

Overall, the factorial design experiments (both 6 hours and 24 hours) showed that the lipase amount (w/w) can have the largest impact on the yield and the methanol: lipid molar ratio (mol: mol) have the lowest impact. The water content (v/v) impact was between these two factors. The methanol: lipid molar ratio (mol: mol) factor and water content factor (v/v) had consistent impacts on the yield when comparing the 6 hours experiments with 24 hours experiments. However, the lipase amount (w/w %) factor had the opposite impact when comparing these experiments. In this case, the ester hydrolysis might cause this situation.

5.6.2 Response surface methodology experiment

According to the FDE experiment, when increasing the methanol concentration, the yield of esters increased. Thus, for the molar ratio of methanol to fatty acids, 9:1-15:1 was examined in the response surface methodology experiment. According to the research, the fatty acid to solvent ratio was another important factor in this experiment. Thus, this ratio was used in this experiment. The fatty acid concentration in this experiment was 25mg/1.3ml to 100mg/1.3ml and the solvent used was hexane. The lipase used was 10%w/w to 50%w/w ((based on amount of the FFAs). The water content was not tested because the impact of it was not high, and in this model, the water volume required was relatively low. Thus, it was difficult to measure the water volume.

5.6.2.1 The results of the response surface methodology experiment

Table 38 showed the results of the response surface methodology experiment after the reaction time of 6 hours and 24 hours.

Table 38. The results of the response surface methodology experiment after the reaction time of 6 hours. C16 and C18 were used in these samples. The three factors were: the FA/ solvent concentration (mg/mL), the methanol: FFA ratio (mol: mol) and the lipase: FFA amount (w/w%). The responses were the esterification yields of MC16, MC 18, and the total esterification yield from GC-FID.

Run	Factor 1 A: FA/Solvent mg/ml	Factor 2 B: Methanol: FFA ratio mol: mol	Factor 3 C: Lipase: FFA amount w/w %	Response 1 MC16 Yield %	Response 2 MC18 Yield %	Response 3 ME Sum Yield %
10	19.23	9.00	30.00	17.97	26.70	22.34
8	19.23	12.00	50.00	17.28	29.33	23.31
13	19.23	12.00	10.00	13.76	19.65	16.71
7	19.23	15.00	30.00	17.48	28.43	22.98
9	38.45	9.00	10.00	43.74	61.20	52.47
16	38.45	9.00	50.00	46.48	71.03	58.78
6	38.45	12.00	30.00	45.24	69.53	57.39
3	38.45	12.00	30.00	40.25	62.29	51.27
15	38.45	12.00	30.00	38.85	59.81	49.33
11	38.45	12.00	30.00	43.16	65.18	54.17
12	38.45	12.00	30.00	44.26	67.31	55.79
1	38.45	15.00	10.00	42.20	60.94	51.57
14	38.45	15.00	50.00	43.91	68.74	56.33
17	76.92	9.00	30.00	35.26	54.49	44.88
2	76.92	12.00	10.00	35.12	49.91	42.52
4	76.92	12.00	50.00	39.20	60.45	49.83
5	76.92	15.00	30.00	44.94	69.94	57.44

Table 39. The results of the response surface methodology experiment after the reaction time of 24 hours. C16 and C18 FFAs were used in this experiment. The three parameters were: the FA/ solvent concentration (mg/mL), the methanol: FFA ratio (mol: mol) and the lipase: FFA amount (w/w%). The responses were the esterification yields of MC16, MC 18, and the total esterification yield from GC-FID.

Run	Factor 1 A: FA/Solvent mg/ml	Factor 2 B: Methanol: FFA ratio mol: mol	Factor 3 C: Lipase: FFA amount w/w %	Response 1 MC16 Yield %	Response 2 MC18 Yield %	Response 3 ME Sum Yield %
10	19.23	9.00	30.00	16.65	29.33	22.99
8	19.23	12.00	50.00	16.94	29.18	23.05
13	19.23	12.00	10.00	20.30	34.95	27.63
7	19.23	15.00	30.00	16.19	28.01	22.10
9	38.45	9.00	10.00	33.44	58.05	48.25
16	38.45	9.00	50.00	36.91	57.08	47.00
6	38.45	12.00	30.00	35.92	56.44	45.18
3	38.45	12.00	30.00	38.16	59.18	48.67
15	38.45	12.00	30.00	35.37	55.31	45.34
11	38.45	12.00	30.00	38.60	59.96	49.28
12	38.45	12.00	30.00	41.93	65.21	53.57
1	38.45	15.00	10.00	48.40	74.59	61.50
14	38.45	15.00	50.00	40.65	65.70	53.18
17	76.92	9.00	30.00	50.32	79.48	64.89
2	76.92	12.00	10.00	50.95	74.85	62.91
4	76.92	12.00	50.00	60.15	92.18	76.17
5	76.92	15.00	30.00	46.38	71.85	59.12

The table above showed the results of the response surface methodology experiment after the reaction time of 6 hours and 24 hours and the results analysis were shown below. To determine the transesterification productivity of each lipids, the mixture of fatty acids was used. 80mg C16 and 20mg C18 FFAs were used. This composition was chosen because after hexane-ethanol extraction, the ratio of C16:C18 was around 4:1.

5.6.2.2 The 6-hour reaction results analysis

The ANOVA for response surface quadratic model was shown below

Table 40. the ANOVA analysis for response surface model after 6 hours' reaction.

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	3201.32	9	355.7	30.7	< 0.0001
A-FA/Solvent	1494.14	1	1494.14	128.95	< 0.0001
C-Lipase: FFA amount	78	1	78	6.73	0.0357
A2	1550.11	1	1550.11	133.78	< 0.0001
Adeq Precision:			16.192		

From table above, The Model F-value of 30.70 implies the model was significant. There was only a 0.01% chance that a "Model F-Value" this large could occur due to noise. Values of "Prob > F" less than 0.0500 indicated model terms were significant. In this case A, C, A^2 were significant model terms. "Adeq Precision" measured the signal to noise ratio. A ratio greater than 4 was desirable. The ratio of 16.192 indicated an adequate signal. This model can be used to navigate the design space.

Final equation was shown below:

ME Sum Yield=53.59+13.67* A+1.23 * B+3.12 * C+2.98* A * B+0.18* A * C-0.39* B * C-19.19* A^2+2.51 * B^2-1.31 * C^2

5.6.2.3 3D surface analysis (first 6 hours reaction)

Because the response surface analysis was used, the 3D surface analysis can show the optimal conditions for the lipase esterification. Figure 33 showed the 3D surface between all the factors for the first 6 hours.

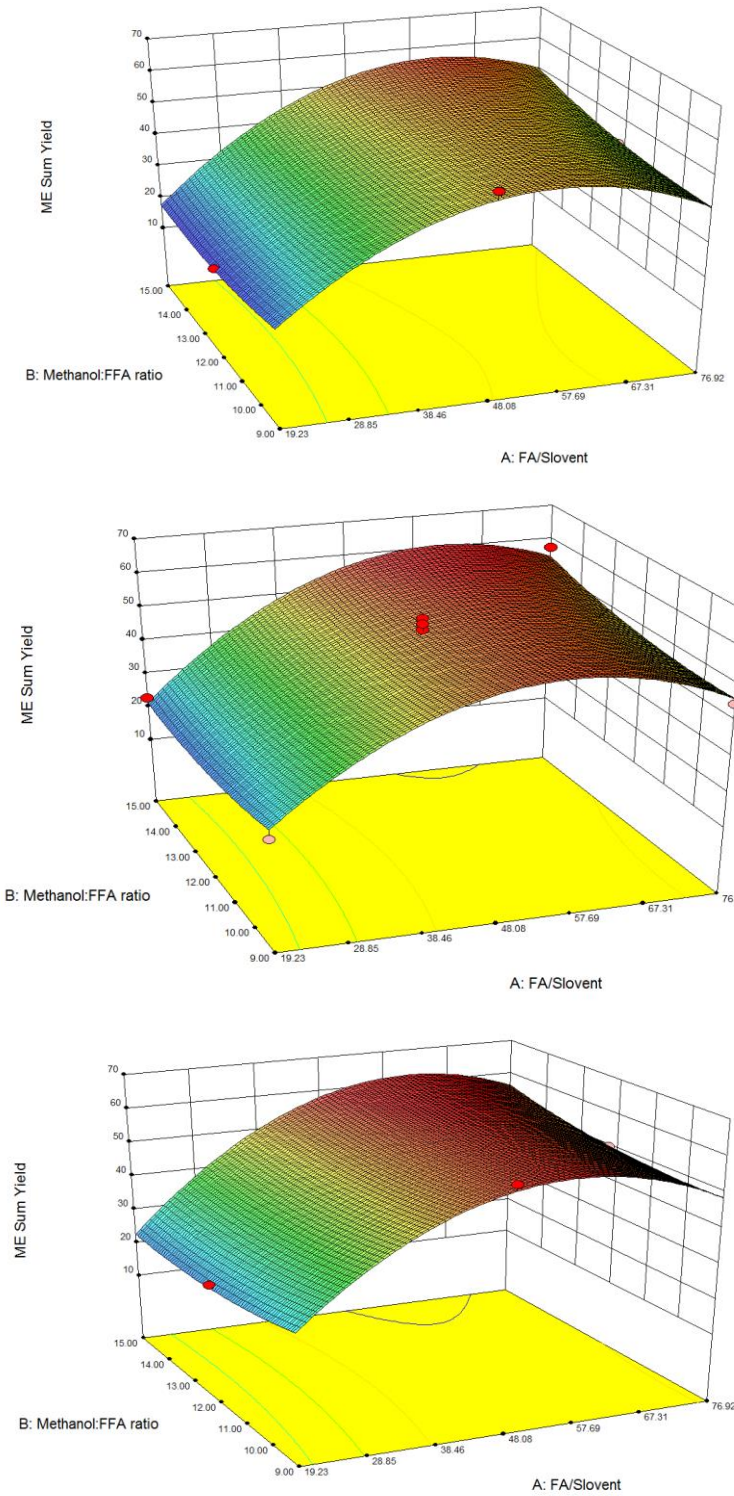

Figure 33. The 3D surface when the methanol: FFA molar ratio (parameter B) and the FA/ solvent (mg/ml) (parameter A) were parameters for the first 6 hours. The lipase: FFA ratios were at 10%, 30%, and 50%.

*The figures were taken from Design Expert software.

Figure 34 showed that when increasing the lipase: FFA ratio (parameter C) from 10% to 50% w/w, the highest yield of all the FFA/solvent concentration and methanol: FFA molar ratio was obtained when the lipase: FFA amount was at 50% w/w.

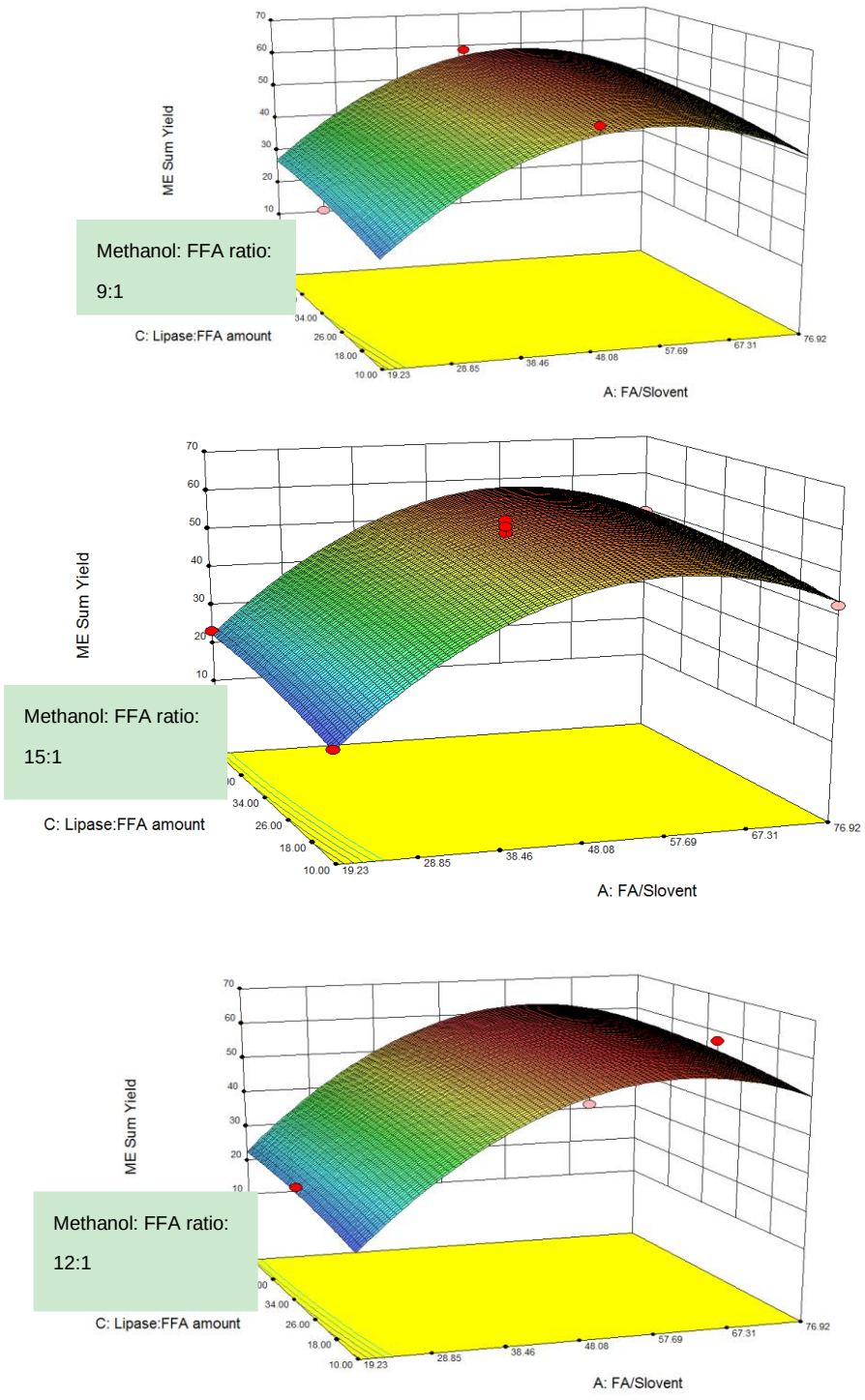

Figure 34. The 3D surface when the lipase: FFA amount (parameter C) and the FA/ solvent (mg/ml) (parameter A) were as parameters. The methanol: FFA molar ratio was at 9:1, 12:1, and 15:1 w/w.

*The figures were taken from Design Expert software.

Figure 35 showed when increasing the methanol to FFA ratio, the yield of all the lipase amounts (%) and the FA concentrations (mg/ml) had insignificant changes. Besides, when the methanol: FFA ratio was at 15:1, the yield reached the highest point.

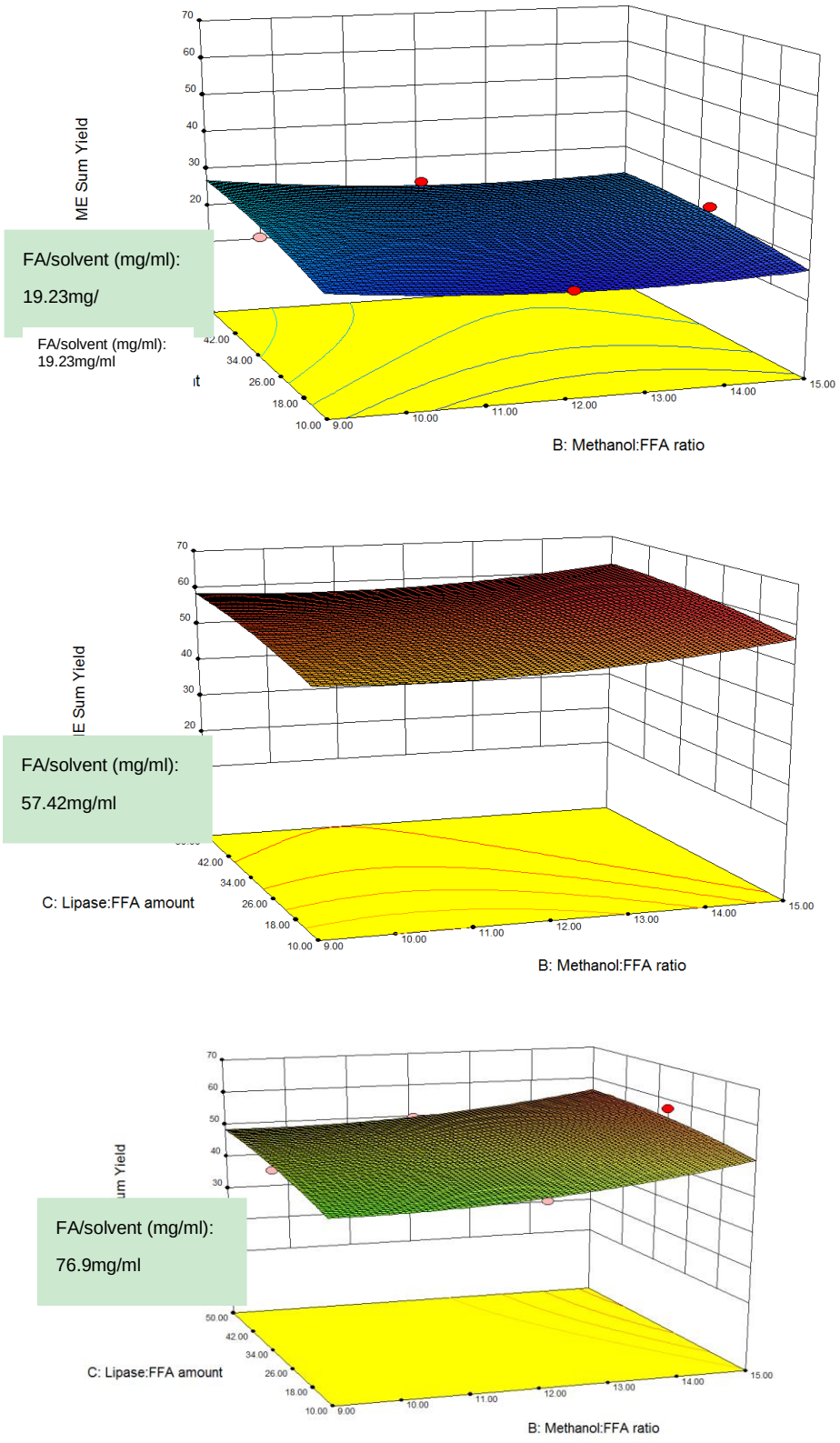

Figure 35. The 3D surface when the lipase: FFA amount (parameter C) and the methanol: FFA molar ratio (parameter B) were the variables. The FA/solvent (mg/ml) (parameter A) were at 19.23mg/ml, 57.42mg/ml, and 76.9mg/ml.

*The figures were taken from Design Expert software.

Figure 35 showed that when the FA/solvent (mg/ml) was at 19.24mg/ml, the yields of all the conditions were low (the colour was blue). When increasing the FA/solvent concentration, the yields of all the conditions were increasing as well. The highest point was reached when the FA/solvent concentration was at 57.42mg/ml. Then, the yield was decreasing when increasing the FA/solvent concentration further.
Thus, the optimal conditions for lipase esterification of the first 6 hours are shown as below

Table 41. The optimal conditions for lipase esterification of the first 6 hours.

FA/solvent (mg/ml)	methanol: FFA molar ratio (%)	lipase: FFA amounts (%)	yield (%)
57.42	15:01	50%	58.76

5.6.2.1 The 24-hour reaction results analysis

The ANOVA for response surface quadratic model was shown below

Table 42. the ANOVA analysis for response surface model after 24 hours' reaction.

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	3831.071	9	425.6745	16.59176	0.0006
A-FA/Solvent	3499.115	1	3499.115	136.387	< 0.0001
Adeq Precision:			13.941		

The Model F-value of 16.59 implies the model was significant. There was only a 0.06% chance that a "Model F-Value" this large could occur due to noise. Values of "Prob > F" less than 0.0500 indicated model terms were significant. In this case, factor A (FA/ Solvent; mg/ml) was significant model terms. "Adeq Precision" measured the signal to noise ratio. A ratio greater than 4 was desirable. This ratio of 13.941 indicated an adequate signal. Thus, this model can be used to navigate the design space.
Final equation was shown below:

$$ME\ Sum\ Yield = 48.61+20.91*A+1.60*B-0.11*C-1.22*A*B+4.46*A*C-1.77*B*C-5.69*A^2-0.65*B^2+4.52*C^2$$

5.6.2.1 3D surface analysis (24 hours reaction)

Because the response surface analysis was used, the 3D surface analysis can show the optimal conditions for the lipase esterification. Figure 36 showed the 3D surface between all the parameters after 24 hours.

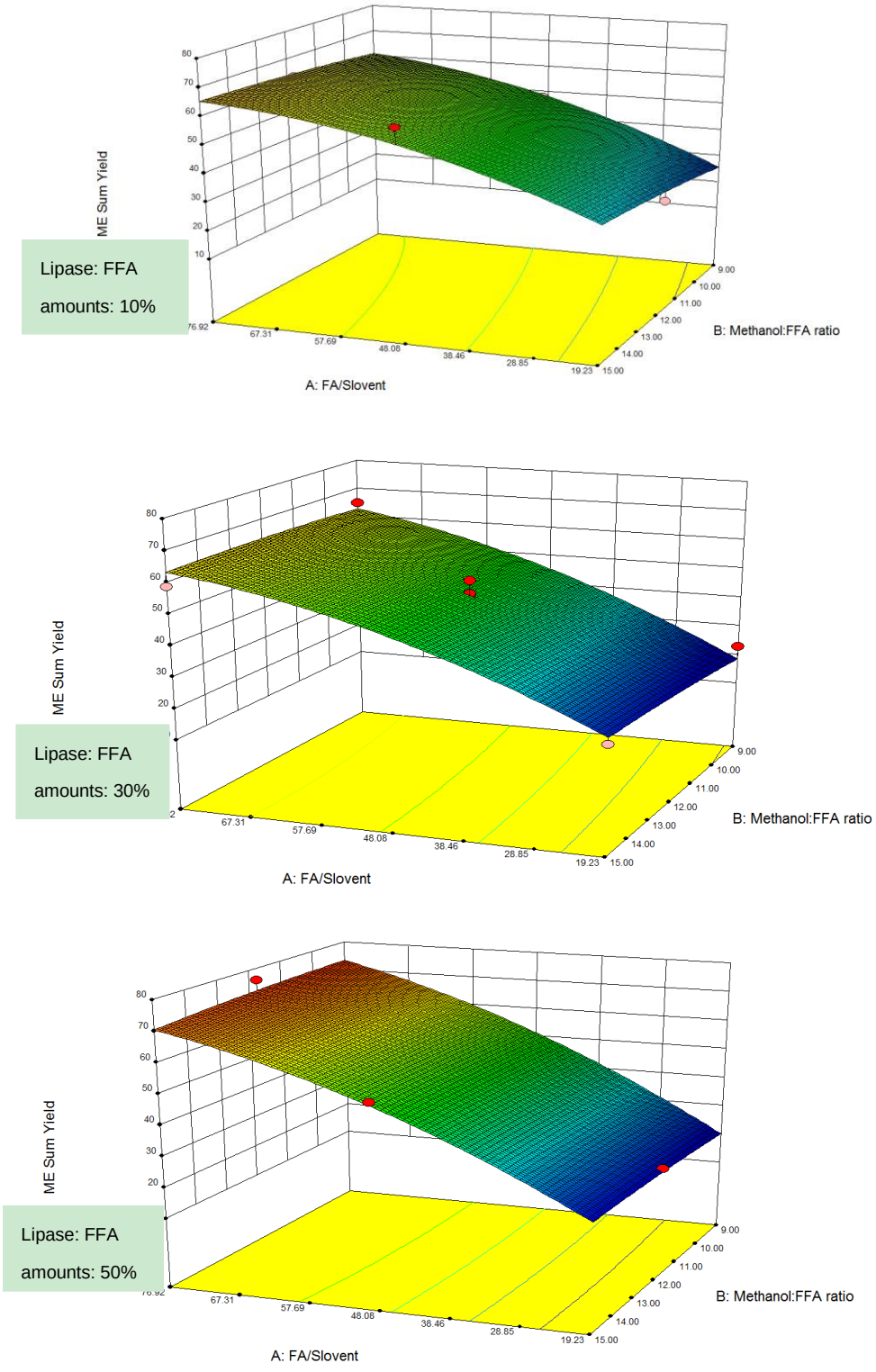

Figure 36. The 3D surface when the methanol: FFA molar ratio (parameter B) and the FA/ solvent (mg/ml) (parameter A) were parameters after 24 hours. The lipase: FFA amounts were at 10%, 30%, and 50%.

*The figures were taken from Design Expert software.

From figure 36, the yield was decreased a little when the lipase: FFA ratio was increasing for the first time for all the conditions. Then, the yield increased when the lipase: FFA ratio was increasing. Then, the highest yield was reached when the lipase: FFA amount was at 50%.

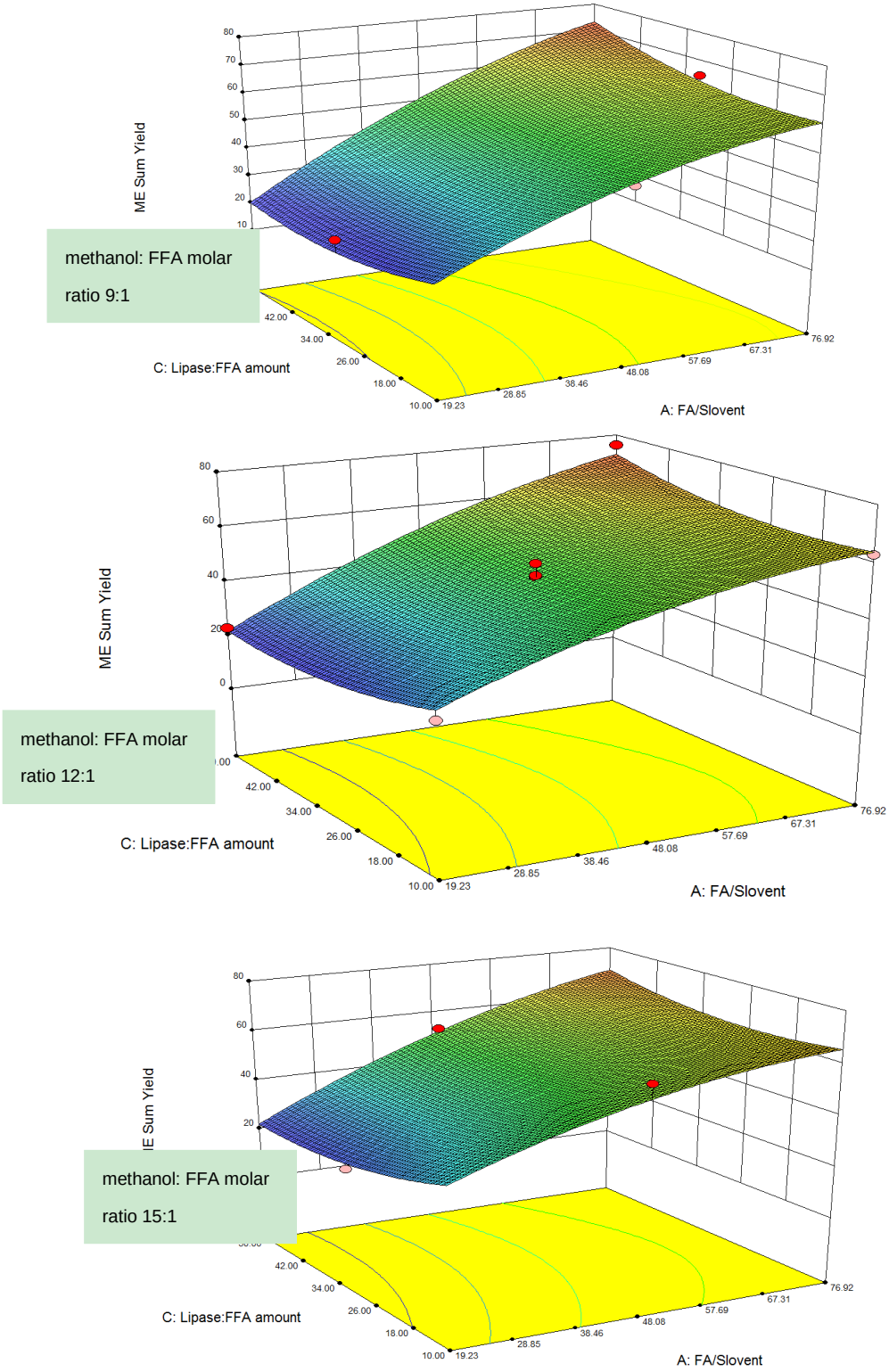

Figure 37. The 3D surface when the lipase: FFA amount (parameter C) and the FA/ solvent (mg/ml) (parameter A) were parameters for 24 hours reaction. The methanol: FFA molar ratio was at 9:1, 12:1, and 15:1.

*The figures were taken from Design Expert software.

Figure 37 showed that when increasing the methanol: FFA molar ratio, the highest yield for all the conditions was decreasing. Thus, the optimal lipase: FFA amount was: 9:1.

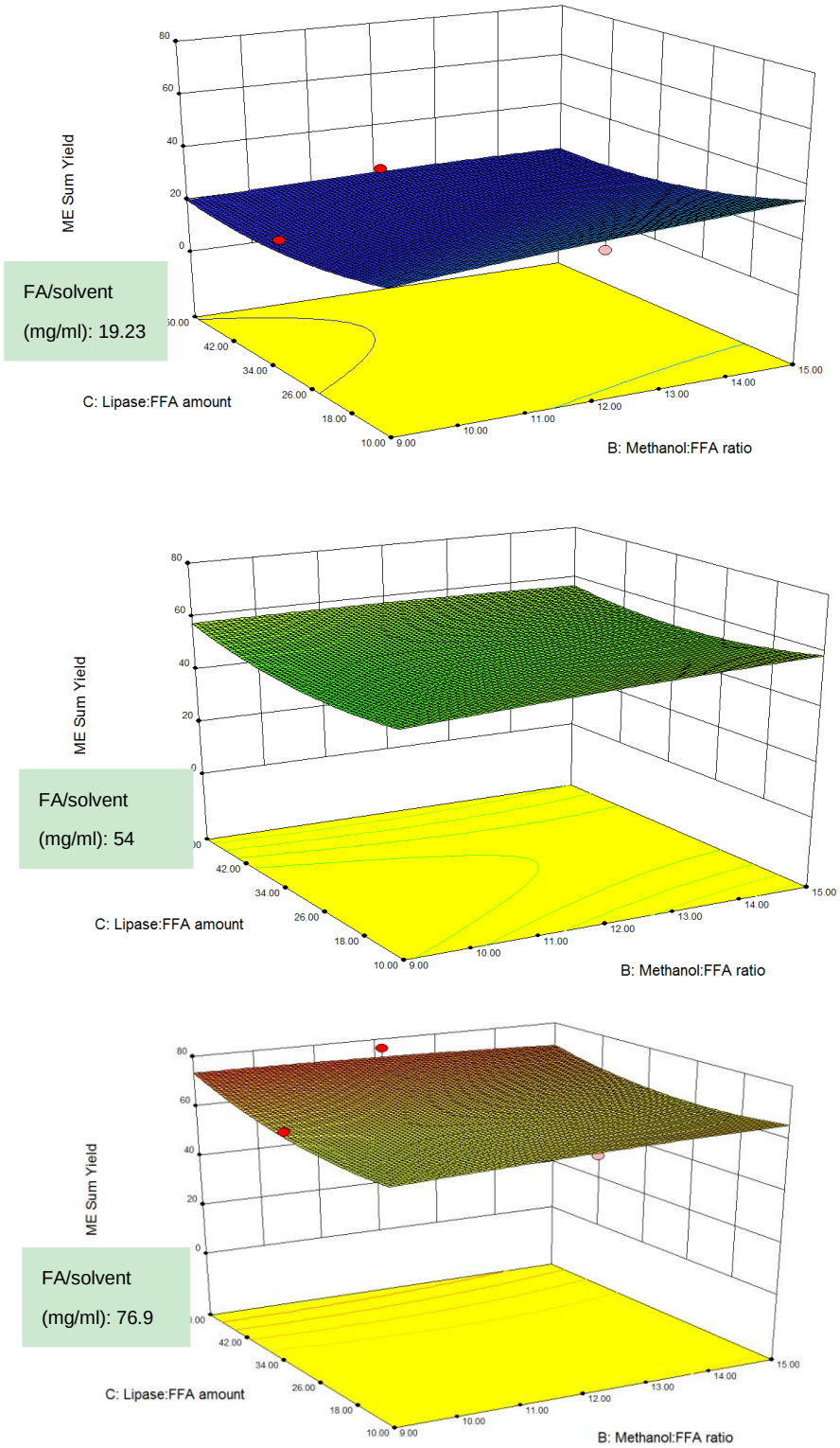

Figure 38. The 3D surface when the lipase: FFA amount (parameter C) and the methanol: FFA molar ratio (parameter B) were parameters. The FA/solvent (mg/ml) (parameter A) were at 19.23mg/ml, 54mg/ml, and 76.9mg/ml.

*The figures were taken from Design Expert software.

Figure 38 showed that when the FA/solvent concentration was increasing, the yield increased. Thus, the optimal FA/solvent concentration was: 76.90mg/ml.
Thus, the optimal conditions for lipase esterification after 24 hours were shown in table 43.

Table 43. The optimal condition for lipase esterification after 24 hours.

FA/solvent (mg/ml)	methanol: FFA molar ratio (%)	lipase: FFA amounts (%)	yield (%)
76.92	9:1	50%	76.17

In this experiment, the reaction time tested was 6 hours and 24 hours. However, it was reported that the reaction yield increased from 84.0% to 99.5% when the reaction time increased from 3 hours to 12 hours (Wu, 2017). Thus, the reaction time (from 1 to 6 hours) should be tested in the future. Besides, Xiong and Li found the optimal molar ratio of substrate to lipid was 3:1 and Wang found that when using Novozyme 435, the optimal molar ratio of methanol to algae lipid was 12:1 and the conversion rate was much higher at 99.1% (Xiong X. and Li M., 2008; Wang L., 2014). The different of the scales might cause these differences. In this project, the yield of table 43 was the highest. Thus, the conditions of the lipase transesterification used was the same as table 43.

5.6.3 Comparison of solvent type and Scale

Aim:
To compare the esterification performances when using different solvents and scale.

5.6.3.1 Impact of scale on transesterification reaction

PTFE plates and Duran bottles were used in this experiment to have a comparison of different scales. The volume in PTFE wells was 1.5mL and the volume in Duran bottles was 20mL. Besides, in this experiment, the hexane was used as a solvent. In experiment 3, lipid extracted from the microalgae was used and the scale was 1.5mL. For each group, the reaction time was 6 hours and 24 hours.

Table 44. The transesterification yield of FFAs (specify) after 6 hours and 24 hours when using PTFE plates and Duran bottles and in experiment 3, the microalgae lipids were used. In all experiments hexane was used as a solvent for lipid extraction.

Sample (reaction volume)	6 hours	24 hours
PTFE FFA well (1.5 mL)	5.76%	3.97%
Duran bottles (20mL)	15.58%	9.31%
PTFE algae sample well (1.5mL)	53.74%	118.64%

From table 44, the esterification yield decreased from 5.8% to 4.0% in PTFE plates when the reaction time changed from 6 hours to 24 hours and this result was lower than the response surface experiment. For the Duran bottles, although the esterification yield was higher than the PTFE plates, the yield still decreased from 15.58% to 9.31% when

increasing the reaction time from 6 hours to 24 hours. For the microalgae lipids, the esterification yield increased from 53.74% to 118.64% and this yield was much higher than that of the other two groups. In this experiment, the microalgae sample apparently had a higher esterification yield but more experiments were needed to verify this conclusion.

5.6.3.2 The comparison of different solvents

In this experiment, 2 different solvents (hexane and t-butanol) were used to dissolve the fatty acids and the esterification yield was tested on the larger scale (Duran bottles). C15 and C16 FFAs were tested in this experiment and the results of those two acids showed similar trends in terms of transesterification yield.
The figure below shows the yield for the first 6 hours in the Duran bottles.

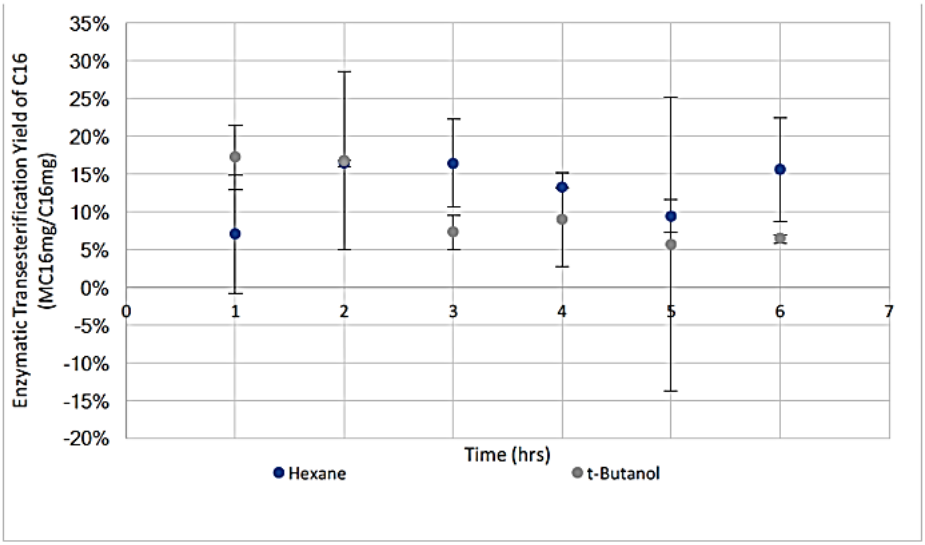

Figure 39. The transesterification yield change after extraction over 6 hours with two different solvents: hexane and t-butanol. The ● represented the yield when using hexane as a solvent and ● represented the yield when using t-Butanol as solvent.

Figure 39 showed the yield change during the first 6 hours of two solvents: hexane and t-butanol and the yield varied from 0% to 15%. After one hour, the esterification after extraction with t-butanol had a higher yield than with hexane. However, after two hours, the two systems had similar results and then, the sample extracted with hexane had a higher yield after 3 hours. The expected trend in this experiment was increasing during the first six hours. However, there were no significant changes seen in figure 39. Besides, the yield was much lower than the last experiment (small scale).

5.7 In-situ transesterification

Aim:
The test of in-situ transesterification yield with the base line.

From the response surface experiment, the optimal conditions for lipase transesterification can be considered as lipase: FFAs: 50% w/w, water content: 0.1%, FA/solvent: 50mg/1.3ml, and Methanol: FFA ratio: 9:1. In experiment 1, the concentration method was centrifugation and the cell disruption method was sonication. Then, hexane and methanol were used as extraction solvent. For the transesterification step, the conditions were the same as described before, and there was no water in the system because the water required was too little to be tested. The transesterification time was 24 hours, and the temperature was 37°C. Experiment 2 was designed for in-situ transesterification. Firstly, the microalgae cells were concentrated by centrifugation. Then, after removing the supernatant, the Viscozyme was added in and the same amount of lipase, solvent, and methanol (lipase: FFAs: 50% w/w, water content: 0.1%, FA/solvent: 50mg/1.3ml, and Methanol: FFA ratio: 9:1) were added to create the transesterification system. For experiment 3, the microalgae cells were harvested by centrifugation and the sonication was used to disrupt the cells. Then, 3mL of methanol: H_2SO_4 mix was added to the samples directly.

Table 45. The FAMEs yield when using enzymes as catalysts during transesterification. In experiment 1, the lipids were extracted by hexane and methanol mixture and then the lipase was added to start the transesterification. In experiment 2, there was not the extraction step, and the lipase was added directly after centrifugation.

No.	Concentration	Cell wall treatment	Extraction	Catalyst	Extraction	Yield*
1	Centrifuge	Sonication	Hexane - Methanol	Lipase	Hexane-methanol (1:1, double extraction)	14.79%±5.41%
2	Centrifuge	Enzymes	/	Lipase		0.94%±0.51%
3	Centrifuge	Sonication	/	Sulfuric acid		87.41±4.53%

*The yields were compared with the Nile red results

In experiment 1, the cells were concentrated by centrifugation and disrupted by sonication. Hexane and methanol were used as the extraction solvents and then, the lipase, methanol, and hexane were added in. This was a non-aqueous system. The yield for experiment 1 was around 14%. There were 2 steps can influence this yield: the extraction step and the transesterification step.

In experiment 2, the cells were concentrated by centrifuge. Then, the enzymes for cell wall treatment and the hexane, methanol, and lipase were added in the same Kimble tube to start the in-situ transesterification step. The yield for this experiment was around 1%. This was because after centrifugation, the biomass was wet, and the amount of the water was relatively high for the transesterification. Besides, the water bath was used during the in-situ transesterification, and the solvents and the enzymes were not well mixed. This can influence the yield as well.

In experiment 3, the in-situ transesterification was catalyzed by sulfuric acid. There were 2.935mL of methanol in the transesterification system. The transesterification yield was 87.4%, which was the highest performance in these three experiments.

Thus, the shaker will be used in the future work to mix the solvents during transesterification.

5.8 The performance comparison between the base line and modified process.

For this project, the steps and the performance of baseline, modified process 1, and modified process 2 were showed as below.

Table 46. The steps and the performance of the processes.

Process	Harvesting	Cell wall treatment	Extraction	Transesterification	Yield
Baseline	Centrifuge	Sonication	Chloroform-methanol	Acid transesterification	48.26%
Modified process 1	Flocculation-centrifuge	Water bath*	Hexane-methanol	Lipase transesterification	17.57%
Modified process 2	Flocculation-centrifuge	Ethanol	hexane	Lipase transesterification	17.26%

*the microalgae cell wall of modified process 1 can be treated with or without enzymes. The yield and the energy requirement were similar under these two conditions. However, when using lipase in cell wall treatment, the sugars were hydrolysed, and the sugars can be recycled for the cultivation. In this project, there was no requirement of recycle sugar for cultivation. Thus, there wan no enzyme in cell wall treatment but just water bath.

The calculations of the yield were showed as below.

For the base line, the extraction yield was 53.63% and the acid transesterification yield was 89.99%. Thus, the performance of the whole process was:

$$1\ X\ 53.63\%\ X\ 89.99\% = 48.26\%$$

For the modified process 1, the extraction yield was 23.07%, and the transesterification yield was 76.17%. Thus, the performance of the whole process was:

$$1\ X\ 23.07\%\ X\ 76.17\% = 17.57\%$$

For the modified process 2, the extraction yield was 22.66%, and the transesterification yield was 76.17%. Thus, the performance of the whole process was:

$$1\ X\ 22.66\%\ X\ 76.17\% = 17.26\%$$

5.9 The energy balance for the base line and modified process.

Aim:

To estimate the base line energy requirement and the modified process energy requirement.

For this project, the modified process was aimed to have a higher FAMEs yield and a lower energy input than the base line. Thus, an energy balance was created.

There were 4 parts in the two processes: the harvesting, cell wall treatment, lipid extraction, and transesterification.

5.9.1 The energy input for baseline process

5.9.1.1 The baseline energy input

For the base line, centrifugation was used for harvesting and Eppendorf 5804R Refrigerated Centrifuge was used. The power consumption of this centrifuge was 1650W with max capacity 1L. Thus, the power input of this step was 330W/g. Because the scale used in the experiment was 5ml, the energy input was 4950J for the bench scale. The sonication was used in the base line for cell breakage the power input was 53280J/L. During lipid extraction, the vortex was used to mix the solvents and the sample. Then, the centrifuge was used again to separate the two parts. The vortex used was Chemical Fixed Speed Vortex Mixer and the power input was 60W with 15ml liquid. The centrifuge used was Eppendorf 5804R Refrigerated Centrifuge. The power input for this step was 14700J. Then, the solvent was evaporated, and the fatty acids were kept. During this step, the machine used was: Genevac EZ-2.3 Plus Mk3 Personal Evaporator. The power input for this step was 213576J. For the base line, acid was used during the transesterification and the temperature required was 60° C for 4 hours. The power required to heat the system and keep the temperature constant were calculated. During the transesterification, there were two chemicals in the system: methanol and fatty acids. Besides, there was some sulfuric acid but the amount of it was quite low. Thus, in this energy balance, the sulfuric acid was not considered. The calculation was shown in the appendix and the power input for methanol was 34797.6J and the power input for fatty acids was 6.9J. After transesterification, 3ml of water and 6ml of hexane were added in the tube and then mixed by vortexing. Centrifugation was used to separate the two phases and then the hexane was evaporated. The conditions and the calculation for these steps were shown in the appendix. The power input for the extraction was 13230J and the power input for the evaporation was 388320J.

From table 46, the total energy input for 5ml of sample was 669581.0J.

Table 47. The input energy of base line process.

Step name	Conditions	Scale	Energy input for bench scale (J， per 5ml)
Centrifugation	10min/3000rpm	5ml	4950
High-pressure homogenization	High-pressure homogenization	0.1ml	0.5
Lipid extraction	Centrifuge: 10min	10ml	14700
	Shake: 2min	10ml	
Evaporation	Genevac	6.6ml, 2hours	213576
Transesterification	60° C, 4h/methanol	3ml	34797.6
	60° C, 4h/lipids	0.00568ml	6.9
FAME extraction	shake: 2min	9ml	13230
	Centrifuge: 10min	9ml	
Evaporation	Genevac	6mL; 2hours	388320
SUM			669581.0

Figure 40 showed the energy input of the base line (per 5mL)

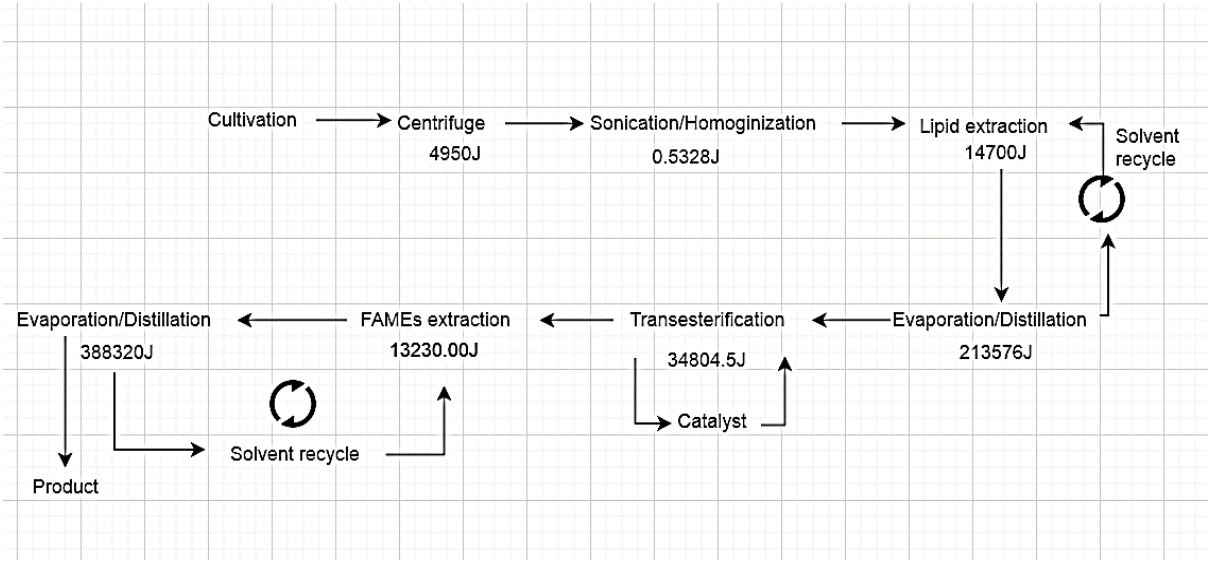

Figure 40. The energy input for base line process (per 5mL).

5.9.2 The energy balance for the modified process

For the modified process, chitosan was used for concentrating the cells and then the Eppendorf 5804R Refrigerated Centrifuge was used. The Vortex was used in the flocculation process to mix chitosan for 30s. The Vortex used was Chemical Fixed Speed Vortex Mixers and the power consumption was 600J. The power consumption of this centrifuge was 1650W with max capacity: 1L. Thus, the power input of this step was 330W/g. Because the scale used in the experiment was 1ml, the energy input was 990J for the bench scale. Viscozyme was used in the modified process for cell wall treatment and the power input was 2.77J. During lipid extraction, the vortex was used to mix the solvents and the sample. Then, the centrifuge was used again to separate the two parts. The vortex used was Chemical Fixed Speed Vortex Mixer and the power input was 60W with 15ml liquid. The centrifuge used was Eppendorf 5804R Refrigerated Centrifuge and the calculation are shown in the appendix. The power input for this step was 588J. Then, the solvent was evaporated, and the fatty acids were kept. During this step, Genevac was used to evaporate the hexane and the machine used was Genevac EZ-2.3 Plus Mk3 Personal Evaporator. The calculation were shown in the appendix.

From the table 47 and 48, the total energy input for the 5ml of sample was 76318.71J (process 1) and 80963.95J (process 2).

5.9.2.1 Modified process energy input

Modified process 1

There were two modified process. Table 47 showed the energy input calculation of process 1, which contained: harvesting (flocculation and centrifuge), cell wall treatment (enzymatic treatment), lipid extraction (methanol-hexane), and enzyme transesterification.

Table 48. The energy input of modified process 1.

Stage name			Conditions	Scale	Energy input (per 5ml)
					J
cultivation			shake/light	/	/
Modified process 1	Harvesting	Flocculation	Vortex	5mL,30s	600
		centrifuge	10min/3000rpm	1ml	990
	Enzymatic treatment (water bath)		37°C， 24h	0.1ml (after centrifuge)	2.77
	lipid extraction (shake/centrifuge)		Centrifuge :10min;	0.2ml （after centrifuge)	588
			Shake :2min	0.2ml	
	Evaporation		Genevac	0.2mL, 1hour	6472
Cooling			Cooling	/	/
transesterification			37°C, 24h/acid	0.00568ml	1.62
			37°C, 24h/methanol	0.00022ml	4.32
FAME extraction			shake	1ml with double extraction	2940
			Centrifuge: 2min	1ml with double extraction	
Evaporation			Genevac	2mL, 1hour	64720
SUM			/	/	76318.71

Modified process 2

There were two modified process. Table 48 showed the energy input calculation of process 2, which contained: harvesting (flocculation and centrifuge), cell wall treatment (ethanol treatment), lipid extraction (hexane), and enzyme transesterification.

Table 49. The energy input of modified process 2.

Stage name			Conditions	Scale	Energy input (per 5ml)
					J
cultivation			shake/light	/	/
Modified process 2	Harvesting	Flocculation	Vortex	5mL,30s	600
		centrifuge	10min/3000rpm	1ml	990
	Cell wall treatment and lipid extraction	Extraction/Cell wall treatment	Magnetic stirrer (28hrs)	0.211ml	1108.8
		Centrifuge	3000rpm; 5min	0.211ml &0.6541ml	891.21
	Evaporation		Genevac	0.6mL, 30ml	9708
Cooling			Cooling	/	/
transesterification			37°C, 24h/acid	0.00568ml	1.62
			37°C, 24h/methanol	0.00022ml	4.32
FAME extraction			shake	1ml with double extraction	2940
			Centrifuge: 2min	1ml with double extraction	
Evaporation			Genevac	2mL, 1hour	64720
SUM			/	/	80963.95

5.9.2.2 The flow chart of the energy input of the modified process

Modified process 1

The harvesting, cell wall treatment, and extraction of modified process 1 were: flocculation, centrifuge, enzymatic treatment, and hexane-methanol as lipid extraction solvent. Figure 41 showed the energy input of the modified process 1 (per 5mL sample).

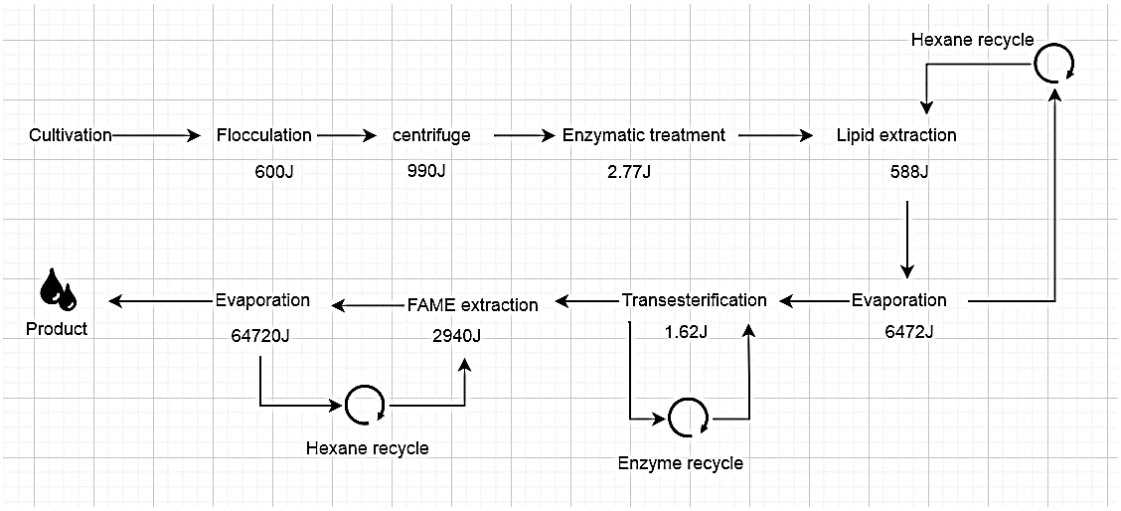

Figure 41. The energy input of the modified process 1 (per 5 mL sample). The energy requirement presented for each step was based on 5mL sample.

Modified process 2

The harvesting, cell wall treatment, and extraction steps of modified process 2 were: flocculation, centrifuge, ethanol cell wall treatment, and hexane as lipid extraction solvent. Figure 42 showed the energy input of the modified process 2 (per 5mL sample).

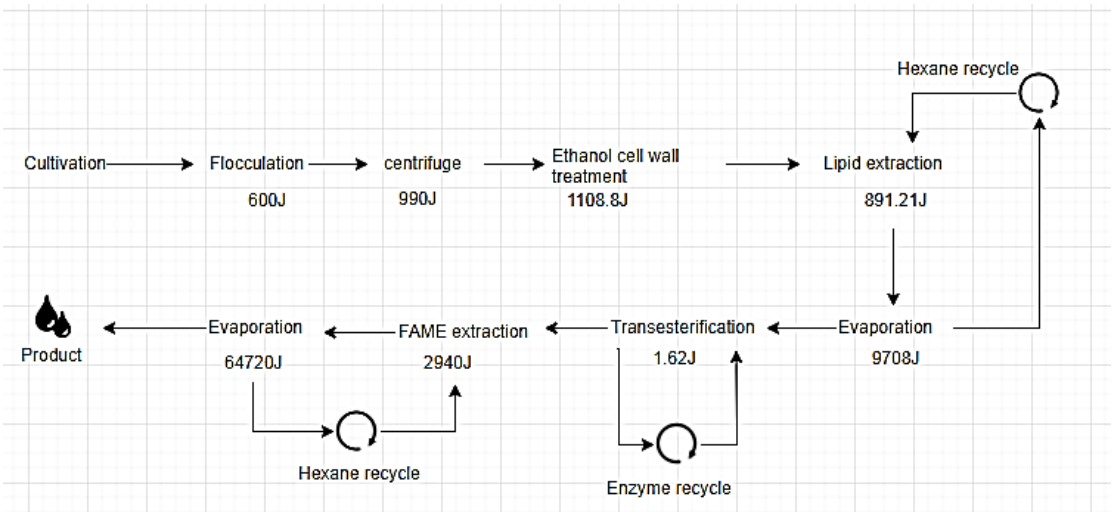

Figure 42. The energy input of the modified process 2 (per 5 mL sample). The energy requirement presented for each step was based on 5mL sample.

5.9.3 The comparison of the energy input between the baseline and modified processes

Because the total energy required for the base line was: 669581.0J and the energy required for the modified process was: 76318.71J (process 1) and 80963.95J (process 2) based on 5 ml sample volume (~28mg of dry biomass).

$$\frac{76318.71\text{J}}{669581.0\text{J}}\text{x}100\% = 11.40\%$$ (process 1)

$$\frac{80963.95\text{J}}{669581.0\text{J}}\text{x}100\% = 12.08\%$$ (process 1)

Thus, the energy required for the modified process was only 11.40% (process 1) and 12.08% (process 2) of the base line process. Those two values were not significantly different.

5.9.4 The calculation of energy output

The products of all the processes were FAMEs. The lower heating value of FAMEs was 37.1MJ/kg and the density of it was 0.88kg/L. The average cell concentration in the calculation was 5g/L. The average of lipid concentration in microalgae culture was 3g/L. Thus, for 5mL sample, the amount of lipid was: 5mL*3mg/mL=15mg=0.015g;

5.9.5 Base line energy output

For the base line, the extraction ratio was: 53.6% and the acid transesterification performance was 89.99%. Thus, the extracted FAME was:

0.015g x 53.6% x 89.99% = 0.0072g

The FAMEs volume:

0.88kg/L=0.88g/mL;

$$\frac{0.0072g}{0.88g/ml} = 0.008ml$$

Thus, the energy output was shown below:

$$37.1\text{MJ/kg} * 0.88\text{kg/L} = 3.26\text{E7J/L}$$

$$3.26E7J/L * 0.008ml = 268.03J$$

Thus, for 5ml of the sample, the baseline output energy was 268.03J.

5.9.6 Modified process energy output

Modified process 1:

For the modified process, the extraction yield was: 23.07% (ethanol extraction) and enzymatic transesterification performance was: 76.17%. Thus, the extracted FAME was:

0.015g x 23.07% x 76.17% = 0.0026g

The FAMEs volume:

0.88kg/L=0.88g/mL;

$$\frac{0.0026g}{0.88g/ml} = 0.003ml$$

Thus, the energy output was shown below:

$$37.1MJ/kg * 0.88kg/L = 3.26E7J/L$$

$$3.26E7J/L * 0.003ml = 97.65J$$

Thus, for 5ml of the sample, the output energy was 97.65J.

Modified process 2:

For the modified process, the extraction yield was: 22.66% (ethanol extraction) and enzymatic transesterification performance was: 76.17%. Thus, the extracted FAME was:

0.015g x 22.66% x 76.17% = 0.0026g

The FAMEs volume:

0.88kg/L=0.88g/mL;

$$\frac{0.0026g}{0.88g/ml} = 0.003ml$$

Thus, the energy output was shown below:

$$37.1MJ/kg * 0.88kg/L = 3.26E7J/L$$

$$3.26E7J/L * 0.003ml = 95.91J$$

Thus, for 5ml of the sample, the output energy was 95.91J.

The energy percentage of output to input was:

Base line:

$$\frac{268.03}{669581.0} = 0.04\%$$

Modified process 1:

$$\frac{97.65}{76318.71} = 0.13\%$$

Modified process 2

$$\frac{95.91}{80963.95} = 0.11\%$$

Thus, the energy output was shown in table 50.

Table 50. The energy output of sample for base line and modified process 1 and 2.

Stage name	Energy output for 5ml of sample (base line process)	Energy output for 5ml of sample (modified process 1)	Energy output for 5ml of sample (modified process 2)
FAME energy output	268.03J	97.65J	95.91J
Energy percentage of output to input	0.04%	0.13%	0.11%

From table 50, the energy output of base line (268.03J) was approximately 3 times higher than the modified process 1(97.65J) and modified process 2 (95.91J). This was because the comparison was based on the FAMEs yield and the yield of the base line was higher than that of the two modified processes. Besides, the energy input of these three processes were not same. To simplify the comparison, the energy input for these processes were assumed as 1 (100%), which was the same of energy input of base line.

Thus, when using 100% energy input, the power output for base line was 0.04%. when using 100% energy input, the power output for modified process 1 was 0.13%, and when using 100% energy input, the power output for modified process 2 was 0.11%.

Thus, when not considering about the used cultivation material, the power output ratio of modified process 1 to baseline was:

$$\frac{0.13\%}{0.04\%} x\ 100\% = 325\%$$

Which means when not considering about the cultivation material and with the same energy input, the power output ratio of modified process 1 to baseline was 325%.

When not considering about the used cultivation material, the power output ratio of modified process 2 to baseline was:

$$\frac{0.11\%}{0.04\%} x\ 100\% = 275\%$$

Which means when not considering about the cultivation material and with the same energy input, the power output ratio of modified process 1 to baseline was 275%.

The power output ratio of modified process 2 was lower than modified process 1 and both modified processes were higher than the baseline. These results indicated that in this project, both modified processes can produce more biofuel than the baseline when using same energy input.

6. Summary of results

1. The TAP medium with glucose (11g/L) in heterotrophic growth produced cells with the highest lipid content (~52.59% w/w DCW) and the highest dry cell concentration (5.6 g/L) after 8 days of cultivation.
2. The enzymatic cell wall treatment method would decrease the microalgae cell size, but the change was insignificant. Some holes were formed on the cell wall because of the enzymes. Besides, the size of microalgae aggregates after flocculation can be 10 times larger than the cell size before flocculation.
3. The clarification levels of two groups (the flocculation followed by enzymatic treatment and the enzymatic treatment followed by flocculation) had significant difference and the clarification level were 91.4% and 89.3% respectively.
4. The sugar concentration of flocculation before enzymatic treatment group was higher than the enzymatic treatment before flocculation group. However, when comparing the enzymatic saccharification yield (% w/w), results showed the latter group had higher hydrolysed sugar yield (11.57%).
5. The extraction yield of flocculated cells before enzymatic treatment (1.11%) was higher than that of the cells undergoing enzymatic treatment before flocculation (0.95%). However, the difference was insignificant.
6. When using ethanol for cell wall treatment, 5ml ethanol with 1 g of wet biomass (~67% water content) was the optimal condition and the extraction yield was around 23%.
7. When using chloroform-methanol as an extraction solvent, the cells processed by centrifugation and sonication had higher extraction yield (~53%) than the cells undergoing flocculation and enzymatic treatment process (~32%). When just using flocculation to harvest microalgae cells, the extraction performance was the lowest (~6%).
8. The optimal conditions of enzymatic treatment to hydrolyse the microalgae cell walls were pH: 3; time: 4 hours, and enzyme concentration: 6μg/g DCW. However, the results showed that during the treatment under the same conditions, the enzymes had an insignificant effect on extraction performance. When the cell wall treatment (without enzyme) presented, the extraction yield increased when comparing to the yields without treatment.
9. The hexane was a suitable replacement solvent in the extraction stage if using methanol or ethanol as co-solvent.
10. The acid transesterification performance was around 90% and the highest lipase transesterification performance was around 76%. The optimal enzymatic transesterification condition were: FA/solvent (mg/ml): 100:1.3, methanol: FFA molar ratio (%):9:1, lipase: FFA amounts (%):50%, and time (hours): 24.
11. When increasing the scale during lipase transesterification from 1.5mL to 20mL, the yield increased around 3 times. The results showed that using hexane as the solvent resulted in a higher transesterification performance compared to tert-butanol.

12. The in-situ acid transesterification resulted in a high performance of around 87%. By contrast, the lipase in-situ transesterification performance was around 1%. This was because the water content was very high.
13. The process yield of baseline was 48.26%. The process yield of modified process 1 was 17.57%. The process yield of modified process 2 was 17.26%. The process yields of modified process 1 and modified process 2 were similar to each other.
14. The energy required for the base line process was 669581.0J for the 5mL of the sample. The energy needed for the modified process 1 was 76318.71J for the 5mL of the sample. The energy needed for the modified process 2 was 80963.95J for the 5mL of the sample. The energy required of the modified process1 and 2 was only 11.40% and 12.08% of the base line process.
15. The lower heating value of FAMEs was used to calculate the energy output of this process. The energy output was 268.03J for 5mL of the sample (base line) and 97.65J for 5mL of the sample (modified process 1). The output energy of the modified process 2 was 95.91J for 5mL sample. The energy output of base line, modified process 1 and modified process 2 were still lower than that of the energy input of base line and the modified process 1 and 2. The percentage of energy input to output was: 0.04% (base line), 0.13% (modified process 1), and 0.11% (modified process 2). Besides, when not considering about the cultivation material, the power output ratio of modified process 1 to baseline was 325%. When not considering about the cultivation material, the power output ratio of modified process 1 to baseline was 275%

7. Conclusion

The aim of this project was to develop an energy-efficient biofuel process that had a similar process yield with the baseline process.

There were two modified processes evaluated in this project. Flocculation, centrifuge, water bath, hexane-methanol as extraction solvent, and enzyme transesterification were included in the modified process 1. The extraction yield of this process was 23.07% and the lipase transesterification yield was around 76%. Flocculation, centrifuge, ethanol treatment, hexane as extraction solvent, and enzyme transesterification were included in the modified process 2. The extraction yield of this process was around 23% and the lipase transesterification yield was around 76%. According to the calculation, the energy required of the modified process1 and 2 was only 11.40% and 12.08% of the base line process. Besides, when not considering about the waste of cultivation material and with the same energy input, the power output ratio of modified process 1 to baseline was 325%. The power output ratio of modified process 2 to baseline was 275%. The power output ratio of modified process 2 was lower than modified process 1 and both modified processes were higher than the baseline. These results indicated that in this project, both modified processes can have higher energy output than the baseline when using same energy input.

8. Future work

8.1 Cell wall treatment

Although there were sugars released after the enzymatic treatment, the results showed that the enzymes had little influence on extraction performance. Thus, more experiments were required to confirm this conclusion. Firstly, the experiments can be based on the modified process. A time course experiment (over 24 hours) will be done in the future. Also, different enzymes can be used in the future experiments. The negative control will be the same condition without enzymes. The extraction method will be chloroform-methanol (2:1 v/v) mixture. Then, the acid transesterification will be used.

If the result shows that the enzymatic treatment has an effect on lipid extraction, then the optimal conditions will be found by DOE.

If the result shows that the enzymatic treatment has no effect on extraction, the mechanical methods will be tested for cell disruption prior to extraction.

8.2 Extraction method

The volume ratio of hexane-methanol can be tested in future work. Moreover, the hexane-ethanol mixture can be used to extract the lipids as well. Then, the extraction time can be tested. A DoE will be used in the future and the factors can be the extraction solvent, cell culture volume ratio, the extraction time, and the numbers of time for extraction.

8.3 Transesterification

The microalgae lipids will be used in lipase transesterification. A DoE will be used in the future experiments for the optimal conditions and the factors can be the lipase: FFA amount, the transesterification time and methanol: FFA molar ratio.

8.4 In-situ transesterification

A dewatering step will be added before the in-situ transesterification and the water content will be detected. A lipase transesterification experiment with the same water content after centrifugation (~58%) will be done to determine the transesterification performance for which the DoE will be used to determine the optimal conditions. The DoE factors can be the water content during the reaction, the methanol: lipid molar ratio, the lipase: lipid amount ratio, and the transesterification time.

Then the energy balance will be established for the in-situ transesterification process and compared with that of the baseline and modified processes.

9. Appendix:

9.1 OD dry mass calibration curve in TAP medium using different methods

The OD-dry mass curve can show the relationship between the OD and microalgae cell dry mass. From figure 43 and 44, the data shows that this relationship is linear. Thus, for a specific medium, when the OD of a microalgae culture is known, the cell dry mass can be calculated directly from this medium's specific OD-dry mass relationship.

Those two OD-dry mass curves below were tested by two different methods using TAP medium. The first one was using the microalgae culture on day 7th and preparing different optical density dilutions to obtain different microalgae concentrations. Finally, the relationship between the concentration of the Chlorella cells and the optical density of the microalgae culture are shown on the graph. The second method was using every day's culture OD and every day's microalgae cell concentration to plot the correlation on the graph. Those two OD-dry mass curves showed little difference to each other, which proves that the optical density and dry mass of C. Sorokiniana in TAP medium have a linear relationship:

Y=0.2031X,

Where Y is C. Sorokiniana cell concentration (g/L) and X is the optical density of the culture.

Because every point was measured triplicates, the error bars are the standard deviation (n=3) for each point.

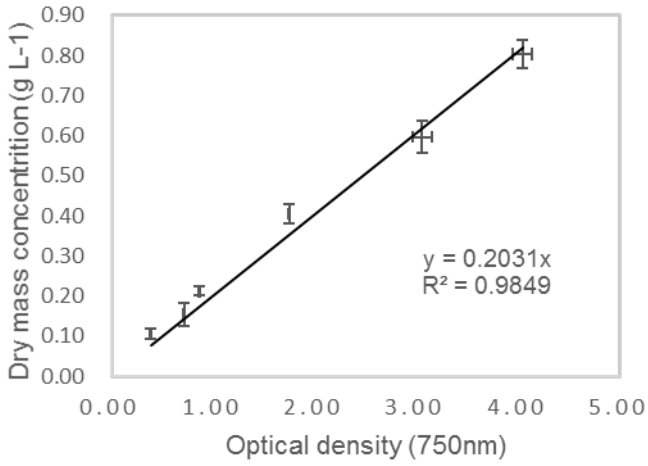

Figure 43. OD-dry mass curve by dilution. The optical density and the dry mass were measured by dilution. The day 7th culture was diluted and the concentration after dilution were: 10%, 20%, 25%, 50%, 75%, and 100% (raw material) to the raw material. Then, the linear ship between these two parameters was: Y=0.2031X, where Y was dry mass (g/l) and X was optical density. The R^2 for this curve was 0.9849.

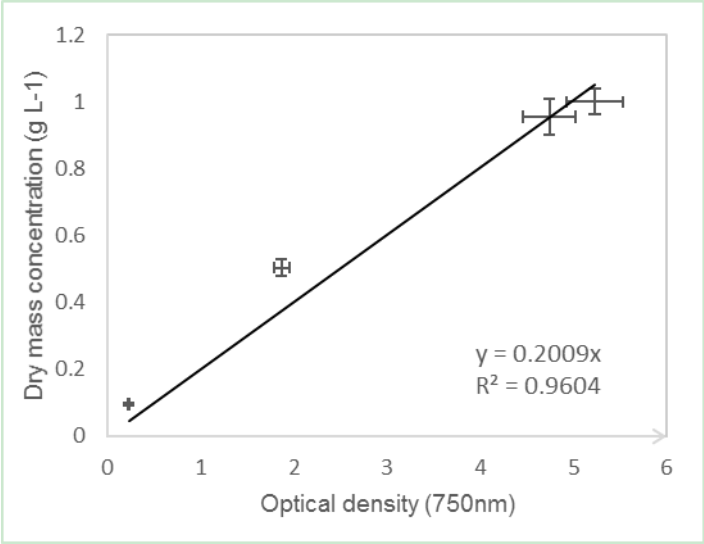

Figure 44. OD-dry mass curve by everyday measuring. The optical density and the dry mass were measured every day during cultivation. Then, the linear ship between these two parameters was: Y=0.2009X, where Y was dry mass (g/l) and X was optical density. The R^2 for this curve was 0.9604.

9.2 One example of the GC-FID analysis figure and the calculation

The FAMEs were analyzed by GC-FID and figure below shows the peaks that have been detected.

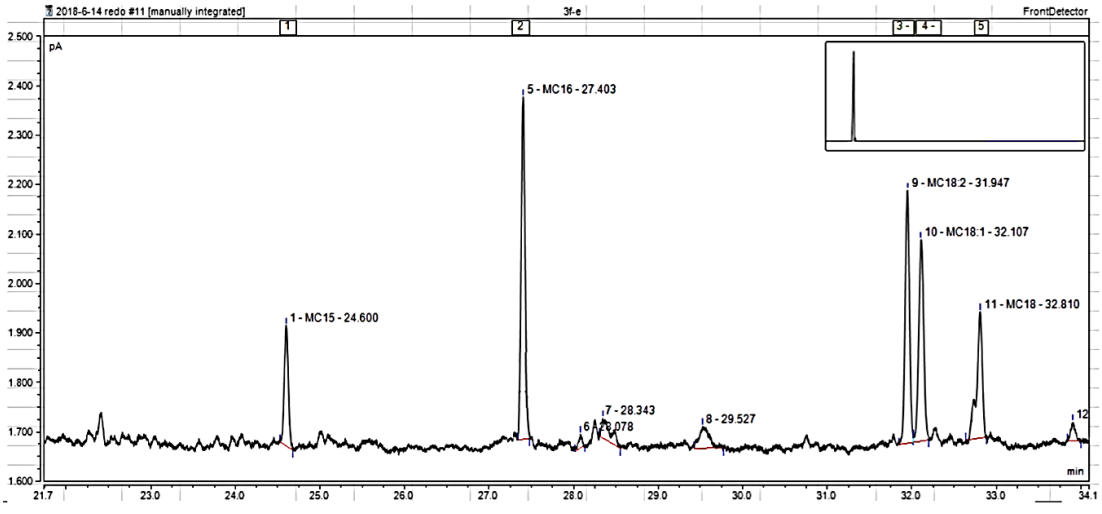

Figure 45. the peaks that has been detected by GC-FID. There were 5 main peaks and some small peaks. The main peaks were: C15 (internal standard), C16, C18, C18:1, and C18:2.

From the chart above, the main peaks can be detected as: C15:0, C16:0, C18:0, C18:1 and C18:2. C15:0 is the internal standard which was added in and C16:0, C18:0, C18:1 and C18:2 were the lipids that has been extracted from the microalgae.
Table below shows the raw data from this GC-FID analysis.

Table 51. The raw data from 2018-06-14, flocculation group, sample 3. The unit of the lipids amount was mg/mL. Peak 1 was MC15 (internal standard). Peak 2 was MC16. Peak 3 was MC18:2. Peak 4 was MC18:1. Peak 5 was MC18

Peak	Peak name	Ret.Time	Amount	Rel.Area	Area	Height	Type	Width (50%)	Asym/	Resol.	Plates
No.		min		%	pA.min	pA		min	EP	EP	EP
1	MC15	24.6	0.0124	8.8	0.0134	0.24	BMB	0.051	1.06	21.42	1292767
3	MC16	27.403	0.0396	25.01	0.0379	0.69	BMB	0.052	1.01	50.17	1559762
4	MC18:2	31.947	0.0284	20.28	0.0308	0.51	BMB	0.055	0.99	1.58	1855133
5	MC18:1	32.108	0.03	18.55	0.0282	0.41	MB	0.064	1.05	6.85	1389232
6	MC18	32.81	0.0214	12.78	0.0194	0.26	BMB	0.057	0.72	n.a.	1830831
Maximum			0.0396	25.01	0.0379	0.69		0.064	1.06	50.17	1855133
Minimum			0.0124	8.8	0.0134	0.24		0.051	0.72	1.58	1292767
Sum			0.1318	85.42	0.1296	2.11					

The calculations of the extraction performance are shown below.
For MC15:
The amount of MC15 in GC-FID is: 0.0124mg/ml and the volume for it is 200μL.
Thus, the amount of MC15 after transesterification is:

$$200\mu L = 0.2ml$$

$$0.2ml\ x\frac{0.0124mg}{ml} = 0.00248mg$$

The molecular weight for MC15 is 256.432g/mol.
Thus, for MC15:

$$\frac{256.432g}{mol} = 256432mg/mol$$

$$\frac{0.00248mg}{256432mg/mol} = 9.67\ X\ 10^{-9}mol$$

According to the previous experiment, the transesterification performance is 90%.
Thus, the C15 before transesterification has:

$$\frac{9.67\ X\ 10^{-9}mol}{90\%} = 1.07\ X\ 10^{-8}mol$$

For C15, the molecular weight is 242.405 g/mol
Thus, the weight for C15 before transesterification:

$$242.405\frac{g}{mol}X\ 1.07E^{-8}mol = 2.60E^{-6}g = 0.0026mg$$

After extraction, the acids were stored in 200μL hexane and 15μL was used in transesterification
So, the C15 concentration in 200μL was:

$$15\mu L = 0.015mL$$

$$\frac{0.0026mg}{0.015ml} = 0.174mg/ml$$

Thus, the amount of C15 after extraction was:

$$200\mu l = 0.2ml$$

$$\frac{0.174mg}{ml}X0.2ml = 0.035mg$$

Because C15 is the external standard and 200μg C15 was added before extraction
Thus, the extraction ratio is:

$$0.035mg = 35\mu g$$

$$\frac{35\mu g}{200\mu g} = 17\%$$

However, C15 is the external standard, which means C15 was added before extraction and it is much easier to be extracted.

For the lipid produced by cells, the extracted weight of C16, C18, C18:1 and C18:2 is calculated by the same procedures.

The molecular weight of these acids and esters are:

Table 52. The molecular weight of the acids

Name	FA	FAME
	g/mol	g/mol
C15	242.405	256.432
C16	256.432	270.459
C18	284.486	298.513
C18:1	282.486	296.513
C18:2	280.486	294.513

The total weight of extracted lipid is:

Table 53. The total weight of extracted lipid

Name	extracted
	mg
C15	0.0347
C16	0.1112
C18	0.0802
C18:1	0.0847
C18:2	0.0604
SUM	0.3712

Because 5ml culture was used and the lipid concentration was: 0.81g/L

Thus, the total lipid before extraction was:

$$\frac{0.81g}{L} = 0.81mg/mL$$

$$\frac{5mLX0.81mg}{mL} = 4.05mg$$

Thus, the extraction ratio for intracellular lipid is:

$$\frac{0.3712mg}{4.05mg} = 0.092 = 9.2\%$$

So, the intracellular lipids extraction ratio is: 9.2%, which is much lower than C15 extraction performance (17%).

Thus, the rest of the results below were used to estimate the cellular lipids extraction performance rather than the internal standard (C15) extraction performance.

9.3 Factorial design experiment

9.3.1 The equations for the other response (6 hours)

The equations of the other response were shown:
1: MC 15 Yield =+85.50+14.52*A+1.90*B-0.47*C-2.43*AB-7.09*AC-0.68* BC-5.65*ABC
2: MC 16 Yield =+34.38-0.24*A-2.52*B +2.09*C- 1.52*AB+1.24*AC+3.93*BC+0.66*ABC
3: MC 18 Yield =+44.42-0.87*A-4.04*B+2.03*C-4.84*AB+1.87*AC+3.91*BC-1.43*ABC
4: MC 18:1 Yield=+56.30+2.06*A-3.82*B+3.04 * C-5.21*AB+3.27*AC+6.36*BC-0.96*ABC
5: MC 18:2 Yield =+49.23+1.07*A-3.80*B+4.24*C-3.89*AB+3.39*AC+5.15*BC+0.06*ABC
6: Sum =+50.49+1.16* A-2.93* B+5.68* C-3.56* AB+4.19* BC
* The factors were coded.
The factor A (methanol: FFA ratio, mol: mol) can positively influence the yields of MC 15, MC18:1 and MC 18:2, and the other yields were negatively influenced. The factor B (water content, v/v) can negatively influence the yields of MC 16, MC 18, MC 18:1, and MC 18:2. Only MC 15 was positively influenced. The factor C (Lipase: FFA amount, w/w) can positively influence the yields of MC 16, MC 18, MC 18:1, and MC 18:2. Only MC 15 was negatively influenced. Because MC 15 was added in the extraction for extraction performance detection, the yield of MC 15 can be ignored for the sum yield. Thus, the factor A can positively influence the yield. The factor B can negatively influence the yield, and the factor C can positively influence the yield. The equation in terms of coded factors can be used to make predictions about the response for given levels of each factor. The coded equation is useful for identifying the relative impact of the factors by comparing the factor coefficients. From this equation, the impact of the factors was: C>BC>AB>AC>B>A.

9.3.2 The equations for the other response (24 hours)

The equations of the other response were shown:
1:MC 15=+102.16+9.47* A+7.62*B+3.19* C+1.58* AB+2.11* AC-2.58* BC-4.87* ABC
2: MC 16=+41.73+1.54* A-0.26* B-2.90* C+0.19* AB-0.38* AC+1.08 * ABC
3:MC 18=+57.45+3.40* A-0.96* B-2.51* C-1.76* AB+1.47* AC
4:MC 18:1=+72.50+7.62* A-0.34* B-4.25* C-2.28 * AB+1.39* AC+0.91* BC-0.66* ABC
5:MC 18:2=+61.67+4.14* A-1.85* B-2.26* C-1.94* AB+1.49 * AC-1.40* BC-1.24* ABC
6: Sum Yield =+60.80+0.75* A-1.48*B-5.03*C+0.15*A*B +2.80*A*B*C
The factor A (methanol: FFA ratio, mol: mol) can positively influence the yields of all the esters. The factor B (water content, v/v) can negatively influence the yields all the esters. Only MC 15 was positively influenced. The factor C (Lipase: FFA amount, w/w) can negatively influence the yields of MC 16, MC 18, MC 18:1, and MC 18:2. Only MC 15 was positively influenced. Because MC 15 was added in the extraction for extraction performance detection, the yield of MC 15 can be ignored for the sum yield. Thus, the factor A can positively influence the yield. The factor B can negatively influence the yield,

and the factor C can negatively influence the yield. The equation in terms of coded factors can be used to make predictions about the response for given levels of each factor. The coded equation was useful for identifying the relative impact of the factors by comparing the factor coefficients. From this equation, the impact of the factors was: C>ABC>B>A>AB.

9.4 Response surface methodology experiment (6 hours)

Figure below shows the interaction between the acid concentration and the esters yield when the methanol: FFA molar ratio was at 9:1 and 15:1 and the lipase amounts were at 10%, 30%, and 50% w/w.

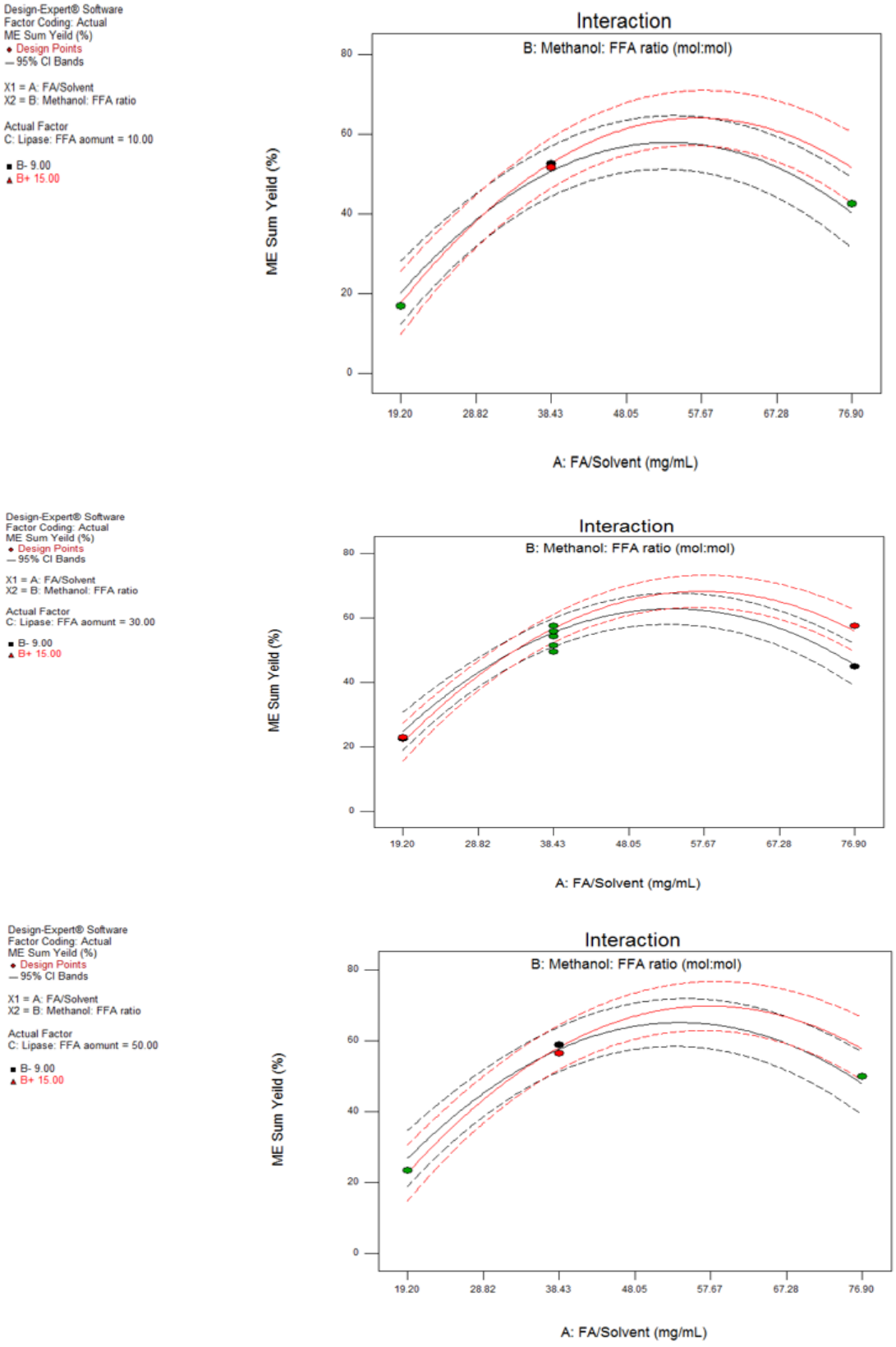

Figure 46. The interaction between the acid concentration and the esters yield when the methanol: FFA molar ratio was at 9:1 and 15:1 and the lipase amounts were at 10%,30%, and 50% w/w.

9.5 Response surface methodology experiment (24 hours)

Figure 47 indicates the interaction between the acid concentration and the esters yield when the methanol: FFA molar ratio was at 9:1 and 15:1 and the lipase amounts were at 10%, 30%, and 50% w/w. From figure 47, the higher acids concentration would always have a higher yield. Besides, although the yield of 9:1 of methanol: FFA molar ratio was lower than that of 15:1, when the lipase: FFA amount increasing, the 9:1 of methanol: FFA molar ratio would have a higher yield than that of 15:1 when the FA/solvent concentration was higher than 40%.

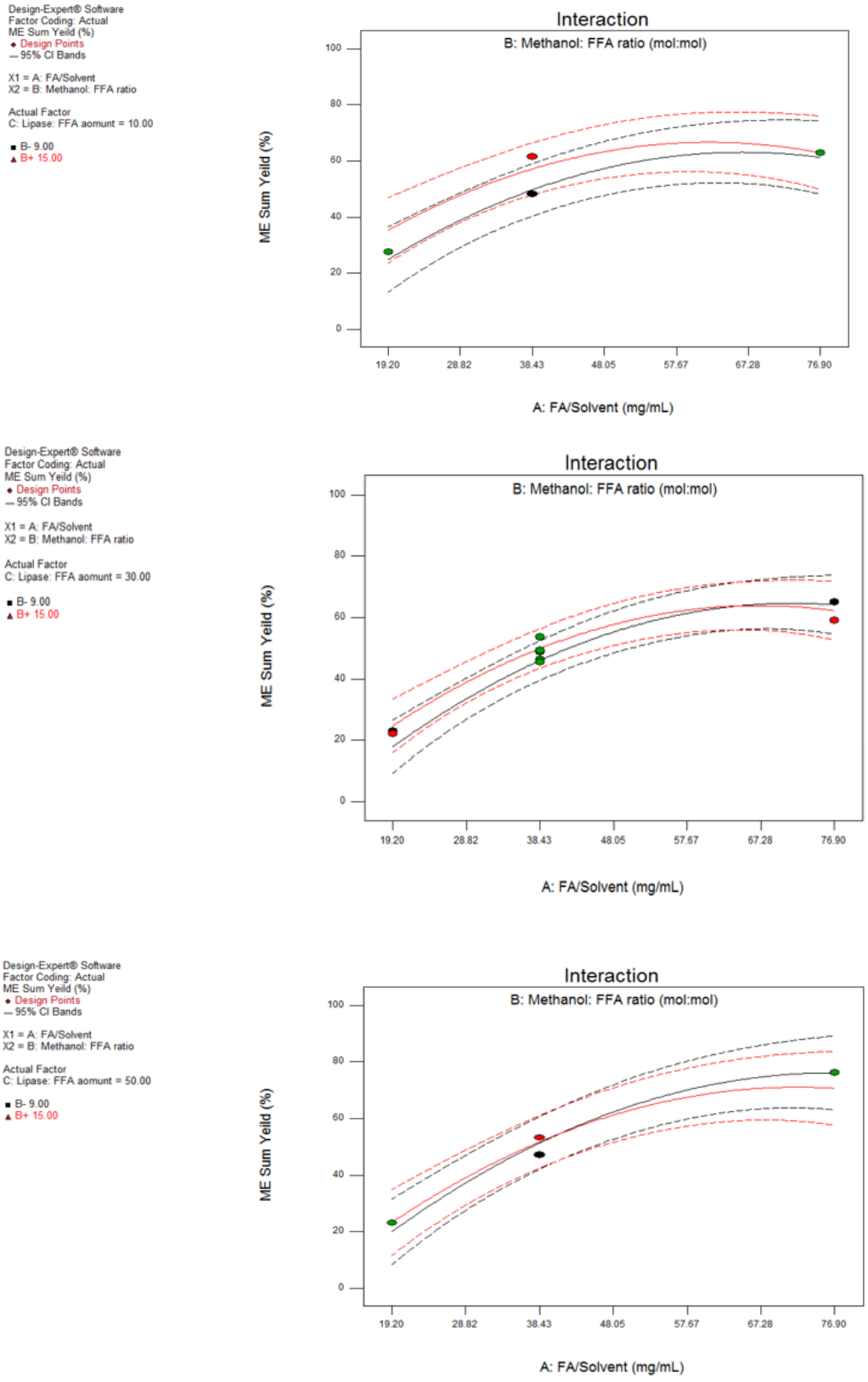

Figure 47. The interaction between the acid concentration and the esters yield when the methanol: FFA molar ratio was at 9:1 and 15:1 and the lipase amounts were at 10%,30%, and 50% w/w.

9.6 Calculation of energy input for base line process

The calculation of the required energy are shown below:

- Centrifuge:

The centrifuge used in this experiment was Eppendorf 5804R Refrigerated Centrifuge with A-4-44 Rotor. The max capacity was: 4 × 250 mL/2 × 5 MTP and the power consumption was :1650W.

Thus: 4x250ml=1000ml=1L; 5g/LX1L=5g; 1650W/5g=330W/g; For 5mL sample: 1650w/l*10min*60s=990000J/L; 990000J/L*0.005L=4950J.

- High pressure homogenization:

After centrifuge, the scale of the samples decreased to 0.1mL. The high pressure used in this calculation was: Industrial High-Pressure Homogenizer 90mpa High Power 2500ltr / Hr SRH2500-40. The motor power was 37kW. For 1 litre sample: 1L÷2500L/h=4*E-4h; energy input:37000w=37000J/s;37000J/s*4*E-4*60*60s=53280J. Because there was 0.1mL of sample, the energy input was: 53280J/L*0.0001L=5.33J.

- Lipid extraction:

During lipid extraction, there were two steps: the shaking (2mins) and the centrifuge (10mins). The vortex used in this experiment to mix the solvents was: Chemical Fixed Speed Vortex Mixers Laboratory Vortex Mixer Lab Orbital Shaking Vortex Mixer Mini Portable Liquid Mixer 110V MX-F. The power of it was: 60W and the capacity was: 15mL. Thus: 60w/15ml=4w/ml=4000w/l. Because the samples required to shake for 2 minutes: 4000w/lx2min*60s/min=480000 J/L.

The centrifuge used was Eppendorf 5804R Refrigerated Centrifuge with A-4-44 Rotor. The max capacity was 4 × 250 mL/2 × 5 MTP and power consumption was: 1650w. Thus: 4x250ml=1000ml=1L; 1650w/1L=1650w/L 1650w/L=1650J/s*L, 1650J/s*Lx10min*60s=990000J/L;

Because there was 10mL of solvents during shaking and centrifuge. Thus: =480000J/L+990000J/L=1470000J/L, 1470000J/L*0.01L=14700J

- Evaporation:

The chloroform was evaporated after extraction. There was 5mL of chloroform: methanol (2:1 v/v) mixture added to extract the lipids during extraction. Thus, in 5mL of chloroform: methanol (2:1 v/v) mixture, 5mL/3*2=3.33mL. The machine used was: Genevac EZ-2.3 Plus Mk3 Personal Evaporator. The capacity was 180ml and the power consumption was1610VA. Thus: 1610va=1610w; 1610w/0.18L=8988.889w/L; 8988.889W/L*120min*60s=64720000J/L. There was 3.33mL of chloroform. Thus: 64720000J/L*3.3*0.001L=213576J.

- Transesterification:

There were two parts during transesterification, the methanol and the lipids. The amount of sulfuric acid was too small that in this step, it was not counted. According to the final product, the volume of the lipids was: 0.00568ml. The characters of lipids were: oil free convention: 50-350 w/m2k; molar ratio: free fatty acid: methanol=1:3; free faty acid heat

capacity: 463.36J/molK (C16); 501.50J/molK (C18); density: 0.895g/ml and molar mass: 282.47g/mol.
The energy required to heat the lipids: 500J/molK*(60-20) K÷282.47g/mol=70.80J/g. Then, the solvents were kept at 60 ° C. Thus, for keep temperature (1L): 200w/m2K*0.01m2*(60-20)K=80w;
total=70.80J/g+80w/L÷895g/L*4*60*60(s)=1357.95J/g,
=1357.95J/g*895g/L=1215365.25J/L. Because there was 0.00568ml of lipids, the energy required was: 1215365.25J/L *0.00568*0.001L=6.9J.
The 3mL of methanol was used and the characters of methanol are: methanol heat capacity:79.9 J/(mol K); molar mass: 32.04 g mol−1; density:0.792 g/cm3, and steam: methanol heat transfer coefficient:1134.893182-3972.126138w/m2k. Thus, the energy required for heating can be calculated as methanol heat capacity: 79.9J/molK ÷32.04g/mol=2.50J/gK; for 1L methanol : 2.50J/gK*792g/L=1980J/L*K; For heat: 1980J/L K*(60-20)K=79200J/L; Then, the solvents were kept at 60° C. Thus, the energy required for keeping the temperature was: 2000w/m2K*(60-20) K*0.01m2=800w; total: 79200J/L÷792g/l+800w/L÷792g/l*4*60*60(s)=14645.45J/g;
14645.45J/g*792g/L=11599200/L. The amount of methanol in transesterification was 3mL. Thus, the energy required during the transesterification was: 11599200/L*0.003L=34797.6J

- FAME extraction:

After transesterification, 3mL of water and 3mL of hexane was used during extraction. Thus, the total volume was: 3mL+3mL+3mL=9mL. The vortex and centrifuge used were the same as before. Thus, the energy required during extraction can be calculated as: 480000J/L+990000J/L=1470000J/L, 1470000J/L*0.009L=12320J.
Then, the hexane was evaporated by Genevac, which was the same as above. Thus, the energy required can be calculated as: 64720000J/L*6*0.001L=388320J.

Thus, the total energy required for base line was: 669581.0J

9.7 Calculation of energy input for modified process

The calculation of the required energy was showed below:

- Harvesting:

Flocculation was used in the cultivation stage, and the vortex used was: Chemical Fixed Speed Vortex Mixers Laboratory Vortex Mixer Lab Orbital Shaking Vortex Mixer Mini Portable Liquid Mixer 110V MX-F. The power consumption of it was 60w and the capacity was 15ml. Thus, 60w/15ml=4w/ml=4000w/l 4000w/lx30s=120000 J/L. There was 5mL of culture in each sample. Thus, the energy consumption was: 120000J/L*0.005L=600J
After flocculation, the sample was concentrated by centrifuge. The centrifuge used was: Eppendorf 5804R Refrigerated Centrifuge with A-4-44 Rotor. The max capacity of it was 4 × 250 mL/2 × 5 MTP and the power consumption was 1650w. Thus:

4x250ml=1000ml=1L; 5g/LX1L=5g; 1650w/l*10min*60s=990000J/L. Because after flocculation, the volume of sample decreased to 1mL, the energy consumption was: 990000J/L*0.0001L=990J.

- Enzymatic treatment:

During enzymatic treatment, the temperature was 37°C and the volume was 1ml. The specific capacity of water was 4.18 J/ (g °C) . The water to air heat transfer coefficient will be depended on the material in transmission surface: cast iron:7.9, mild steel11.3, copper:13.1 (w/m2*K). After flocculation, for 1 L sample, the area that has water-air heat transfer will be: 0.01 m2. Thus, 4.18J/g°C÷1s*(37-20) ° C=71.06w/g; 11.3 w/m2K*(37-20) K=192.1w/m2; for 1L sample: 192.1w/m2*0.01m2=1.921w. For 4 hours reaction: 1.921w/L*4h*60min*60=27662.4J/L. For 0.1ml sample: 27662.4*0.1*0.001=2.77J.

- Ethanol cell wall treatment

The cell concentration of samples was 5.5g/l and the water content after centrifuge was 65%. Thus, when using 5ml of cell culture, the wet biomass was: 5ml * 5.5g/l/65%=0.042g. 1g wet biomass required 5ml of ethanol. Thus, 0.042g wet biomass required 0.042g*(5ml/1g) =0.211ml ethanol. The stirrer used was Stuart® UC152 Ceramic Coated Hot Plate Stirrer (Findel, UK) and the capacity of it was10L with 500J/s power. Thus, for 0.211ml of sample, the power required was :500J/s/1L*0.000211L=0.011J/s. The ethanol treatment and hexane extraction required 28hours' mixing. Thus, 0.011J/s*28hr*60min/hr*60s/min=1108.8J

- Lipid extraction:

Then vortex and the centrifuge were used during lipid extraction and the machines used were the same as used in the base line. Thus, during shaking, 60w/15ml=4w/ml=4000w/l 4000w/lx2min*60=480000 J/L. During concentration: 4x250ml=1000ml=1L; 1650w/1L=1650w/L 1650w/L=1650J/s*L, 1650J/s*Lx10min*60s=990000J/L. The volume during extraction was 0.2ml. Thus, the energy consumption was: 480000J/L+990000J/L=1470000J/L, 1470000J/L*0.00002L=294J. For double extraction: 294J*2=588J

- Evaporation:

Genevac was used in evaporation to evaporate the hexane and the machine used was Genevac EZ-2.3 Plus Mk3 Personal Evaporator. The capacity was 180ml and the power consumption was 1610VA. Thus, the energy consumption can be calculated as: 1610va=1610w; 1610w/0.18L=8988.889w/L; 8988.889W/L*60min*60s=32360000.4J/L. For 0.2Ml of hexane: 32360000.4J/L*0.2*0.001L 6472.00J

- Transesterification:

Enzymes were used in transesterification and 0.00022ml of methanol was used. The characters of lipids were the same as base line: oil free convention: 50-350 w/m2k; molar ratio: free fatty acid: methanol=1:3; free fatty acid heat capacity: 463.36J/mol*K (C16); 501.50J/mol*K (C18); density: 0.895g/ml; molar mass: 282.47g/mol. Thus, the energy consumption was: methanol heat capacity: 79.9J/mol*K ÷32.04g/mol=2.50J/g*K; for 1L

methanol : 2.50J/g*K*792g/L=1980J/L K; for heat: 1980J/L K*(37-20)K=33660J/L; For keep temperature (1L): 2000w/m2K*(37-20)K*0.01m2=340w;total:=33660J/L +340w/L*6*60*60(s)= 7377660J/L. For 0.00022ml: 7377660J/L*0.00022*0.001L=1.62J.
For lipids heating: 500J/mol*K*(37-20) K÷282.47g/mol=30.09J/g For keep temperature (1L): 200w/m2K*0.01m2*(37-20) K=34w; total=30.09J/g+34w/L÷895g/L*6*60*60(s)=850.65J/g, 850.65J/g*895g/l=761331.75J/L. For 0.00568ml lipids: 761331.75J/L*0.00568*0.001L=4.32J.

- FAME extraction:

The centrifuge and vortex used in this step were the same as before. Thus: 480000J/L+990000J/L=1470000J/L, 1470000J/L*0.002L=2940J

- Evaporation:

The machine used in this step was Genevac. Thus, 32360000.4J/L*2*0.001L=64720J

Thus, the total energy required for modified process was: 76318.71J

10. References

1 Allard, B., & Templier, J. (2000). Comparison of neutral lipid profile of various trilaminar outer cell wall (tls)-containing microalgae with emphasis on algaenan occurrence. Phytochemistry, 54(4), 369-380.

2 Amini Z., Ilham Z., Ong H C., et al (2016). State of the art and prospective of lipase-catalyzed transesterification reaction for biodiesel production[J]. Energy Conversion and Management, 141(JUN.):339-353.

3 Antonio Ramírez Fajardo, Luis Esteban Cerdán, Medina, A. R., Fracisco Gabriel Acién Fernández, & Grima, E. M.. (2007). Lipid extraction from the microalga phaedactylum tricornutum. eur j lipid sci tech. European Journal of Lipid ence and Technology, 109(2), 120-126.

4 Araki, N., & Takeda, H.. (2010). Species-specificity of the lytic activity of the cell wall in the genus chlorella. Physiologia Plantarum, 85(4), 704-709.

5 Bajaj, A., Lohan, P., Jha, P. N., & Mehrotra, R. (2010). Biodiesel production through lipase catalyzed transesterification: an overview. Journal of Molecular Catalysis B Enzymatic, 62(1), 9-14.

6 Bligh, E. and Dyer, W. (1959). A rapid method of total lipid extraction and purification. Canadian Journal of Biochemistry and Physiology, 37(8), pp.911-917.

7 Boyle, N.R. and Morgan, J.A. (2009) Flux balance analysis of primary metabolism in Chlamydomonas reinhardtii. BMC Syst. Biol. 3, 4.

8 Braun, E. and Aach, H. (1975). Enzymatic degradation of the cell wall of Chlorella. Planta, 126(2), pp.181-185.

9 Brennan, L. and Owende, P. (2010). Biofuels from microalgae—A review of technologies for production, processing, and extractions of biofuels and co-products. Renewable and Sustainable Energy Reviews, 14(2), pp.557-577.

10 Carvalho, A., Meireles, L. and Malcata, F. (2006). Microalgae Reactors: A Review of Enclosed System Designs and Performances. Biotechnology Progress, 22(6), pp.1490-1506.

11 Chen, G. and Chen, F. (2006). Growing Phototrophic Cells without Light. Biotechnology Letters, 28(9), pp.607-616.

12 Chen, W., Zhang, C., Song, L., Sommerfeld, M. and Hu, Q. (2009). A high throughput Nile red method for quantitative measurement of neutral lipids in microalgae. Journal of Microbiological Methods, 77(1), pp.41-47.

13 Cheng, Y., Juang, Y., Liao, G., Ho, S., Yeh, K., Chen, C., Chang, J., Liu, J. and Lee, D. (2010). Dispersed ozone flotation of Chlorella vulgaris. Bioresource Technology, 101(23), pp.9092-9096.

14 Chisti, Y. (2007). Biodiesel from microalgae. Biotechnology Advances, 25(3), pp.294-306.

15 Chojnacka, K. and Noworyta, A. (2004). Evaluation of Spirulina sp. growth in photoautotrophic, heterotrophic and mixotrophic cultures. Enzyme and Microbial Technology, 34(5), pp.461-465.

16 Christopher, L. P., Kumar, H., & Zambare, V. P.. (2014). Enzymatic biodiesel: challenges and opportunities. Applied Energy, 119(APR.15), 497–520.

17 Cristina G., and Fernandez B. S. (2011). Impact of microalgae characteristics on their conversion to biofuel. Part II: Focus on biomethane production. Biofpr Pages 205-218.

18 Daroch, M., Geng, S. and Wang, G. (2012). Recent advances in liquid biofuel production from algal feedstocks. Applied Energy Pages 1371-1381

19 Demirbas, A. and Fatih Demirbas, M. (2017). Importance of algae oil as a source

of biodiesel. pp. 23-29.

20 Divakaran, R., & Pillai, V. N. S.. (2002). Flocculation of algae using chitosan. Journal of Applied Phycology, 14(5), 419-422.

21 Divakaran, R., Sivasankara Pillai, V. (2002). Flocculation of algae using chitosan. Journal of Applied Phycology 14, 419–422.

22 Edwin K. Eckhard L. Werner R. (1992). Cell wall composition of virus-sensitive symbiotic Chlorella species. Phytochemistry pp. 3103-3104.

23 Eriksen, N. (2008). Production of phycocyanin—a pigment with applications in biology, biotechnology, foods and medicine. Applied Microbiology and Biotechnology, 80(1), pp.1-14.

24 Estevez, M., Malanga, G. and Puntarulo, S. (2001). Iron-dependent oxidative stress in Chlorella vulgaris. Plant Science, 161(1), pp.9-17.

25 F. Chen, Y. Zhang, S. Guo. Growth and phycocyanin formation of Spirulina platensis in photoheterotrophic culture. Biotechnology Letters, 18 (5) (1996), pp.603-608

26 Falkowski, P. and Raven, J. (2007).3rd. Aquatic photosynthesis. Princeton, Princeton Univ. Press. pp 375.

27 Fangfang Yang, Changhong Cheng, Lijuan Long, Qunju Hu, Qikun Jia, Hualian Wu, and Wenzhou Xiang (2015) Extracting Lipids from Several Species of Wet Microalgae Using Ethanol at Room Temperature Energy & Fuels, 29 (4), 2380-2386

28 Frappart, M., Massé, A., Jaffrin, M., Pruvost, J. and Jaouen, P. (2011). Influence of hydrodynamics in tangential and dynamic ultrafiltration systems for microalgae separation. Desalination, 265(1-3), pp. 279-283.

29 Geciova, J., Bury, D. and Jelen, P. (2002). Methods for disruption of microbial cells for potential use in the dairy industry—a review. International Dairy Journal, 12(6), pp.541-553.

30 Gerken, H. G., Donohoe, B., & Knoshaug, E. P.. (2013). Enzymatic cell wall degradation of chlorella vulgaris and other microalgae for biofuels production. Planta 237, pages 239–253

31 Ghaly, M. A. H. A. (2015). Microalgae oil extraction pre-treatment methods: critical review andcomparative analysis. Journal of Fundamentals of Renewable Energy and Applications, 05(4).

32 Gong, Y., & Huang, J.. (2020). Characterization of four untapped microalgae for the production of lipids and carotenoids. Algal Research, 49, 101897.

33 Gonzalez, L. and Bashan, Y. (2000). Increased Growth of the Microalga Chlorella vulgaris when Coimmobilized and Cocultured in Alginate Beads with the Plant-Growth-Promoting Bacterium Azospirillum brasilense. Applied and Environmental Microbiology, 66(4), pp.1527-1531.

34 Guldhe, A., Singh, B., Mutanda, T., Permaul, K., & Bux, F. (2015). Advances in synthesis ofbiodiesel via enzyme catalysis: Novel and sustainable approaches. Renewable andSustainable Energy Reviews, pp. 1447–1464.

35 H. Kanda, P. Li, T. Ikehara, M. Yasumoto-Hirose (2012). Lipids extracted from several species of natural blue–green microalgae by dimethyl ether: extraction yield and properties. Fuel Volume 95, pp. 88-92

36 Halim, R., Harun, R. , Danqua, M. K. , & Webley, P. A. . (2012). Microalgal cell disruption for biofuel development. Applied Energy, 91(1), 116-121.

37 Hanotu, J., Bandulasena, H. and Zimmerman, W. (2012). Microflotation performance for algal separation. Biotechnology and Bioengineering, 109(7), pp.1663-1673.

38 Harrison, S. (1991). Bacterial cell disruption: A key unit operation in the recovery of intracellular products. Biotechnology Advances, 9(2), pp.217-240.

39 Huang, A., Sun, L., Wu, S., Liu, C., Zhao, P., Xie, X. and Wang, G. (2016). Utilization of glucose and acetate by Chlorella and the effect of multiple factors on cell composition. Journal of Applied Phycology, 29(1), pp.23-33.

40 Huntley, M. and Redalje, D. (2006). CO2 Mitigation and Renewable Oil from Photosynthetic Microbes: A New Appraisal. Mitigation and Adaptation Strategies for Global Change, 12(4), pp.573-608.

41 Ivana, L., Kesi, E. , Zduji, M. , & Skala, D. . (2016). Solid Acids as Catalysts for Biodiesel Synthesis. Heterogeneous Catalysts: Design, Applications and Research Insights pp. 21-136.

42 J.M. Romero García, F.G. Acién Fernández, & J.M. Fernández Sevilla. (2012). Development of a process for the production of l-amino-acids concentrates from microalgae by enzymatic hydrolysis. Bioresource Technology, 112(none), 164-170.

43 JIANG, Y., PETRIER, C. and WAITE, T. (2006). Sonolysis of 4-chlorophenol in aqueous solution: Effects of substrate concentration, aqueous temperature and ultrasonic frequency. Ultrasonics Sonochemistry, 13(5), pp.415-422.

44 Jiménez, C., Cossío, B., Labella, D. and Xavier Niell, F. (2003). The Feasibility of industrial production of Spirulina (Arthrospira) in Southern Spain. Aquaculture, 217(1-4), pp.179-190.

45 Ji-Yeon, Park, Min, S., Park, & Young-Chul. (2015). Advances in direct transesterification of algal oils from wet biomass. Bioresource Technology.

46 John PIRT, S. (1986). The thermodynamic efficiency and dynamics of photosynthetic growth. New Phytologist, 102(1), pp.3-37.

47 Katsumi Yamaguchi, Hiroshi Nakano, Masahiro Murakami, Shoji Konosu,Osamu Nakayama, Midori Kanda, Akihiro Nakamura & Hiroaki Iwamoto (1987) Lipid Composition of a Green Alga,Botryococcus braunii, Agricultural and Biological Chemistry, 51:2, 493-498,

48 Kumar, K., Dasgupta, C. N., & Das, D. (2014). Cell growth kinetics of Chlorella Sorokiniana and nutritional values of its biomass. Bioresource Technology, 167, 358–366.

49 Kumari, V., Shah, S., & Gupta, M. N.. (2007). Preparation of biodiesel by lipase-catalyzed transesterification of high free fatty acid containing oil from madhuca indica. Energy & Fuels, 21(1), 368-372.

50 Lea, P. J., & Miflin, B. J.. (2003). Glutamate synthase and the synthesis of glutamate in plants. Plant Physiology & Biochemistry, 41(6-7), 555-564.

51 Lee, O., Kim, A., Seong, D., Lee, C., Jung, Y., Lee, J. and Lee, E. (2013). Chemo-enzymatic saccharification and bioethanol fermentation of lipid-extracted residual biomass of the microalga, Dunaliella tertiolecta. Bioresource Technology, 132, pp.197-201.

52 Lee, R. (2009). 3rd. Phycology. Cambridge: Cambridge University Press.

53 Mata, T., Martins, A. and Caetano, N. (2010). Microalgae for biodiesel production and other applications: A review. Renewable and Sustainable Energy Reviews, 14(1), pp.217-232.

54 Meher, L. C., Vidya Sagar, D., & Naik, S. N. (2006). Technical aspects of biodiesel production bytransesterification - A review. Renewable and Sustainable Energy Reviews, 10(3), 248–268.

55 Miao, X. and Wu, Q. (2006). Biodiesel production from heterotrophic microalgae oil. Bioresource Technology, 97(6), pp.841-846.

56 Miller, M., Miller, D. and Brayman, A. (1996). A review of in vitro bioeffects of inertial ultrasonic cavitation from a mechanistic perspective. Ultrasound in Medicine & Biology, 22(9), pp.1131-1154.

57 Molina Grima, E., Belarbi, E., Acién Fernández, F., Robles Medina, A. and Chisti, Y. (2003). Recovery of microalgae biomass and metabolites: process options and economics. Biotechnology Advances, 20(7-8), pp.491-515.

58 Moreno, L. M, Muñoz Prieto, E, & Casanova, H. (2015). Flocculatin with chitosan of

microalgae native of the colombian plateau. Ciencia En Desarrollo, 6(1).

59 Mubarak M , Shaija A , Suchithra T V (2017) . A review on the extraction of lipid from microalgae for biodiesel production. Algal Research, 7:117-123.

60 N. Azcan, & O. Yilmaz. (2012). Microwave irradiation application in biodiesel production from promising biodiesel feedstock: microalgae (chlorella protothecoides). Lecture Notes in Engineering & Computer ence, 2201(1), 1309.

61 Nayak, M., Karemore, A., & Sen, R.. (2016). Performance evaluation of microalgae for concomitant wastewater bioremediation, co2 biofixation and lipid biosynthesis for biodiesel application. Algal Research, 16, 216-223.

62 Nguyen, Thanh. B., Sorres, J., Tran, M. Q., Ermolenko, L., & Al-Mourabit, A.. (2012). Boric acid: a highly efficient catalyst for transamidation of carboxamides with amines. Organic Letters, 43(12), 3202-3205.

63 Olaizola, M. (2003). Commercial development of microalgae biotechnology: from the test tube to the marketplace. Biomolecular Engineering, 20(4-6), pp.459-466.

64 Parsons, S., Raikova, S. & Chuck, C.J. (2020). The viability and desirability of replacing palm oil. Nat Sustain 3, pp. 412–418.

65 Perez-Garcia, O., Escalante, F. M. E., De-Bashan, L. E., & Bashan, Y.. (2011). Heterotrophic cultures of microalgae: metabolism and potential products. Water Research, 45(1), 11-36.

66 Phukan, M. M., Chutia, R. S., Konwar, B. K., & Kataki, R.. (2011). Microalgae chlorella as a potential bio-energy feedstock. Applied Energy, 88(10), 3307-3312.

67 Ranjith Kumar, Ramanathan， Hanumantha Rao, Polur， Arumugam, Muthu (2015) Lipid extraction methods from microalgae: a comprehensive review Frontiers in Energy Research 2(61):1-9

68 Ríos, S., Salvadó, J., Farriol, X. and Torras, C. (2012). Antifouling microfiltration strategies to harvest microalgae for biofuel. Bioresource Technology, 119, pp.406-418.

69 Salim, S., Vermuë, M. and Wijffels, R. (2012). Ratio between autoflocculating and target microalgae affects the energy-efficient harvesting by bio-flocculation. Bioresource Technology, 118, pp.49-55.

70 Sheehan, J., Dunahay, T., Benemann, J. and Roessler, P. (1998) A Look Back at the U.S. Department of Energy's Aquatic Species Program—Biodiesel from Algae. National Renewable Energy Laboratory, Report NREL/TP-580-24190, pp. 294-298.

71 Sheng, J., Vannela, R. and Rittmann, B. (2012). Disruption of Synechocystis PCC 6803 for lipid extraction. Water Science & Technology, 65(3), p.567.

72 Supatchalee Sophonthammaphat, & Edyvean, R., (2015). Flocculation C.vulgaris using chitosan. International Congress and Expo on Biofuels & Bioenergy. Renewable Energy Appl, 5:5

73 Terry, K. and Raymond, L. (1985). System design for the autotrophic production of microalgae. Enzyme and Microbial Technology, 7(10), pp.474-487.

74 Toh, P. Y., Yeap, S. P., Kong, L. P., Ng, B. W., Chan, D. J. C., & Ahmad, A. L., et al. (2012). Magnetophoretic removal of microalgae from fishpond water: feasibility of high gradient and low gradient magnetic separation. Chemical Engineering Journal, s 211–212(none), 22–30.

75 Ugwu, C., Aoyagi, H. and Uchiyama, H. (2008). Photobioreactors for mass cultivation of algae. Bioresource Technology, 99(10), pp.4021-4028.

76 Vanoni, M. A., & Curti, B.. (2005). Structure–function studies on the iron–sulfur flavoenzyme glutamate synthase: an unexpectedly complex self-regulated enzyme. Archives of Biochemistry and Biophysics, 433(1), 193-211.

77 Vazhappilly, R. and Chen, F. (1998). Heterotrophic Production Potential of Omega-3 Polyunsaturated Fatty Acids by Microalgae and Algae-like Microorganisms. Botanica Marina, 41(1-6), pp.553-558.

78 Wang Pu, Yang Li-rong & Wu Jian-ping. (2001), Kluwer Academic Publishers. Printed in the Netherlands. Biotechnology Letters 23: 1429-1433.

79 Wen, Z. and Chen, F. (2003). Heterotrophic production of eicosapentaenoic acid by microalgae. Biotechnology Advances, 21(4), pp.273-294.

80 Wilhelm, C., Claudia Büchel, Fisahn, J., Goss, R., Jakob, T., & Laroche, J., et al. (2006). The regulation of carbon and nutrient assimilation in diatoms is significantly different from green algae. Protist, 157(2), 91-124.

81 Yang, C., Hua, Q., & Shimizu, K. (2000). Energetics and carbon metabolism during growth of microalgal cells under photoautotrophic, mixotrophic and cyclic light-autotrophic/dark-heterotrophic conditions. Biochemical Engineering Journal, 6(2), 87-102.

82 Yang, F., Xiang, W., Sun, X., Wu, H., Li, T. and Long, L. (2014). A Novel Lipid Extraction Method from Wet Microalga Picochlorum sp. at Room Temperature. Advances and New Perspectives in Marine Biotechnology. pp. 1258-1270

83 Yangli, Y., Yun, H., Ao, X., Qian, F., Qiang, L., & Weida, Z., et al. (2018). Optimizing culture conditions for heterotrophic-assisted photoautotrophic biofilm growth of chlorella vulgaris to simultaneously improve microalgae biomass and lipid productivity. Bioresource Technology, 270, 80-87.

84 Young, G., Nippen, F., Titterbrandt, S. and Cooney, M. (2011). Direct transesterification of biomass using an ionic liquid co-solvent system. Biofuels, 2(3), pp.261-266.

85 Zhang, X., Hu, Q., Sommerfeld, M., Puruhito, E. and Chen, Y. (2010). Harvesting algal biomass for biofuels using ultrafiltration membranes. Bioresource Technology, 101(14), pp.5297-5304.

86 Zhang, X., Zhang, Y. and Chen, F. (1999). Application of mathematical models to the determination optimal glucose concentration and light intensity for mixotrophic culture of Spirulina platensis. Process Biochemistry, 34(5), pp.477-481.

87 Zheng, H., Gao, Z., Yin, J., Tang, X., Ji, X. and Huang, H. (2012). Harvesting of microalgae by flocculation with poly (γ-glutamic acid). Bioresource Technology, 112, pp.212-220.

88 Zheng, H., Yin, J., Gao, Z., Huang, H., Ji, X. and Dou, C. (2011). Disruption of Chlorella vulgaris Cells for the Release of Biodiesel-Producing Lipids: A Comparison of Grinding, Ultrasonication, Bead Milling, Enzymatic Lysis, and Microwaves. Applied Biochemistry and Biotechnology, 164(7), pp.1215-1224.

89 Zimmerman, W., Tesař, V. and Bandulasena, H. (2011). Towards energy efficient nanobubble generation with fluidic oscillation. Current Opinion in Colloid & Interface Science, 16(4), pp.350-356.