CHARACTERISATION OF *CHLORELLA VULGARIS* CELL WALL BREAKDOWN TO IMPROVE ANAEROBIC HYDROLYSIS

A THESIS SUBMITTED IN FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN ENVIRONMENTAL ENGINEERING

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Declaration

I, Morley Muse, declare that the PhD thesis entitled '**Characterisation of *Chlorella vulgaris* cell wall breakdown to improve Anaerobic Hydrolysis**' is no more than 100,000 words in length including quotes and exclusive of tables, figures, appendices, bibliography, references and footnotes.

This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma. Except where otherwise indicated, this thesis is my own work.

Signature:  **March 2021.**

Conference presentation relevant to the scope of this thesis

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Abstract

Microalgae can be used to polish secondary treated wastewater by removing nutrients and carbon without the addition of oxygen making it a reduced energy treatment compared to traditional extended aeration systems. The recovered microalgae in turn can be used for biofuels applications such as biogas production via anaerobic digestion and biodiesel production via lipid transesterification. Anaerobic digestion is a more feasible option due to its low energy requirement and on-site power generation ability for water utilities. Nevertheless, anaerobic digestion of microalgae has several challenges with the most difficult being the recalcitrant nature of the cell wall of most microalgae resisting microbial attack during digestion. This has resulted in low methane yields after long retention times during anaerobic digestion. Also, the rigidity of the cell walls has led to low lipids release from microalgae cells due to difficulty in extracting the intracellular cell components, affecting other biofuels production processes. Due to this, several authors have suggested a pretreatment process as a means to disrupt the cell wall structure and improve degradation of microalgae. To determine the efficiency of microalgae pretreatment, a proper quantitative technique is useful to analyse cell disruption rate. This research began by comparing different pretreatment technologies using a light microscope. The light microscope was fitted with a Neubauer haemocytometer cell counter, in addition to the use of image-J cell counting software for visual analysis to quantify cell wall disruption using *Chlorella vulgaris (C.vulgaris)* as the model alga. *C.vulgaris* was selected as the microalgae species in this project as it has been widely established as a suitable species for biofuel production and anaerobic digestion due its dominance, being a local species in Australia, higher growth rates and higher lipids content when compared to other species. Pretreatment techniques compared included thermal processes using a water bath and autoclave, mechanical processing using a high-speed homogeniser, combinations of water bath and high-speed homogeniser as well as enzymatic pretreatment using lysozyme. The results of the experiments conducted showed over 80% cell disruption using high speed homogeniser and lysozyme enzyme. Thermal pretreatment using Autoclave produced the lowest cell disruption results at 42%. The results of the combination of water bath at roiling boil for 5 minutes and 5 minutes high speed homogeniser treatment at 4,000rpm showed a 50% cell disruption rate. For the water bath thermal pretreatment alone, 20 minutes was found to be most effective producing a 65% disruption rate.

However, using microscopic analysis, although effective, is time-consuming for larger cell counts, making industrial pretreatment efficiency determination a challenge. Besides, the degree of pretreatment necessary to disrupt the cell is affected by the mechanical strength of the cell wall. Currently, there is little or no test for cell wall strength measurement that is shown to impact cell wall disruption and using anaerobic digestion to quantify cell strength can be slow due to long retention times. Understanding microalgae mechanical strength would enable better selection of microalgae pretreatment methods and improve energy production from microalgae, making it a more efficient process resulting in improvements in subsequent anaerobic digestion rates. From the study, a reproducible technique using high-speed homogeniser (at speeds between 4,000rpm to 33,000rpm) to evaluate the relative cell wall strength of

C.vulgaris was developed and cell disruption was determined from lipid concentration following extraction. During the technique development, several solvents including diethyl ether, hexane and dichloromethane were investigated and compared for their use in extracting broken-only algae cells from solution. Dichloromethane proved to be the most suitable solvent for wet algae lipids extraction. From the results, it was determined that significant lipids extraction was from 8,500 rpm, which was identified as the critical speed with shear rate of 18,227s^{-1}. The maximum shear rate at 33,000rpm was found to be 70,765s^{-1}. Total lipids available in the cell was calculated using a modified Bligh and dyer method of dichloromethane to methanol of 2:1. It was found that the percentage of lipids from broken only cells compared to the total lipids in the cells was about a quarter at maximum cell disruption speed of 33,000rpm. Experimental verification was conducted using chlorophyll analysis and lysozyme addition which displayed a similar trend as the lipid extraction results show that the critical speed was also observed at 8,500rpm. Lysozyme enzymatic pretreatment was investigated for cell wall disruption and its impact in anaerobic hydrolysis as previous research had shown its ability to degrade *C.vulgaris* cells for biofuel processes. Lysozyme was later deduced in this project to initiate cell disruption, making further cell degradation by other hydrolytic enzymes easier, leading to improved lipids extraction and better anaerobic hydrolysis. The novel technique developed will assist biofuel technologies to determine the efficiency of microalgae pretreatment and has also provided knowledge on the critical shear rate when disruption occurs.

Furthermore, microalgae cells showed resistance to microbial hydrolysis during previous anaerobic digestion studies using recovered microalgae from wastewater systems. Commercial anaerobic digestion using microalgae from wastewater utilises bacteria inoculum already present in the wastewater system. The effectiveness of this, however, has been low generating low yields of methane. Researching and identifying key micro-organisms in microalgae anaerobic digestion will promote the technology and improve bio-methane yields. To achieve this, bacteria such as *Escherichia coli (E.coli), Streptococcus thermophilus (S.thermophilus), Lactobacillus plantarum (L.plantarum), Acetobacter aceti (A.aceti),* as well as hydrolytic enzymes such as lysozyme, amylase, cellulase, pectinase, and *Aspergillus oryzae (A.oryzae)* fungus were utilised in separate and combined experiments' to show the effectiveness of microbial selection and enzymes as inoculum for degrading *C.vulgaris* cell wall during anaerobic hydrolysis to produce volatile fatty acids (VFA) as intermediates. The amount of VFAs produced was used as a means of experimental process efficiency and to predict potential bio-methane production. Two separate experiment batches were conducted with batch 1 having retention times of 30, 45 and 60 days. Batch 2 had a retention time of 15 days as the results from batch 1 showed optimum VFA production at 30 days retention time. From the results, optimum total VFA concentration was obtained after 15 days retention using inoculum containing mixed enzymes (lysozyme, cellulase, pectinase and amylase) at 195 mg/l. This is followed by mixed bacteria containing *E.coli, S.thermophilus* and *L.plantarum* at total VFA concentration of 161 mg/l. Literature review on the selected bacteria shows the capability of these bacteria being able to produce the selected hydrolytic enzymes. Hence, the efficiency

of the bacteria in producing total VFA results close to the values obtained from the mixed enzymes. Lowest VFA production was observed in test containing *A.oryzae* alone at 23mg/l. The low digestion efficiency observed from the fungus has been suggested to be as a result of the possibility of no cellulose wall detected in *C.vulgaris* cell. Another possibility is the aerobic property of the fungus limiting its growth efficiency during digestion.

To investigate this further, *C.vulgaris* was flocculated with *A.oryzae* for 24 hours as well as 72 hours and used to harvest the microalgae. The harvested *C.vulgaris* cells were then subjected to high-speed homogeniser treatment using the technique developed earlier before undergoing anaerobic digestion using a retention time of 13 days with sampling every two days in a separate experiment. The initial tests involving harvesting of the microalgae by flocculation shows 72 hours to produce greater flocculation efficiency with almost 100% of the cells observed to flocculate and clump together under visual observation using a motic light microscope at 400X magnification. For the cell strength tests, 72-hours flocculated algae also displayed better performance with lipids extraction efficiency of 27% more than the control containing *C.vulgaris* alone. The 24-hour flocculated microalgae also showed good results with 20% more lipids production compared to the control containing *C.vulgaris* alone. However, when the flocculated microalgae at 72-hours was investigated for anaerobic hydrolysis, the results were again low providing only 14.7mg/l of total VFA at peak observed at day 5. The results confirm the earlier findings of the possibility of the absence of cellulose in the cell wall of *C.vulgaris*. Hence, the use of fungus *A.oryzae* maybe useful only in microalgae harvesting technology and not anaerobic digestion.

In addition, the project provides a detailed energy calculation of the different pretreatment strategies employed and discussed the amount of energy consumed. Thermal pretreatment was found to have a lower energy consumption at 86kJ/L feed with energy recovery for both autoclave and waterbath compared. Also, without energy recovery, thermal pretreatment was still quite low at 497 kJ/feed for autoclave and 393 kJ/L feed for waterbath. Contrarily, high speed homogeniser was found to be energy intensive at maximum speed of 33000rpm with energy consumption of 1,080 kJ/L. However, at the critical speed of 8,500 rpm, energy consumption of the high speed homogeniser was low and close to thermal pretreatment with energy recovery utilising only 88.7 kJ/L feed.

Moreover, potential biomethane to be produced from the optimum anaerobic hydrolysis experiment conducted at 15-days was evaluated. An energy balance and cost analysis were documented from the various biological and enzymatic pretreatments employed. A positive energy balance was observed across the various inoculum employed. Optimum net energy production was recorded by inoculum containing mixed enzymes (lysozyme, pectinase, cellulase and amylase) at 3362 J/L feed. This is followed by mixed bacteria (*E.coli, S.thermophilus* and *L.plantarum*) inoculum with net energy production at 2769.5 J/L feed. Investigating and proposing an effective method of microalgae digestion will enable microalgae disposal from wastewater and promote energy recovery making microalgal treatment of wastewater more likely in water and waste treatment facilities.

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CHAPTER 3 COMPARISON OF PRETREATMENTS FOR IMPROVED *CHLORELLA VULGARIS* DISRUPTION EFFICIENCY

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BBM	Bolds basal medium
CO_2	Carbon dioxide
COD	Chemical oxygen demand
DW	Dry weight
HRT	Hydraulic retention time
ISR	Inoculum to substrate ratio
N	Nitrogen
N_2	Nitrogen gas
OD	Optical density
P	Phosphorus
PTFE	Polytetrafluoroethylene
GC	Gas chromatograph
Std Dev	Standard deviation
TS	Total solids
TSS	Total suspended solids
TVFA	Total volatile fatty acids
VFA	Volatile fatty acids
VS	Volatile solids.

CHAPTER 1 INTRODUCTION

1.1 Background

Due to the current issues of global warming and climate change, the need for alternative energy is imminent. Current climate financing from public funds is estimated to be US$66.8 billion by 2020 (Pandey et al., 2018). However, making renewable energy systems energy and cost efficient is a key strategy in addressing the climate change dilemma. Biofuels as a renewable energy form have received considerable attention in mitigating the greenhouse effect and producing cleaner fuels. There are different forms of biofuels including biodiesel from lipids transesterification, bioethanol from fermentation, and biogas from anaerobic digestion. Different feedstock such as corn, palm, rapeseed, soy, wheat among others can be used for biofuel production such as palm and rapeseed oils are used for biodiesel production. However, the use of these feedstocks has huge implications due to their competition with food. This has led to research in developing alternative feedstock for biofuel production.

Recently, anaerobic digestion using microalgae is becoming more accepted as a result of its waste to energy capability in a net carbon neutral system making wastewater treatment more feasible (Park et al., 2011). In addition to biogas production, other intermediates of the anaerobic digestion process such as hydrogen and volatile fatty acids are in high demand for various industry applications including demand as bulk chemicals, bioplastics, food additives, textiles and pharmaceutical industries (Wainaina et al., 2019, Tampio et al., 2019, Lukitawesa et al., 2020).

Microalgae are capable of utilising nutrients' from wastewater for their growth (Wrede, 2019b) without extra cost due to wastewater systems being rich in nitrogen and phosphorus, which are essential nutrients for algae growth (Beuckels et al., 2015). In addition, the nutrients can be recovered in the form of digestate, which is a by-product of anaerobic digestion and re-used (Torres et al., 2013).

Incorporating anaerobic digestion technology into wastewater systems will improve the economic sustainability of microalgae biofuel production to produce on-site electricity with reduced operational costs.

However, microalgae wastewater treatment must overcome one of its greatest challenge, which is the cell wall digestibility, to maximise biomethane production from biogas. Cell wall digestibility is a challenge as microalgae have recalcitrant walls, which is a defence mechanism in their aquatic environment. Unfortunately, this inhibits bacterial hydrolysis limiting biomethane production. Microalgae pretreatment has been recommended as an effective approach in cell wall disintegration, improving microbial attack during anaerobic digestion, leading to higher methane production.

This research compared various pretreatment methods to determine their effectiveness in cell wall disruption for biofuel applications including anaerobic digestion. This study also developed a reproducible technique to evaluate the mechanical strength of the microalgae cell wall in solution to test the effect of certain algae pretreatment methods for improving microalgae cell disruption. In addition, the research identified feasible inoculum including bacteria, fungus and enzymes for increasing potential bio-methane yield via anaerobic hydrolysis. Finally, an energy balance calculation was computed to determine the net energy involved using the different pretreatment strategies outlined in this project.

1.2 Research Development: Thesis Outline and Chapters' description

The central theme of this research was to characterise the effectiveness of pretreatments of *Chlorella vulgaris (C.vulgaris)* as a microalgae for anaerobic hydrolysis to improve anaerobic digestion and maximise potential bio-methane production. This initial chapter introduces microalgae biofuels as an alternative energy and a means of mitigating global warming due to its ability to produce cleaner fuels in a net carbon system. This chapter also discusses the various chapters in this thesis providing a summary of the work conducted.

Chapter 2 provides a review of current literature in anaerobic digestion technology using microalgae. Emphasis on algae types including macroalgae and microalgae were reviewed with a focus on their intracellular composition and use in biofuel production. Microalgae was shown to be beneficial over macroalgae for biofuel production with emphasis on anaerobic digestion. Microalgae cultivation using nutrients available in wastewater system was established to be a better strategy to convert waste to energy via anaerobic digestion for water utilities. Different cultivation technologies such as open ponds, raceways and photobioreactors were introduced and their use in laboratory or large-scale applications was discussed including their advantages and disadvantages. Microalgae dewatering and harvesting was determined to be of utmost importance in biofuel applications. Several harvesting techniques such as gravity sedimentation, filtration, flotation, flocculation, centrifugation and electrical approaches were compared, highlighting their pros, cons and applicability. Next, the benefit of wastewater being used as a feed for microalgae and its benefit in anaerobic digestion was highlighted. Then, the history of wastewater treatment was discussed and the need for lower energy treatment processes. Anaerobic digestion is seen to be an efficient wastewater treatment technique and its benefits over other biofuel applications such as biodiesel using microalgae was emphasised. Following that, anaerobic digester configurations, design and chemical reactions involved in the process were evaluated. Next, factors limiting the use of anaerobic digestion technology such as pH, temperature, VFA concentration, C/N ratio, toxicity effect, organic loading rate and HRT were discussed. Moreover, the literature identified knowledge gaps in previous research including low biomass concentration, ammonia

inhibition, lipid concentration, digestate and nutrient recycling and cell wall digestibility. Of the issues highlighted, cell wall digestibility is currently the most challenging problem associated with microalgae digestion. This research aims to address the microalgae cell wall degradation problem. Also, criteria for selection of microalgae for anaerobic digestion was examined and *C.vulgaris* was determined to be an efficient species due to its availability, high lipid content and rapid growth rates compared to other microalgae. Details of the *C.vulgaris* cell, including its morphology, structure and composition, was reviewed to provide a detailed understanding of the digestibility challenge. In addition, cell wall pretreatment which has been considered by several researchers as a key strategy in resolving cell wall digestibility was discussed in detail, highlighting the various types of pretreatment, current advancement, their challenges as well as merits. Finally, the major aims and objectives of this research were outlined with research questions being posed forming the contents of the subsequent chapters.

Following the gaps identified in chapter 2, microalgae pretreatment has been suggested by previous researches to resolve the difficulty in cell wall degradation of microalgae. Chapter 3 had the main objective of comparing thermal, mechanical and biological pretreatments such as autoclave, water bath, high-speed homogeniser, combination of high-speed homogeniser and water bath as well as lysozyme enzymatic by conducting experimental investigations on the effectiveness of these pretreatment techniques on microalgae cell disruption. Their disruption efficiencies were analysed using light microscopy for visual examination as well as cell counting by using a Neubauer haemocytometer counter and image-J software. The experiments were conducted in duplicate and the cell disruption was given as an average of both tests. The objective of the chapter was achieved as lysozyme enzymatic pretreatment was concluded to be most suitable pretreatment technique followed by high-speed homogeniser.

Chapter 4 developed a reproducible technique using high-speed homogenisation to analyse the cell wall strength of *C.vulgaris* as a model microalgae. Evaluating the ability to break the cell wall using mechanical shear can be useful in measuring the effectiveness of pretreatments to anaerobic hydrolysis and subsequently digestion. The chapter begins by providing a background on the mechanical property of microalgae and the need to understand the cell wall and its correlation to lipids release efficiency. This chapter describes the development of the technique to measure the relative cell wall strength of microalgae and the impact of various pretreatment on the cell wall strength was determined by measuring the amount of lipids extracted following each pretreatment. Experiments using high-speed homogeniser to break the cells were conducted and lipid extraction was performed using solvents. Several single solvents were evaluated for their efficiency in extracting lipids from broken-only wet *C.vulgaris*

cells and compared with the total available lipids in the microalgae. Solvents compared were dichloromethane, hexane and diethyl ether. Dichloromethane was found to be most efficient of the three solvents trialled. Effect of time and enzymes on the cell wall strength were further used to confirm and develop the technique. Experimental verification using chlorophyll analysis was conducted and shear force calculations from the results was shown. The graphical trend for the experiments' shows a direct proportionality of lipids with homogeniser speeds. Significant disruption efficiency was observed from 8,500 rpm across all experiments conducted. This speed was deduced as the critical speed with a shear rate of 18,227s^{-1} . The shear force required for cell disruption is useful in microalgae biofuel processes to understand mechanical energy requirement for pretreatments.

Chapter 5 aimed to address the use of selective microbes and enzymes effect on anaerobic hydrolysis of *C.vulgaris* cells. Earlier researchers focused on anaerobic digestion of microalgae grown in wastewater using bacteria already present in the system as inoculum. Results from previous studies have shown several limitations including low volatile fatty acids production and subsequently reduced biomethane yield with microalgae cells passing through the digester and coming out unbroken even after long hydraulic retention times (HRT). Investigations on maximising volatile fatty acids and biomethane production have focused on pretreating the cells. Since microbial hydrolysis is the rate limiting step of anaerobic digestion, this chapter involved the use of microbes and/or enzymes to facilitate cell hydrolysis and improve anaerobic digestion to maximise potential bio-methane production. The fungus, *Aspergillus oryzae (A. oryzae)*, enzymes (including lysozyme, amylase, cellulase, pectinase), *Acetobacter aceti* and hydrolytic bacterial cultures of *Escherichia coli, Streptococcus thermophilus, Lactobacillus plantarum* were used in various experiments to demonstrate the effect of selective microbial isolation and enzymes as inoculum for degrading *C.vulgaris* during anaerobic hydrolysis. The objective of improving anaerobic hydrolysis and maximising volatile fatty acids is to increase anaerobic digestion efficiency. Results showed the highest volatile fatty acids released from the inoculum containing mixed hydrolytic enzymes and mixed hydrolytic bacteria with both results displaying over 95% more volatile fatty acids than the control culture with *C.vulgaris* alone. The hydrolytic bacteria used were investigated via previous literature and have been shown to contain the hydrolytic enzymes used in the project. Hence, the effective release of VFAs in the process. Since, the addition of purified enzymes may not be cost effective on a commercial scale, the results from the bacterial inoculum were promising and showed potential for improving commercial anaerobic digestion and maximising biomethane production in an energy efficient manner.

Previous anaerobic hydrolysis tests using fungi as inoculum with the *C.vulgaris* substrate recorded in Chapter 5 showed the fungus *A.oryzae* to produce low VFA levels compared to bacteria and enzymes. The hypothesis was inferred that the results were due to the absence of cellulose in *C.vulgaris* cell wall as well as the aerobic nature of the fungus. To understand *C.vulgaris* cell strength further and confirm the findings, Chapter 6 had the major objective of demonstrating the relationship between microalgae cell strength and anaerobic hydrolysis using *C.vulgaris* harvested by flocculation using *A.oryzae. A.oryzae* has shown strong suitability in harvesting microalgae in previous studies with harvesting efficiencies of over 90%(Prajapati et al., 2016, Wrede et al., 2014). The chapter also had the aim of investigating the effect of time on flocculation ability of the algae cells prior to hydrolysis. To achieve the objectives outlined, the chapter conducted two separate experimental investigations. Initial experiments focused on the effect of time on *fungus* flocculation efficiency with microalgae by testing a combination of fungus *A.oryzae* and high-speed homogeniser pretreatment on *C.vulgaris* cells. Fungi flocculation was tested for 24-hour and 72-hour timeframes prior to high-speed homogenisation. The cells were also observed for visual changes using a light microscope. Process verification was conducted by analysing the lipids extracted using the high-speed homogeniser technique introduced earlier in chapter 4. The cell strength results showed 72-hour flocculated *C.vulgaris* to extract more lipids by 6% more than the 24-hour test sample. When these results were compared with cell strength pretreatment using high-speed homogeniser alone, 21% more lipid extraction was recorded by flocculated microalgae.

Following this, a separate anaerobic hydrolysis experiment was conducted using *C.vulgaris* harvested via *A.oryzae* flocculation for 72-hours and pretreated using high-speed homogeniser at varying speeds from 4000 to 33,000 rpm to determine the effect of fungi on hydrolysis of *C.vulgaris* cells. Process efficiency was determined from volatile fatty acids released recorded every two days over a 13-day period. The results showed a shorter hydraulic retention time to be more beneficial for fungus-algae hydrolysis as peak VFAs were observed day 5 HRT. However, VFAs recorded were still significantly lower as compared to other bacteria or enzyme inoculums considered in Chapter 5. This supported the idea that the cell wall of *C.vulgaris* may indeed be lacking cellulose, as *A.oryzae* secretes cellulase and the absence of the appropriate substrate to act upon may have resulted in the low concentration of final VFA produced. The results from chapter 6 concludes that *A.oryzae* may be useful for *C.vulgaris* harvesting as established by several studies due to its efficient flocculation ability but not for anaerobic hydrolysis or digestion as a result of the lack of cellulose synthesizing ability.

Chapter 7 provided an energy calculation of the various pretreatment techniques used in this project. Thermal pretreatment using autoclave and water bath was found to consume less energy than mechanical pretreatment using high-speed homogeniser. Also, an estimate of

potential biomethane from the biological pretreatment was evaluated and a positive energy ratio was recorded. Enzymatic pretreatment using enzyme mixture of lysozyme, cellulase, pectinase and amylase were the most energy efficient. However, when enzyme cost is considered, biological pretreatment using bacteria (*E.coli, S.thermophilus* and *L.plantarum*) were found to be the most energy and cost efficient due to their ability to secrete desired enzymes mentioned.

Chapter 8, being the concluding chapter determined if the aims of the project had been attained. It also provided discussions on the significance of the research and scientific knowledge contributed. In addition, the challenges encountered, and limitations of the project were also highlighted. Finally, this chapter provided a summary of the key findings and identified opportunities and recommendations of future research. As part of the key findings, the project was able to establish a novel technique to determine and compare pretreatment efficiency. The project also identified microbes such as *E.coli, S.thermophilus, L.plantarum* as key additional bacteria inoculum for wastewater microalgae anaerobic hydrolysis in saving cost and energy. In addition, lysozyme enzyme was seen as an effective enzyme in *C.vulgaris* cell wall disruption, initiating hydrolysis of the microalgae. However, its potency is more effective when used in enzyme mixture containing lysozyme, cellulase, pectinase and amylase as the lysozyme can begin cell degradation and the cells can be further be degraded by the other enzymes.

In addition, fungal pretreatment showed efficiency in algae flocculation which can further be employed as a harvesting technique but showed little improvement in VFA production for anaerobic hydrolysis. Energy calculations showed that biological pretreatment employing bacteria and enzymes were more energy and cost efficient for anaerobic hydrolysis and digestion.

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CHAPTER 2 LITERATURE REVIEW

2.1 Introduction to biofuels

With the growing world population estimated to reach 9.7 billion people by 2050 (UnitedNations, 2019) and the associated increasing environmental concerns, there has never been a greater need of alternative energy sources. Biofuels have gained prominence in combating the issue of climate change and global warming due to their potential to turn waste into energy and production of cleaner fuels when compared to fossil fuels. Globally, biofuel production was 1,841 thousand barrels per day in 2019 compared to 187 thousand barrels in 2000 with the United States leading the production and market price estimated to be 153.8 billion US dollars by 2024 (Tiseo, 2020a). Other countries leading production are Brazil, Indonesia, Germany, France, China, Argentina, Thailand, Netherlands and Spain (Tiseo, 2020b).

Biofuels refer to solid, liquid and gaseous fuels such as bioethanol, biodiesel and biogas produced from bio-renewable feedstocks (Demirbas, 2009). They can be produced from food crops and have environmental benefits such reduction in carbon footprint and generation of cleaner fuels. However, due to competition for use as food, some biofuels have been viewed less favourably for use in energy production. Classification of biofuels may be grouped based on their technologies including first, second, third and fourth generation biofuels. First generation biofuels are fuels extracted directly from the sugars or oils of traditional crops such as maize, rape seed, sugar cane or other similar crops (Murphy et al., 2015). Second and third generation biofuels maybe called “Advanced biofuels” and includes non-food crops as production feed or inedible components of crops such as wood, straw, corn and algae (Murphy et al., 2015). Fourth generation biofuels are comprised of genetically engineered crops that produce fuels that are made to extract more carbon dioxide from the environment than they will emit using technology such as pyrolysis or, gasification (Demirbas, 2011). Some advanced fuels are bioethanol made from cellulose and hemicellulose in addition to biogas and biodiesel from other non-food crop sources (Altun, 2011 2011, Ertas and Alma, 2011).

Algal biofuels are increasing in popularity due to being non-competitive with food crops unlike terrestrial crops (Schenk et al., 2008) like corn, soybean and palm tree oil. Microalgae in particular has gained research and industrial attention as an alternative biomass feedstock due to their use of non-arable land, high oil yield and carbon neutral benefit (Collet et al., 2011, Stephens et al., 2013, De Schamphelaire and Verstraete, 2009).

Algae are a group of aquatic plants that can be unicellular or multicellular in nature and containing chlorophyll, but they have no true stem or root like traditional plants (Milledge et al., 2014). Algae can be classified as either microalgae or macroalgae. Microalgae are

microscopic and unicellular green algae found in water bodies is a common example, while macroalgae form large multi-cellular organisms such as seaweed.

2.1.1 Macroalgae biofuels

Both macroalgae and microalgae have been evaluated for their ability to produce biofuels. (Milledge et al., 2014) conducted research focusing on energy extraction methods from seaweeds. The study identified direct combustion, pyrolysis, gasification and trans-esterification to be the energy extraction or production methods for dry macroalgae, whereas anaerobic digestion, fermentation and hydrothermal treatments can be used for wet macroalgae. The drying step involved in most macroalgae energy extraction has contributed to its low overall energy efficiency. Macroalgae has low lipid content of about 0.3% to 6% when compared to microalgae which can be up to 70% (Milledge et al., 2014). Also, due to their low oil content, Macroalgae are not suitable in biodiesel production but are rather used for food production and extraction of alginates, agars and carrageenan (Pandey et al., 2013). When evaluated for bioethanol production, macroalgae is inefficient as its yield of 90L per dry metric ton of seaweed is very low compared to traditional crops such as corn (Milledge et al., 2014). Anaerobic digestion has been suggested to be the most probable macroalgae energy extraction technology for industrial scale application (Milledge et al., 2014). The need to utilise macroalgae as feedstock was due to the constant fouling of coastal waterways with macroalgae and as a result of their rapid growth rate (Ward et al., 2014). Studies have considered anaerobic digestion of macroalgae (Briand and Morand, 1997, Moen et al., 1997), and there were plans to have large-scale macroalgae farms which were unfortunately not successful due to economic reasons, as well as dependence then on fossil fuels with little focus on renewables (North, 1980, King et al., 1985). Besides its lack of cost effectiveness, macroalgae for anaerobic digestion biofuels have had little success due to other reasons. Firstly, as macroalgae are abundant in sulphated polysaccharides, they produce hydrogen sulphide during anaerobic digestion which may adversely affect methane production in addition to making the process more costly as the biogas produced requires further treatment for removal of hydrogen sulphide (Pandey et al., 2013, Moen et al., 1997). Also, macroalgae are highly recalcitrant due to the presence of substances such as polyphenols, cellulose and lignin in their cell walls resisting bacterial attack and resulting in low biogas yields (Briand and Morand, 1997, Bird et al., 1990). In addition to these, their growth is seasonal making biomass feedstock difficult for anaerobic digestion (Moen et al., 1997, Briand and Morand, 1997, Milledge et al., 2014).

2.1.2 Microalgae and its advantages in biofuel production

Microalgae are microscopic algae found in aquatic systems that are unicellular and simple (Thurman and Burton, 1997). They are photosynthetic in nature requiring sunlight, carbon dioxide, nitrogen and phosphorus and consist of carbohydrates, proteins and lipids contained in their cell wall and membranes. They range between 2-50μm in size (Sharma et al., 2013a), and have a very high cell oil content ranging between 25% to 77% of their dry biomass (Tang et al., 2020). They are grouped into diatoms and green algae (Demirbas, 2011). They are prokaryotic or eukaryotic and can be unicellular or simple multicellular (Mata et al., 2010).

Microalgae are gaining popularity for use in production of biofuels over traditional plants. They are able to produce 20,000 to 80,000 L of crude bio-oil per acre in one year, which is 7 to 31 times greater than conventional plants such as oil palm (Tang et al., 2020). They have rapid reproductive rates, are adaptive to different growth conditions including temperature changes from seasonal cycles unlike traditional plants, and are easy to cultivate (Pandey et al., 2013). Other advantages microalgae have for biofuel production over other crops include: their lower complexity requiring reduced water for growth, are capable of converting 6% of the total incident radiation into biomass (Benemann, 2008, Stucley et al., 2012b) , CO_2 mitigation ability, more cost effective cultivation as they can be grown in wastewater and have no competition with food production (Demirbas, 2011, Wagner, 2007). Also, the photosynthetic efficiency of microalgae in engineered systems can reach 4 to 6% of solar energy compared to 1-2% of other terrestrial crops (Shilton and Guieysse, 2010). Moreover, every microalgae cell is able to undergo photosynthesis unlike traditional plants for which only the above ground portion performs photosynthesis (Pandey et al., 2018). In addition, due to the microscopic size of microalgae, nutrient absorption from the environment is simple as each individual cell absorbs nutrients unlike higher plants with extended roots making nutrient absorption more time consuming due to long nutrient transport distances. Furthermore, CO_2 utilisation is performed by each algal cell, but traditional plants can only access CO_2 through the stomata in the leaves (Pandey et al., 2013).

Due to their many advantages and non-competition with terrestrial plants, microalgae have been deemed a favourable feedstock for energy generation processes such as biodiesel production using trans-esterification, biogas production from anaerobic digestion, and bioethanol production from fermentation (Pandey et al., 2013). Of these fuels and energy processes, anaerobic digestion has been suggested to potentially be the most cost-effective energy generation process (Zabed et al., 2020, Passos et al., 2013a). This is due to several factors ranging from utilisation of wastewater, to supply and recycle nutrients for the anaerobic digestion process, low sludge production, low operating costs, reduced energy requirement (from saved costs of drying of microalgae like in biodiesel production), sustainable biogas generation, production of intermediates such as volatile fatty acids and hydrogen, as well as

digestate to be used as fertiliser (Zabed et al., 2020, Lukitawesa et al., 2020, Wainaina et al., 2019). However, certain constraints have been associated with production of biogas from anaerobic digestion including recalcitrant cell wall degradability, low lipids production, low carbon to nitrogen (C/N) ratio, and accumulation of volatile fatty acids (VFAs) in the digester (Ward et al., 2014, Zabed et al., 2020).

2.1.3 Types and characteristics of microalgae species

When considering anaerobic digestion of microalgae, some options for process specification includes; algae species, culture methods, ease of harvesting, ease of cell lysis, lipid content and extraction, as well as increased biogas production (Ward, 2015, Moheimani et al., 2015a). Certain algal strains have been shown to produce more lipids and are able to be produced in large scale such as *Botryococcus braunii, Dunaliella tertiolecta, Haematococcus, Spirulina, Euglena gracilis, Isochrysis albana, Nannochloris sp., Neochloris oleoabundans, Phaeodactylum tricornutum, Chrysotila carterae, Prymnesium parvum, Scenedesmus dimorphus, Tetraselmis chui, and Tetraselmis suecica* (Santhanam, 2009, Pandey et al., 2013, Pandey et al., 2018). However, most of these species are adapted to salt-water systems and would rather grow slowly in freshwater. Common algae species located in wastewater systems are *Chlorella*, *Euglena, Scenedesmus, Chlorococcum, Selenastrum,* Microcystis, Phormidium, Ankistrodesmus, Micractinium, Oocystis, Phytoconis, Chlamydomonas, Oscilitoria, Synechocystis and Actinastrum (Moheimani et al., 2015b, Moheimani et al., 2015a, De Pauw and Van Vaerenbergh, 1983). Of the freshwater algae listed, *Chlorella* species are very promising in terms of oil content but have high cell lysis resistance (Gerken et al., 2013, Zheng et al., 2011, Moheimani et al., 2015a). One of the key recommendation for future research suggested by Moheimani et al. (2015a) is to develop a suitable technology to disrupt the cell wall of *Chlorella* species to harness its full lipid potential and digestion capability for improving methane production. Figure 2.1 shows microscopic imaging of a mixed algae culture obtained from the Western water treatment plant in Melbourne Australia identified using standard methods (Bellinger and Sigee, 2015, York and Johnson, 2002). The Table 2.1 illustrates the potential bio-methane production from various microalgae substrates used in anaerobic digestion. From the table, Dunaliella and *Chlorella vulgaris (C.vulgaris)* may be seen as producing the greatest amount of methane.

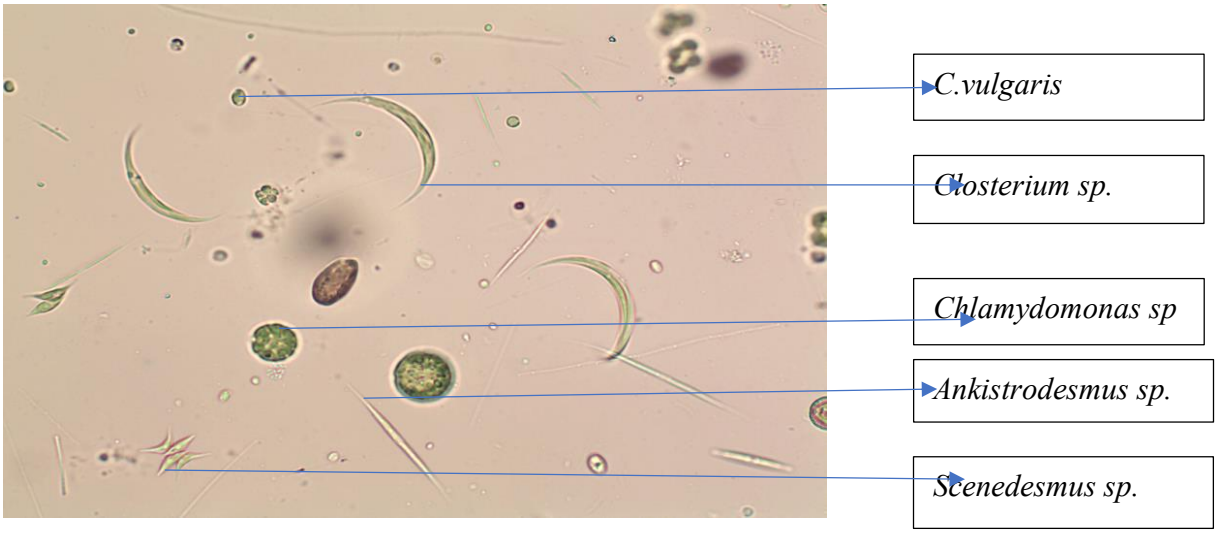

Figure 2.1: Different microalgae species obtained from Western water treatment plant, Australia

Table 2.1 Microalgae species with methane yield

Microalgae Species	**Digestion Temperature (°C)**	**Methane produced (L/kg Volatile Solids (VS))**	**Reference**
Arthrospira platensis (A.platensis)	-	293	(Mussgnug et al., 2010)
Chlamydomonas reinhardtii (C.reinhardtii)	-	387	(Mussgnug et al., 2010)
Chlorella kessleri (C.kessleri)	-	218	(Mussgnug et al., 2010)
Chlorella vulgaris	31	350	(Hernández and Córdoba, 1993)
Euglena gracilis (E.gracilis)	-	325	(Mussgnug et al., 2010)
Spirulina maxima	35	260	(Samson and Leduy, 1982)
Scenedesmus obliquus (S.obliquus)	-	312	(Mussgnug et al., 2010)
Dunaliella salina (D.salina)	-	323	(Mussgnug et al., 2010)
Tretraselmis	35	250-310	(MARZANO et al., 1982)

2.2 Criteria for selecting microalgae species for anaerobic digestion: Why *Chlorella vulgaris* (*C.vulgaris*)?

Microalgae can be used as whole cells or degraded during pre-treatment steps to make available more intracellular components such as carbohydrates and lipids for biofuel applications like anaerobic digestion (Pandey et al., 2018). The significance of microalgae strain selection for biofuel application, particularly anaerobic digestion, is dependent on the desired product, commercial scalability, extraction of intracellular components such as lipids, process requirements such as culture methods, cell wall configuration, growth rate, lipid content and biogas maximisation capability.

It is estimated there are 350,000 species of microalgae (Brodie, 2007). However, researched species have focused on about 20 species obtained from culture collections (Larkum et al., 2012). Major research in terms of strain selection has been based on isolation and screening of a microalgae that is scalable for biofuel technology and grows continually, however, increase in growth rate has been shown to be influenced more by environmental conditions (Pandey et al., 2013). Directions for improvement in microalgae strain selection has been suggested to focus on factors such as ability to contain increased desirable compounds such as lipids, manipulations to growth conditions e.g. temperature, salinity, stress, and harvesting techniques (Vandamme et al., 2013).

A review investigation conducted by Moheimani et al.(2015b) reporting methane production from anaerobic digestion of various microalgae species showed *C.vulgaris* to generate significant methane yield ranging from 189 mL/g/volatile solids (VS) up to 450 mL/g/VS. However, it is important to note that methane production varied for the same *C.vulgaris* in different studies highlighting that other factors such as pre-treatment, digester configuration and microbial species also affect the final biogas yield (Moheimani et al., 2015b).

C.vulgaris has been widely established as a model algae species for biofuel production as well as anaerobic digestion, as it is dominant, common, and has higher growth rates and lipid content when compared to other microalgae (Gerken et al., 2013) . Hence, *C.vulgaris* is a suitable species of microalgae for detailed further study. In addition, *C.vulgaris* are commonly found in waste water systems (Sydney et al., 2014). For example, a study conducted at Western water treatment plant identified a significant amount of chlorella species as one of the most dominant species present in the treatment pond (Wrede, 2019b).

2.2.1 *C.vulgaris* morphology and composition

C.vulgaris are simple nonmotile freshwater unicellular eukaryotic green microalgae that can survive in environments that have viruses, bacteria, and fungi. They have a thick cell wall of approximately, 100 to 200 nm which is a major characteristic of this species (Sydney et al., 2019, Pandey et al., 2013). This corresponds with measurements by (Martinez et al., 1991) of 120-130 nm cell wall thickness. The cell wall gives them mechanical and chemical protection, making them resistance to various substances including heavy metals and microbes (Pandey et al., 2013).

The photosynthetic pigments found in *C.vulgaris* are chlorophyll a and b, and carotene, as well as several xanthophylls (Darzins et al., 2010). *C.vulgaris* is unicellular, spherical, subspherical or ellipsoid in shape, and has a diameter of 2-10 micrometres with no flagella (Safi et al., 2014, Pandey et al., 2013, Scragg et al., 2003, Ru et al., 2020). The cells appear as single cells or in colonies of up to 64 cells and they reproduce asexually by dividing mother cell into 2 to 32 autospores (Ru et al., 2020, Safi et al., 2014). Its chloroplast is cup-shaped with or without pyrenoids (Ru et al., 2020). In its chloroplast, it contains chlorophyll-a and –b. *Chlorella* has a photosynthetic efficiency of about 8% which exceeds other terrestrial crops of about 1 to 2% (Shilton and Guieysse, 2010, Zelitch, 2012).

C.vulgaris cells are composed of proteins, carbohydrates, pigments, minerals and vitamins (Safi et al., 2014, Ru et al., 2020). The carbohydrates in C.vulgaris are mainly made up of amylose and amylopectin with a carbohydrate dry weight of 12 to 55% (Ru et al., 2020). Its total protein content is estimates to be 43 to 58% of its dry weight using growth conditions from (Safi et al., 2014). In terms of lipids, *C.vulgaris* contains 5 to 58% of lipids of its dry weight (Safi et al., 2014, Gerken et al., 2013), which can be increased to 60 to 68% when cultivated in mixotrophic conditions (Ru et al., 2020). Its high lipid content makes it favourable for anaerobic digestion and biofuels generation. In addition, the cell contains compounds such as glycolipids, waxes, hydrocarbons, phospholipids and free fatty acids (Safi et al., 2014) which can be used for different industrial applications like biofuel production.

The cell wall of *C.vulgaris* cell is complex, consisting of various components, such as; a fibrillar layer (which is like a skeleton made up of cellulose, mannon, and xylan), an amorphous layer (which houses the fibrillar), and a trilaminar sheath consisting of high protein in form of glycoprotein (Aarthy A, 2018). The trilaminar sheath also contains a substance called ‘algaenan’, a lignin-like biopolymer formed from hydroxylated fatty acids and phenolics. This algaenan compose of hair-like fibers are very resistant to bacterial activity and subsequently slows the rate of bacterial hydrolysis. Other constituents of the cell includes carbohydrate such as hemicellulose, some fructose, rhamnose, and glucose (Aarthy A, 2018). The rigidity of the cell comes from the presence of glucosamine (Safi et al., 2014).

The image in Figure 2.2 depicts the ultrastructure of *C.vulgaris* cell.

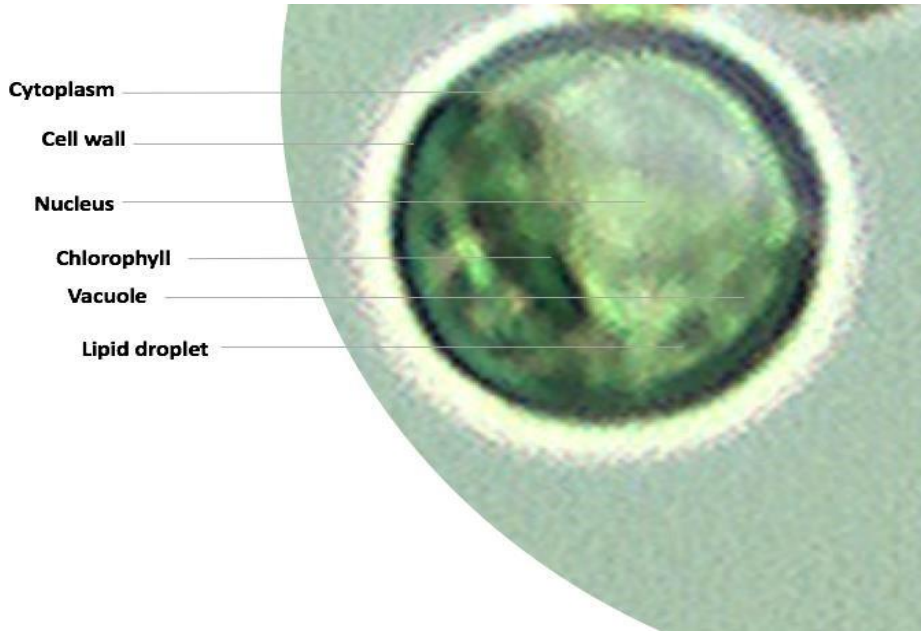

Figure 2.2: Microscopic Image (X1000 magnification) of C.vulgaris using a motic BA310 light microscope.

2.3 Microalgae Cultivation Technology

For production of biofuels, a great deal of microalgae needs to be produced. Microalgae can be cultivated in open ponds or raceways, closed systems and photobioreactors (Slade and Bauen, 2013). Microalgae growth requires adequate amounts of sunlight, water, nitrogen and phosphorus. Wastewater systems are already enriched with nutrients making algal grown of waste water feasible (Beuckels et al., 2015). In laboratory scale cultures, several recipes such as MLA, bolds basal medium, modified zarrouk medium, BG-11, SWES, D Medium, DYIY medium, Jaworski's medium, K medium, MBL medium-Woods hole (CSIROMarineResearch, Ahlgren et al., 1992, Moheimani et al., 2015a) are used to supply nutrients for growth. Thoughtful formulation, monitoring and control of culture are crucial in ensuring proper growth of the microalgae.

2.3.1 Open ponds

Open ponds are the oldest form of mass cultivation (Sun et al., 2016). They are shallow ponds sometimes known as extensive ponds. Nutrients used in this configuration often come from sewage or waste treatment plant effluents. The ponds usually cover a large surface area and rely on natural agitation from the wind making it an unreliable means of cultivation. They are the most common cultivation setting for large-scale, outdoor algae growth (Sing et al., 2013).

Open ponds are beneficial for low-cost algae cultivation and operation (Tredici and Materassi, 1992). Nevertheless, the possibility of microbial contamination is very likely. Besides, this system has reduced algae growth rates due to inadequate mixing and low light penetration (Moheimani et al., 2015a). Figure 2.3 depicts an Open pond system locate at the Western treatment plant, Werribee Australia.

Figure 2.3: Western treatment Plant, Werribee Australia (Melbourne-Water).

2.3.2 Raceways

Raceway ponds are sometimes called intensive ponds that are used to grow algae for biofuel production. They are one of the most popular commercial algal cultivation technologies (Richmond et al., 1993, Pandey et al., 2018). The raceway is made by digging into the earth, pouring concrete as reinforcement or lining with plastic to avoid the liquid seeping into the ground (Yousuf, 2019). Installations of raceways are usually around 0.2 ha to 0.5ha in size and 15cm to 35cm deep (Darzins et al., 2010, Pandey et al., 2013). They have a paddlewheel which agitates and distributes nutrients, mixing the algae cells within the pond, adding CO_2 via mixing with the paddle wheel and enabling water circulation. The designs are usually shallow in order to allow sufficient penetration of sunlight (Demirbas, 2011). The incoming nutrients arrive from a single point in the raceway, usually in front of the wheel, and are released slowly distributing evenly across the pond. The algal biomass is harvested behind the wheel and this can be performed continuously. Algae production rates in raceways are 10 times higher than open ponds (Darzins et al., 2010). Some examples of algae grown commercially using raceway ponds are *spirulina, D.salina, C.vulgaris* and *Haematococcus pluvialis* (Darzins et al., 2010). The raceway ponds are more practical compared to photobioreactors as they do not have any issues of microalgae adhering to container walls. However, they are shallow and do require considerable land. Besides, they are prone to contamination by other algae and microorganisms (Pandey et al., 2013, Moheimani et al., 2015a). Figure 2.4 is a pilot scale

raceway pond taken onsite at the Parwan Western Water Treatment Facility in Melbourne Australia.

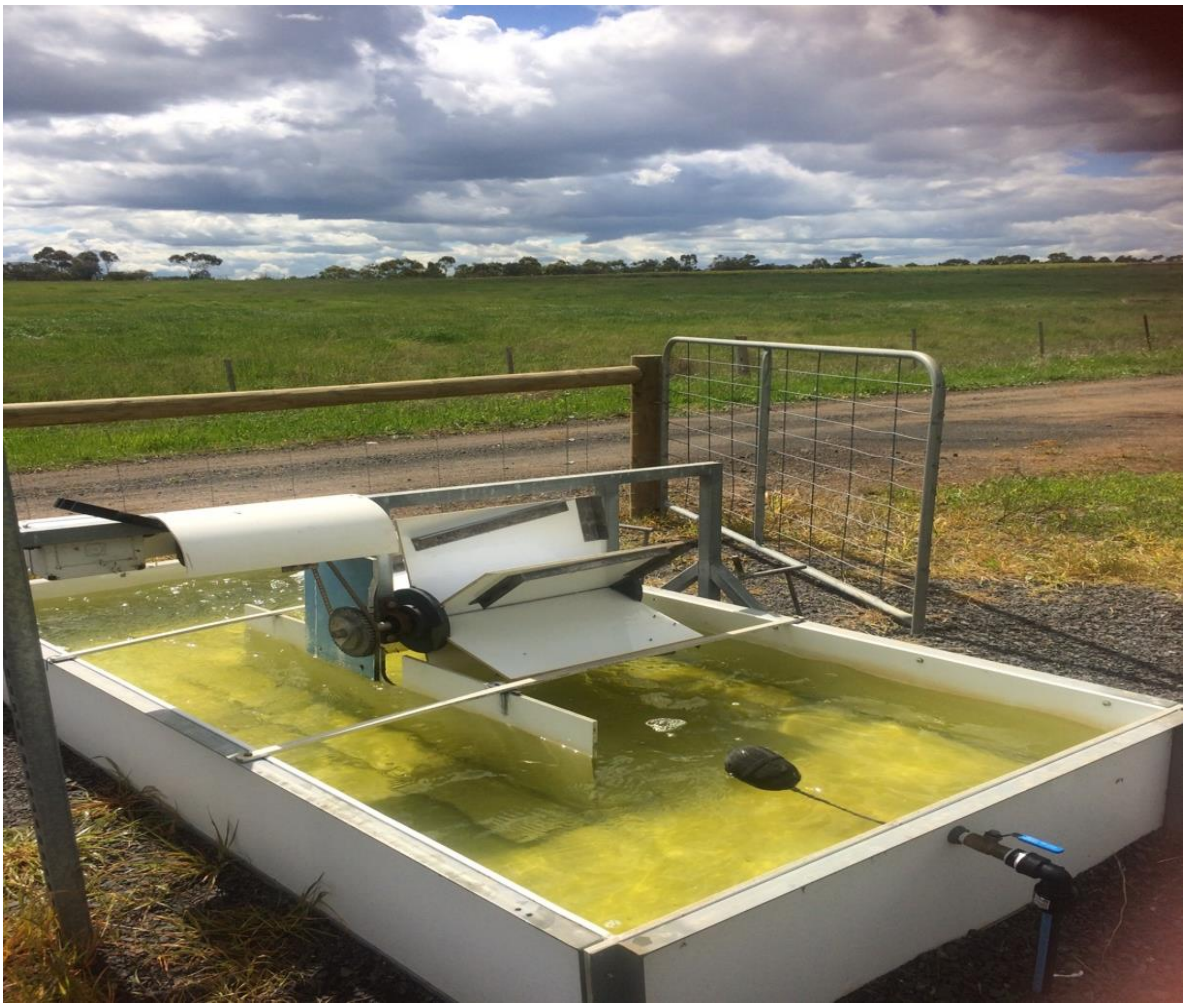

Figure 2.4: Pilot raceway pond, Parwan Western Water Treatment Facility Australia.

2.3.3 Photobioreactor (PBR)

Microalgae can also be cultured in an enclosed system using a photobioreactor (PBR), which is typically one of the growth mechanisms used on a laboratory scale. PBRs are transparent tubes capable of absorbing sunlight or artificial light with a batch or continuous flow of sterilised water, nutrients', air and carbon source for growth of an algal suspension within the tubes. PBR comes in different sizes and laboratory scale systems are usually of 5L, 10L and 20L or greater (Pandey et al., 2013). Algae would usually be harvested after days or weeks of residence time depending on the use and growth phase. One of the key advantages of photobioreactors is their ability to reduce evaporation (Dodd, 2017, Moheimani et al., 2011). In addition, the risk of contamination by other micro-organisms is minimal with this system of cultivation (Moheimani et al., 2015a). Moreover, enclosed photobioreactors reduce CO_2 losses providing consistent cultivation conditions and improved utilisation of CO_2 (Jeffrey, 1994).

Photobioreactors are efficient because growth parameters such as temperature, pH, agitation, nutrients and light intensity can be monitored and adjusted to obtain the desired growth pattern in a controlled environment.

However, one of the main challenges with this system is the cost of construction and operation when compared to open ponds, as PBRs require specialised media preparation for algae as well as cost of purchasing equipment (Borowitzka, 1996, Yousuf, 2019). In addition, scaling is limited using PBR. To resolve this issue, it is recommended to use wastewater to save cost of acquiring nutrients. Other cons of PBR are the cost of scaling up, energy costs and system maintenance (Moheimani et al., 2015a). Some studies have aimed to resolve some issues associated with photobioreactors including: optimising the light path and oxygen concentration, controlling the temperature and resolving issues with turbulence (Moheimani et al., 2015a). Figure 2.5 shows the PBR laboratory set-up used for algae growth in this project.

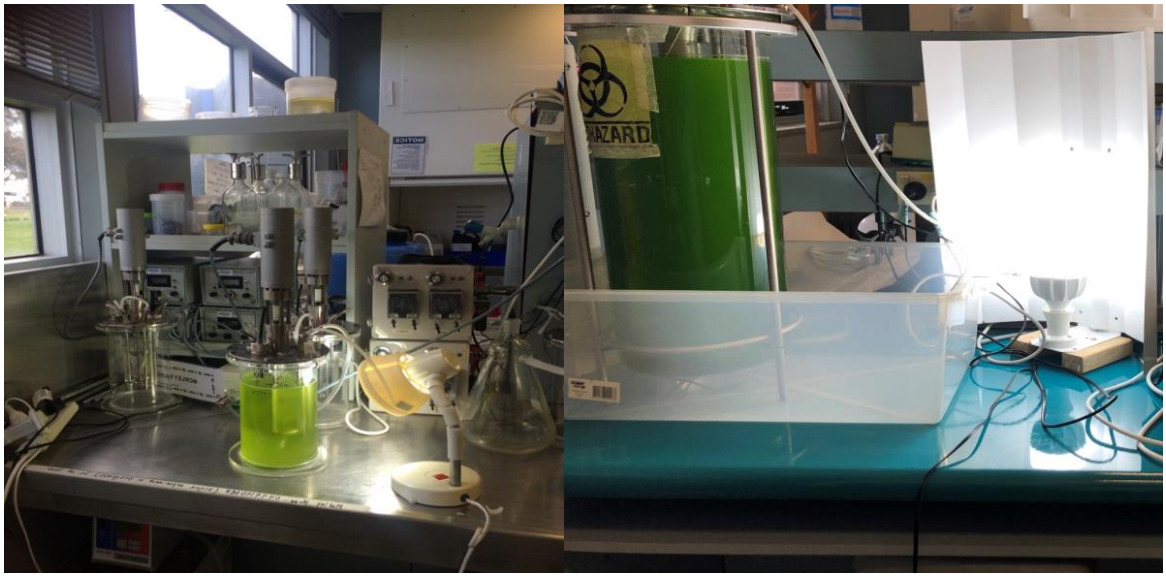

Figure 2.5: 5L and 20L photobioreactors. Victoria University, Werribee Australia.

2.4 Microalgae Wastewater-Source of Nitrogen and Phosphorous

An interesting concept about algae growth is their increased requirement of nitrogen and phosphorous (which are the main constituents' of fertilisers) more than traditional plants due to their higher growth rate (Pandey et al., 2018). Nitrogen is essential for protein and amino acid synthesis (Varjani et al., 2017). Other essential nutrients microalgae need for growth are carbon and phosphorus. Carbon is used during photosynthesis to breakdown the complex polysaccharides into simples sugars whilst phosphorus serves as a fertiliser improving storage and transfer of energy within the microalgae cells. The required C:N:P ratio for microalgae growth has been stated as 100:16:1 known as the Redfield ratio (Redfield, 1958).

According to Borowitzka and Moheimani (2013), 14,447 tons of nitrogen in the form of $NaNO_3$ and 219 tons of phosphorus in the form of Na_2HPO_4 are required to generate 100,000bbl of algal oil annually. A study by Komolafe et al. (2014) states the average estimated cost of synthetic fertilisers to be $450 per ton, so the cost of supplying nutrients for algae growth can be high.

To avoid the high cost of fertilisers, wastewater can be used to supply nutrients for microalgae growth. Wastewater systems have been known to possess high amounts of nitrogen and phosphorus making it a feasible means of microalgae cultivation as the algae can be grown by extracting these nutrients, and treating the wastewater in the process (Pandey et al., 2018). Wastewater systems are gaining considerable attention for biofuel applications due to the abundance of nitrogen, phosphorus and other nutrients in wastewater systems at no cost (Beuckels et al., 2015).

In most water treatment facilities, microalgae may be considered a nuisance and an unwanted species. Cleaning these water systems and disposing the algae has cost implications. According to Samcotech (2016), an engineering wastewater company, a wastewater facility can cost anywhere between US$500,000 to $1.5 million, including the design, installation and start-up. An alternative cost and energy savings technology that has been recommended for wastewater utilities will be to extend their facility to accommodate a biofuel unit, such as an anaerobic digestion plant, where microalgae can be recovered and processed to provide biogas and on-site electricity leading towards carbon neutrality (Golueke et al., 1957, Hussainy, 1979, Brennan and Owende, 2010). Microalgae treatment of wastewater systems have been shown to cut operational cost by over 80% compared to sludge wastewater treatment (Park et al., 2011). In addition, microalgae can be used to remove and concentrate heavy metals and form attachments with harmful chemicals in wastewater systems providing an added advantage in wastewater treatment (Wrede, 2019b, Park et al., 2011, Lizarralde et al.). Moreover, the organic matter present in wastewater systems requires bacterial oxidation prior being expelled to the environment, utilising oxygen from electromechanical air blowers and consuming a substantial amount of energy in the process (Moheimani et al., 2015a). Incorporating microalgae cultivation for biofuel production in wastewater systems would enable the algae to supply some oxygen required for bacterial oxidation rather than the use of electromechanical air blowers which is energy intensive (Moheimani et al., 2015b). This in turn will lower the operating cost of wastewater facilities (Owen, 1982, Craggs et al., 2013). In addition, carbon dioxide generated from organic matter degradation can be used as carbon supply for microalgae photosynthetic reactions (Moheimani et al., 2015a), moving the system towards carbon neutrality and helping to mitigate global warming. Moreover, a joint microalgae-wastewater facility can provide an improved energy balance creating more sustainable production (Moheimani et al., 2015a). To optimise microalgae-wastewater

efficiency, advances in microalgae degradation, improving the disruption rate and extraction of intracellular components of the cells, such as the lipids, needs more research in order to encourage anaerobic digestion and biodiesel production from algae.

2.5 Microalgae Dewatering and Harvesting Techniques

Microalgae concentration in solution is usually very low. Microalgae harvesting can be difficult because algae densities in most cultures range between 0.3 to 1.0gL^{-1} (Stucley et al., 2012a). As a result, harvesting microalgae for use in biofuel applications can be challenging. Deciding on the method of harvesting would depend on the algae species. (Barros et al., 2015) stated that harvesting microalgae should be done in a two-step process; thickening and dewatering to reduce the energy costs involved. Thickening is carried out using a chemical coagulant or allowing gravity sedimentation concentrating the algae to 2-7% total suspended solids, while dewatering further concentrates the algae by an extra 15% to 25% using a centrifuge to remove excess moisture (Barros et al., 2015). The essence of dewatering is to reduce the water content and increase the algae concentration. Dewatering of microalgae is a necessary step for converting algae into useful products. When dewatering microalgae, the aim is usually to increase the algae concentration to around 5 to 10% by volume making it easier for subsequent processing of the algae. Due to the high amount of energy required in dewatering microalgae, some researchers have identified dewatering as one of the major challenges to industrial scale microalgae production (Borowitzka and Moheimani, 2013, Benemann, 2013, Ward et al., 2014). Indeed, the energy requirement is estimated to be responsible for 20% to 30% of the total cost of microalgal biodiesel processing (Girard et al., 2014).

Common microalgae harvesting techniques are centrifugation, filtration, gravity sedimentation, flotation, flocculation and electrophoresis (Pandey et al., 2013). Sometimes, harvesting methods are combined to achieve a lower-cost process. (Smith and Davis, 2012) suggested combining flocculation with gravity sedimentation to save cost.

When determining a suitable harvesting technique, certain considerations such as desired end products, risk of contamination, morphological changes of the algae cell, costs, processing time, algae species, biomass quantity and quality, as well as recycling of culture medium should be considered (Barros et al., 2015, Grima et al., 2003, Uduman et al., 2010, Singh and Patidar, 2018).

2.5.1 Gravity Sedimentation

Separation of the algae from the water solution can be done via gravity sedimentation, where quiescent conditions allow the algae to settle to the bottom of the containing vessel (Pandey et al., 2013). The water can then be easily removed, and algae recovered in a more concentrated form. It is the most common, simple and inexpensive form of microalgae harvesting technique in wastewater systems due to the large volume of microalgae suspension that can be processed for low value end-products such as biofuels (Brennan and Owende, 2010, Barros et al., 2015). It is widely used for large microalgae sizes of around 70μm for species such as Spirulina sp. (Munoz and Guieysse, 2006) . This is because settling microalgae can be difficult due to its low concentration during its photosynthetic growth of 0.5 to 5 g/L, its size of 2-30 μm in diameter and its negative surface charge from its peptidoglycan layer (Pandey et al., 2013, Wrede et al., 2014, Ummalyma et al., 2017). In addition, algae can control their buoyancy allowing them to go up and down the water column by releasing gas. However, algae settling is slow with settling rates of 0.1-2.6 cm/hr, and the concentrate has low algae concentration due to extended settling causing the algae to lose some useful intracellular components such as lipids and proteins (Barros et al., 2015).

2.5.2 Filtration

Another method of microalgae harvesting, and dewatering is via filtration (Barros et al., 2015). Filtration can be effective by the use of membranes. However, one of the key issues with membrane is fouling from prolonged use, resulting in the need for consistent backwashing and cleaning and for a change in membranes making the technology not cost-effective (Barros et al., 2015). Membrane fouling has been attributed to the interaction between the surface chemistry of the algae and the membranes, especially in polymeric membranes (Wrede, 2019b). To minimise the impact of fouling, researchers have begun exploring other types of membranes such as ceramic, metal and PTFE. Ceramic membranes are now gaining more traction in water research due to their improved cost effectiveness, durability and efficient selectivity (Zhang et al., 2013). (Wrede, 2019b) investigated and compared ceramic, metal PTFE membrane filtration harvesting techniques to harvest microalgae and found PTFE to be the most effective membrane as a result of its low fouling, and lower capital cost.

2.5.3 Flocculation

Besides the use of filtration to harvest microalgae, another common harvesting technique is flocculation. Flocculation is the addition of a chemical, physical or biological coagulant to the algae suspension that causes the algae to form bonds with the coagulant resulting in large clusters or flocs. Flocculation has been suggested to be a feasible method of microalgae

harvesting when efficiency, economic cost, and energy consumption is the focus (Li et al., 2020).

Chemical flocculation entails addition of chemical compounds' such as metals or organic flocculants to induce flocculation (Vandamme et al., 2013). It is used for large scale microalgae processing, concentrating the algae by 20 to 100 times, and reducing energy requirement of concentration in the process (Uduman et al., 2010, Vandamme et al., 2013, Salim et al., 2011, Grima et al., 2003).

Physical flocculants include the use of ultrasound and electro flocculation to separate microalgae from solution (Vandamme et al., 2013). In addition, they are highly cost effective (Vandamme et al., 2013, Wrede, 2019b, Li et al., 2020).

On the other hand, biological flocculation uses biological agents such as bacteria or fungi to cause microalgae to form clumps by creating a bond with the algae leading to distinct separation of the algae-flocculant from the water (Miranda et al., 2015, Leite et al., 2013, Wrede et al., 2014). Biological flocculation is one of the most efficient forms of microalgae harvesting as studies show close to 100% harvesting of microalgae using fungi within 3 hours to 24 hours (Bhattacharya et al., 2017, Prajapati et al., 2016, Wrede et al., 2014). In addition, biological flocculants can be grown alongside the microalgae as seen in wastewater systems. Moreover, the use of bacteria and fungi can further release hydrolytic enzymes that can reduce the recalcitrant property of microalgae cell walls as they are a form of pretreating the cells (Li et al., 2020). This project further explores fungi biological flocculation in chapter 6.

2.5.4 Centrifugation

Centrifugation is a method of harvesting algae that produces a thick concentrated alga by separating microalgae from its growth media, reducing the water content by 90% to 95%. It is regarded as the fastest harvesting method (Barros et al., 2015). Centrifugation uses high speeds of 1,500 to 5,000 rpm to spin down the algae. The separation of microalgae relies on factors such as cell size, density of the culture, algal species and centrifuge type (Singh and Patidar, 2018, Heasman et al., 2000). Centrifugation has been identified as one of the most effective methods for removing algae from suspension and is used for the manufacture of high value products (Singh and Patidar, 2018, Pragya et al., 2013, Barros et al., 2015). The use of centrifuge can also damage microalgae cells due to the intense shear force expended on the cells (Wrede, 2019b, Griffiths et al., 2011, Barros et al., 2015, Singh and Patidar, 2018). This may actually be useful in biogas production as some microalgae feed going into the anaerobic digester may have disrupted leading to improved biomethane production.

Although centrifugation is highly effective, has a high recovery rate, is chemical free and works on most algae species, it is also very energy intensive and can have a high cost of maintenance.

2.5.5 Flotation

Flotation uses dissolved air to form micro-bubbles that attach to algae and transport them to the top of a collection tank where they concentrate. It is employed for large scale algae harvesting, uses minimal space, its highly adaptable and is low cost (Wrede, 2019b). It is widely used in wastewater systems and applied after flocculation (Rubio et al., 2002). Flotation requires bubble-particle collision and adhesion (Barros et al., 2015). Smaller microalgae cells have been shown to be readily taken up by the introduction of air bubbles (Uduman et al., 2010, Show and Lee, 2014). Factors that influence flotation harvesting method includes bubble flux and size. Smaller bubbles have been known to possess higher surface area to volume ratio, resulting in rapid bonding with particles (Barros et al., 2015).

There are four types of flotation techniques namely; dissolved air flotation, dispersed air flotation, electrolytic flotation and ozonation-dispersed flotation (Barros et al., 2015).

Dissolved air flotation uses a bubble diameter of <100um and is the most common. It involves dissolving air into the algae suspension at high pressure making the solution supersaturated and resulting in bubble formation as the pressure decreases across a nozzle causing the formed bubbles. The bubbles float to the surface of the suspension with attached algae. This process uses a high amount of energy for operation (Barros et al., 2015) driven by the need to supersaturate the suspension with air. Dispersed air flotation creates bubble via the introduction of air through a permeable material using a higher pressure. It also requires lesser energy (Barros et al., 2015). In addition, it may be seen as a less effective method due to the larger bubble sizes.

Electrolytic flotation on the other hand creates hydrogen bubbles via electrolysis while ozone-dispersed flotation forms charged bubbles (Show and Lee, 2014, Rawat et al., 2011). During electro-flotation, the hydrogen when introduced causes a separation between the algae and the culture media, making the hydrogen to bond with the algae and transporting it to the surface of the water (Singh and Patidar, 2018). Electro-flocculation utilises flocculants produced from the electrolytic oxidation of specific electrode which agitates the microalgae solution forming flocs with algae removal rate of 80 to 95% (Chen et al., 2011).

The use of electrical techniques for microalgae harvesting are useful due to their versatility for multiple algae strains as well as their non-chemical approach (Uduman et al., 2010, Zenouzi et al., 2013). In addition, electrical methods are easy to regulate and utilise lesser energy than centrifugation (Vandamme et al., 2013, Show and Lee, 2014).

Electro-flocculation is affected by electrode material, electrolysis time, density, pH and biomass composition (Singh and Patidar, 2018). Also, wear and tear of electrode has been stated to be the major drawback of this process as well as changes in temperature and pH which can alter the algae suspension (Vandamme et al., 2013). In addition, implementation of electro-flotation may be challenging due to the explosive and toxic nature of the gases used

like hydrogen and ozone. Hence, there will be a lot of health and safety concerns associated with this harvesting technique. Of the different metals used as electrode, aluminium has been selected as the most efficient (Lee et al., 2013b, Xu et al., 2010).

2.6 Anaerobic Digestion- An energy efficient option for microalgae wastewater treatment

Anaerobic digestion technology has demonstrated to serve as a solution to waste management while at the same time providing a renewable form of energy. Some advantages anaerobic digestion offers in biological wastewater treatment are; energy provision via methane production, reduced production of sludge, lower energy consumption, raw waste stabilisation, production of fertiliser, and lower space requirement (Demirel and Yenigün, 2002, De Mes et al., 2003).

In traditional wastewater treatment, nutrients removal such as nitrogen and phosphorus are achieved via denitrification and precipitation (Molazadeh et al., 2019). Nitrogen is released directly into the environment whilst phosphorus is precipitated by combination with cations such as calcium, aluminium or iron making it a cost effective means of treating wastewater (Tchobanoglous et al., 2003).

Microalgae is useful for wastewater treatment as the algae can effectively remove nitrogen and phosphorus without the addition of oxygen making it a lower energy process (Jankowska et al., 2017). The recovered microalgae can be used for biofuel production due to its high lipid content in applications such as biogas production via anaerobic digestion and biodiesel production through lipids transesterification as seen in figure 2.6 below. However, the means to effectively recover energy from microalgae remains an outstanding issue (Ward et al., 2014).

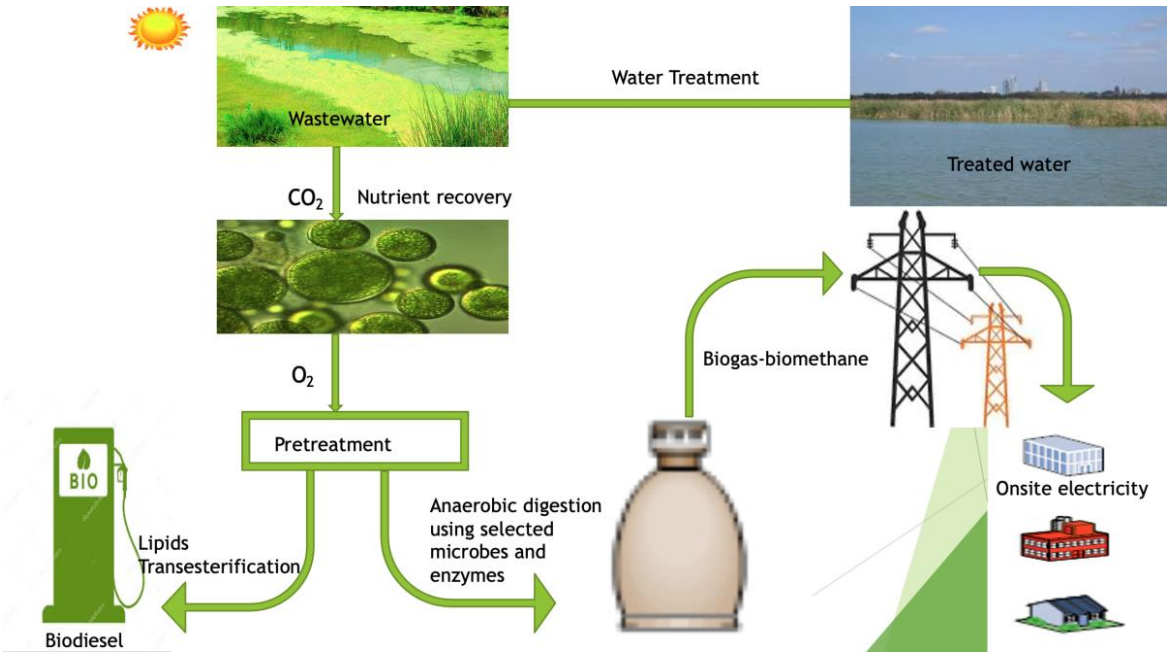

Figure 2.6 Pictural description of nutrient recovery from wastewater for biofuel production.

The use of algae biomass for biofuel production has been researched extensively, with recent studies degrading *C.vulgaris* for biofuel production (Benemann, 2013, Brennan and Owende, 2010, Benemann, 2008, Craggs et al., 2013, Dvoretsky D., 2016, Tang et al., 2020). The drawback, however, for biofuel production from algae biomass has been the energy used for production involved in the entire process. It has been found that the cost of generating biofuel from algae biomass is equivalent to the energy produced (Passos et al., 2014a). Biofuel applications like biodiesel production have downstream processes that are cost and energy intensive. Hence, the need to explore degradation of algal biomass using less energy intensive options such as anaerobic digestion.

Anaerobic digestion technology incorporated into waste water treatment eliminates most of the downstream processes such as drying, extraction and conversion (Ferrell and Sarisky-Reed, 2010). This makes anaerobic digestion a less expensive energy generating technology to implement than biodiesel for water utilities (Ward, 2015). Sialve et al., (2009) recommended anaerobic digestion to be less energy consuming than lipids extraction. Besides, anaerobic digestion reduces the amount of sludge, efficiently producing renewable energy by utilising the biomethane obtained from biogas generated from the decomposition process for electricity production. One of the main benefits of anaerobic digestion is treatment of biodegradable waste and sewage (McCarty, 1964). In addition, anaerobic digestion helps in carbon emission reduction, reducing odours, pathogens and organic matter concentration (Ward, 2015).

However, there are factors such as cell wall digestibility, low C/N ratio among others, that limit the use of anaerobic digestion technology which will be discussed later in this chapter.

2.7 Brief History and Development of Anaerobic Digestion

Microalgae wastewater treatment using anaerobic digestion has been on-going since as early as the 1950's (Golueke et al., 1957, Benemann et al., 1977, Chuka-ogwude et al., 2020, Zabed et al., 2020).

Anaerobic digestion is a natural occurrence in certain soils, lakes and ocean basin sediments (Koyama, 1963). It occurs as marsh gas which was discovered by Alessandro Volta in 1776 (Zehnder and Mitchell, 1978, MacGregor and Keeney, 1973). Anaerobic decomposition of organic matter has been studied since the 18th century. Most of the work done in early research was focused on liquefaction of wastewater solids in the absence of oxygen. The findings led to early anaerobic wastewater treatment (Hughes, 1982). With time, methane's advantage in heating, lighting and operation of gas engines began gaining popularity which drove the need for further research (Hughes, 1982, Buswell, 1939). Modern anaerobic digestion research has focused on investigations on reducing the factors that adversely affects the anaerobic digestion process in order to maximise biogas production.

Anaerobic digestion processes can be conducted in a covered pond or in a digester, a temperature regulated enclosed vessel capable of breaking down organic matter in the absence of oxygen to produce biogas. Early digesters used the Imhoff tank model which was later replaced by closed tank systems (Grando et al., 2017). The advantages of the modern digesters over natural anaerobic processes are waste treatment, nutrient recycling and odour control (Ostrem and Themelis, 2004). When designing an anaerobic digester, certain factors need to be considered including the type of waste to be used, the rate of waste generation and temperature of the environment (Igoni et al., 2008).

2.7.1 Anaerobic Digester Technologies

There are different digester technologies used in anaerobic digestion which depends on the waste to be treated, that is, food waste, animal manure or wastewater (Allen and Isom, 2012). The four main categories of digester types are covered anaerobic lagoon, plug-flow, complete-mix and dry digester (Igoni et al., 2008, Cowley and Brorsen, 2018). The covered anaerobic lagoon is a digester model that is sealed to enable methane recovery mitigating greenhouse gas emissions. Plug flow digesters are long narrow tanks, built with concrete in a rudimentary manner to reduce the need for additional heat requirement (Hamilton, 2014). Complete mix digesters are enclosed vessels with heat supply from a mechanical, hydraulic or gas mixing system. Dry digesters are made up of concrete and steel in a silo-style manner.

2.7.2 Anaerobic Digester Configurations

Anaerobic digesters may be designed according to their operational mechanism including batch or continuous, mesophilic or thermophilic temperature range, solids content, and the number of process stages (Rabii et al., 2019). Batch digesters, feedstock is loaded per batch, intermittently discharged and reloaded after each digester run (Zabed et al., 2020). On the other hand, continuous digesters have the feedstock constantly added to the digester and the digestate continuously emptied (Zabed et al., 2020). Most laboratory scale experiments are conducted in batch modes while industrial application uses continuous modes.

Mesophilic digesters run at operating temperatures between 30°C to 40°C, while thermophilic digesters operate between 50°C to 60°C (Ogejo et al., 2009, Khalid et al., 2011, Zabed et al., 2020). Thermophilic digesters are known to have increased reaction rate due to the raised temperatures, however, mesophilic digesters have reduced energy requirement as the temperature of the reactor is lower resulting in improved stabilization (Gebreeyessus and Jenicek, 2016). Mesophilic bacteria can survive readily than thermophilic bacteria in smaller digesters, poorly insulated digesters or digesters in cold climates, as mesophilic bacteria require lower temperature ranges than thermophilic bacteria. Moreover, thermophilic digesters are not always cost or energy effective, as they require more heat for operation so mesophilic operation is more common and reliable.

2.8 Anaerobic digestion process

Anaerobic digestion is a series of biological processes in which microorganisms break down biodegradable material in the absence of oxygen via complex and synergistic interactions of hydrolytic, fermentative, acidogenic and methanogenic bacteria (Parawira, 2004). The end products of this process are biogas and digestate, a solid component used as bio-fertiliser and soil conditioner (Amigun et al., 2012). The estimates of biogas produced from anaerobic digestion depending on the feedstock are as follows: methane CH_4 (50–75%), carbon dioxide CO_2 (25–45%), hydrogen sulphide H_2S (0-1%), hydrogen H_2 (0-1%), carbon monoxide CO (0–2%), nitrogen N_2 (0–2%), ammonia NH_3 (0-1%), oxygen O_2 (0–2%), and water H_2O (2–7%) (De Graaf and Fendler, 2010). The relative percentage composition of the trace gases relies on the feed material and the overall anaerobic digestion process. The biogas can be used for heating and electricity production by biogas combustion, and as transport fuel after purification. Analysis of past experiments conducted shows that $1m^3$ of biogas may produce 2.1kWh of electrical energy and 2.9 kWh of heat (Ali Shah et al., 2014).

Early research viewed anaerobic digestion as a three stage process that comprises hydrolysis, fermentation and methanogenesis to produce biogas (Parkin and Owen, 1986). Recent studies have broken the fermentation process into hydrolysis, acidogenesis and acetogenesis making it a four-stage process (Adekunle and Okolie, 2015, Christy et al., 2014, Ward, 2015).

2.9 Anaerobic digestion equations

There are four main reactions that take place during anaerobic digestion: hydrolysis, acidogenesis, acetogenesis and methanogenesis. A pictorial representation of the reactions is shown below, Figure 2.7.

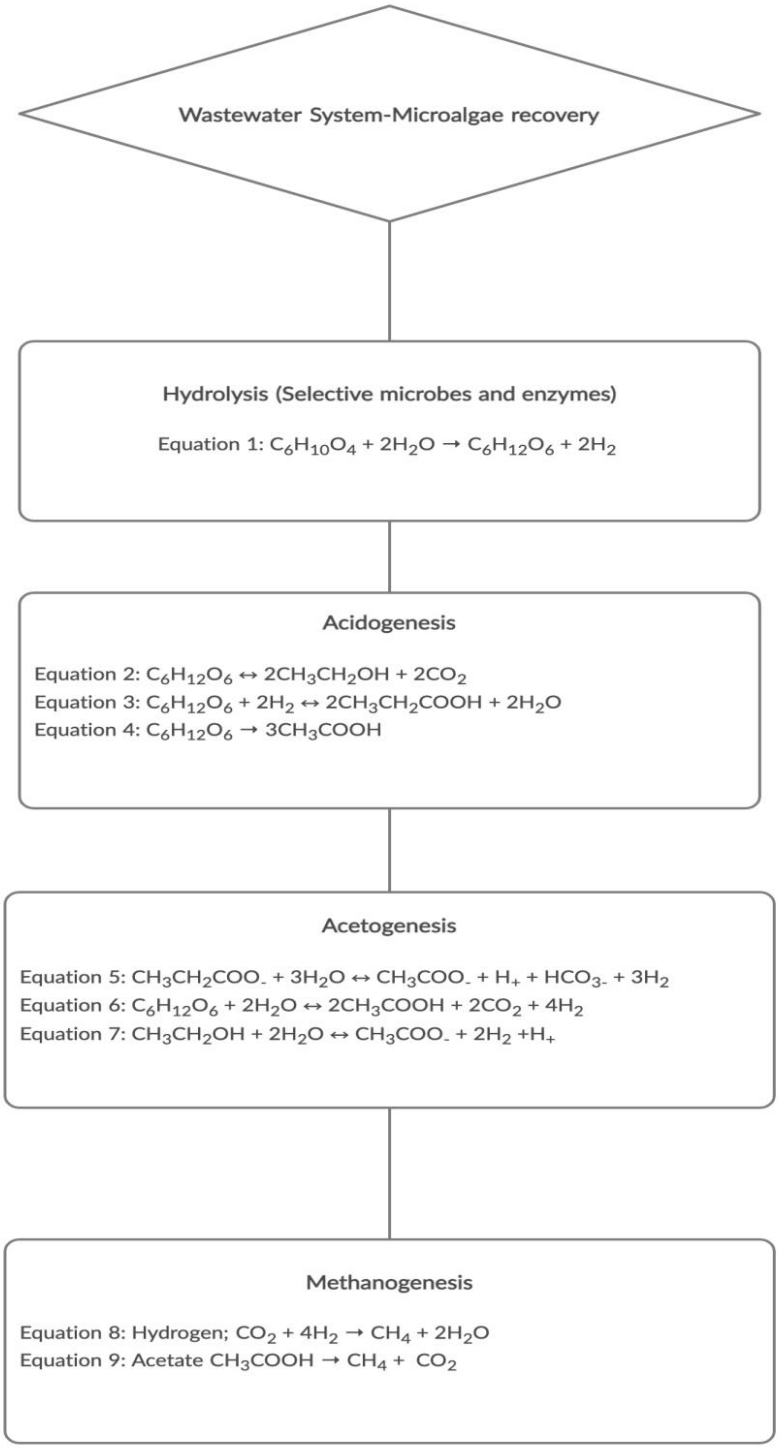

Figure 2.7 Anaerobic digestion process equations.

2.9.1 Hydrolysis

The initial step of the anaerobic digestion process is a reaction known as hydrolysis, also known as de-polymerisation. This is where hydrolytic bacteria break down insoluble complex organic substrates like carbohydrates, proteins, and lipids into simple sugars, amino acids, long-chain fatty acids and alcohols (Varjani et al., 2017). Hydrolytic bacteria do this as a result of secretion of various hydrolytic enzymes such as cellulase, protease, lipase that enable degradation by increasing the reaction rate.

Hydrolysis occurs in two stages (Christy et al., 2014, Vavilin et al., 1996).

I. **Bacterial colonization:** This is characterized by hydrolytic bacteria causing surface degradation, producing monomers and releasing enzymes.
II. **Surface degradation:** Once a surface has been covered by bacteria, degradation occurs at constant rate in the reaction.

Equation 1: $C_6H_{10}O_4 + 2H_2O \rightarrow C_6H_{12}O_6 + 2H_2$

Hydrolysis is the rate-limiting step for anaerobic digestion of microalgae with high suspended solids and COD ratio as a result of the lignocellulosic nature of the algae substrate causing difficulty with accessibility of hydrolytic microbes (Mata et al., 2010, Varjani et al., 2017). It is usually carried out by facultative bacteria that are capable of surviving with or without oxygen (Mata et al., 2010, Schlüter et al., 2008, Botheju et al., 2010).

To promote penetration by bacteria and extracellular enzymes, pre-treatment of the algae cell is necessary. During digestion of solid waste, only 50% of organic material is biodegraded due to lack of enzymes to participate in the degradation process (Ziemiński and Frąc, 2012, Ali Shah et al., 2014). Hydrolytic degradation is affected by particle size, pH, and enzyme production by bacteria species, diffusion and adsorption of enzymes (Ali Shah et al., 2014). In addition, hydrolysis is also inhibited by low temperature affecting the overall reaction and reactor design (Lew et al., 2011). The products of hydrolysis form the substrates for the next reaction, acidogenesis. Below is Table 2.2 showing hydrolytic bacteria involved in anaerobic digestion.

Table 2.2: Various bacteria identified in reactor after hydrolysis

Bacteria genera	**Risk group**	**Anaerobic reaction**	**Reactor temperature condition**	**Enzyme produced**	**References**
Clostridia	2	Hydrolysis Acidogenesis	Thermophilic	Lysozyme	(Christy et al., 2014, Public-Health-Agency-of-Canada, 2010, Alouf et al., 2005, Zabed et al., 2020, Cirne et al., 2007a, Adekunle and Okolie, 2015)
Bacteroides	2	Hydrolysis Acidogenesis	Mesophilic	Fibrinolysin, Penicillinase, Lysozyme, Lecithinase, Deoxyribonuclease, Phosphatase, protease, and Lipase.	(Zabed et al., 2020, Citron, 2007, Christy et al., 2014, Euzéby, 2010, Rudek and Haque, 1976, Sattler, 2011, Adekunle and Okolie, 2015)
Cellulomonas	1	Hydrolysis Acidogenesis	Mesophilic	Hydrolases, Cellulases, Hemicellulases, Xylanases, Cellobiase	(Zabed et al., 2020, Leibniz-Institute, Rajoka and Malik, 1999)
Bifidobacterium	1	Hydrolysis Acidogenesis	Mesophilic	β-galactosidase, fructose-6-phosphate phosphoketolase	(Zabed et al., 2020, Moo-Young, 2019, Robinson, 2014, Fuquay et al., 2011)
Butyrivibrio	1	Hydrolysis Acidogenesis	Mesophilic	Xylanase	(Zabed et al., 2020, Leibniz-Institute, Bajpai, 2014, Christy et al., 2014, Cirne et al., 2007a)
Thermomonospora	1	Hydrolysis Acidogenesis	Mesophilic	Xylanase	(Zabed et al., 2020, Parte et al., 2020, Toldra and Kim, 2017, Leibniz-Institute)
Ruminococcus	2	Hydrolysis Acidogenesis	Mesophilic	Xylanase, Avicellase, Cellulase, Cellobiosidase	(Zabed et al., 2020, Leibniz-Institute, EKİNCİ et al., 2001)
Erwinia	1	Hydrolysis Acidogenesis	Mesophilic	Cellulase, Protease, Xylanase	(Zabed et al., 2020, Barras et al., 1994, Leibniz-Institute)

Acetovibrio	1	Hydrolysis Acidogenesis	Mesophilic or Thermophilic depending on substrate	Cellulase, xylanase	(Zabed et al., 2020, Dassa et al., 2012, Khan, 1980)
Microbispora	1	Hydrolysis Acidogenesis	Mesophilic or Thermophilic depending on species	Cellulase, Xhitinase	(Zabed et al., 2020, Whitman, 2015, Nawani et al., 2002, Parte et al., 2020)
Pseudomonas	1	Hydrolysis Acidogenesis	Mesophilic	Lipase, Protease	(Zabed et al., 2020, Leibniz-Institute, Rajmohan et al., 2002)
Bacillus	1	Hydrolysis Acidogenesis	Mesophilic	Protease, Amylase	(Zabed et al., 2020, Leibniz-Institute, Danilova and Sharipova, 2020, Barros et al., 2013, Divya et al., 2015)
Streptococcus	1	Hydrolysis Acidogenesis	Mesophilic	Proteinase, Peptidases, Streptokinase	(Zabed et al., 2020, Cui et al., 2016, Delorme et al., 2010, Christy et al., 2014, Divya et al., 2015, Ali Shah et al., 2014, Adekunle and Okolie, 2015, Ziemiński and Frąc, 2012)
Lactobacillus	1	Hydrolysis Acidogenesis	Mesophilic	Lysozyme Proteases, Peptidases, Ureases, Amylase, Esterases	(Christy et al., 2014, Leibniz-Institute, Padmavathi et al., 2018, Lentsner et al., 1975)
Escherichia coli	1	Hydrolysis Acidogenesis	Mesophilic	Cellulase Amylase	(Divya et al., 2015, Christy et al., 2014, Leibniz-Institute, Amraini et al., 2017, Gao et al., 2015, Pang et al., 2017, Haryanto et al., 2018)
Micrococcus	1	Hydrolysis	Mesophilic	Protease Peptidase	(Christy et al., 2014, Leibniz-Institute, Ilori et al., 1995, Bhowmik and Marth, 1988)

2.9.2 Acidogenesis

The next step of the process known as 'Acidogenesis'. It is the fastest step in liquid phase anaerobic digestion where acidogenic bacteria such as *Clostridium, lactobacillus spp. , pseudomonas, streptococcus, E.coli, salmonella, bacillus,* or *micrococcus flavobacterium* (Ali Shah et al., 2014) transforms the products of hydrolysis into simple organic compounds mainly volatile acids such as propionic, formic, lactic, butyric or succinic acids as well as ketones, alcohols, H_2, CO_2 and NH_3 (Zeeman et al., 1997, Ostrem and Themelis, 2004). The same bacteria active in hydrolysis reaction undertakes the acidogenic phase of the process as seen in Table 2.2. Acidogenesis sometimes works simultaneously with acetogenesis. In fact, previous research viewed both reactions as a single stage 'fermentation' reaction (Parkin and Owen, 1986) as the end-products rely on the conditions of the reactor medium (Varjani et al., 2017). For example, the end-product will be acetate if H_2 is removed by H_2 consuming methanogens (Van Lier et al., 2008) such as *Methanobrevibacter spp..* Nevertheless, if H_2 builds up, products such as propionate and butyrate may be found in the reactor (Varjani et al., 2017). Therefore, if products such as H_2, CO_2 and acetic acid are produced in acidogenesis given the right conditions of the reactor, then the products may skip acetogenesis and directly undergo methanogenesis (Varjani et al., 2017).

The equations below display the steps involved in acidogenesis.

Equation 2: $C_6H_{12}O_6 \leftrightarrow 2CH_3CH_2OH + 2CO_2$

Equation 3: $C_6H_{12}O_6 + 2H_2 \leftrightarrow 2CH_3CH_2COOH + 2H_2O$

Equation 4: $C_6H_{12}O_6 \rightarrow 3CH_3COOH$

2.9.3 Acetogenesis

In this stage, the products of acidogenesis are transformed by acetogenic bacteria (*Acetobacterium, Sporomusa etc. (Zabed et al., 2020))* into hydrogen, carbon dioxide and acetic acid.

Acetogenic bacteria come in two forms, namely; hydrogen-producing and homoacetogens (Varjani et al., 2017). Hydrogen-producing acetogenic reaction takes place when the partial pressure of hydrogen is low, having been consumed by hydrogen scavenging bacteria (Hattori, 2008, Ostrem and Themelis, 2004). This reduced partial pressure of hydrogen is also a required factor for the digester design.

On the other hand, homoacetogenesis occurs from dissolved H_2 and CO_2 (Varjani et al., 2017). Other acetate bacteria comprise those belonging to *Syntrophomonas* and *Syntrophobacter* classes (Schink, 1997). Other bacteria such as *Methanobacterium suboxydans* and *Methanobacterium propionicum* are capable of converting pentanoic acid to acetic acid and propionic acid to acetic acid respectively (Ali Shah et al., 2014).

Overall, the efficiency of biogas production is largely influenced by acetogenesis as 70% of methane is produced from the reduction of acetates (Ali Shah et al., 2014). The reactions below occur in acetogenesis:

Equation 5: $CH_3CH_2COO^- + 3H_2O \leftrightarrow CH_3COO^- + H^+ + HCO_3^- + 3H_2$

Equation 6: $C_6H_{12}O_6 + 2H_2O \leftrightarrow 2CH_3COOH + 2CO_2 + 4H_2$

Equation 7: $CH_3CH_2OH + 2H_2O \leftrightarrow CH_3COO^- + 2H_2 + H^+$

2.9.4 Methanogenesis

In final stage of the anaerobic digestion process, methanogens in the absence of oxygen, convert hydrogen and acetic acid into methane gas and carbon dioxide. Methanogens are sensitive to changes in temperature and pH. The suitable pH for methanogens is 6.8-7.2 (Ali Shah et al., 2014). Methanogens do not function if the pH level is less than 6.0 (Christy et al., 2014). They are also able to utilize CO_2 as a carbon source (Ziemiński and Frąc, 2012). It is important to note that methanogenesis is the rate controlling step of the anaerobic process (Ostrem and Themelis, 2004, Christy et al., 2014). Methanogenesis pathways are reliant on the inoculum sludge, substrate and experimental conditions (Demirel and Scherer, 2008).

Methanogenesis occurs in two pathways (Parawira, 2012, Cavinato, 2011, Ali Shah et al., 2014);

a. **Acetoclastic methanogenesis**: direct breakdown of acetic acid into methane and CO_2
b. **Hydrogenotrophic methanogenesis:** reduction of carbon dioxide with hydrogen into methane.

Acetoclastic methanogenesis produces 70% of methane, derived from acetic acid conversion by heterotrophic methane bacteria while hydrogenotrophic methanogenesis produces 30% methane from CO_2 reduction by autotrophic methane bacteria where H_2 is consumed (Cavinato, 2011, Ali Shah et al., 2014, Ostrem and Themelis, 2004).

Acetoclastic methanogens are those belonging to *Methanosarcina sp*. and *Methanosaeta sp* (Demirel and Scherer, 2008, Zabed et al., 2020). *Methanosarcina barkeri* and *Methanosarcina sp.* are efficient decomposers of acetates (Ali Shah et al., 2014).

It should be noted, however, that methanogens of the genera *Methanosarcina sp*. are able to undergo both Acetoclastic and hydrogenotrophic pathways compared to *Methanosaeta sp*. (Thauer et al., 2008, Conklin et al., 2006, Liu et al., 2011). The advantages *Methanosarcina sp*. have over other methanogens in the anaerobic digestion process are their rapid doubling times, high tolerance to pH changes, tolerance to high concentrations of salt, ammonia and VFAs (Ali Shah et al., 2014).

Hydrogenotrophic methanogens are those belonging to *Methanobacteriales* and *Methanomicrobiales* (Goberna et al., 2010). Some examples of hydrogenotrophic

methanogens include *Methanobacterium, Methanothermobacter, Methanospirillum, Methanibrevibacter and Methanoculleus* (Zabed et al., 2020, Christy et al., 2014).

Methane produced is mainly from substrates which are by-products of previous reactions such as acetic acid, H_2, CO_2, formate, methanol, methylamine or dimelthyl sulphide as shown in the reactions below (Demirel and Scherer, 2008):

Main methanogenic reactions:

Equation 8: Hydrogen; $CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O$

Equation 9: Acetate $CH_3COOH \rightarrow CH_4 + CO_2$

Other methanogenic reactions:

Equation 10: Formate: $4HCOOH \rightarrow 3\ CH_4 + 3CO_2 + 2H_2O$

Equation 11: Methanol: $4CH_3OH \rightarrow 3\ CH_4 + CO_2 + 2H_2O$

Equation 12: Carbon monoxide: $4CO + 2H_2O \rightarrow CH_4 + 3\ H_2CO_3$

Equation 13: Trimethylamine: $4(CH_3)_3N + 6H_2O \rightarrow 9CH_4 + 3\ CO_2 + 4NH_3$

Equation 14: Dimethylamine: $2(CH_3)_2NH + 2H_2O \rightarrow 3CH_4 + CO_2 + 2NH_3$

Equation 15: Monomethylamine: $4(CH_3)NH_2 + 2H_2O \rightarrow 3CH_4 + CO_2 + 4NH_3$

Equation 16: Methyl mercaptans: $2(CH_3)_2S + 3H_2O \rightarrow 3CH_4 + CO_2 + H_2S$

Equation 17: Metals: $4Me^0 + 8H^+ + CO_2 \rightarrow 4Me^{++} + CH_4 + 2H_2$

2.10 Calculating Theoretical Methane Potential

During microalgae wastewater treatment using anaerobic digestion, it is possible to calculate the potential methane yield from a wastewater sample if C, N, H and O compositions are known using the equations (Nielfa et al., 2015, Zabed et al., 2020).

$$(C_nH_aO_bN_c) + \left(\frac{4n - a - 2b + 3c}{4}\right) H_2O \rightarrow \left(\frac{4n + a - 2b - 3c}{8}\right) CH_4 + \left(\frac{4n - a + 2b + 3c}{8}\right) CO_2 + cNH_3$$

Assuming n, a, b, c represents the C, H, N O contents of the substrate, methane yield in litres/g VS becomes:

$$\left(\frac{4n + a - 2b - 3c}{12n + a + 16b + 14c}\right) \times Vm$$

Where Vm is the molar volume of methane given as 22.14 L at $0^{O}C$ and 1 atm (Sialve et al., 2009, Tchobanoglous et al., 2003).

2.11 Overview of Anaerobic Bacteria and enzymes

Anaerobic digestion is brought about by anaerobic bacteria that produce enzymes from hydrolysis to methanogenesis. Since, hydrolysis is the rate limiting step for the anaerobic digestion process, improving the rate of hydrolysis is vital in the overall reaction rate. During hydrolysis, hydrolytic bacteria attack the cell wall breaking down complex polysaccharides to simple forms.

Bacteria are prokaryotes, microscopic single-celled organisms without a nucleus, which are neither plants or animals, existing in the environment and in their host organism (Brazier, 2019). They exist as spherical, rod-shaped or spiral forms (Hogan, 2010). A typical bacterial cell consists of a capsule, a peptidoglycan cell wall, plasma membrane, cytoplasm, ribosomes, flagellum, pili and DNA (Brazier, 2019). Bacteria receive nutrition via consumption of organic carbon from their host known as 'Heterotrophic bacteria' or via synthesizing their own nutrition through photosynthesis or chemosynthesis.

Anaerobic bacteria are micro-organisms that survive in the absence of oxygen. They are able to undergo anaerobic metabolism that involve anaerobic respiration and fermentation producing volatile fatty acids and methane in the process as end-products (Hogan, 2010).

In terms of broad classification, bacteria survival would depend on the ability to utilise oxygen. There are three main classifications namely; facultative, microaerophilic and obligate anaerobic (Vazquez-Pertejo, 2019). Facultative bacteria are bacteria that are capable of growing aerobically or anaerobically. Microaerophilic bacteria are bacteria that need minimal oxygen concentration of around 2 to 10%. Then, obligate anaerobic are bacteria that are not able to undergo aerobic metabolism but can tolerant oxygen.

Anaerobic bacteria can be thermophilic or mesophilic as demonstrated in Table 2.2 depending on the digester configuration. In wastewater anaerobic digestion, bacteria characterisation and identification in the media is limited due to the difficulty and associated costs (gene sequencing, morphology, physiology, biochemical characterisation). Anaerobic digestion occurs in such systems using the wastewater with the assumption that the bacteria present would adjust to the digester configuration and conditions from hydrolysis to methanogenesis. This quite often has led to low methane yields as a result of rigidity with the cell wall of most microalgae, as well as low volume of bacteria to microalgae biomass (Ward et al., 2014). Recent studies have been investigating the use of selective microbes for anaerobic digestion, which is one of the major focus of this research. Studies have shown increases in methane yield by 25 to 96.3% when agricultural residues including corn straw, cotton stalks and manure are pre-treated with complex microbes, cellulolytic bacteria and clostridium under aerobic conditions and 20 to 55^{o}C and HRT of 12 hrs to 20 days (Lu et al., 2009, Bruni et al., 2010, Zhong et al., 2011, Zhang et al., 2011, Chun and Peng, 2010). It is, therefore, important to choose bacteria that are able to accommodate the conditions of the reactor.

2.11.1 Cell wall digestion enzymes

Microalgae substrate breakdown by bacteria is made possible via the use of enzymes, which are proteinaceous molecules that catalyse biochemical reactions (Christy et al., 2014). The rate limiting step of the anaerobic digestion process is hydrolysis (Liew et al., 2019). This involves the release of enzymes from hydrolytic bacteria to breakdown complex polymers such carbohydrates, proteins or lipids, into simpler compounds such as sugars, amino acids and peptides with lower molecular weight.

To maximize methane production in anaerobic digestion, the use of enzymes has been encouraged (Gerken et al., 2013, Demuez et al., 2015, Liew et al., 2019). Enzymes are cost efficient when produced by bacteria consortium found within the anaerobic system.

Enzymes are classified into endoenzymes and exoenzymes. Both endo and exoenzymes are produced by bacterial cells. Endoenzymes act on the breakdown of internal components of microalgae while Exoenzymes acts on the cell wall of the algae cells to solubilise the cells. The first step is an extracellular reaction involving release of extracellular enzymes on the outermost layer of the bacterial cell to soften the algae cell wall in order for the bacteria to successfully attack the cell leading to cell disruption (Christy et al., 2014). In cell degradation, the activity of extracellular enzymes is carried out by exoenzymes, which are released through the cell slime (Zhong et al., 2011) to the insoluble layer (Delgenes, 2003). During enzymatic hydrolysis, exoenzyme act on cell wall to solubilise the cell whist the endoenzymes disrupt the soluble substrate within the cell. Enzymatic hydrolysis depends on chemical interaction of the enzyme to solubilise the cells, making cell disruption easier and lipids extraction more efficient (Show et al., 2015). Since enzymes act on different parts of the microalgae, having variety of bacteria consortia is important to provide endo and exoenzymes.

These enzymes have been identified in the reactor after anaerobic co-digestion of *C.vulgaris* with bacterium *flammeovirga yaeyamensis* for efficient microalgal oil extraction (Chen et al., 2013). Also, studies have shown lysozyme and cellulase to be effective in the degradation of microalgae cell wall (Aarthy A, 2018, Gerken et al., 2013) with lysozyme degrading the outermost cell layer. In addition, cellulase is crucial in anaerobic digestion as microalgae's rigid cell wall is made up of about 45% cellulose (Aarthy A, 2018). Besides, cellulase production has been observed to enhance methane production by over 50% during anaerobic digestion of *Chroococus sp*. microalgae (Prajapati et al., 2016). However, the experiments were not based on *C.vulgaris* species.

2.12 Factors and Conditions that Affect Microalgae Anaerobic Digestion

From the initial studies of microalgae digestion by (Golueke et al., 1957), there has been identified issues such as composition of the substrate, temperature, pH, effect of C/N ratio, OLR, hydraulic retention time, substrate to inoculum ratio (SIR), lipids concentration and cell wall digestion that can impact digestion performance. Several authors have conducted reviews of the factors that may affect anaerobic digestion rate, particularly for microalgae, which are detailed in this section (Varjani et al., 2017, Ward et al., 2014, Kwietniewska and Tys, 2014).

2.12.1 Substrate composition

Microalgae cultivation is known to affect the physiochemical and biological composition of the microalgae harvested (Kwietniewska and Tys, 2014). This may be due to changes in the cultivation such as light adjustment, nutrient addition or salinity (Ruangsomboon, 2012, Heredia-Arroyo et al., 2010). Although microalgae cell composition is reliant on the species used, environmental conditions such as stress conditions, changes in biochemical composition, nitrogen deficiency can influence digestion (Sialve et al., 2009).

2.12.2 Temperature

Anaerobic digestion can be operated at different temperatures including psychrophilic (<20^{o}C), mesophilic (25-40^{o}C) and thermophilic (45-60^{o}C) temperature ranges (Khalid et al., 2011, Mathew et al., 2015, Ogejo et al., 2009, Ostrem and Themelis, 2004). Although mesophilic reactors are optimum at 35^{o}C, they can function well between 30^{o}C to 35^{o}C (Ostrem and Themelis, 2004).

The reactor temperature has been known to affect the physical and chemical properties of the substrates, as well the biological processes affecting methane-forming bacteria in the system (Ogejo et al., 2009, Varjani et al., 2017). For example, thermophilic digesters can permit higher pathogen destruction rate and better substrate disruption while mesophilic bacteria tolerate more environmental changes, including temperature variations, during anaerobic digestion (Ostrem and Themelis, 2004).

Increasing the reactor temperature may have positive effects on the anaerobic reaction rate including improving the rate of hydrolysis, causing greater accessibility for microbial breakdown and affecting the overall HRT of the system (Abdelgadir et al., 2014). A study conducted by Ehimen et al.(2011) showed a 61% increase in methane yield in the mesophilic temperature range when the temperature was increased from 25^{o}C to 35^{o}C. Also, Ward et al. (2008) reported more fatty acids were released at 55^{o}C than at 38^{o}C during anaerobic digestion with 95% more methane production. In addition, this was further demonstrated in

the study by Ward et al.(2008) where 95% of methane yield was produced after a HRT of 11 days in the thermophilic range compared to 27 days with mesophilic temperatures. Moreover, at higher digester temperature, fluid movement is faster due to lower fluid viscosity, and there is reduced gas solubility that also results in easier phase separation (Van Lier et al., 2008). On the other hand, higher temperatures may have adverse effects on anaerobic efficiency including ammonia inhibition which affects the overall reaction rate (Varjani et al., 2017). Also, thermophilic reactors are known to be unstable and often require additional energy to stabilise the temperature (Karagiannidis and Perkoulidis, 2009, Chen et al., 2008). Modern anaerobic digesters are conducted in mesophilic range due to the stability such system present. However, they also have longer retention times (Ostrem et al., 2004).

2.12.3 pH

pH has been known to be a determining factor in the anaerobic digestion process (Kwietniewska and Tys, 2014). The range of acceptable pH during anaerobic digestion process is between 5.5 to 8.5, while the optimum pH range has been suggested to be 6.8 to 7.2 (Ogejo et al., 2009, Ostrem and Themelis, 2004). Anaerobic digestion process is known to have a series of reactions including hydrolysis, acidogenesis, acetogenesis and methanogenesis. These different reactions operate at different pH optimums. Methanogenesis reactions display sensitivity to changes in pH and function best at pH of 7.0 to 7.2, as methanogenetic bacterial growth rate slows below pH of 6.6 (Ogejo et al., 2009, Ward et al., 2008, Ostrem and Themelis, 2004, Ali Shah et al., 2014). Acidogenetic bacteria, however, can cope in a wider pH range between 4.0 to 8.5 (Ogejo et al., 2009, Ward et al., 2008, Appels et al., 2008). It is worthy to note however that biofilms can protect micro-organisms including bacteria from extreme pH (Wen et al, 2019). These fluctuations in pH sometimes result in two-stage anaerobic digester designs due to the different by-products of each reactions which have separate effects on the pH (Ward et al., 2008). For example, acidogenesis and acetogenesis reactions would produce acids and CO_2 causing the pH to decrease. Methanogenesis on the other hand, consumes the acids in further reactions to produce methane leading to a rise in the pH value that eventually stabilizes (Ogejo et al., 2009). When the rate at which acids are produced in the system is higher as a result of increased organic loading rate, with lower acid removal rate, the anaerobic system can sour (Yuan and Zhu, 2016). Digester failure can occur from acid build-up in the system due to higher volatile fatty acids being accumulated causing the pH to drop drastically below 5.0 which has a negative effect on methanogenic bacteria (Lusk, 1999, Ostrem and Themelis, 2004). When this occurs, it is important to allow the pH to stabilize before adding more feed to the system. In addition, to maintain pH stability, it is sometimes important to introduce an alkali such as sodium or potassium hydroxide to neutralise the acid, in the case of limited

alkalinity required to buffer pH in the feedstock. This can also induce solubilisation of the substrate making it easier for enzymatic degradation during digestion (Ostrem and Themelis, 2004).

The pH interaction in the digester is relevant as it affects the ratio of ionized and non-ionized inhibitors of methanogenesis. By-products like fatty acids, ammonia and hydrogen sulphide are toxic in their non-ionized state affecting the rate of anaerobic digestion adversely (Ward et al., 2008).

2.12.4 Carbon to Nitrogen ratio

The carbon and nitrogen ratio show the amount of organic carbon and nitrogen in the feed. A feedstock high in carbon is usually balanced by adding more nitrogen like fertiliser (Ostrem and Themelis, 2004). Also, excess nitrogen in a system would mean more ammonia, so it is important to monitor the feedstock entering into the system. Fluctuations in the C:N:P ratio during anaerobic digestion may have a negative effect on the buffering capacity (Varjani et al., 2017). The optimum ratio of C/N is between 20 to 30 for promotion of anaerobic activity (Ostrem and Themelis, 2004, Ward et al., 2008, Parkin and Owen, 1986, Muthudineshkumar and Anand, 2019).

2.12.5 Effect of Moisture Content in Feedstock

Anaerobic digester design can be determined by the amount of moisture in the feed (Kwietniewska and Tys, 2014), as this affects the anaerobic process and methane production indicting that an optimum amount of moisture content is required in the process (Varjani et al., 2017). Due to this, digesters may be designed as wet or dry systems. Dry digesters have 30 to 40% dry matter in the feed, while wet digesters can handle 10% to 25% dry matter (Karagiannidis and Perkoulidis, 2009). For microalgae biomass, pre-treatment is highly significant, as freshly harvested algae may sometimes contain just 2% to 10% dry matter (Golueke et al., 1957, Patil et al., 2011). Hence, dewatering to reduce the moisture content becomes relevant in maximising methane production. A study conducted by De Schamphelaire and Verstraete (2009) inferred that concentrating microalgae after harvesting is a crucial process in improving anaerobic digestion. Other authors suggested the use of a settling tank or a gravity settling model as a means of algae concentration (McCarty, 1964, Collet et al., 2011). One of the major concerns of high moisture content in the digester is the issue of bacteria wash-out as a result of low digestible content of the feedstock (Ward et al., 2014). In fact, there has been instances where the anaerobic digester has shut down in midst of an experiment due to the mixed liquor being low in solids (De Schamphelaire and Verstraete, 2009). To overcome this, techniques involving harvesting, concentrating and

dewatering the microalgae have been recommended as pre-treatments to anaerobic digestion (Moheimani et al., 2015a).

2.12.6 Organic Loading Rate (OLR)

The amount of volatile solids loaded into the anaerobic digester per unit volume at a specific time in a continuous process is known as the Organic Loading Rate (OLR) (Kwietniewska and Tys, 2014, Zabed et al., 2020). OLR balance is important for a reliable anaerobic digestion process as it maintains the stability of the acidogenic and methanogenic reactions in the system (Zabed et al., 2020). If the OLR is raised above the optimum limit, an issue of overloading may develop leading to a reduction in volatile solids breakdown and subsequently low biogas yield (Babæe and Shayegan, 2011, Rincon et al., 2007).

In addition, overly high OLR above the optimum may cause organic acid build-up in the anaerobic digester (Liu and Tay, 2004). The amount of feedstock loaded onto the digester during the initial phase of anaerobic digestion is important, as high initial loading of feedstock may slow bacterial activity (Golueke et al., 1957). This may result in increased by-products of the hydrolysis-acidogenesis reactions. Consequently, this may lead to increased volatile fatty acids, resulting in reduction in pH (in case of insufficient alkalinity), as well as the inability of methanogens to convert the acids to methane (Kwietniewska and Tys, 2014).

2.12.7 Retention Time

Retention time of anaerobic digestion processes can refer to either solid retention time (SRT) or hydraulic retention time (HRT) (Kwietniewska and Tys, 2014). The solid retention time is known as the mean time the bacterial solids are in the digester (Kwietniewska and Tys, 2014). It is significant as it influences the biochemical characteristics of organic matter in the digester (Varjani et al., 2017). It relies on the property of the feedstock and affects methanogenic activity within the digester (Varjani et al., 2017). A study conducted by Halalsheh et al. (2005) shows methanogenesis occurring at SRT range of 5 to 15 days at 25^{o}C and 30 to 50 days at 15^{o}C. HRT is defined as the volume of the reactor per the influent flow rate and represents the time the liquid phase remains in the digester (Henze et al., 2008). It is influenced by the substrate and loading rate. It is beneficial for the HRT to be at optimum rate because reducing the HRT by increasing the feed flowrate may lead to build-up of VFA in the system and to system wash out and eventual collapse of the anaerobic process (Nagamani and Ramasamy, 1999, Ogejo et al., 2009). Also, using HRT less than 10 days may lead to reduction in methane production (Kwietniewska and Tys, 2014). The study conducted by Ostrem and Themelis (2004) identified key research areas in reducing the retention time including; use of multi-stage digestion for bacterial population optimalization for specific anaerobic reactions, improved

circulation, introduction of constant bacteria supply to minimise bacteria wash-out, controlling environmental parameters, and pretreatment of feedstock.

2.12.8 Inoculum to substrate ratio (ISR)

Anaerobic digestion involves the breakdown of biomass by bacteria. The composition of biomass and bacteria within the system is important as too many bacteria leads to starvation of the microbes and insufficient bacteria would slow the anaerobic process due lack to bacteria to act on the substrate. Hence, having a balanced ratio of the inoculum and substrate is key in maintaining a smooth anaerobic digestion process. Experiments conducted by Zeng et al. (2010) concluded that the highest amount of methane yield was reported when the inoculum to substrate ratio was 2.0 from anaerobic digestion of *Microcystis spp*., however, there was a 45% increase in methane composition of the total biogas when the ISR was reduced to 0.5.

2.12.9 Lipids concentration

Microalgae cell composition are made up of carbohydrates, proteins, nucleic acids and lipids. Lipids are gaining attention in biofuel processes as they have a higher theoretical methane production than carbohydrates or proteins (Zamalloa et al., 2011). Lipids can be removed for other biofuel applications before anaerobic digestion. This has been suggested as an advantage to anaerobic digestion as increased lipid concentration can inhibit anaerobic digestion as a result of low alkalinity and buffering (Park and Li, 2012, Cirne et al., 2007b). It should be noted that lipid extraction technology used for extracting lipids can also influence microalgae digestion as residual solvent can adversely affect the process and decrease methane production (Ward et al., 2014, Ehimen et al., 2009, Bligh and Dyer, 1959, Thiel, 1969). Besides, lipids are in form of fatty acids, triacylglycerols, glycolipids and sterols located inside the cell bound membranes and cell walls are difficult to extract as a result of inefficient cell wall degradability, which is an attribute of the high mechanical strength of the algae cell wall (Lee et al., 2012).

2.12.10 Cell wall digestibility

Perhaps the most challenging issue affecting the rate of anaerobic digestion from various studies conducted is the cell wall digestibility of microalgae biomass. Algal cells like plants, have cell walls composed of polysaccharides such as cellulose, hemicellulose and pectin (Passos et al., 2013b, Kendir and Ugurlu, 2018) with the addition of glycoproteins (or combination of both) which differentiates algae from land plants. Intracellular components such as lipids have to be extracted from the cells for biofuel applications including anaerobic digestion.

Several studies have reported instances where microalgae have undergone anaerobic digestion, but the cells remain intact and undigested due to their recalcitrant property demonstrating their resistance to bacterial attack (Golueke et al., 1957, Mussgnug et al., 2010, Zhou et al., 2009, Hernández and Córdoba, 1993). These reports have observed intact cells after retention times of 30 days, 45 days, 64 days and up to 6 months during anaerobic digestion (Hernández and Córdoba, 1993, Zhou et al., 2009, Mussgnug et al., 2010). The rigidity of the cell wall is due to the carbohydrate structure of most microalgae cells containing hemicellulose and biopolymers (Ward et al., 2014). A study conducted by Mussgnug et al. (2010) reported increased methane production using microalgae species without a cell wall (such as *D.salina* (Borowitzka, 2018)) or species containing protein (*C.reinhardtii, A. platensis and E. gracilis*) than from species with a carbohydrate cell wall such as *C. kessleri and S. obliquus.* For example*, C.reinhardtii* produced 587 ml biogas g/VS compared to *S.obliquus* with only 287 ml biogas g/VS produced.

Due to this, microalgae pre-treatment has been suggested to degrade the cell wall and increase bacterial hydrolysis, as hydrolysis is the rate limiting step in the anaerobic digestion process (Ward, 2015, Ras et al., 2011, Mussgnug et al., 2010, Chen and Oswald, 1998).

Pretreatment techniques to disrupt and break cells can be physical, mechanical, thermal, chemical and biological. Pretreating microalgae cells may involve optimising parameters such as temperature, pressure, shear force, chemical addition or biological agents on the cells. Pretreatment can be costly and some authors have reported that the energy requirement for pre-treating microalgae is similar to the energy derived from the process (Lee et al., 2013a, Sialve et al., 2009, Yen and Brune, 2007, Lü et al., 2013). As a result of this, current research and investigations in the use of biological pre-treatment such as enzymes and bacteria have been encouraged (Ward et al., 2014).

2.13 Overview of Microalgae Pre-treatment methods

Industrial and laboratory scale microalgal pre-treatments are broadly classified into five types: mechanical, physical, thermal, chemical and biological as illustrated in Figure 2.8. Mechanical pre-treatment covers ultrasound, sonication, microwave, bead milling, beat beating, high pressure press, high speed and high-pressure homogenisation as well as pulsed electric field treatments. Physical treatment is quite similar to mechanical and involves freeze drying, manual grinding, and osmotic shock. For heat or thermal treatment, classification includes thermal, thermal with steam explosion and hydrothermal. Also, some examples of thermal treatment are water bath and autoclave as they involve application of high temperature generating heat that disrupts the algae. Chemical treatment involves the addition of chemicals such as sulphuric acid or base such as sodium hydroxide or even oxidation to degrade the algae cells. Finally, biological treatment involves use of micro-organisms such as bacteria and fungi or enzymes secreted from these microbes to degrade the cells.

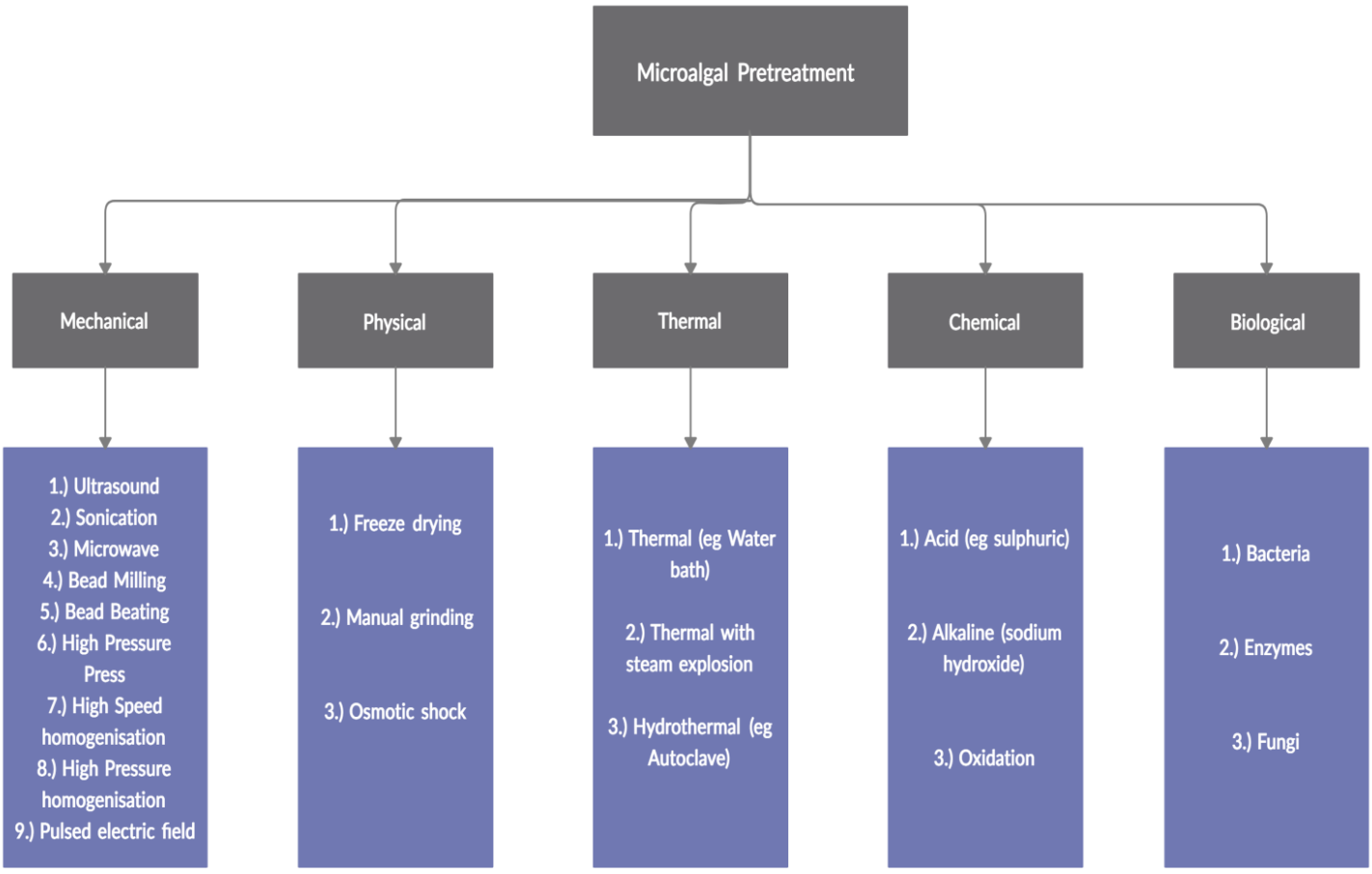

Figure 2.8: Types of Microalgae Pretreatment.

2.13.1 Mechanical Pretreatment

Mechanical pretreatment includes the use of methods such as sonication, bead milling, ultrasound, homogenisers and microwaves (Torres et al., 2013, Aarthy A, 2018) to break microalgae cells. Mechanical pretreatment is one of the most common and preferred treatment method as they are almost independent of the microalgae species used (Jankowska et al., 2017). Experiments conducted by Gonzalez-Fernandez et al. (2012) report a 44% increase in methane production when microalgae biomass was subjected to ultrasound at a frequency of 20Hz for 15 minutes treatment time. Also, a 26% increase in biomass solubilisation was recorded by freezing (Harun and Danquah, 2011). Mechanical pre-treatments have higher energy requirement when compared with other methods such as chemical, thermal and biological treatment (Lee et al., 2012).

It is a pretreatment method that also includes irradiation. Ultrasound, microwave, gamma rays and electron beam are forms of irradiation pretreatment (Zheng et al., 2014). Mechanical treatments such as bead-beating, ultrasonication and homogenisation are appropriate for laboratory scale experiments of microalgae cell disruption, however, large scale applications remains challenging due to the high energy demand required for operation (Lari et al., 2019, Steriti et al., 2014).

To improve anaerobic digestion, it has been suggested to combine pre-treatment methods. There was an 82 to 106% recorded increase in methane yield when homogenisation and ultrasonic pre-treatment were combined in the laboratory (Lee et al., 2013c). A study using mechanical pre-treatment on *C.vulgaris* microalgae cells at conditions of 3,500 rpm for bead milling, 2,450 MHz for microwave, and 50 Hz for ultrasonication showed 0.15, 0.18 and 0.2 g lipid content release per 0.5 g/l dry weight respectively (Prabakaran and Ravindran, 2011).

(Lee et al., 2012) reported that mechanical pretreatment is less likely to contaminate the lipids derived from algae compared to chemical pretreatment. In addition, mechanical treatments may release lipids that can form compounds with proteins and cell debris making lipid separation labour intensive (Harrison, 1991).

2.13.1.1 Ultrasound Pretreatment

Ultrasound pretreatment can be used to increase microalgal cell solubilisation and disruption. It employs a short operational time of approximately 2 minutes, have higher reproducibility and provides purity to the desired product (Aarthy A, 2018).

Ultrasound treatment involves generation of sonic waves via quick compression and decompression cycles at frequency of 50kHz and above. The compression cycles create cavitation as liquid molecules move in the cell by acoustic waves generated within the fluid (Suslick and Flannigan, 2008). With increased ultrasound intensity and pressure, the cells

begin to compress and implode generating heat and eventually causing disruption of the algal cells (Suslick and Flannigan, 2008). Cavitation aids mixing of the fluid components via micro-turbulence as well as degradation of the cells from the shockwaves generated due to the shear forces created in the liquid (Aarthy A, 2018). This enables microbial hydrolysis and volatile fatty acid formation which can be used in later reactions to produce methane (Rodriguez et al., 2015). Ultrasonic pretreatment is influenced by factors such as ultrasound intensity, characteristics of the air bubble, proximity of the cells to the bubble, frequency, power, and exposure time of sonic waves (Krehbiel et al., 2014, Lari et al., 2019)

It is influenced by microalgal species and specific energy applied (Passos et al., 2014b). Other parameters that determine its effectiveness are exposure time, output power, biomass concentration and temperature (Passos et al., 2014b, Rodriguez et al., 2015). For example, a study by Passos et al. (2014a) found that there was a linear relationship between the applied specific energy and methane production for various pretreated algae including *Clamydomonas sp., Scenedesmus sp., Nannocloropsis sp., Acutodesmus obliquus, Oocystis sp.,* and *Microspora sp.* The results showed biomass solubilisation and methane improvement by 16 to 100% and 6 to 33% respectively when ultrasonic treatment was applied with a specific energy range of 16 to 67 MJ/kg Total solids (TS). The results for methane production of up to 100% confirms a similar study for ultrasound pretreatment which showed methane improvements' of 91% (Park et al., 2013).

2.13.1.2 Sonication

Sonication and ultrasound are very similar but vary in frequency and pretreatment volumes, with sonication operating at frequencies of 25kHz (Chandler et al., 2001, Fykse et al., 2003). Sonication is a mechanical pretreatment method that operates by producing sonic waves and involves a fast compression and decompression that creates transient and stable cavitation. The transient cavitation occurs from unsteady oscillations which causes the cell wall and membranes to break generating shock waves and shear forces (Brujan et al., 2001, Hosikian et al., 2010). There are mainly two types of sonicators; the horn and bath (Aarthy A, 2018). The horn sonicators uses piezoelectric generator made up of zirconate titanate crystals that vibrate at amplitude between 10μm to 15 μm whilst bath employs the use of transducers that cause ultrasonic waves when placed at the bottom of a reactor (Joannes et al., 2015). Both types of sonicators are used for batch processes but can be improved to undergo continuous processes via the implementation of flow cells (Borthwick et al., 2005, Gogate et al., 2006). Sonicators are used when pure products are desired (Chemat and Khan, 2011). The cell disruption efficiency in sonication can be affected by temperature, reaction time, addition of glass beads and viscosity of the cell suspension (Lee et al., 2012). Lower temperature results in rapid bubble collapse and increased shear rate during sonication. Also, extended reaction

time generates more energy that can be used for cell disruption in the process. Moreover, addition of glass beads has been seen to improve the rate of crushing (Bhaduri and Demchick, 1983). In addition, reduced viscosity leads to rapid cavitation, releasing intracellular components of the cell and resulting in efficient disruption (Popinet and Zaleski, 2002).

Factors that affect microalgae disintegration using sonication method include algae size, shape, density, concentration and time of harvest (Lari et al., 2019). In a study conducted by Kurokawa et al. (2016) a co-relationship between frequency and disruption rate was identified. The study showed algae cells totally disrupted at 2.2MHz over 2 minutes whereas 40% disruption was recorded at 0.4MHz for 10 minutes. Also, frequencies of 20 to 100 kHz have been observed for chemical reaction induction and cell wall disruption (Yamamoto et al., 2015).

2.13.1.3 Microwave Pretreatment

Microwave heating occurs as a result of fast oscillating electric field from a dielectric material inducing heat due to frictional force of the molecules in motion leading to higher temperature of the substrate and resulting in cell disintegration (Terigar et al., 2010). Here, energy is produced by an electromagnetic field and passed on to the material been heated providing heat evenly with minimal thermal gradient (Zheng et al., 2014). Microwave frequency varies between 300MHz to 300 GHz and is able to break the hydrogen bonds present in a fluid leading to a destructing of the cell proteins (Passos et al., 2013b). During microwave pretreatment of microalgae cells, the generation of pressure and heat within the cells force intracellular disruption to occur (Ranjith Kumar et al., 2015). There has been reports of microwave assisted degradation of microalgae exhibiting higher production of lipid due to micro-cracks in the cell walls (Šoštarič et al., 2012). Microwave has been reported as an effective technique for cell disruption of *C.vulgaris* when compared to chemical, sonication, bead beating and autoclave pre-treatments (Lee et al., 2010). Parameters influencing microwave pretreatment are dielectric properties algae mixture, solid-liquid ratio, microwave frequency, output power, exposure, process time, biomass concentration, temperature, microalgae species and penetration depth (Aarthy A, 2018, Passos et al., 2014b, Jankowska et al., 2017, Lari et al., 2019). Microwave treatment has efficient disruption and reduced processing time. It also results in improved energy transfer, selective heating, and increased reaction rate (Aarthy A, 2018).

In addition, increasing and accelerating heat generation has been reported to affect lipid quality (Balasubramanian et al., 2011). Microwave pretreatment is capable of saving time on treating the cells as large fluid volumes can be heated within the fluid rather than the use of thermal gradients, however, the depth of penetration of the microwaves remains a limitation of this method.

2.13.1.4 Bead Milling

Cells are disrupted by agitated glass beads in this pretreatment method. Cell degradation is promoted by increased acceleration and rotation of fine beads mixed within the microalgal biomass (Aarthy A, 2018). Several factors may influence the disruption rate using bead milling. Some of these factors include: residence time, bead size, cell size and cell strength (Geciova et al., 2002, Doucha and Lívanský, 2008). Bead milling disruption method is more useful in laboratory scale applications (Aarthy A, 2018). In industrial applications, a dyno-mill (a large bead milling equipment) is more effective as it rotates faster and has discs for stimulation of the beads (Krisnangkura, 1986, Munir et al., 2013). A study conducted by Postma et al. (2017) on cell disruption of *C.vulgaris* showed that the rate of release of intracellular carbohydrates and protein was inversely proportional to the energy required for smaller sized beads. Another study revealed that the rate of disruption is higher with increased volume of beads in a particular suspension (Hopkins, 1991).

2.13.1.5 Bead Beating

This pretreatment method uses fast accelerating motion of glass or steel beads placed in a cylindrical container to disrupt microalgae cells by rigorously grinding the cells against the surface of the beads (Show et al., 2015, Halim et al., 2012). Bead beating relies on certain factors to improve disruption efficiency such as bead shape, size, composition, distance from cells to beads and rigidity of the microalgae cell wall (Show et al., 2015, Lari et al., 2019). Bead beating has been suggested as an effective technique for lipids extraction from nonpolar lipids (Ryckebosch et al., 2012). The method can be used in conjunction with other cell disruption methods like chemical or mechanical processes (Lari et al., 2019). Bead beating technique has been demonstrated to be efficient for biomass concentrations of around 100 to 200 g/L (Greenwell et al., 2010). To achieve this, it has been suggested to dewater the biomass and concentrate it upon harvesting (Lari et al., 2019). Bead beating is a recommended technique for large scale operations; however, it can also be applied in laboratory use (Show et al., 2015, Lee et al., 2010, Geciova et al., 2002).

2.13.1.6 High Pressure Press

This involves using high pressure using instruments such as screw, expeller or piston to break the cell wall of microalgae releasing the intracellular components (Show et al., 2015). High pressure press has been used in conjunction with other cell disruption techniques increasing the lipid yield by 70% to 75% (Schwede et al., 2011). Microalgae pre-treated using high pressure press showed 33% improvement in biogas production when compared with untreated microalgae (Schwede et al., 2011).

2.13.1.7 High Pressure Homogenisation

High pressure homogenisation acts by pumping microalgae paste under high pressure leading to rapid liquid movement via shear forces resulting in collapse of the cell cavities and releasing internal cell components (Show et al., 2015). The working mechanism of this method involves the use of positive displacement pumps under high pressure between the range of 150MPa to 400MPa that pushes the microalgae cell suspension through an orifice making it collide with the valve scattering the cells around the impact surface (Aarthy A, 2018). Cell disruption using this method is greatly influenced by high energy densities, high working pressure and number of cycles, applied pressure and cell wall strength (Günerken et al., 2015, Greenwell et al., 2010). High pressure homogenisation is influenced by the following factors including: the impingement of cells located on surfaces of valve seat and impact ring, turbulence, viscosity, high pressure shear, decrease in pressure and escape of gas bubbles, and collapse of cavitation bubbles (Lee et al., 2012).

2.13.1.8 High Speed Homogenisation

This method employs a stirring device at high speeds. A high-speed homogeniser is a mechanical stirring device composed of a stator and rotor that uses hydrodynamic cavitation at mixing points of the fluid interphase, disrupting the cells as a result of shear force distribution and turbulence within the liquid mixture (Lee et al., 2012). (Shirgaonkar et al., 1998) have stated mechanical shear to be responsible for cell disruption at homogeniser speeds less than 4,870 rpm but cavitation to be the main force over this speed. (Aarthy A, 2018) reports 8500 rpm as the critical speed that reduces the surrounding pressure to the vapour pressure of the fluid collapsing the cavities.

High speed homogenisation is a simple mechanical disruption method with short contact times capable of extracting various biochemicals from cells (Günerken et al., 2015). This method is useful for determining the mechanical strength of microalgae cells as it has short contact time and can cause rapid disruption in the cell. Moreover, the varying speeds can provide an insight to the degree of rigidity and predict energy consumption leading to energy savings. High speed homogenisation is capable of disrupting cells in suspension with a higher dry cell weight concentration of 2 to 6%w/w resulting in reduced water footprint and less downstream processing cost (Aarthy A, 2018). In addition, high speed homogenisation is useful for extracting intracellular lipids from the cells. A study carried out by Wang and Wang (2012) reported up to 76% of lipid extraction from *Nannochloropsis sp* using high speed homogenisation.

High speed homogenisers come in two types: rotor-stator and blade homogeniser. The rotor-stator homogeniser has speed rotating blade with a static tube as well as slots, grooves or teeth to improve shear rate (Lee et al., 2012). They are suitable for homogenising fluid

samples with medium to low viscosity of about 10Pascal-second (Pa.s) and are also used when finer products are desired (Lee et al., 2012). It should be noted however, that blade homogenisers can have issues with aeration and foaming (Show et al., 2015). High speed homogenisers can be used in batch, semi-batch and continuous processes (Maa and Hsu, 1996). Factors that influence disruption efficiency using this method includes design and size of the rotor-stator, viscosity of the fluid, flow rate and concentration of the algae cells (Lee et al., 2012). Laboratory scale high speed homogeniser can be more impactful for liquids with low to medium viscosity.

High speed homogenisation is a technique that needs further research as very little experimentation has been conducted on their performance. Due to this, process development, scale-up, and operation of high speed homogenisers requires further investigation (Lee et al., 2012).

2.13.1.9 Pulsed Electric Field Treatment

This method uses an external electric field to activate electrical charges within the cell wall or membrane of cells (Aarthy A, 2018). The electric field generated creates pores within the cell membrane that are proportional to electrical pulses as a result of electromechanical compression and electroporation (Fernandes, 2015, Qin et al., 2014). Electrical conductivity during treatment causes metabolites to be released from the disrupted cells (Aarthy A, 2018). A study by Eing et al. (2013) showed lipid yield increase by 9 times when *Auxenochlorella protothecoides* was pretreated using pulsed electric field prior to solvent extraction. Another experiment conducted by Zbinden et al. (2013) using pulsed electric field treatment to treat *Ankistrodesmus falcatus* algae at conditions of 45kV, 360 ns 1/e pulse duration resulted in over 50% more extraction efficiency of lipids.

2.13.2 Physical Pretreatment

Physical pretreatment generally refers to non-chemical and non-biological treatment. Physical pretreatment is a form of mechanical treatment but is mainly employed in laboratory scale techniques. Some physical treatments include freeze drying, manual grinding and osmotic shock which will be discussed further.

2.13.2.1 Freeze Drying

Freeze drying is a laboratory scale analytical technique for disrupting microalgae. Mainly used to determine the dry weight of algae in conjunction with solvent treatment. Freeze drying involves the use of vacuum to dry frozen algae at -80^{o}C by sublimation, (Guldhe et al., 2014). Freezing wet algae cells causes ice formation, sometimes in form of microneedles internally piercing the cells. During freeze drying, the water expands within the container, pressing against the cell wall of the microalgae leading to further disruption (Chisti and Moo-Young, 1986).

Freeze drying is used when total lipids are being measured from microalgal biomass. Since lipids are volatile substances, upon freezing there is no lipid loss due to evaporation (Pourmortazavi and Hajimirsadeghi, 2007). After freeze drying, the Bligh and dyer method can be used to obtain total lipids from algae cells. Freeze drying can be used in combination with other treatment methods such as grinding, ultrasonication and microwave to improve efficiency (Prakash and Raja, 2014).

2.13.2.2 Manual Grinding

Manual grinding can be used for lipid extraction. There are various means of manual grinding. (Zheng et al., 2011) investigated three different methods of manual grinding for disrupting *C.vulgaris* cells for lipids extraction. The first method involved harvesting of the algae from suspension and addition of liquid nitrogen. The mixture was then allowed to thaw and then ground. The second method included mixing quartz sand to separate alga harvested from suspension and ground directly. The third method involved drying of the algae upon harvesting in an oven, then adding quartz sand to the algae before grinding. All three test samples were then washed with distilled water afterwards. The results of the experiments' show grinding in liquid nitrogen to be the most effective disruption (Zheng et al., 2011).

2.13.2.3 Osmotic Shock

Osmotic shock occurs as a result of sudden variations in water concentrations in cell membranes due to solute additions like salts, neutral polymers or mineral substrates. It can also occur from dilution of external cell surfaces (Rój et al., 2015). The interruption of osmotic pressure of the cells may result in cell wall disintegration, liberating intracellular components of the cells (Lari et al., 2019). Previous work conducted to disrupt *C.vulgaris* cells using osmotic shock with 10% NaCl, 1-minute vortex and 48 hours incubation shows similar efficiency in cell disruption when compared with bead beating although osmotic shock needed a higher retention time (Lee et al., 2010, Amaro et al., 2011).

2.13.3 Thermal pretreatment

Thermal pretreatment methods involve the subjection of microalgae biomass to high temperatures in order to solubilize the cells and have long been investigated for pre-treating algal biomass. Thermal pretreatment is the most researched algal pretreatment method and is used in continuous reactors resulting in net energy production.

Thermal pretreatment may further be divided into thermal, hydrothermal, steam explosion with the addition of various chemicals resulting in thermo-chemical pre-treatment. Thermal pretreatment occurs when the temperature is below 100^{o}C under atmospheric pressure. Low temperature pretreatment is useful in conserving energy use. Moreover, heat is a requirement in promoting bacterial activity during anaerobic hydrolysis in thermophilic or mesophilic reactors. Thermal pretreatment is reliant on temperature as well as exposure time of the algae, as temperature is a key determinant of anaerobic biodegradability and algae degradation (Chen and Oswald, 1998).

Following experiments conducted by Golueke et al. (1957) that concluded a pre-treatment step was necessary for cell wall disruption, there have been several attempts of disrupting algal cells using thermal processes. Golueke et al.(1957) observed that algal cells were disrupted when the temperature was raised to 50^{o}C, from 30^{o}C which was the reactor temperature. Experiments conducted by Zelitch (2012) displayed a significant increase in methane production of about 60-120% using thermal pre-treatment at 120-140^{o}C for 15-30 minutes. Also, a study carried out by Mendez et al. (2014) showed a 93% increase in methane production with thermal pretreatment of *C.vulgaris* at 120^{o}C for 40 minutes.

2.13.3.1 Hydrothermal Pretreatment

Hydrothermal pretreatment takes place at temperatures range of 100 to 250^{o}C with gradual release of pressure following pretreatment at shorter retention times (Rodriguez et al., 2015). An example of hydrothermal pretreatment is an autoclave operating at over 100^{o}C. Hydrothermal treatment is affected by factors such as temperature, pressure, and exposure time (Rodriguez et al., 2015). A study conducted by Mendez et al. (2014) showed a 1.5 fold methane production when *C.vulgaris* cells were treated hydrothermally at 120^{o}C for 10 minutes and 6 bar. Results of hydrothermal pretreatment has shown degradation in the outermost layer of the microalgae cell (Passos and Ferrer, 2015). (Passos et al., 2015a) showed a 28% total methane yield when microalgae was pretreated using hydrothermal treatment in an autoclave at 130^{o}C, 1.7 bars and 15 minutes treatment time.

Some advantages of hydrothermal pretreatment includes: increased biomass solubilisation from the exposure time, high methane production and sterilisation of feedstock (Rodriguez et al., 2015). However, this treatment method may also lead to thickened biomass with high energy consumption (Rodriguez et al., 2015).

2.13.3.2 Steam Explosion

When operating thermal pretreatment, the pressure is directly proportional to temperature. However, at above 160^{o}C, steam explosion can provide a sudden drop in pressure to ambient pressure via a pressure reduction valve. This is known as thermal hydrolysis (Passos and Ferrer, 2015) or autohydrolysis (Zheng et al., 2014). This reduction in pressure results in disruption of the cells as the biomass cells go through an explosive decompression. The typical pressure drops range for steam explosion is 0.69 to 4.83 Pa with temperature up to 260^{o}C. Steam explosion pretreatment is more common in pre-treating lignocellulosic biomass than algae.

2.13.4 Chemical Pretreatment

Chemical pretreatment involves the use of acids (e.g. sulphuric acid), alkalinity (e.g. sodium hydroxide) or organic solvents (e.g. chloroform, ether or alcohols) to solubilise cell walls and membranes effecting microalgae cell disruption (Jankowska et al., 2017, Lari et al., 2019). Chemical pretreatment results in chemical reactions involving the strong covalent bonds of functional groups found on cell wall surfaces resulting in release of intracellular components (Show et al., 2015). This method has shown effectiveness in hydrophobic molecule extraction such as plant pigments, as they are extracted by solvents (Lari et al., 2019). Extracellular cell components influence chemical pretreatment as solvent uptake transforms the chemistry of the membrane, enhancing microalgae disruption process (Lari et al., 2019). Chemical treatments are much easier for smoother cell walls and enables exposure of cells to chemical attack leading to better lipid extraction efficiency (Brennan and Owende, 2010, Miranda et al., 2012). Chemical pretreatment has been shown to increase biomass solubilisation of hemicellulose-based cell walls (Torres et al., 2013), and bioethanol and biohydrogen production are mainly enhanced via chemical pretreatment (Harun et al., 2010).

Studies have shown improvement in bioethanol production when microalgae cells were pre-treated sulphuric acid. A study conducted by Nguyen et al. (2009) showed ~29 wt% (g ethanol/g microalgae) ethanol yield after pre-treating *Chlamydomonas reinhardtii* biomass with 3% sulphuric acid at 110 °C for 15–20 min reaction time. Furthermore, experiments carried out by (Harun and Danquah, 2011) displayed highest bioethanol concentration at 7.20 g/L when pre-treatment was performed using 15 g/L of microalgae at 140 °C and 1% (v/v) of sulphuric acid for 25 minutes.

Also, hydrogen peroxide and ferrous sulphate have been effective in the disruption of *C.vulgaris,* making available double the amount of lipid via the formation of hydroxyl groups targeting the components of the cell wall leading to cell fragmentation (Steriti et al., 2014). Moreover, treating *Nannochloropsis oculata* microalgae for 2 hours with hydrochloric acid (HCl) at a pH of 2.0 , shaken for 1hour, 2 hour and 3 hour at 180 rpm has shown to be effective for lysing the cells after enzymatic hydrolysis leading to increased lipid release (Surendhiran and Vijay, 2014).

(Zheng et al., 2014) stated the drawback with chemical pretreatment is the cost involved in the process. Overcoming this difficulty would require utilising an affordable chemical to transform lignocellulose into a biodegradable substrate. Also, studies have shown the formation of undesired by-products when using sodium hydroxide. This can alter the products of microalgae oil extraction process interacting with value-added metabolites and contaminating the lipids released (Mendez et al., 2014, Lee et al., 2013c). Another limitation of chemical treatment is possible corrosion of cultivation equipment. Factors that influence the efficiency of chemical treatment includes concentration of algae, time of treatment and

temperature (Lari et al., 2019). Studies have shown that the amount of lipids released during chemical pretreatment using free nitrous acid is directly proportional to concentration of nitrous oxide and treatment time (Bai et al., 2014). Another example of temperature dependency on chemical treatment showed better cell disruption of microalgae at a temperature of 160°C when compared to a lower temperature of 120°C. At 160°C, the C/C_0 (intact cell count of the sample, C to initial cell count, C_0) ratio was 0.33 with 8 vol% of acid for 45 minutes. Then, at 120°C, the C/C_0 ratio was 0.97 using 3 vol% of acid for same 45 minutes (Halim et al., 2012). Utilising mechanical, thermal, chemical and physical methods can be energy demanding, as the energy consumption for pre-treatment of biomass may be equal to or higher than the energy derived from the microalgae cell (Ward et al., 2014). Modern pretreatment research has been focused on utilising biological treatments as they can be more cost and energy efficient.

2.13.5 Biological Pretreatment

Biological pretreatment involves the use of biological substances such as enzymes, bacteria producing particular enzymes and fungi to induce solubilisation of microalgae cells and degrade the cell wall aiding anaerobic hydrolysis. Biological pretreatment methods of agricultural residuals and other plant components have been previously studied for improving biogas production, although little work has been done on microalgae (Zheng et al., 2014). This alternative pretreatment is very promising, as it involves minimal use of energy, thus enabling net energy production.

Anaerobic digestion rates can be improved by degrading recalcitrant compounds in the cell wall such as carbohydrate into simpler forms leading to substrate accessibility (Liew et al., 2019). Biological pretreatment has been known to improve the degradability of cellulose in lignocellulose biomass to access intracellular lipids, maximising lignin removal, leading to more biogas production (Zheng et al., 2014). For example, same study showed 15% to 5 folds increase in methane yield when agricultural residuals like chestnut and sisal leaves were used as feedstock with fungi pretreatment. Also a 25% to 96.63% improvement in methane yield was observed when corn stalks and cassava residues were digested with yeast and cellulolytic bacteria (Zheng et al., 2014).

There has been recent attraction for biological pretreatment due to its low energy use, no chemical input and environmental friendliness. The main issue around them, however, relates to the long pre-treatment time required to solubilise cells. In the case of microalgae, biological pretreatment can be used to solubilise the cells and also act as inoculum for substrate digestion using the algae as feed and releasing volatile fatty acids, improving biogas production.

2.13.5.1 Bacterial Pretreatment

Bacterial pretreatment has recently begun to gain considerable attention for biofuel applications; however, it is still in its inception phase. (Zhang et al., 2011) pre-treated autoclaved corn straw with complex microbial agents and saw a 75% increase in methane yield. It has been noted that microbes have high cellulose and hemicellulose degradation potential (Zheng et al., 2014).

Commercial biogas production from microalgae wastewater system utilises bacteria consortium acclimatized to the wastewater system, which has been co-cultivated with the algae as inoculum. Low biogas production has been recorded from wastewater considering the potential that high lipid microalgae promises to deliver. One explanation to this limitation is the difficulty in the degrading the cell wall (Ward et al., 2014). Also, it should be noted that the presence of destructive bacteria during co-incubation in the wastewater system may be a contributing factor to the low biomass productivity levels, as the bacteria could inhibit microalgal growth (Lee et al., 2017). A study has been done on co-cultivation of *C.vulgaris* with bacterium *Flammeovirga yaeyamensis* for efficient microalgal oil extraction. The results obtained showed the presence of hydrolytic enzymes such as amylase, cellulase and xylanase from these bacteria (Chen et al., 2013).

Since co-cultivation has showed possible inhibition to microalgae growth, the breakthrough in utilising bacteria for microalgae anaerobic digestion may be in selectively choosing bacteria that produce hydrolytic enzymes as an additional inoculum for microalgae anaerobic digestion to maximise biogas production.

2.13.5.2 Fungal Pretreatment

In anaerobic digestion of lignocellulosic materials, one of the major concerns is the degradation of lignin and hemicellulose. There has been investigations on fungal classes to degrade these components. (Sun and Cheng, 2002) investigated the use of different fungi for biogas production and found white-rot fungi to be the most effective in lignocellulose pretreatment. Also, (Mackuľak et al., 2012) observed a 15% increase in biogas when *Auricularia auricala-judae* was pre-treated with sweet chestnut leaves and hay. Other authors have observed increased methane production when lignin agricultural residual biomass are pretreated with fungi (Muthangya et al., 2009, Zheng et al., 2014, Zhao, 2013).

Several studies using fungi pretreatment with agricultural residues and hardwood as feedstock between 28 to 37^{o}C temperature and HRT of 12 to 21 days aerobic condition have realised improvement in methane production by 15% and up to 5 fold (Take et al., 2006, Amirta et al., 2006, Mackuľak et al., 2012, Muthangya et al., 2009).

Other studies conducted showed the potential of fungus *aspergillus sp.* to degrade lignocellulose, producing cellulase which can help in cell wall degradation of microalgae

(Pérez et al., 2002, Phutela et al., 2011, Taseli, 2008, Xie et al., 2013). Fungi pretreatment has also been observed during fungi flocculation with microalgae. Fungal flocculation of algae occurs in nature and can be demonstrated in lichens (Wrede, 2019b), however, scientific research for fungal microalgae pretreatment and anaerobic digestion is in the early stages. (Wrede, 2019a) investigated the capability of different fungi to flocculate algae and the results showed *Aspergillus oryzae* to be the most effective species with 95% algae removal in monoculture flocculation. When tested on mixed algal communities from wastewater system, the algae removal value was over 70%. This shows fungi's potential in pre-treating algae for wastewater systems. (Prajapati et al., 2016) used *Chroococcus sp.* algae flocculated with *Aspergillus lentulus* fungus to show 100% flocculation efficiency in under 6 hours with significant cellulase production, release of sugars from the algal cells and a 54% increase in anaerobic digestion as well as a 50% improvement in methane production. While Prajapati et al. (2016) investigated the use of fungi for improvement in methane production, information on its effect on anaerobic hydrolysis (the rate limiting step of anaerobic digestion) and its effect on the cell wall strength is yet to be explored.

2.13.5.3 Enzymatic Pretreatment

Since enzymes act on specific substrates sites within a cell, hydrolytic enzymes will catalyse complex polysaccharide compounds within the microalgae cell to simpler molecules. For example, cellulase acts on cellulose to produce glucose, protease acts on proteins to produce amino acids whilst lipase will act on lipid components of a cell (Aarthy A, 2018).

Enzyme pretreatment is greatly influenced by enzyme dose, pH, temperature, agitation and exposure time (Passos et al., 2014b). (Liew et al., 2019) reported a 7 to 76% increase in biogas production after biological enzyme pretreatment of high strength wastewater using lipase, amylase, protease, and ligninolytic enzymes. The study stated that it is important to optimize pretreatment conditions to improve hydrolysis and suggested that increased enzyme loading may be needed to attain similar hydrolytic rate when enzymes are not in optimal operation. In addition, results from the tests conducted showed that conditions of pH 7-8, 30 to 55^{o}C temperature and 1 to 2% w/w enzyme concentration were optimal for enzymatic pretreatment (Liew et al., 2019).

Enzymatic hydrolysis is beneficial as it can occur at low temperatures preventing oil oxidation and requiring reduced energy for operation (Lari et al., 2019). In fact, a study conducted by Surendhiran and Vijay (2014) showed enzymatic hydrolysis as a more efficient cell disruption technique than mechanical treatments. Enzymatic lysis using cellulase and lysozyme enzymes to pretreat *Chlorella sp*. at an enzyme dose of 5 mg/l, a temperature of 55^{o}C for 10 hours resulted in a lipid released concentration of 24% and 22% of dry weight respectively (Zheng et al., 2011). Also, there are studies that have shown increase in lipid recovery when

enzymes have been used in addition to other pretreatment methods. For example, there was 90% increase in lipid extraction from *Nannochloropsis sp*. when enzyme was used following alkaline treatment (Wu et al., 2017). In addition, 92.6% lipid recovery was observed when enzymatic treatment was utilised alongside high pressure homogenisation (Wang et al., 2015). Although enzymes have been identified as a feasible alternative pretreatment method, there are still issues regarding its cost effectiveness. Current research and investigations around enzyme pretreatment have been aimed at identifying low-cost enzymes that are environmentally friendly with reduced application cost.

2.14 Challenges and Merits in Microalgal Pretreatment Methods

Although there have been several advancements in microalgal pretreatment techniques leading to algae cell disruption and improved lipid extraction for biofuel production, there are still challenges that limit the use of some of the pretreatment methods highlighted above.
Table 2.3 below summarises the challenges and merits of various pre-treatment processes for disruption of algae cells.

Table 2.3 Challenges and merits of various microalgal pretreatment methods

Pretreatment Method	**Merits**	**Challenges**	**References**
1.) Bead Milling	Effective energy utilisation when operating with biomass feed concentrations of 100 to 200 g/l.	Difficult to upscale process as an extensive cooling system is required.	(Lee et al., 2012, Aarthy A, 2018)
2.) Bead Beating	Effective for disrupting cells and extracting non-polar lipids with minimal contamination, has low operating cost and use for small or industrial applications	Requires high energy, useful for dense biomass concentration, requires an algae concentration step after harvesting.	(Show et al., 2015, Lari et al., 2019, Greenwell et al., 2010)
3.) High Pressure homogenisation	Lower cooling cost, lower heat formation, no dead volume in reactor, easy	Uses a high amount of energy	(Aarthy A, 2018, Lee et al., 2012)

	scale up, low risk of thermal degradation.		
4.) High Speed homogenisation	Easy, effective with short contact time	Aggressive technique splitting all intracellular components	Aarthy A, 2018
5.) High Pressure Press	Effective pretreatment method	Energy intensive and costly especially for large scale applications	(Show et al., 2015)
6.) Ultrasonication	Provide purity to product, requires short residence time to disrupt cells, high reproducibility, operates at low temperature, simple, economical, prevents protein denaturation	Requires high electricity demand which may unbalance the net energy input and output	(Lee et al., 2013c, Passos et al., 2015a)
7.) Freeze Drying	Easy, ensures no loss of lipids, improved efficiency	Expensive and requires high energy consumption	(Pourmortazavi and Hajimirsadeghi, 2007, Aarthy A, 2018)
8.) Osmotic Shock	Effective in enhancing lipid extraction	May require longer treatment time	(Lari et al., 2019)
9.) Chemical Treatment	High efficiency, less energy requirement	Possible chemical corrosion, may have chemical reaction forming undesired products, chemicals may be expensive.	(Steriti et al., 2014, Lari et al., 2019)
10.) Microwave	Low cost, useful in algae lipid extraction process, simple and time saving	Possible product damage, requires high energy use for operation	(Lari et al., 2019)
11.) Enzymatic Hydrolysis	Relatively low energy consumption, short extraction time and higher productivity, more efficient disruption	High cost of enzymes	(Aarthy A, 2018)

12.) Pulsed Electric field	Can improve pore formation leading to cell wall solubilisation	Less efficient due to release of metabolites from disrupted cells	(Joannes et al., 2015, Aarthy A, 2018)
13.) Thermal (e.g., waterbath <100^{o}C)	Low energy consumption	Increased exposure time	(Passos et al., 2014b)
14.) Biological pretreatment	Low energy requirement, no chemical needed, environmentally friendly, cost effective, constant release of hydrolytic enzymes hence very practical	Possible low efficiency in some instances as various bacteria and fungi secrete different enzymes which have diverse effects on pretreatment. Also, bacteria and fungi may compete for nutrients with microalgae if grown together, long pretreatment time, knowledge of cell wall structure of microalgae and enzyme secreted by the bacteria or fungi to know target site.	(Zhang et al., 2020, Barati et al., 2021).

2.15 Microalgae cell wall degradation issues

It has been postulated that the presence of algaenan (an aliphatic non-hydrolysable polymer found in a thin algae cell wall of about 10-20 nm with a trilaminar structure (TLS)) may be responsible for bacterial resistance during hydrolysis or degradation (Gerken et al., 2013, Sander and Murthy, 2009). The ultrastructure of *C.vulgaris* reveals two distinct layers as shown earlier in Figure 2.2, with the inner layer consisting of a lower electron density than that the outer layer, which has long protruding hair like fibers that act as hydrophilic surface preventing attachment to the cells (Gerken et al., 2013). Degrading this outer layer will enable better attachment of bacteria and fungi leading to biomass solubilisation and eventual breakdown of the inner cell layer (Gerken et al., 2013).

An experiment showing changes in *C.vulgaris* cell structure following enzyme degradation conducted by Gerken et al. (2013) using fluorescent DNA staining and transmission electron microscopy reveals that *C.vulgaris* is sensitive to lysozyme enzymes which is responsible for degrading N-acetylglucosamine. The lysozyme enzyme usually acts on bacterial peptidoglycan and functions in such a way that it increases the outer surface of the electron-dense layer, removing the hair-like fibers in the process. The hair like fibers on the surface are composed of polysaccharide chains. In other words, lysozyme causes hydrolysis of peptidoglycan in *C.vulgaris* cell wall (Dvoretsky D., 2016), providing room for further bacterial hydrolysis of cell. Although this study provided insight on lysozyme effect on *C.vulgaris* for biofuel production, there was no specific information on its effect on the cell wall strength and anaerobic hydrolysis to improve volatile fatty acids production and subsequently digestion. Therefore, experiments involving lysozyme and a mixture of bacteria normally found in the anaerobic digestion process for their impact on hydrolysis could provide a means to increase methane production from anaerobic digestion of microalgae.

Also, current anaerobic digestion in wastewater systems occurs using resident microbial community already acclimatized in the system. There have been reports, however, of low biogas production resulting from the rigidity of microalgae cells (Ward, 2015). In spite of this, there is limited knowledge on the use of additional microbes to the wastewater system to improve the bacterial community leading to efficient digestion.

2.16 Key Research Questions and Aims

The gaps identified in this literature review have led to the following questions:

- What pre-treatment methods are most effective to weaken *C.vulgaris* cell wall by a cost and energy efficient means?
- Can measurement of the ability to break *C.vulgaris* cells mechanically be used to determine the effectiveness of pre-treatment prior to anaerobic digestions? Is there a current technique to measure cell wall strength of wet *C.vulgaris*?
- Could lysozyme be a key enzyme in degrading *C.vulgaris* cell wall for anaerobic hydrolysis?
- What is the effect of enzymatic pre-treatment using enzyme combination of lysozyme, amylase, pectinase & cellulase on *C.vulgaris* cells?
- Can selective bacteria improve the efficiency of anaerobic digestion?
- Are fungi useful in weakening *C.vulgaris* cell wall for improved anaerobic hydrolysis?
- Estimate the energy efficiency of the pretreatment techniques mentioned above.

Since the project was focused on anaerobic hydrolysis of microalgae with process efficiency determination from estimation of VFA production, only hydrolysis and acidogenesis stages were utilised assuming a two-stage digestion process.

Selective microbes including *L.plantarum, E.coli, S.thermophilus A.aceti* and *Aspergillus oryzae fungus* were used in various experiments during this study to investigate the use of isolated microbes for increasing the solubilisation of microalgae and to foster bacterial hydrolysis. The microbes selected for this project considered factors such as the risk group category, type of anaerobic reaction required, i.e. hydrolysis and acidogenesis, reactor temperature condition and enzyme produced as specified in Table 2.2.

Firstly, *E.coli* was selected as it's safe and within risk group 1 category. *E.coli* is used as a hydrolytic bacterium to promote bacterial hydrolysis due to its secretion of amylase enzyme. Moreover, most *E. coli* strains do not cause disease as they are free living in human and animal intestines (MayoClinic, 2016). Also, they are able to operate in mesophilic temperatures. *E. coli* has an optimum growth of 37^{o}C and evidence from laboratory test growth shows they can withstand temperatures of up to 49^{o}C (Fotadar et al., 2005). Next, *E. coli* is easy to grow and has a long shelf life. When the conditions for growth are favourable, it has a doubling time of 20 minutes.

Further bacterial hydrolysis might be accomplished by using *Streptococcus thermophilus*, as Genus Streptococci that is a major hydrolytic anaerobic bacterium as documented by several authors (Ziemiński and Frąc, 2012, Christy et al., 2014, Adekunle and Okolie, 2015).

S.thermophillus has an optimum growth temperature of 42^{O}C.They also do not form spores which helps to limit contamination and reproduction. In terms of compound production, *Streptococcus*, *Lactobacillus*, and *Bacillus*, for example, produce lactic acid, while *E.coli* produce ethanol, lactic acid, succinic acid, acetic acid, CO_2, and H_2 (Delorme et al., 2010). Furthermore, *A.aceti* is a major producer of acetic acid in anaerobic hydrolysis, which would eventually promote Acetoclastic reaction in the production of methane. Acetic acid accounts for 70% methane production during anaerobic digestion (Ali Shah et al., 2014). So, low volumes of acetic acid with this bacterium would confirm the products of hydrolysis have quickly transformed into further products, including methane production, which is, however, not the key focus of this study. In addition, it is beneficial to have different bacteria strains go into the reactor so in case one strain dies off, the other strain can still continue the hydrolytic process.

Lactobacillus was chosen as it's a hydrolytic bacteria and various species are capable of secreting lysozyme enzyme (Saygusheva et al., 2013). Lysozyme has been found to degrade the outermost hair like layer of *C.vulgaris* (Gerken et al., 2013), so removal of the outermost cell wall of *C.vulgaris* with *Lactobacillus* could be possible.

Finally, *Aspergillus oryzae* has been identified as secreting cellulase which is a hydrolytic enzyme in breaking down cellulose, a major component of the thick cell wall of most microalgae (Prajapati et al., 2016, Bhattacharya et al., 2017, Wrede, 2019b). Therefore, investigating the potential of using this fungus to degrade the cell wall of *C.vulgaris* for anaerobic hydrolysis will contribute additional knowledge to microalgae cell degradation during anaerobic digestion.

2.17 References

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CHAPTER 3 COMPARISON OF PRETREATMENTS FOR IMPROVED *CHLORELLA VULGARIS* DISRUPTION EFFICIENCY.

3.1 Introduction

Over the last decade, microalgae have been researched extensively as a source of biofuel production for products such as biodiesel, biogas, bioethanol and bio-hydrogen that can further be converted into electricity, energy generation, or used as transportation fuels (Benemann, 2013, Benemann, 2008, Brennan and Owende, 2010, Demirbas, 2011, Moheimani et al., 2015b). The focus on microalgae has been mainly due to their use of non-arable land for cultivation thus reducing the competition for growing food crops, their high photosynthetic efficiency, their high lipid/oil content and carbon neutrality (as the algae require carbon to grow creating balance in atmospheric carbon and reducing greenhouse emissions) especially in wastewater systems (Craggs et al., 2013, Moheimani et al., 2015a, Slade and Bauen, 2013). Microalgae use in biofuel research has mainly been focused on biodiesel production due to increasing global demand of transport fuels. This has nevertheless been limited due to the large energy demand for biomass harvesting, dewatering and processing. Anaerobic digestion has been investigated as a much lower energy intensive process for biogas production, (Wiley et al., 2011). Anaerobic digestion is a biological process in which organic matter is broken down in the absence of oxygen to produce biogas. (Rodriguez et al., 2015). Hence, anaerobic digestion of microalgae uses microbes such as bacteria and fungi as inoculum to breakdown down microalgae substrates saving energy and reducing carbon emissions.

Anaerobic digestion of microalgae has been researched for biogas production (Passos et al., 2014b), as the process has the potential to use all the degradable components of the microalgae cell including carbohydrates, proteins and lipids to produce biogas (Ward, 2015). However, a major challenge for anaerobic digestion is the hard cell wall of the microalgae because it prevents penetration by microorganisms and extracellular enzymes (Christy et al., 2014).

To resolve this issue, it has been suggested that a pre-treatment step is required to disrupt microalgae cell walls, resulting in microbial access to the inner microalgae contents and an increase in microalgae hydrolysis and subsequent biogas production for further digestion (Ward et al., 2014). There are different types of microalgae pretreatment including mechanical (e.g., ultrasound, sonication, microwave, bead milling, beat beating, high pressure press, high speed homogeniser e.g., freeze drying, manual grinding, and osmotic shock), heat or thermal treatment (e.g., waterbath, autoclave, microwave), chemical treatment (e.g., acid or base treatment) and biological treatment (e.g., bacteria, fungi and enzymes).

Microalgal pretreatment is influenced by certain traits of the cell, such as its structure and composition. The major factors affecting the pre-treatment of microalgae biomass and subsequently methane production are; the amount of cellulose/hemicellulose in the cell wall structure and composition of cell membrane containing carbohydrates (Passos et al., 2014b, Gerken et al., 2013). The complexity of the microalgal cell wall leads to resistance to bacterial or microbial attack during degradation. Studies have shown that microalgae without a thick cell wall produce higher methane levels than those with thick, complex walls (Mussgnug et al., 2010).

Most microalgal cells have been shown to have a trilaminar cell wall structure (as seen in Figure 2.2 in chapter 2), which contains polysaccharides such as cellulose, uronic acid, protein, mannose, and xylan (Lee et al., 2012)

In addition, the cells contain 'algaenan', a biopolymer that contributes to the resistant property of the cell (Aarthy A, 2018). Algaenan serves as an extra protective coating on the cell wall and its polymeric property resists microbial attack, and results in the inability of microbes to penetrate the microalgae cell wall efficiently leading to low digestion rates and making anaerobic digestion ineffective.

Transmission electron microscope imaging has shown disruption of microalgae cell walls after hydrothermal pretreatment (Passos and Ferrer, 2015). The images have also shown improvements in cell solubilisation as the images displayed swollen microalgae cells and the experimental results after anaerobic digestion showed improved hydrolysis and methane production (Passos et al., 2014b, Passos and Ferrer, 2015). (Liew et al., 2019) highlighted that effective pretreatment involves; formation of sugars directly or through hydrolysis, degradation of cells during hydrolysis, and limiting inhibitory products by energy and cost-efficient means.

The overall objective of microalgae pretreatment is to efficiently disrupt the cell wall and release or make accessible intracellular components such as lipids in the form of triglycerides, carbohydrates and glycoproteins that can be broken down via anaerobic digestion and used for biogas generation. The efficiency of the cell disruption process is species specific and is influenced by the cell disruption method chosen whether mechanical, thermal, chemical or biological. However, some microalgae pre-treatments have high energy and cost implications. A pretreatment method is economical if the energy and cost generated is greater than the energy and costs utilised during pretreatment (Lari et al., 2019). Besides cost, another factor that can limit a pretreatment method is scalability. Certain cell disruption methods such as French press, grinding, microfluidizers and bead mills are not effective for large scale industrial applications (Lee et al., 2012).

Microalgae pretreatment is necessary to improve cell disruption efficiency. The degree of cell disruption of microalgae cells can be determined using quantitative and qualitative means

such as visual observation via cell counting, lipids extraction, chlorophyll analysis, volatile fatty acids production and estimation of biogas yield. Upon pretreatment, cells can be viewed under a microscope and differentiated by estimating the number of intact and disrupted cells. A percentage disruption of the cells can then be estimated. To improve this counting assay, it is sometimes helpful to employ a staining technique using trypan or methylene blue dye so that the inside of the disrupted algae cell is also stained with the dye, enabling easier identification of algae with cell walls that have been broken. Also, a haemocytometer may be used to determine the number of disrupted cells and intact cells. A haemocytometer is a calibrated glass slide used for direct counting by placing a culture drop on the grid chamber and counting the number of cells per grid using a light microscope (Liu, 2016).

There are several study reviews on comparisons of microalgae pretreatment methods including mechanical, thermal, chemical and biological (Passos et al., 2015a, Cho et al., 2013, Jankowska et al., 2017). However, most compare mixed microalgae species. (Passos et al., 2014b) suggested that comparison of microalgae pretreatment techniques is more effective if the same microalgae biomass is used as algae pretreatment is species-specific. The study also stated that optical microscopic examination upon pretreatment is necessary in understanding pretreatment effect on the cell structure. In addition, there is little information on efficiency of high-speed homogenisation treatment in comparison to other methods.

The aim of this chapter was to evaluate and compare various microalgae pretreatment techniques such as mechanical (high speed homogenisation), thermal (waterbath and autoclave), thermo-mechanical (waterbath-high speed homogeniser) and enzymatic (lysozyme) using *Chlorella vulgaris* (*C.vulgaris)* microalgae as a model system and to determine their efficiency in breaking the cell wall using microscopic analysis and cell counting techniques. The results are expected to help identify a suitable technique for achieving cell disruption and to identify a suitable pretreatment to anaerobic digestion of algae.

3.2 Materials and Methods

3.2.1 Microalgae cultivation

The microalgal strain, CS-42 *C.vulgaris* Beijerinck was obtained from CSIRO I.U.C.C ex Cambridge, strain CCAP 211/11. The strain was originally cultured in MLA medium and re-cultured in Bolds basal medium (CSIROMarineResearch) in 2L conical flaks with continuous stirring under artificial lights on a 16:8-hour light intensity and ambient temperature. Air was bubbled into the flasks at 200 ml/min. Biomass growth was monitored using a UV biochrom libra s22 spectrophotometer at wavelength of 750 nm.

For quality control, various batches of microalgae were used in separate experiments and harvested towards the end of the exponential growth phase using optical density value range of 1.7 to 2.0 scanned at 750 nm, which is an acceptable wavelength for spectrophotometrical absorbance (Rodrigues et al., 2011).

3.2.2 Microalgae Pretreatment Methods

Several means of disrupting the *C.vulgaris* cells using autoclave, waterbath, high speed homogenisation, waterbath-high speed homogenisation and lysozyme enzyme pretreatment methods were compared, and their efficiencies analysed to evaluate the strength of the cell wall. Experimental techniques were verified by observing morphological changes on the cells showing visual cell disruption using a light microscope and measuring the percentage cell disruption using cell counting techniques.

Each pretreatment technique used a different batch of algae. All pretreatment methods conducted were in duplicates with disruption rate recorded as mean values. Algae concentration was measured using a wavelength at 750 nm with absorbance values given in table 3.1 below. The variance and standard deviation of the absorbance values were found to be 0.0077 and 0.087 for the different algae batches employed.

The total solids of the microalgae were determined by drying 100 ml of the algae in the oven at 105 ^{o}C overnight and measuring the dry microalgae biomass by calculating the difference in weight of the beaker without algae and with the dried algae content. The values also given in table 3.1.

Table 3.1 Optical densities and dry weight (total solids-g/100ml) of the various pretreatment techniques.

Pretreatment	**OD_{750nm}**	**Dry weight (Total solids in g/100ml)**
Waterbath	1.775	2.7754
Autoclave	1.796	3.0096
High-speed homogeniser	1.932	3.402
Lysozyme enzyme	1.733	2.6042
Waterbath-High speed homogeniser	1.913	3.396

In each experiment, 100 ml of sample from the original culture flask was pre-treated using autoclave at 121°C for 15 minutes, water bath at roiling boil, waterbath(roiling boil)-high speed homogeniser (for 5 minutes 4,000 rpm) combination, or lysozyme enzymatic treatments respectively. The tests were performed in duplicates and the average cell disruption was calculated.

The percentage disruption was evaluated using a motic BA310 John Morris Scientific light microscope and a Neubauer hemocytometer was used to visualise and count the cells , an established method outlined by (SELVAKUMARAN and JELL, 2005) as well as image-J image analysis software. Prior to visual examination, the hemocytometer and coverslip were sprayed with 70% v/v ethanol and cleaned with a tissue. After pretreatment, 1 ml of the algae solution was collected and mixed with 0.1 ml of 1:10 dilution of 0.4% trypan blue and kept at room temperature for 2 to 3 minutes to allow thorough mixing and to prevent prolonged exposure to cells. Then, the sample was pipetted into the hemocytometer chamber ensuring no formation of air bubbles and viewed using a light microscope. Initial cell examination and imaging was conducted at a low magnification of 40X, then increased to 400X for the cell counting images. At 1000X, oil was added to the lens for clarity in viewing the images to obtain a better image of the cell structure.

Under the microscope, broken and disrupted cells were stained, retaining dye from the trypan blue and enabling easy identification of visible cell disruption, while the unbroken cells were not stained blue. The number of stained and unstained cells were then counted in the centre square to estimate the percentage cell disruption for each pretreatment technique. Finally, images of each sample were taken at 400X magnification.

3.2.2.1 Autoclave Thermal Pretreatment

Thermal pretreatment was undertaken using an Atherton Cyber CEB9612 model autoclave by autoclaving 100 ml of *C.vulgaris* cells using wet sterilisation at 121^{o}C for 15 minutes, with 15 minutes heating and cooling down. The *C.vulgaris* cells were harvested at the end of their exponential growth phase at optical density of 1.796 at 750 nm. The cells were allowed to cool to room temperature after autoclaving before observing visual changes before and after pretreatment.

3.2.2.2 Waterbath Thermal Pretreatment:

C.vulgaris grown in 2L conical flask was harvested at optical density of 1.775 at 750 nm. Prior to the test, 1 ml of sample was extracted for visual observation to ensure intact cells. Duplicate pretreatment experiments of four separate 100 ml algae suspensions placed in a 250 ml laboratory beaker were subjected to thermal pretreatment using a Thermoline NB6T model waterbath at intervals of 5, 10, 15 and 20-minutes incubation times respectively. Thermolysis was induced at roiling boil of 100^{o}C to allow solubilisation of the microalgae and cell disruption. The samples were allowed to cool to room temperature and 1 ml collected for microscopic analysis. Furthermore, the sample was stained with trypan blue and allowed to mix for 2 to 3 minutes to ensure dye absorption by broken cells. A drop of the stained mixture was placed on a Neubauer haemocytometer slide to determine percentage cell disruption by counting the number of stained cells as a percentage of the total cell number.

3.2.2.3 High Speed Homogeniser Mechanical Pretreatment

C.vulgaris cells harvested at optical density of 1.932 at 750 nm was pre-treated using a CAT unidrive x1000 high-speed homogeniser at various speeds of 4,000, 8,500, 15,000, 20,000, 25,000 and 33,000 rpm respectively. For the tests, duplicate 100 ml algae solution were treated at each speed for 5 minutes. After pretreatment, image analysis and cell counting were conducted for homogenised and unhomogenised cells to evaluate percentage cell disruption.

3.2.2.4 Combined Waterbath- High Speed Homogeniser Pretreatment

A pretreatment combination using water bath and high-speed homogeniser was further investigated. *C.vulgaris* cells at optical density of 1.913 at 750 nm were used. Duplicate 100 ml algae cells in solution were subjected to heat treatment using a water bath at rolling boil for 20 minutes and further homogenised for 5 minutes using the same high-speed homogeniser at 4,000 rpm. Cell solubilisation and degradation were then identified using the light microscope and percentage disruption calculated by counting the number of stained cells.

3.2.2.5 Lysozyme Enzymatic Pretreatment

Enzymatic pretreatment using lysozyme enzyme to solubilise *chlorella vulgaris* cells was investigated. Lysozyme from chicken egg white obtained from Sigma Aldrich, material number L6876-10G was used as enzyme for the test. The algae cells were harvested at optical density of 1.733 at 750 nm. An enzyme dose of 20 mg/ml stock solution of lysozyme was prepared in distilled water. The effect of lysozyme enzyme concentration and time was evaluated. Various volumes of enzyme stock solution including 25 μl, 50 μl, 75 μl and 100 μl, were added to 1 ml samples of *C. vulgaris* and each sample allowed to react over time intervals of 1-hour, 2-hour, 3-hour and 24-hour respectively. Light microscope images were taken before and after pretreatment to observe visual changes and cell disruption was evaluated by counting the number of stained cells.

3.2.3 Cell counting method

To verify the experimental process, in addition to microscopic analysis, a cell counting technique was employed. To count the number of broken cells from the pre-treatment methods used, the cells were first stained with trypan blue dye after cell disruption. The broken cells absorbed the dye after bursting open and became darker in colour showing evidence of cell disruption. Intact cells remained un-dyed and retained their natural green colour.

To quantify the number of disrupted and total cells, cell counting was performed using a Neubauer cytometer grid of 0.0025mm^2 using 400X magnification. Initial cell counting was performed manually by counting the total cells and darker cells of a selected square containing 16 individual squares using the motic BA310 light microscope.

For cell images taken at 1000X*, image J* cell counting software was used by quantifying the number of dead cells from live cells. Images analysed are provided in the appendix section of this study. To count the number of disrupted cells, the equation below was used.

Equation 3.1 showing cell viability

$$Cell\ viability = \frac{Number\ of\ intact\ cells}{Total\ cells\ (Number\ of\ intact + Number\ of\ broken\ or\ dead\ cells)} \times 100$$

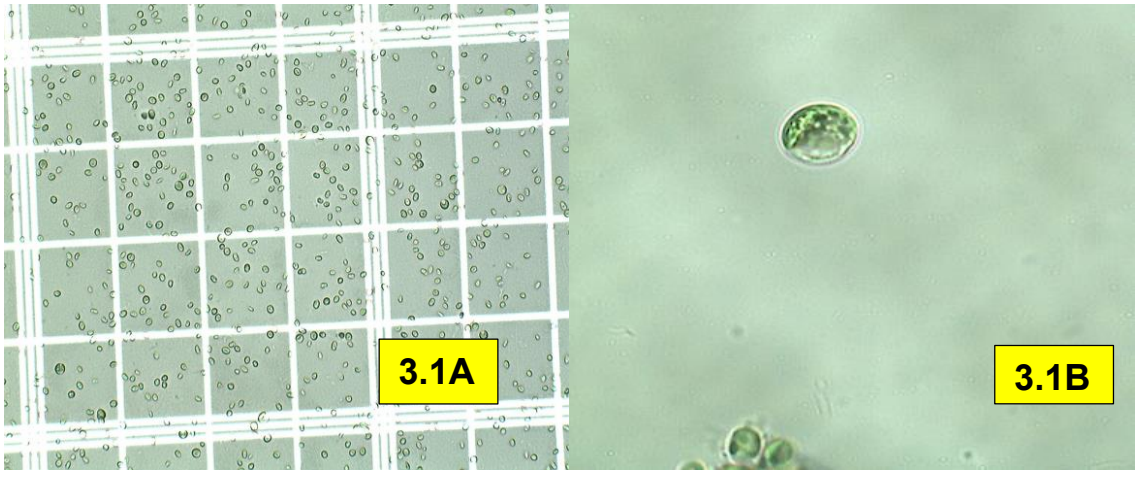
$$Disruption\ rate\ (\%) = 100 - cell\ viability.$$

3.3 Results and Discussion

3.3.1 Autoclave Thermal Pretreatment Imaging

Autoclave pretreatment showed low average disruption rate compared to all other methods used at 42%. This may be as a result of the effect of heating and cooling during warm -up and cooling down stages in the process resulting in inefficient heat circulation. Previous studies using autoclave for 100 ml algae suspension at 121^{o}C for 30 minutes have also recorded low disruption efficiency owing to the slow rate of heat diffusion from the environment into the algae cell walls, making heat distribution inefficient (Lee et al., 2010, Kaufmann et al., 2001). Another explanation may be the low algae concentration in the drop sample that was analysed under the microscope. Besides, the temperature of the metallic container holding the sample in the autoclave during the cooling stage may also have contributed to lowering the application of heat treatment effect on the cells.

Upon pretreatment, there were visual changes to the cell structure observed under the light microscope. After staining with trypan blue, there was visible staining of some of the cells observed showing dark colouration of the cells from a bright green to a darker green colour indicating broken cells with dye accessible to the cell interior. Also, some of the cells appeared more swollen after autoclaving. Moreover, images obtained from autoclave treatment showed disfigured cells with damaged intracellular organelles upon heat application as shown in Figure 3.1 below. Total average cell disruption recorded from autoclave pretreatment using the image-J software was 42% with a standard deviation of 0.78.



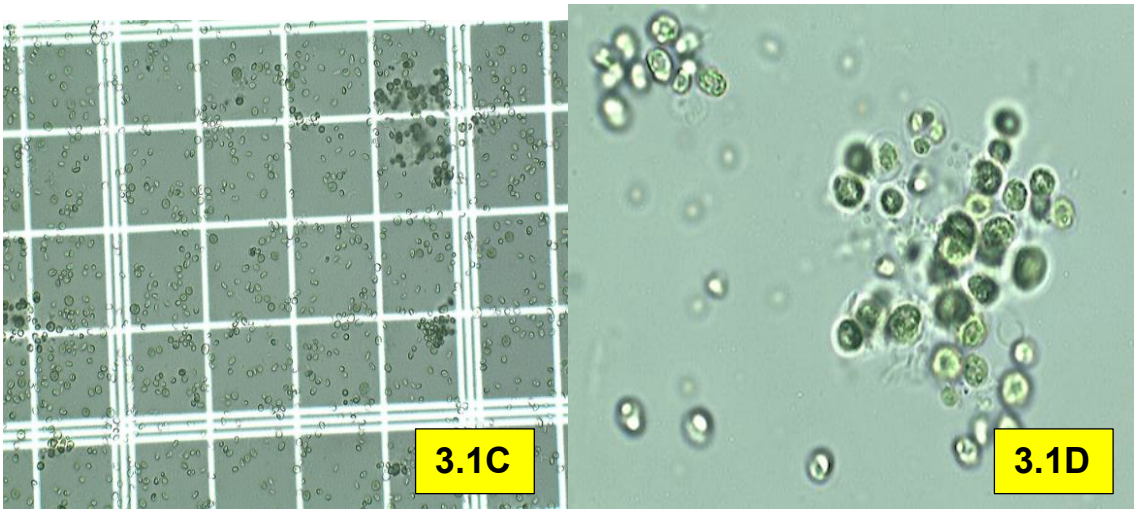


Figure 3.1: Autoclave Pretreatment showing control image (without treatment) in Figure 3.1A (400X) & 3.1 B(1000X) and after autoclave pretreatment on the right at 121°C for 15 minutes in Figure 3.1C(400X) and 3.1D (100X).

3.3.2 Waterbath Thermal Pretreatment Imaging

Water bath pretreatment showed 20 minutes to be the most effective treatment time for cell disruption with a mean rate of 65%. However, 10-minute and 15-minute displayed almost equal results at 58 and 61% disruptions. When compared to 5-minute water bath treatment, there was a 15% increased disruption rate from 5 minutes to 20 minutes. A graph showing the average disruption and error bars for water bath pretreatment at different treatment times at 100°C is shown in Figure 3.2 below.

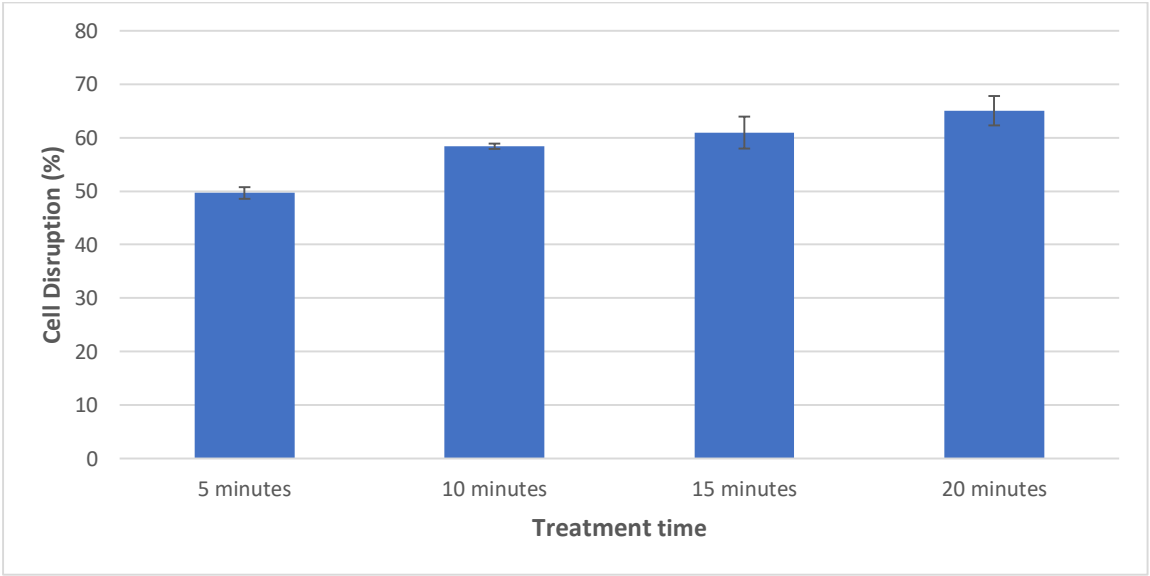


Figure 3.2 Water bath pre-treatment cell disruption at different contact times.

Images taken after the waterbath treatment looked intact but swollen and took up the dye readily as shown in Figure 3.3. There were several dead cells allowing dye to penetrate the cells, staining the cells in the process. Disruption efficiency increased with increase in pretreatment time with mean disruption rates of 50%, 58%, 61% 65% and standard deviations of 1.09, 0.5, 2.99, 2.75 for 5, 10, 15- and 20-minutes treatment times respectively.

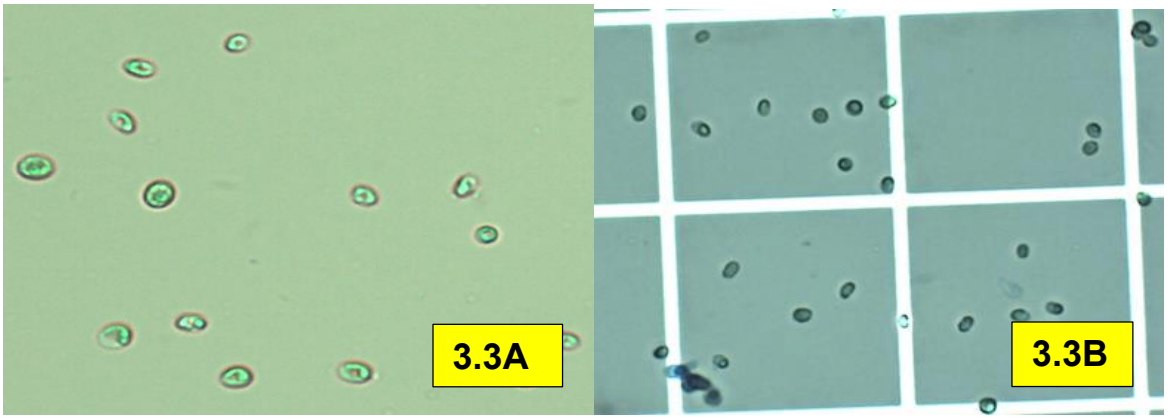


Figure 3.3 Waterbath Pretreatment showing control image in Figure 3.3A(1000X) and after pretreatment at 400X, 100°C for 20 minutes in Figure 3.3B.

3.3.3 High Speed Homogeniser Mechanical Pretreatment Imaging

Mechanical pretreatment using the high-speed homogeniser showed a continuous increase in disruption rate as higher speeds were applied, with maximum disruption at 33,000 rpm resulting in almost 80% cell disruption. There was just a 57% increase in cell disruption when increasing the homogeniser speed from 4,000 rpm to 33,000rpm. Cell disruption climbed gradually from 23% at 4000rpm to 80% at 33,000rpm. A sharp spike in disruption began from 15000rpm with disruption rate increasing by 41% from 8,500 rpm to 15000rpm. Comparing disruption rates; there was a 30% increase in cell disruption from 15,000 rpm to 33,000 rpm. Figure 3.4 shows the cell disruption rate using high speed homogeniser pretreatment.

After high-speed homogenisation, the cells were damaged with split internal components showing visible separation of the thin membrane from the rest of the cell. The external cell layers were cleaved with significant shearing and rupture to the cell displaying cell degradation. The shapes of some cells also appeared disfigured and became irregular. Some broken cells clearly showed oil within them that was presumably lipids. The broken cells collected the trypan blue dye and were stained in the process.

The mean disruption rate increased with increase in homogeniser speed providing values of 23% at 4,000 rpm, 33% at 8,500 rpm, 56% at 15,000 rpm, 57% at 20,000 rpm, 66% at 25,000 rpm and 80% at 33,000 rpm. The standard deviations were as follows: 2.59, 0.07, 0.44, 0.0, 0.51, 0.73 according to the increasing speeds.

Another observation during treatment was a noticeable increase in temperature as homogeniser speed increased, peaking at 68.5°C at maximum homogeniser speed of 33,000 rpm that may have contributed to the disruption efficiency. The increase in temperature also corresponds with the increase in shear rate. The temperature increase was steady, starting at 3°C between 4,000 rpm and 8,500 rpm. This continued across all speeds climbing to 5°C between 8,500rpm and 15,000 rpm, 15°C between 15,000 rpm and 20,000 rpm, 9°C between 20,000 rpm and 25,000 rpm and finally 11.5°C between 25,000 rpm to 33,000 rpm. The images in Figure 3.5 below depicts the visual effect of high-speed homogenisation at 4,000 rpm for 5 minutes.

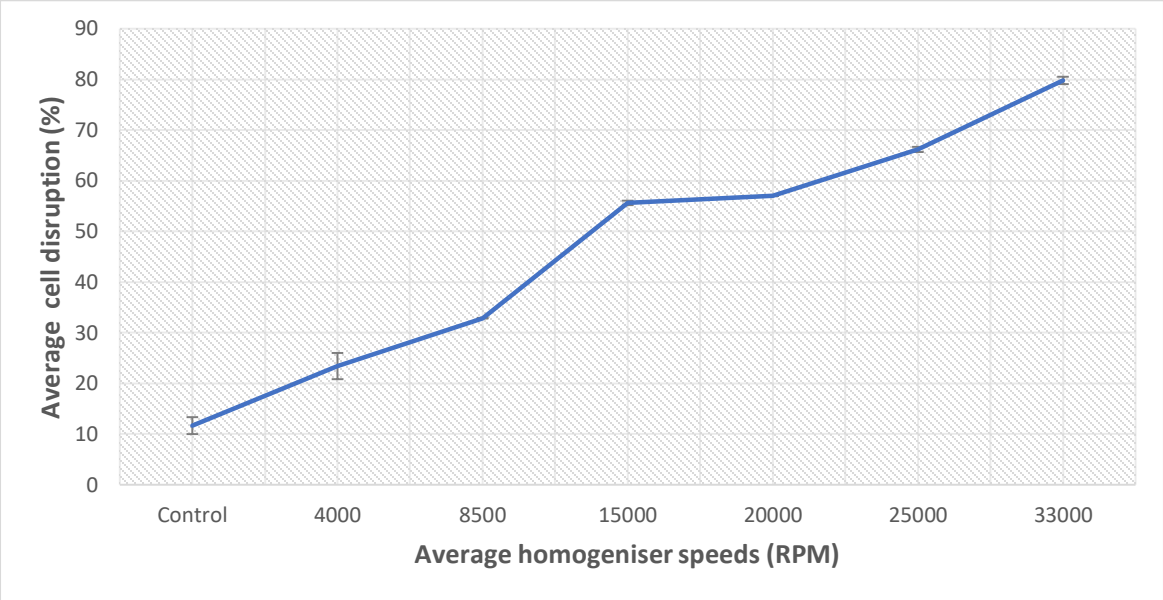


Figure 3.4 Pretreatment cell disruption for various homogeniser speeds.

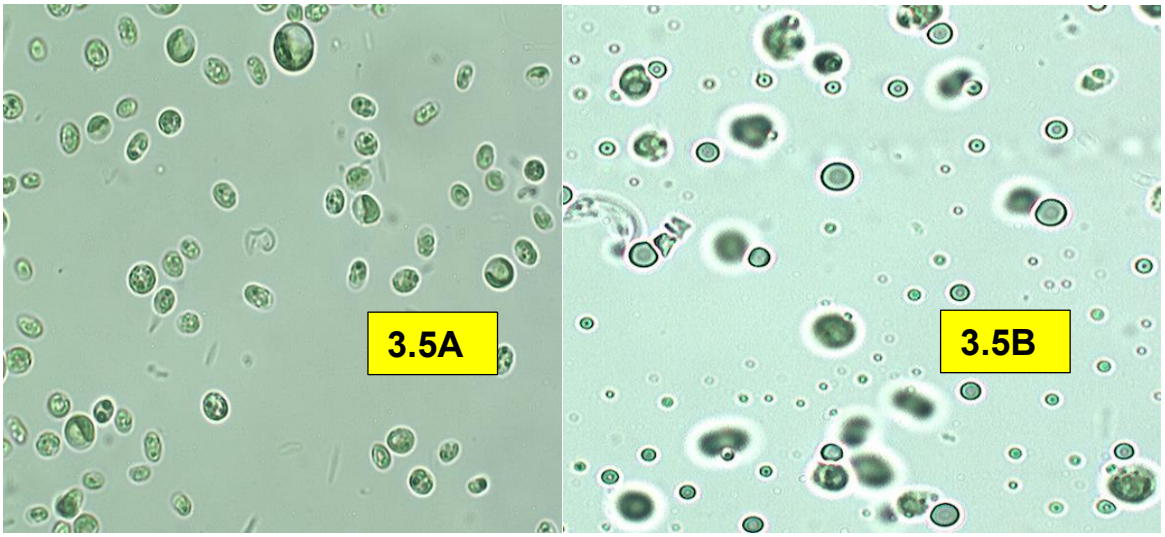


Figure 3.5 showing high speed homogenisation before (Figure 3.5A) and after (Figure 3.5B) pretreatment for 5 minutes at 4,000 rpm at 1000X

3.3.4 Combined Waterbath-High Speed Homogeniser Pretreatment Imaging

Combined treatment of water bath for 5 minutes with high-speed homogeniser at 4,000 rpm for 5 minutes showed a 50% cell disruption rate which was the same as cell disruption of waterbath alone for 5 minutes at 50%. The effect of the waterbath treatment showed more swollen dead cells than fragmented cells. In the future, disruption can be experimented and investigated for 20 minutes water bath and 5 minutes high speed homogeniser.

The microscopic images obtained after combined thermal and mechanical treatment using waterbath at roiling boil for 5 minutes and high-speed treatment for 5 minutes at 4,000 rpm shows a great deal of dead cells from heat application and damaged membranes from shear force from the homogeniser as shown in Figure 3.6. The mean disruption rate recorded was 50% with a standard deviation of 1.95, which is almost 8% more cell disruption than autoclave treatment. There was also a colour difference upon treatment as treated cells became very visibly darker green as a result of temperature increase during treatment killing the cells.

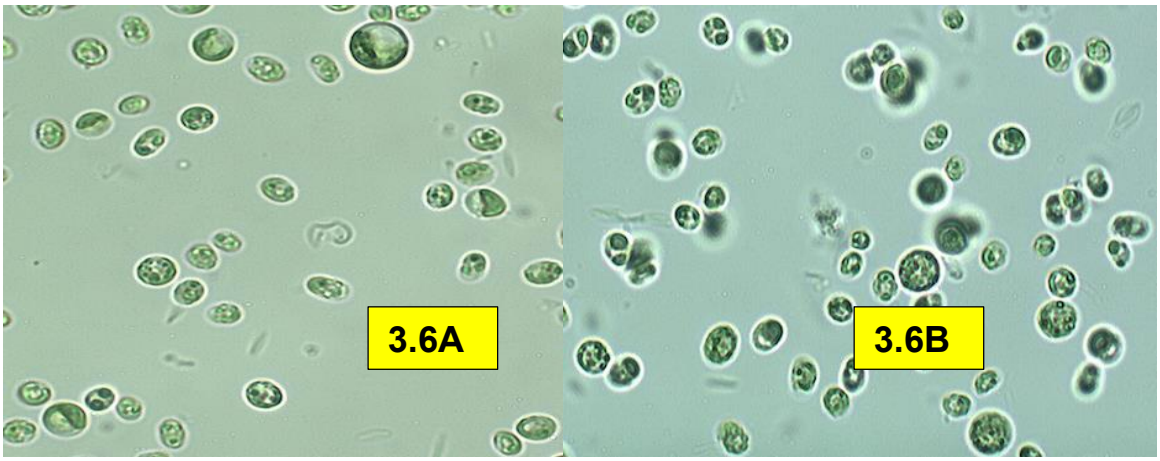


Figure 3.6 showing waterbath-high and speed homogenisation treatment before (Figure 3.6A) and after (Figure 3.6B) at 1000X.

3.3.5 Lysozyme Enzymatic Pretreatment Imaging

All volumes of lysozyme enzyme used had a dose of 20 mg/ml. For 25 µl volume, the disruption rates obtained were: 50% at 1 hour, 55% at 2-hour, 61% at 3-hour and 69% at 24-hour. For addition of 50 µl lysozyme solution the recorded disruption rates were 51% at 1-hour, 55% at 2-hour, 70% at 3-hour, and 76% at 24-hour. Addition of 75 µl of lysozyme solution further increased cell disruption rates as follows: 57% at 1-hour, 68% at 2-hour, 74% at 3-hour and 80% at 24-hour. The most disrupted cells were recorded for additions of 100 µl lysozyme solution with rates of 63% at 1-hour, 74% at 2-hour, 78% at 3-hour and 82% at 24-hour. The results are the mean disruption rates of the duplicate tests conducted with standard deviations range of 0.07 to 3.63%. The results of this test are shown in Figure 3.7.

Lysozyme pretreatment results showed maximum overall disruption at 82% at 100 µl for 24-hour providing the most effective treatment. From the results, 24-hour enzyme incubation time proved to be the most effective timeframe when compared to 1-hour, 2-hour, and 3-hour respectively. The disruption rate increased with treatment time. However, the difference in disruption rate between 3-hour and 24-hour for 25 µl, 50 µl, 75 µl and 100 µl were 14%, 9%, 10% and 5% respectively, which is not a great increase considering the extra time provided for enzyme incubation.

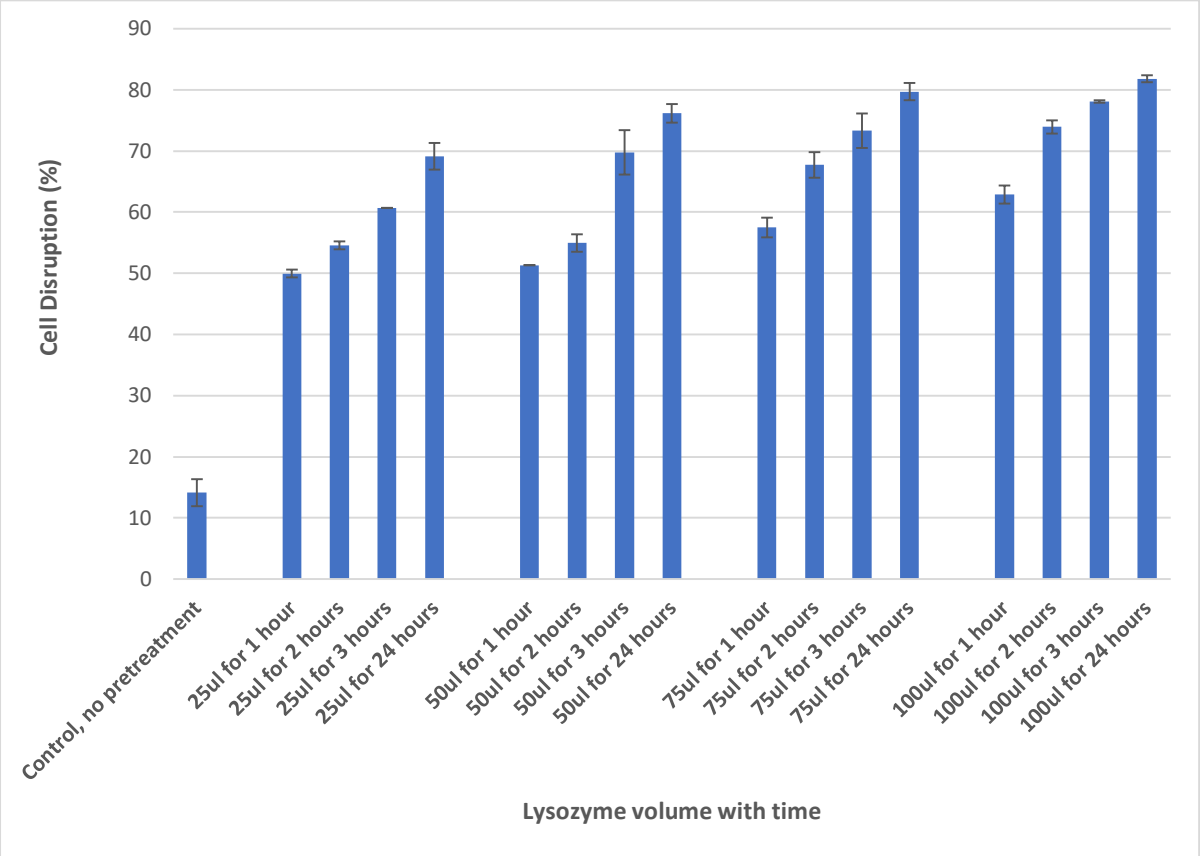


Figure 3.7: Lysozyme pretreatment using different enzyme doses and treatment times.

Lysozyme pretreatment was very effective at disrupting the cells and displayed visible degradation as many algae cells appear to lose their outer covering after lysozyme treatment and were swollen. The cells readily stained upon dye application. Also, cell disruption increased with increased volume of lysozyme application and increased treatment time.
Enzyme treatment from this research has been shown to be affected by time and enzyme volume for the same enzyme dose. Lysozyme's effect on the cell wall of *C.vulgaris* had previously been studied by (Gerken et al., 2013) as a means of degrading the outermost layer of the cell wall removing some hair-like structures on the cell for biofuel application.

From the image analysis obtained in this study, the results agree with (Gerken et al., 2013) and can confirm lysozyme as an effective means to disrupt *C.vulgaris* cells with some of the cells losing their outermost protection as seen in the microscopic images obtained in Figure 3.8.

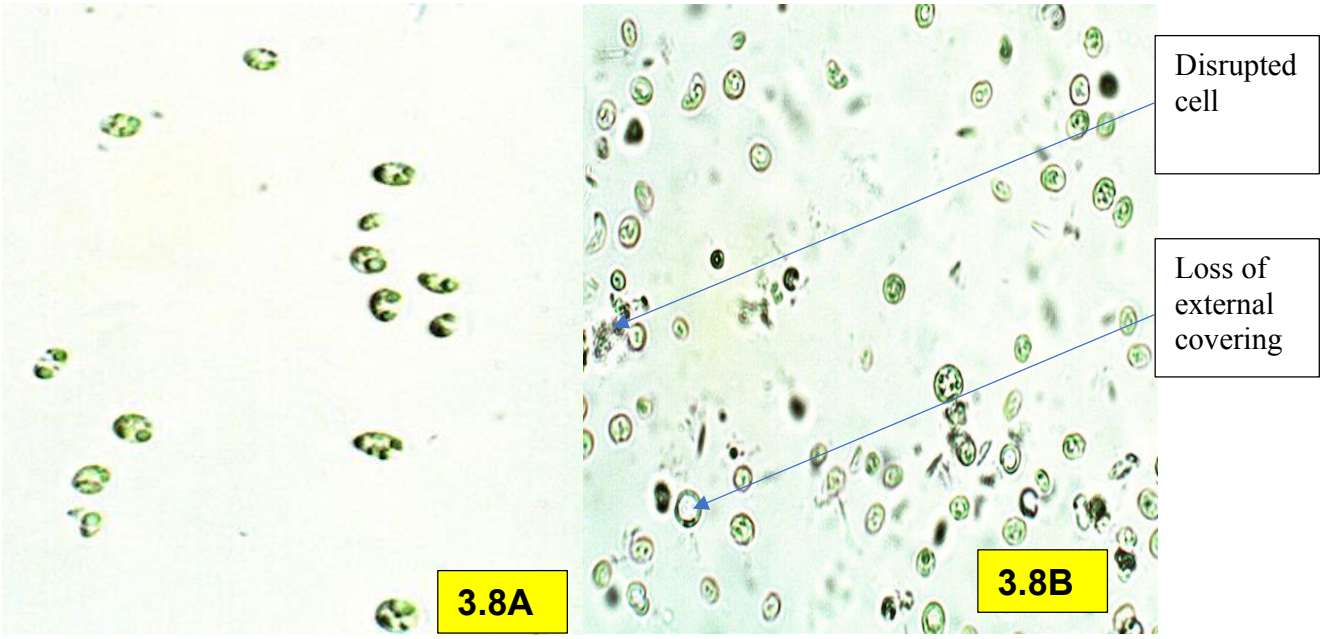


Figure 3.8: Lysozyme control image in figure 3.8A and lysozyme using 100 µl for 24-hour in Figure 3.8B at 1000X.

3.3.6 Comparison of Pretreatment Techniques

Comparing the disruption rates of the various techniques applied during this study, enzymatic treatment using lysozyme enzyme at an applied concentration of 1.8 mg/l (20 mg/L x 0.1 ml/ 1.1 mL =1.8 mg/L) for 24-hour proved to be the most effective technique producing 82% of disrupted cells. Nevertheless, due to the cost of enzymes, 50 µl for 24-hours may be a lower cost alternative volume as the disruption rate was 80%, indicting only a 2.5% increase in disruption rate from 50 µl to 100 µl volume. For greater cost effectiveness of enzyme treatment, research into microbes producing the specific enzymes required for cell disruption and anaerobic digestion should be considered as a means to avoid purchasing purified enzyme. This idea has been explored further and investigated in chapter 5.

Of the cell disruption methods used, autoclave at 121°C for 15 minutes recorded the lowest disruption efficiency. When compared to water bath pre-treatment, the autoclave was a less

effective thermal pretreatment method. There was a 44.48% decrease in disruption rate when comparing autoclave at 121^{o}C for 15 minutes and waterbath at 100^{o}C for 15 minutes. It is possible that the rate of thermal increase and decrease would have affected the cell breakage as the autoclave spent 15 each during the warm-up and cool down stages unlike the waterbath that heated up from room temperature to roiling boil.

Comparing mechanical treatment using high-speed homogeniser and thermal pretreatment using waterbath as well as autoclave, high speed homogeniser showed better performance producing almost 80% disrupted cells at the maximum disruption speed of 33,000 rpm.

Control samples used for all experiments had about 90% viability with mean disruption rates of 11.6%, variance of 2.53 and standard deviation of 1.59. The overall average disruption rate of all methods employed was 56% measured using the Neubauer cell counter and image-J cell counting software.

3.4 Conclusion

The study compared the cell disruption rates of common microalgae pretreatment methods including mechanical, thermal, thermo-mechanical and enzymatic on C*.vulgaris* using microscopic analysis and cell counting technique.

In summary, cell counting, and microscopic analysis are simple but effective techniques to quantify cell disruption efficiency.

Overall, high speed homogeniser and lysozyme enzyme pretreatment offered the most disrupted cells. However, it is also important to analyse the total energy costs involved to determine if these methods are energy efficient. This will be discussed further in Chapter 7.

Following the results obtained from this chapter, research to understand the cell strength of *C.vulgaris* was conducted using the pretreatment methods outlined, except the autoclave, to understand the strength of the cell wall using lipids extraction and chlorophyll analysis in chapter 4. Also, further investigation on the energy and cost utilised versus the disruption efficiency is conducted and detailed in chapter 7.

In conclusion, each pretreatment method is useful for anaerobic digestion. However, lysozyme and high-speed homogenisation were the most effective with 80% cell disruption recorded.

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CHAPTER 4 EVALUATION OF THE CELL WALL STRENGTH OF *CHLORELLA VULGARIS* USING HIGH-SPEED HOMOGENISATION.

4.1 Introduction

Several studies have highlighted the need for microalgae pretreatment as a significant approach in improving anaerobic digestion. In Chapter 3, visual observation of the microalgae cells was conducted using a Neubauer haemocytometer and image-J software as a cell-counting tool to quantify disruption of the cells after pretreatment. However, this method is time-consuming for large cell volumes, hence a better method of quantifying pretreatment effectiveness that utilises larger sample volumes that are more representative of the entire biomass is necessary for improving anaerobic digestion technology and other biofuel processes. This is important for industrial application of microalgae pretreatment.

This chapter focuses on the development of a measurement technique using a high-speed homogeniser to evaluate the mechanical cell wall strength of *C.vulgaris* microalgae. The results from the test can be further used for assessment of microalgae pretreatment strategies as a guide to verify cell disruption efficiency. The effect of homogeniser shear time on the cell strength of microalgae was deduced from this study using varied homogeniser time intervals. The study also evaluated the effect of enzymatic pre-treatment prior to cell homogenisation on lipids extracted using lysozyme and a combination of other hydrolytic enzymes including lysozyme, pectinase, amylase and cellulase. The lysozyme enzyme is a hydrolytic enzyme that can act on microalgae peptidoglycans resulting in cell hydrolysis by removing the hair-like fibers made of polysaccharide chains and in the process provides greater opportunity for further degradation of the cell by anaerobic microbes (Graves et al., 1999, Dvoretsky D., 2016). Microscopic results from experiments conducted in chapter 3 show lysozyme's ability to degrade the outer layer of *C.vulgaris* cells, which is useful for improving the rate of anaerobic hydrolysis. In addition, the results of chapter 3 proved lysozyme enzymatic treatment and high-speed homogenisation as more effective cell disruption techniques with 80% disruption compared to thermal treatments using waterbath and autoclave.

In this chapter, pre-treatment using enzymes was employed along with homogenisation to further understand the strength of the cell. The use of a single enzyme was compared with an enzyme combination to determine any variation in lipids extraction, using this as a surrogate for cell wall strength. Established methods using lipids extraction efficiency and chlorophyll analysis was used to provide an indication of the degree of cell disruption and technique verification.

Traditionally, lipids extraction is done using two established methods mainly Folch method (Chloroform to methanol 2:1) and Bligh & Dyer method (Chloroform to methanol, 1:2) with 1

M NaCl for phase separation (Kumar, 2015). The Bligh & Dyer method is more widely recognised but is limited in estimating concentrations of lipids in samples over 2% (Axelsson and Gentili, 2014). This is significant as microalgae lipids can be over 25% and up to 45% under mechanical stress conditions (Axelsson and Gentili, 2014, Mohan and Devi, 2014, Ratnapuram et al., 2018). However, the use of chloroform has been considered more hazardous and highly toxic due to its higher carcinogenic property than dichloromethane (Li et al., 2014, Ren et al., 2017, Cequier-Sánchez et al., 2008). In addition, chloroform is 15% to 18% more expensive than dichloromethane and has adverse environmental effects (Cequier-Sánchez et al., 2008). Additionally, its use in workplaces has been limited to 2ppm compared to dichloromethane which is 50ppm (Cequier-Sánchez et al., 2008). Moreover, the use of chloroform as an extraction solvent for lipids has been deemed time-intensive and laborious (Axelsson and Gentili, 2014). Earlier investigations by Li (2014) proved that the Bligh & Dyer method was not statistically disparate to dichloromethane/methanol. In fact, lipids yield efficiency followed this trend dichloromethane/methanol > Propan-2-ol/cyclohexane > chloroform/methanol > supercritical-CO2 extraction > Ethanol/KOH for *Tetraselmis sp.* microalgae. Other research conducted by Jeon et al. (2013) reports dichloromethane/ethanol lipids extraction produced 25% more lipids than Bligh & Dyer method. Also, a study conducted by Cequier-Sánchez et al.(2008) demonstrated that chloroform can be replaced by dichloromethane as the results of the study showed that total lipids had insignificant differences with no discrepancy in the lipid classes when dichloromethane/methanol and chloroform/methanol were used as solvents for total lipids extraction.

In this study, dichloromethane was compared to hexane and diethyl ether to determine a suitable solvent for wet lipid extraction after microalgae pretreatment. Then, total lipids determination was conducted using the modified Bligh and Dyer method outlined by Cequier-Sánchez et al. (2008)

4.1.1 Force and energy requirement for mechanical strength of *Chlorella vulgaris*

In order to evaluate the mechanical properties of *C.vulgaris* microalgae, it is important to understand the force and energy requirement needed to disrupt the cells. (Lee et al., 2012) suggested that the energy required for microalgae cell disruption can be optimized by choosing disruption processes that combine cell concentration with solvent extraction as solvents can weaken the cell structure reducing energy needed for disruption.

Mechanical properties of dry microalgae cells have previously been conducted by Lee et al. (2013) using atomic force microscope (AFM) to indent and disrupt individual cells of *Tetraselmis suecica*. From the results obtained, it was discovered that the average force and energy required to indent and break the cells was 673 $J.kg^{-1}$. However, there is currently no

technique to measure cell strength following pretreatment (Lee et al., 2012), which is the central research problem this study aims to address.

To answer the research question, attempts to disrupt wet *C. vulgaris* microalgae cells using the atomic force microscope was initially conducted. It was found that the cells were too thin, small and aspherical making indentations and force measurements difficult.

Algae cell disruption is made possible by shear forces in the form of solid shear and liquid shear. Solid shear uses forces from high-speed homogenisers and bead milling while liquid shear uses high pressure homogenisation and micro-fluidization. Homogenisation has been regarded as the most suitable industrial cell disruption technique (Yap et al., 2015, Günerken et al., 2015). High speed homogenisation has been recommended as a highly effective method of cell disruption releasing biochemicals in the process (Wang and Wang 2012).

Hence, a method to determine the mechanical properties of wet *C. vulgaris* cells was developed using a unidrive X1000 high speed homogeniser as described in the methods section.

The high-speed homogeniser was used to measure the rotational speed and determine the shear rate required to break *C.vulgaris* cells from the amount of extracted lipids obtained at various homogeniser speeds between 4,000 rpm to 33,000 rpm, and changes in the cell strength following pretreatment methods were implied by changes in the extracted lipid versus homogeniser speed curves.

4.1.2 Objective of Study

The main objective of this study was focused on developing a reproducible technique of measuring the mechanical strength of *C.vulgaris* cell walls using high speed homogenisation. A comparison of suitable non-polar solvents efficient for lipid extraction was conducted. Then, the impact of either single or mixed enzymes on *C.vulgaris* cell wall strength was investigated.

4.2 Materials and Methods

4.2.1 Microalgae growth and sample preparation

C.vulgaris (Strain obtained from the I.U.C.C. ex Cambridge. CCAP 1988 strain) was obtained from the Australian National Algae Collection, CSIRO and re-cultured for growth in photobioreactors. The algae were then re-cultured for growth in 2 L conical flasks using bolds basal growth media with continuous stirring at 100 rpm under artificial lights on a 16:8-hour light intensity and ambient temperature (CSIROMarineResearch). Air was bubbled into the flasks at 200 ml/min. The algae was concentrated initially by gravity settling (Ras et al., 2011) overnight to avoid cell stress that might result from centrifugation.

Bolds basal recipe was obtained from (CSIROMarineResearch) using the original recipe and measurements from (Nichols and Bold, 1965, Nichols, 1973). The reagents were sourced from Sigma Aldrich and prepared in solution using milli Q water. Stock solutions used were made in Schott bottles using measured dry weights of each chemical. Two sets of chemical solutions were used to make up the bolds basal media.

To make set one, the following chemicals were used: sodium nitrate (NaNO3) -25 g/L, magnesium sulphate heptahydrate ($MgSO_4.7H_2O$)- 7.5 g/L, sodium chloride (NaCl) -2.5 g/L, potassium phosphate dibasic (K_2HPO_4) -7.5g/L, potassium phosphate monobasic (KH_2PO_4)- 17.5 g/L and calcium chloride dihydrate ($CaCl_2.2H_2O$) -2.5 g/L.

For set two, the following chemicals were employed: boric acid (H_3BO_3) – 11.42 g/L, ethylenediaminetetraacetic acid tetrasodium salt (EDTA) – 50 g/L and potassium hydroxide (KOH) – 31 g/L, ferrous sulphate heptahydrate ($FeSO_4.7H_2O$) - 4.98 g/L and concentrated sulphuric acid (H_2SO_4) – 1 ml/L. Then micronutrients were prepared separately in 800 ml on milli Q water with: zinc sulphate ($ZnSO_4.7H_2O$) - 8.82 g/L, manganese chloride tetra hydrate ($MnCl_2.4H_2O$) - 1.44 g/L, molybdenum oxide (MoO_3) - 0.71g/L, copper sulphate pentahydrate ($CuSO_4.5H_2O$) - 1.57 g/L and cobalt nitrate hexahydrate (Co $(NO3)_2.6H_2O$) - 0.49 g/L.

To make 1 litre of bolds basal stock solution, 10 ml of each set one stock solutions and 1 ml of set two solutions were added to 940 ml of milli Q water. The bolds basal medium was autoclaved at $121^{O}C$ for 15 minutes and allowed to cool to room temperature before transferring to the bioreactor.

4.2.2 Experimental process flowchart description

Figure 4.1 below shows a flow description of the experimental process for the novel cell strength technique developed in this study which is discussed in detail in section 4.2.3 to 4.2.5.

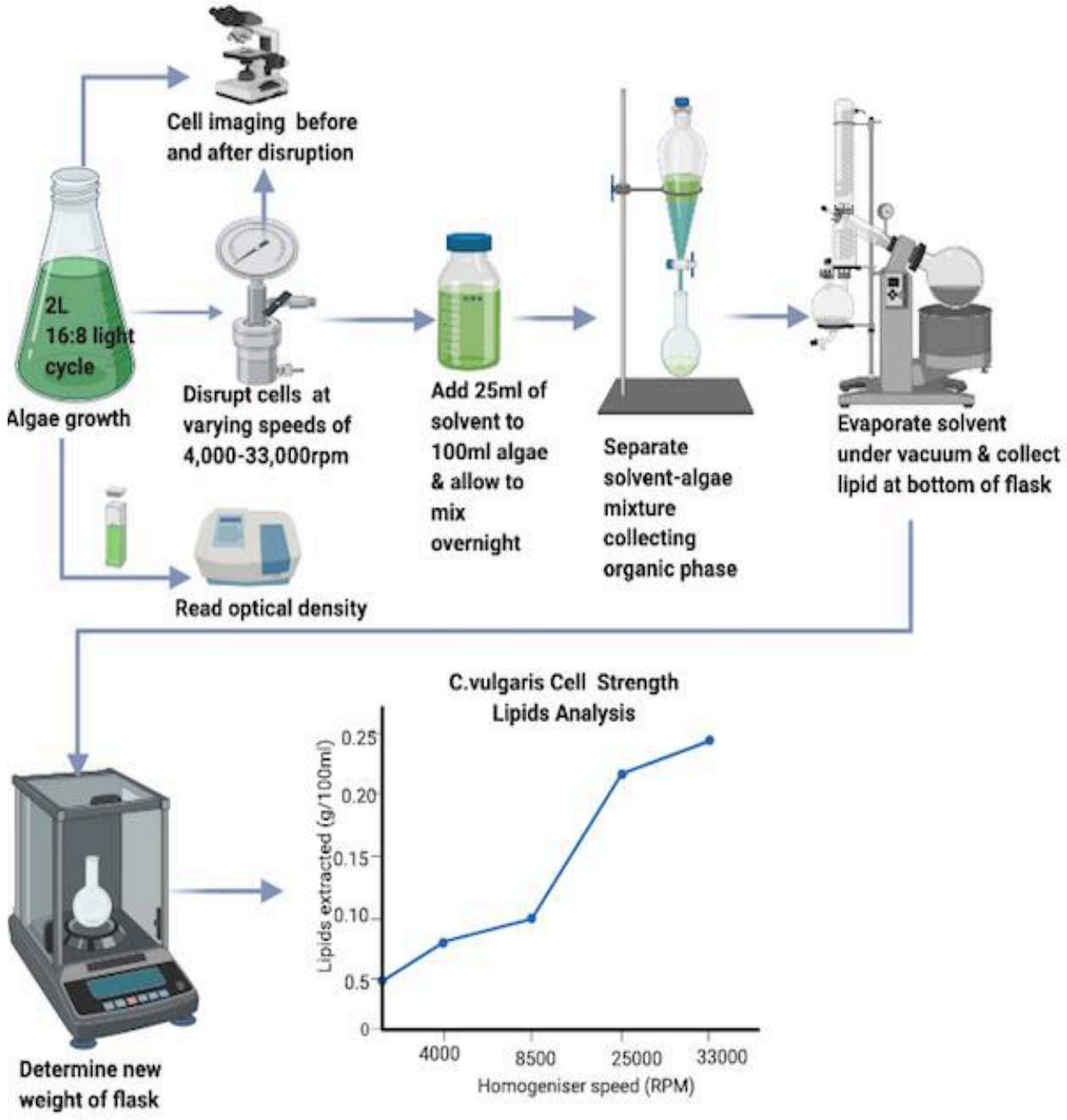


Figure 4.1: Illustration of the proposed process flowsheet for the cell disruption measurement technique.

4.2.3 High speed homogenisation method development

A cell strength measurement technique using a CAT unidrive X1000 high speed homogeniser was developed using laboratory grown *C.vulgaris*. The shear rate of the homogeniser can be expressed in terms of shear rate given in equation (4-1) as:

$$\gamma = \frac{2RoRi\omega}{Ro^2 - R_I^2};\ \omega = \frac{2\pi n}{60} \quad (4\text{-}1)$$

$where\ \gamma\ is\ the\ shear\ rate\ (s^{-1}, \omega\ is\ the\ angular\ velocity\ of\ the\ outer\ cylinder, (s^{-1}), Ro\ is\ the\ outer\ cylinder\ inner\ radius\ (m)\ and\ Ri\ is\ the\ inner\ cylinder\ outside\ radius\ (m), n\ is\ the\ rotational\ speed\ (rpm)\ of\ the\ shear\ cylinder.$

To determine the homogeniser treatment time, two separate time intervals of 5 minutes and 15 minutes contact times were employed at the initial phase of technique development. Two separate algae batches were used in duplicate tests. Optical densities at a wavelength of 750 nm were recorded and readings of 1.644 for the 5 minutes sample and 1.501 for the 15 minutes sample were obtained. 5 minutes homogeniser time produced similar graphical trend curve as 15 minutes showing cell disruption, which is outlined in the results section of this chapter, and was used in all subsequent experiments.

The study focused on lipids extracted from only broken cells as the objective was to determine the cell strength by quantifying the degree of disruption of the cells of the microalgae upon pretreatment. During the method development, preliminary tests investigated the use of methanol as solvent for lipids extraction. However, due to the polarity of methanol, lipids from unbroken cells were being extracted which did not meet the criteria for the technique being developed, as the aim was to determine the amount of lipids from broken cells relative to the total lipid content.

To analyse the total lipids in the cells, a modified Bligh & Dyer method was used where dichloromethane was replaced with chloroform using method provided by (Cequier-Sánchez et al., 2008). This study compared dichloromethane/methanol and chloroform/methanol for total lipids extraction.

For determination of lipids from broken-only cells, a range of solvents were employed such as hexane, diethyl ether and dichloromethane to determine their effectiveness in extracting lipids from broken-only cells. The extraction of lipids from broken cells was performed after high-speed homogenisation at various speeds ranging 4,000 rpm to 33,000 rpm. The lipids were extracted by dissolving the lipids in a solvent and separating the solvent phase from the water phase by settling. A Heidolph rotary evaporator vv2000 at 38°C and 150 rpm was then used to remove the organic solvent from the lipids. The amount of lipids was calculated by weighing the difference between the washed and dried round-bottom flask used with the rotary evaporator, and the round-bottom flask with evaporated solvents and lipids residue.

The speed where rapid disruption begins to occur indicating breakage of the cell wall was evaluated from the lipids-homogeniser speed curve and a critical shear rate for disruption was deduced. Additional cell strength verification tests included: effect of enzymes (including either single or mixed enzymes) before homogenisation and chlorophyll analysis rather than lipid analysis. Data from Chapter 3 had previously shown promising results for cell wall disruption using lysozyme, hence its use in this chapter in comparison with mixed hydrolytic enzymes of cellulase, pectinase, amylase and lysozyme.

4.2.4 Total lipids Determination Technique

50 ml of algae cells were collected in falcon tubes and concentrated using a centrifuge at 4,000 g for 15 minutes to dewater algae. The water was drained, and algae paste was collected. The algae paste was further frozen overnight at -20^{o}C. The frozen algae were transferred into a vacuum chamber to be freeze dried at -80^{o}C for 48 to 72 hours to remove remaining moisture from algae using a Christ LCG freeze dryer by John Morris scientific.

To conduct total lipids extraction, the freeze-dried microalgae sample was crushed into fine powder using a laboratory mortar and pestle. The weight of the microalgae mass was recorded to calculate the total solids as dry weight in g/100ml according to standard methods (Baird, 2017). Then, 25 ml volume of solvent consisting of dichloromethane/ methanol in 2:1 ratio was added to the algae powder and allowed to mix by vortex for 2 minutes. The microalgae-solvent mixture was then centrifuged at 1500 rpm at 4^{o}C for 5 minutes to allow phase separation. The separated organic phase was further collected and filtered using a GF/C glass microfilter with 47 mm diameter (Whatman GF/C healthcare life sciences). The sample was further rinsed with 5 ml of KCl solution (0.88% w/v) to ensure proper phase layering (Ren, 2017). The solvent was later evaporated under nitrogen (Cequier-Sánchez et al., 2008) in the fume hood to remove excess solvent. The sample was dried afterwards in the oven for 15 minutes at 105^{o}C and allowed to cool in the desiccator for 15 minutes. Final weight of the sample was recorded to calculate total lipids available in cells.

4.2.5 Cell Strength Experiment Technique

Prior to the start of the experiments', microscopic imaging of the *C.vulgaris* cells was conducted using a motic BA310 light microscope (manufactured by John Morris Scientific Pty Ltd) to see intact cells.

A single batch of the 2L algae stock was used for the full range of homogenisation speeds in each separate experiment to ensure that a curve of lipid released versus homogenisation speed could be constructed from one batch of microalgae. The experiments were conducted in duplicates. For the solvent comparison tests, the algae batch used had an optical density

of 1.544 at 750 nm wavelength. The algae batch to examine solvents and the impact of lysozyme pretreatment had an optical density of 1.556 at 750 nm.

100 ml of *Chlorella vulgaris* placed in a 250 ml container to avoid spillage was homogenised at speeds of either 4,000 rpm, 8,500 rpm, 15,000 rpm, 20,000 rpm, 25,000 rpm or 33,000 rpm for 5 minutes, and the homogenised suspension poured into 500 ml Schott bottles. Additionally, separate Schott bottles of un-homogenised microalgae suspension was used as the controls. Microscopic light imaging of the cells was taken at 400X after each homogeniser speed to observe visual changes. Further imaging was conducted at 1000X to provide clarity of disrupted cells.

25 ml of the solvent was added to the *C.vulgaris* following homogenisation, hand-shaken vigorously and inverted for 30 seconds to allow mixing and left to settle overnight. After 24 hours, the lipids were released into the solvent phase and a sample of the clear solvent was collected using a separating funnel for determination of the lipid concentration using a rotary evaporator. Before using the rotary evaporator, the 100ml round bottom flasks used were washed, oven dried at 70^{o}C and allowed to cool in the desiccator a day prior to experiments. Then, the weights were measured using an AND GR 200 analytical balance. With the solvent phase in the round bottom flask, the solvent was evaporated under vacuum using a Heidolph vv2000 rotary evaporator leaving the lipid as residue. The temperature of the water used to heat the solvents were maintained at temperatures slightly below the boiling points of the solvents: at 38^{o}C for dichloromethane, 32^{o}C for diethyl ether and 65^{o}C for hexane respectively. The lipid content was calculated by measuring the difference between the new weight of the flask and the original weight.

For estimating the effect of enzyme pre-treatment on cell strength tests, the *C.vulgaris* batch used had an optical density of 1.633 at 750 nm. Enzyme stock solution was prepared at a concentration of 20 mg/ml using milli Q water. Then, the enzyme stock was added to the *C.vulgaris* at a rate of 100 µl/ml for 24-hours prior to high-speed homogenisation. Earlier results in Chapter 3 showed maximum cell disruption of 82% for an enzyme stock addition of 100ul per ml of algae, hence its use in this chapter.

For chlorophyll determination, a UV biochrom libra s22 spectrophotometer was used for analysis. Chlorophyll a was investigated as it's a blue-green pigment which is also found in *C.vulgaris.* The absorbance range for its determination was 660 nm to 665 nm (Hosikian et al., 2010). In one study, Chlorophyll a was determined at exactly 663nm (Schagerl and Künzl, 2007). For this project, spinach obtained from Sigma Aldrich lot # SLBV3064 at 893.49g/mol and stored at -20^{O}C was used for comparison purposes. Chlorophyll a was extracted from the spinach using solvent extraction method with 90%v/v ethanol, which is the ISO:10260 1992 standard (Pápista et al., 2002). The frozen spinach was thawed, immersed into the ethanol solution, vortexed for 2 minutes and filtered using a filter paper. Next, the mixture was

centrifuged for 10 minutes at 1000g using a Beckman Coulter Avanti model J-26S XPI centrifuge to allow phase separation. Spectrophotometric analysis was conducted by scanning 1 ml of the organic phase of the spinach extract at absorbance range of 660 to 665 nm and a peak was observed at 664 nm.

Next, duplicate 100 ml samples of *C.vulgaris* cells were homogenised at the various speeds of 4,000 rpm to 33,000 rpm. Optical density of the algae used was 1.483 at wavelength of 750 nm. 25 ml each of 90%v/v ethanol was then added to the homogenised algae to allow phase separation. 1 ml of the organic phases of each 100 ml mixture was collected and absorbance measured at wavelengths 400 nm to 800 nm and peaks were observed in comparison to the spinach extract.

To further visualise breakage of the cells, the light microscope and a staining technique using trypan blue to stain broken cells was used to observe changes in cell morphology. 0.4% solution of trypan blue was prepared. 0.1 ml (100 µl) of trypan blue dye was added to 1 ml of *C. vulgaris* and imaged using the motic BA310 light microscope. It was expected that the broken cells would absorb the dye into its internal structure and becomes darker in colour. Each experiment was performed in duplicate. The cell density of the cell line suspension was determined using a haemocytometer. The mixture was placed on a Neubauer chamber glass slide and examined under the microscope at low magnification of 400X. The number of stained cells was counted to determine disrupted cells using a Neubauer cytometer (grid surface of 0.0025 mm^2 and chamber depth of 0.100 mm). Cell counting was performed manually by counting darker cells (indicating dead cells) and light cells (showing alive cells) to achieve the total cell numbers of a selected square containing 16 individual squares using the light microscope. For cell counting, the cell disruption rates were calculated using equation (4-2) from (Huang et al., 2016). Liberated lipids were used as means of determining process efficiency.

$$Dt = \frac{Ci - Ct}{ci} \times 100 \qquad (4\text{-}2).$$

D_t=represents the cell disruption proportion at point t

C_i=initial cell quantity of unbroken cells

C_t=cell quantity of unbroken cells at time t.

4.3 Results and Discussion

4.3.1 Growth Curve

Biomass growth was monitored by optical density values scanned at 750 nm using a UV biochrom libra s22 spectrophotometer (Federation and Association, 2005). 750 nm was chosen as it's above the standard wavelength for chlorophyll absorption to avoid interference of results (Pápista et al., 2002). An example growth curve from a monitored algae batch is plotted in figure 4.2. For subsequent experiments', *C.vulgaris* suspension was used when the growth phase was close to the end of the exponential growth at around day 8 to 10 to maximize lipids production (Yadavalli et al., 2014) as seen in figure 4.2 below.

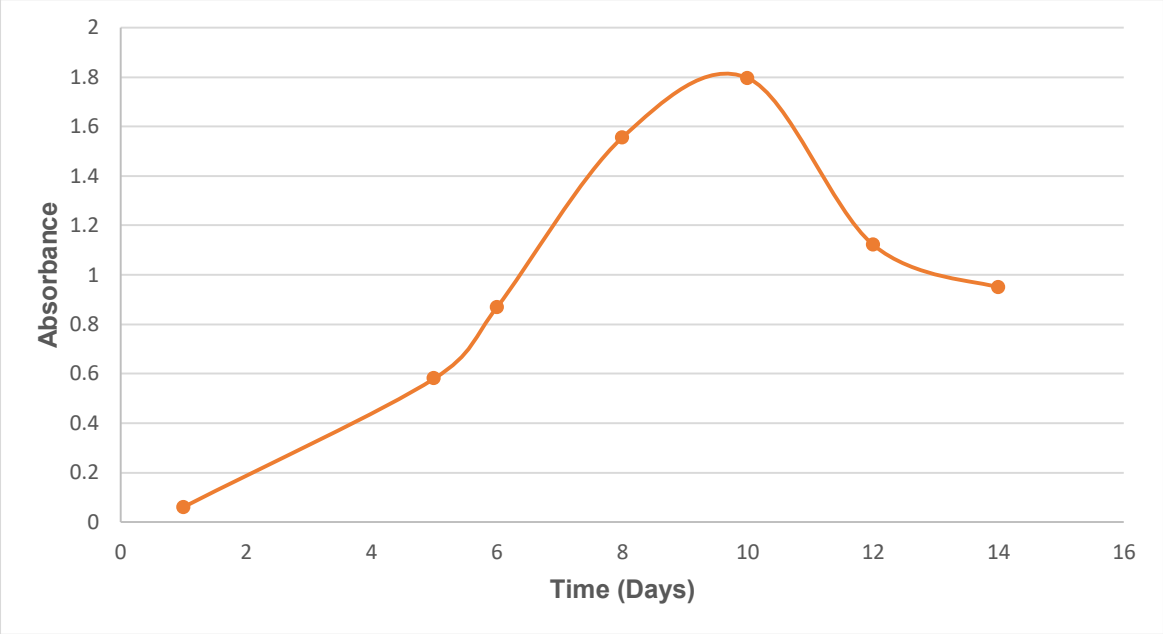


Figure 4.2: Microalgae Growth Curve at wavelength of 750 nm.

4.3.2 Microscopic imaging analysis

From the samples of *C.vulgaris* homogenised at different speeds (4,000 rpm to 33,000 rpm) for 5 minutes, images were taken before and homogenisation at 400X and 1000X to investigate cell disruption as seen in Figures 4.3 and 4.4. Microscopic imaging of samples taken before and after homogenisation shows significant disruption of cells. From 8,500 rpm, there was over 50% disrupted cells. At 15,000 rpm , there was 70% of the total cells stained and 85%of total cells stained at 33,000 rpm using equation (4-2) indicating the percentage of disrupted cells. However, even though the majority of the cells were stained by the dye, not all of the cells appear broken. Some cells stained but still remained intact without much visual deformity to the shape under microscopic observation.

When microalgae cells were incubated with lysozyme before homogenisation for 24-hour, the results showed visible degradation with over 95% of the total cells stained as seen in Figure 4.5. The cells also became darker as they absorbed more of the trypan blue dye. In addition, the cells possibly became more permeable as they appeared swollen after lysozyme addition. Moreover, the cells seemed to aggregate after lysozyme addition. It is possible that lysozyme addition affects the outer cell layer causing digested proteins to become sticky leading to aggregation. This may be attributed to the lysozyme effect in separating the cell components due to increase in permeability of the outer cell wall resulting in the cells readily absorbing trypan blue dye and thereby inducing the dark appearance of cells. Besides, lysozyme has been known to degrade the outermost layer of *C.vulgaris* cells (Gerken et al., 2013).

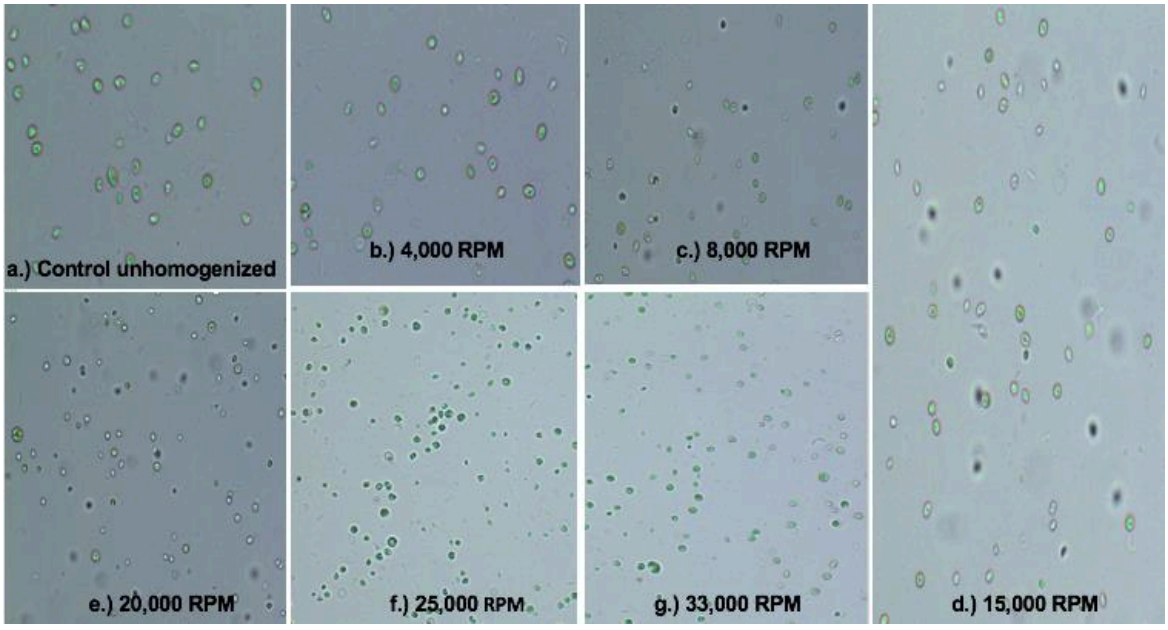


Figure 4.3: Homogeniser Pretreatment with Trypan blue dye at 400x magnification.

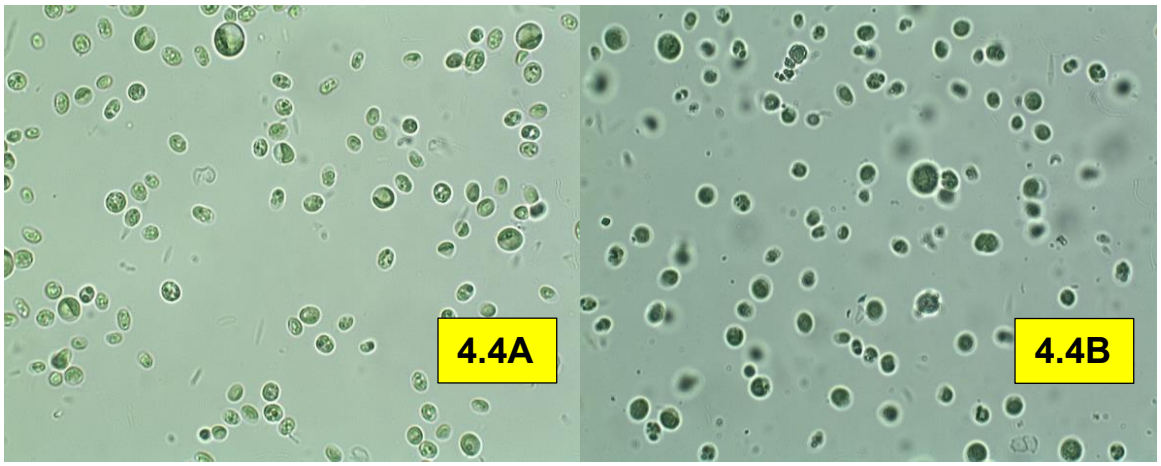


Figure 4.4: Microscopic imaging cell count at 1000X of unhomogenised and trypan blue-stained homogenised C.vulgaris cells at maximum disruption of 33,000 rpm.

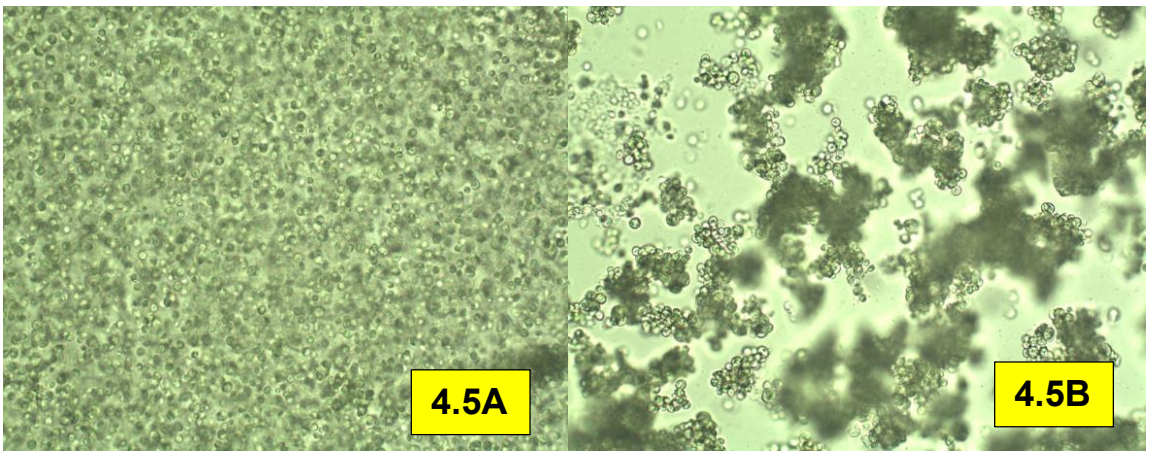


Figure 4.5: Lysozyme enzyme treatment with trypan blue dye at 400X before and after 24-hour contact time.

4.3.3 Effect of homogeniser time on *C.vulgaris* cell strength

Time intervals of 5 minutes and 15-minutes treatment time using high speed homogenisation were initially employed using dichloromethane as the lipid extraction solvent, as lipids from broken-only cells were desired. Other solvents like ethanol or methanol will extract lipids from all cells (Yang et al., 2014).

From the results shown in Figures 4.6a-c, although the 15 minutes extracted more lipids than the 5 minutes time interval, the error bars were larger with standard deviation range of 0.0042 to 0.0175.

Dry weight of the *C.vulgaris* batch used was found to be 1.1126 g/100ml. Total available lipids was calculated to 0.5600 g/100ml, which was 50.33% of the dry weight. This confirms with studies that have stated *C.vulgaris* to have lipids between 5% to 60% (Canelli et al., 2020, Bernaerts et al., 2018).

An estimation of the percentage of lipids from broken-only cells to total lipids in the cell is plotted in Figure 4.6c. Also, rapid and consistent lipids extraction began at 8,500 rpm as seen in Figure 4.6a. This may be taken as the critical speed, an inflection point determined by the rapid and consistent increase in lipids extracted.

5 minutes was selected as the contact time for homogeniser as it showed a similar trend for lipids extracted at 15 minutes, providing same indication of strength of the cell wall. In addition, the reduced contact time meant reduced energy consumption from the device run-time discussed further in Chapter 7.

In addition, there was a gradual increase in temperature from heat generated from the homogeniser device as the homogeniser speed increased, which is also consistent with results observed from (Shirgaonkar et al., 1998). A plot of homogeniser speeds versus temperature change at 15 minutes and 5 minutes is given in figure 4.6b.

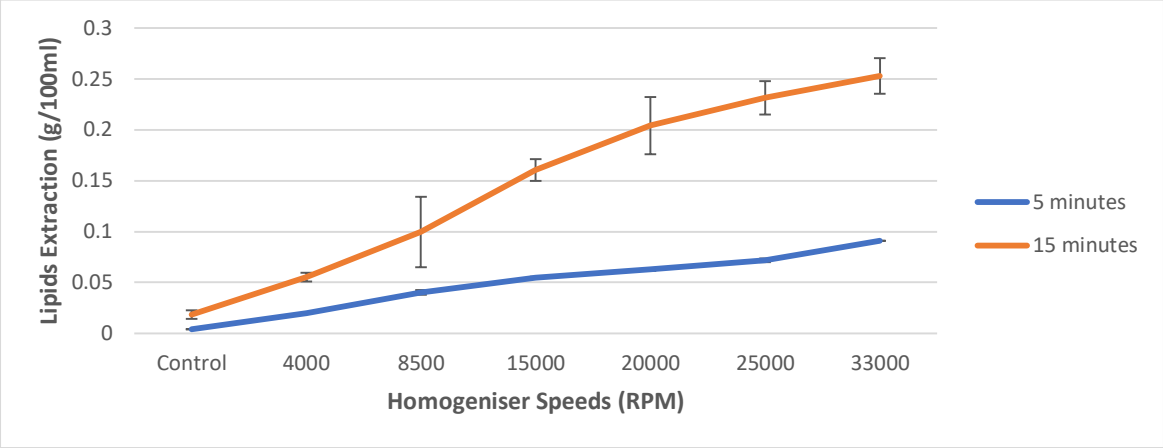


Figure 4.6a: Effect of temperature of homogeniser speeds.

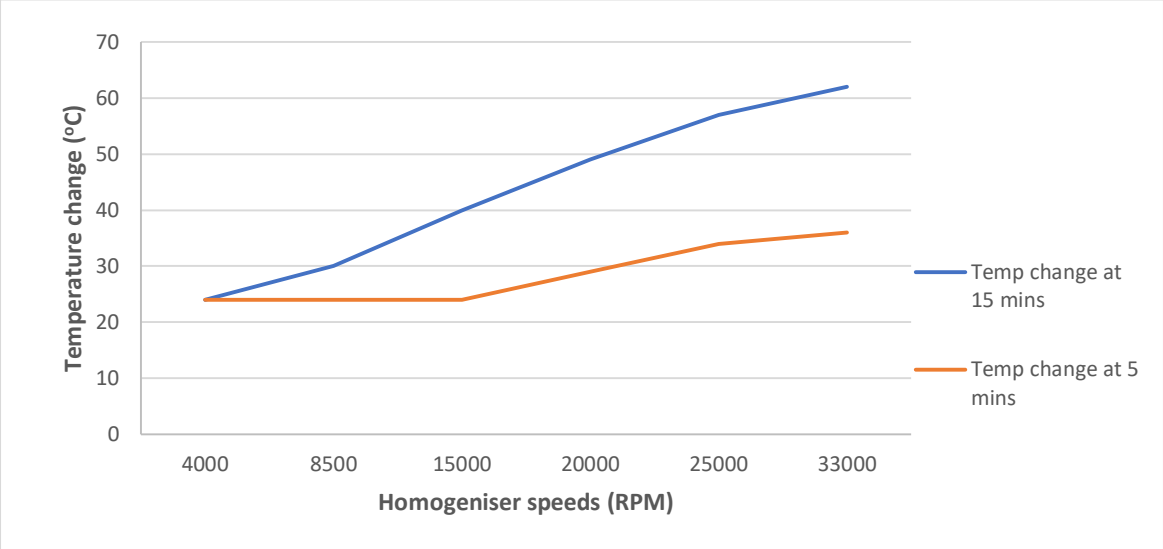
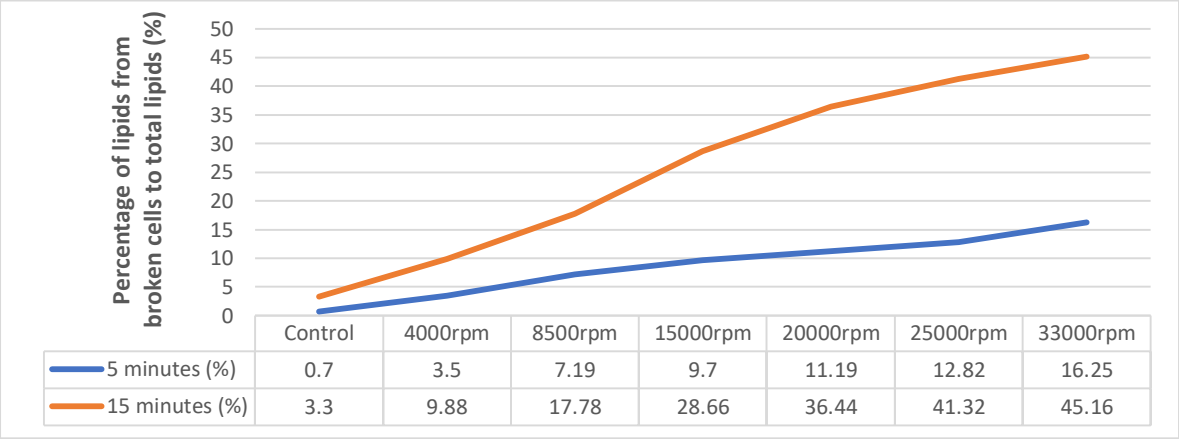


Figure 4.6b: Temperature change versus homogeniser speeds.



	Control	4000rpm	8500rpm	15000rpm	20000rpm	25000rpm	33000rpm
5 minutes (%)	0.7	3.5	7.19	9.7	11.19	12.82	16.25
15 minutes (%)	3.3	9.88	17.78	28.66	36.44	41.32	45.16

Figure 4.6c: Percentage of lipids from broken cells to total available lipids during time-effect on C.vulgaris tests (DW=1.1126 g/100ml; total lipids=0.56 g/100ml).

4.3.4.1 Comparison of Solvents for Lipids Extraction using Diethyl ether, hexane and dichloromethane.

Dichloromethane, diethyl ether and hexane were used as solvents (Lee et al., 2012, Halim et al., 2011, Ramluckan et al., 2014) to extract the lipids from the homogeniser disrupted *C.vulgaris* cells as seen in figure 4.7a. From the results obtained in Figure 4.7a, the solvents had similar trends, but more lipids extraction and lower standard deviation meant the results from dichloromethane are more reliable than hexane and diethyl ether.

Comparing the lipids from the solvent extraction using the three single solvents, dichloromethane extracted 36% more than hexane and 33% more than diethyl ether at maximum disruption of 33,000 rpm as seen in Figure 4.7a. Even at the lowest speed of 4,000 rpm, dichloromethane's lipid extraction was more than double the hexane and an additional 37% of the diethyl ether extracts. In addition, dry weight and total lipids used during the solvent comparison tests were analysed. For solvents only, dry weight and lipids were found to be 0.8048 g/100ml and 0.4204 g/100ml. The total lipids were 52% of the dry weight. A percentage of lipids from broken-only cells to total available lipids in the cell during the solvents' comparison tests is plotted in Figure 4.7b. From Figure 4.7b, at the critical speed of 8,500 rpm, percentage of lipids from broken-only cells to total lipids was found to be 3.52%, 3.24% and 4.40% for diethyl ether, hexane and dichloromethane. At maximum speed of 33,000 rpm, ratio of lipids extracted by the solvents to total lipids became 18.01%, 17.65% and 24% for diethyl ether, hexane and dichloromethane respectively.

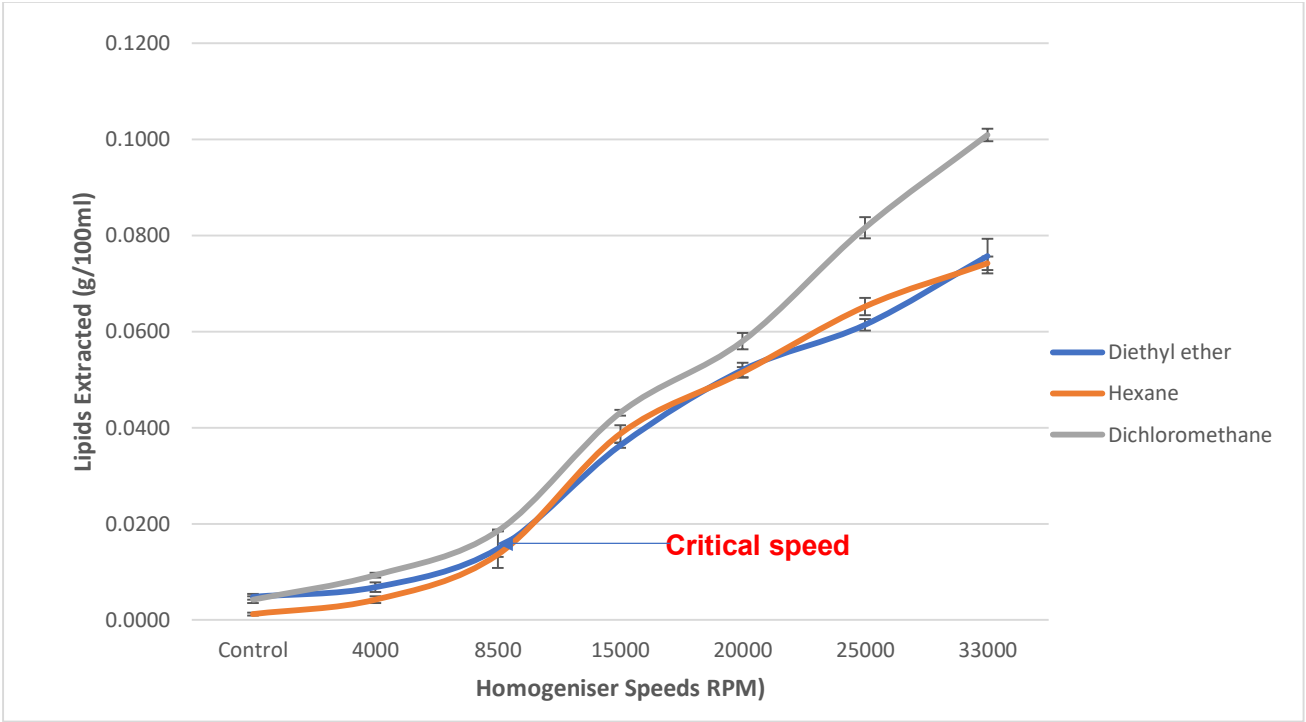
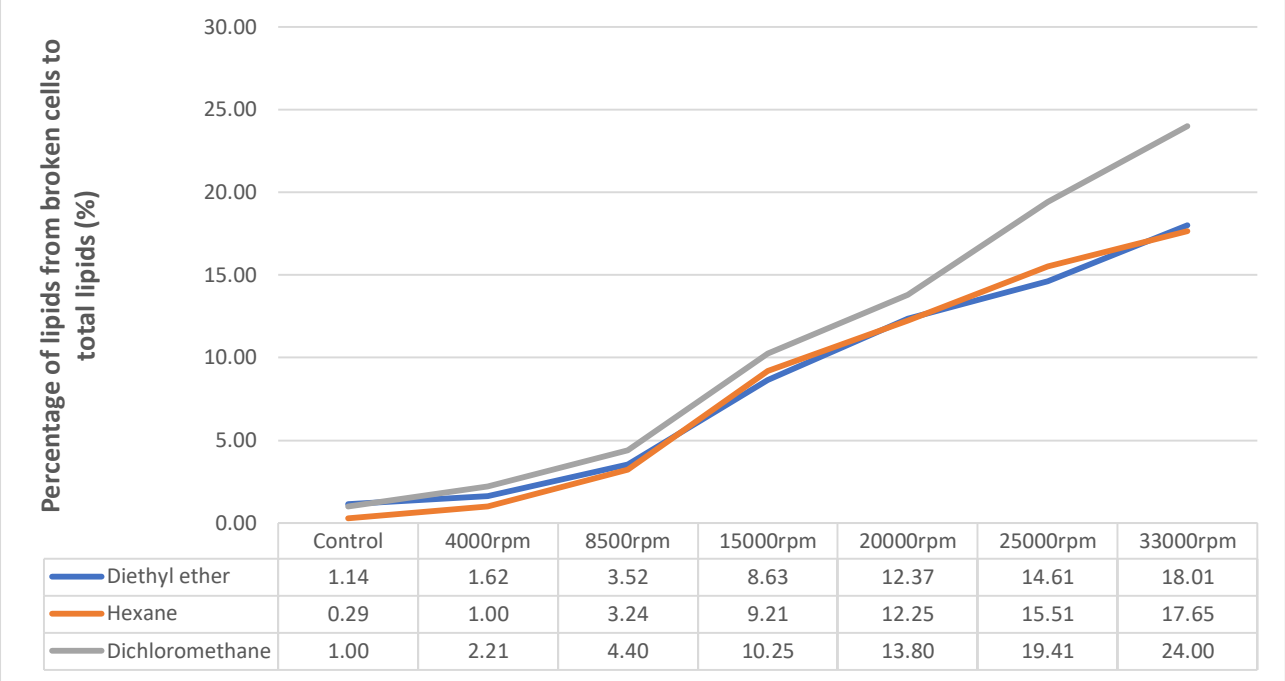


Figure 4.7a: Comparison of solvents for lipids extraction.



	Control	4000rpm	8500rpm	15000rpm	20000rpm	25000rpm	33000rpm
Diethyl ether	1.14	1.62	3.52	8.63	12.37	14.61	18.01
Hexane	0.29	1.00	3.24	9.21	12.25	15.51	17.65
Dichloromethane	1.00	2.21	4.40	10.25	13.80	19.41	24.00

Figure 4.7b: Percentage of lipids from broken cells to total available lipids during solvents-only comparison tests (DW=0.8048 g/100ml; total lipids=0.4204 g/100ml).

4.3.4.2 Comparison of Solvents-Lysozyme Analysis using Diethyl ether and dichloromethane.

Following the results in Figure 4.7a, dichloromethane and diethyl ether were further compared with lysozyme addition before homogenisation. Lysozyme had earlier been deduced from Chapter 3 to improve cell wall disruption of *C.vulgaris* by 82% using a concentration of 20 mg/ml enzyme stock solution with volume of 100µl per ml of algae. Hence, its use to understand the strength of the cell wall from extracted lipids.

The results using solvents-lysozyme extraction are shown in figure 4.8a, with dichloromethane again extracting more lipids than diethyl ether. At the maximum cell disruption speed of 33,000 rpm, lipid from dichloromethane was 0.1048 g/100ml, which is 17% more lipids than that extracted using diethyl ether at 0.0897 g/100ml.

Also, dry weight and total lipids for the solvent-lysozyme tests were given as 0.9766 g/100ml and 0.4800 g/100ml respectively. When deducing the percentage of lipids from broken only cells to total lipids available in the cell at the critical speed of 8,500 rpm, the results were 2.9% and 3.85% for diethyl ether and dichloromethane respectively as seen in Figure 4.8b.

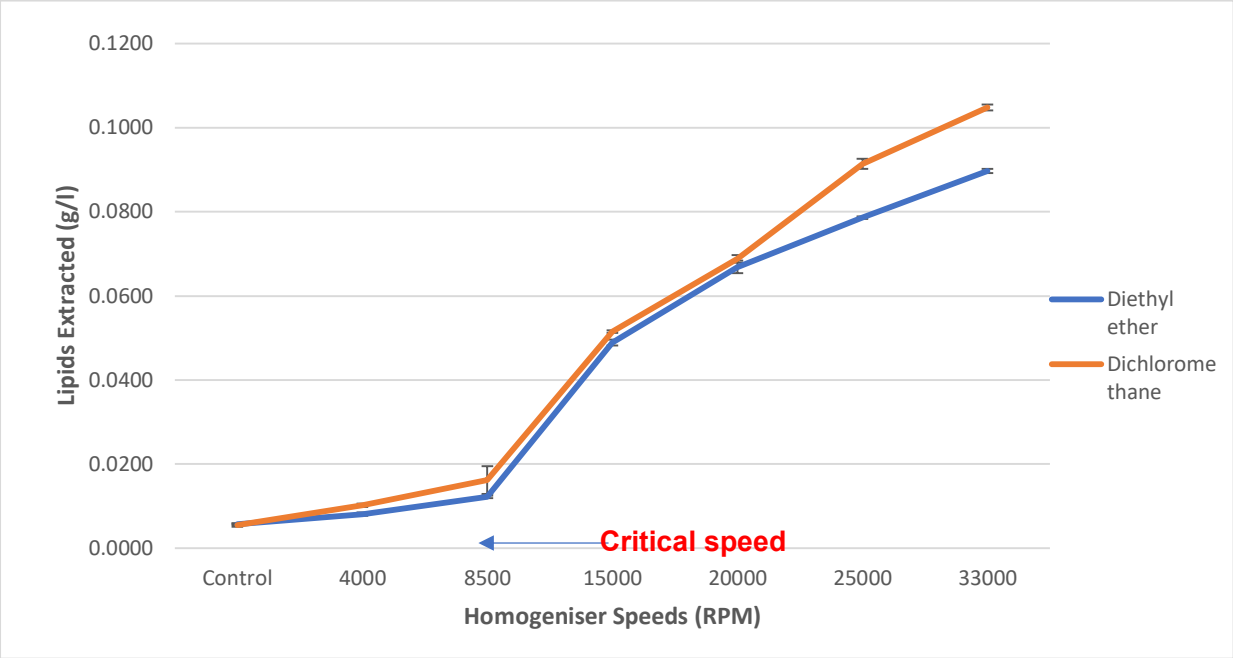
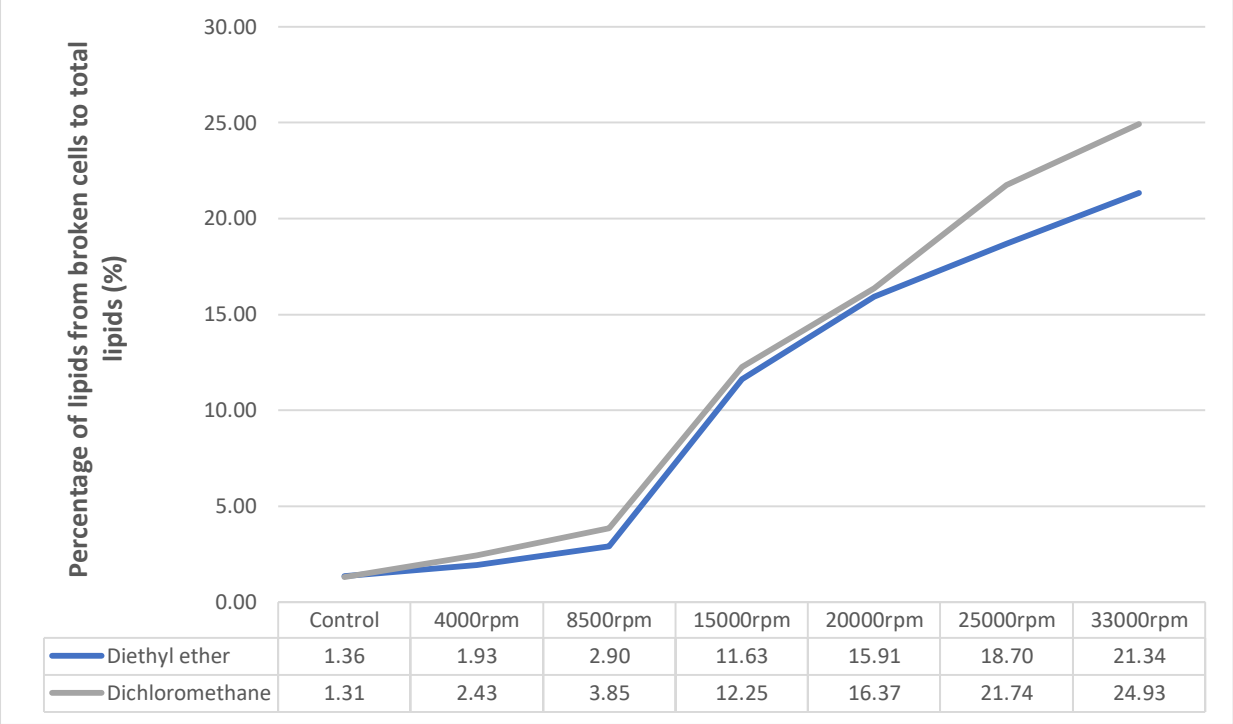


Figure 4.8a: Comparison of solvents for lipid extraction following Lysozyme treatment at concentration of 20mg/l and volume 100µl per ml algae with contact time of 24-hour.



	Control	4000rpm	8500rpm	15000rpm	20000rpm	25000rpm	33000rpm
Diethyl ether	1.36	1.93	2.90	11.63	15.91	18.70	21.34
Dichloromethane	1.31	2.43	3.85	12.25	16.37	21.74	24.93

Figure 4.8b: Percentage of lipids from broken cells to total available lipids during solvents-lysozyme comparison tests (DW=0.9766 g/100ml; total lipids=0.4800 g/100ml).

4.3.5 Solvent-Enzyme Extraction

This study also looked at the effect of single (lysozyme) and combined enzymes (lysozyme, amylase, cellulase and pectinase) on the mechanical strength of *C.vulgaris* by quantifying the amount of lipids extracted by varying homogeniser speeds between 4,000 rpm to 33,000 rpm. From Figure 4.9a, as the homogeniser speed increased, the amount of lipids extracted increased in both single enzyme and combined enzymes' experiments.

Comparing both tests, the enzyme combination displayed greater lipid extraction than the single enzyme lipid extraction. The combined enzymes tests had increased lipid extraction values compared to the lysozyme only tests, with differences in lipids extraction values given in the table 4.1 below:

Table 4.1: Difference in lipids extraction values between combined enzymes and lysozyme-only tests.

Homogeniser Speeds (rpm)	**Lipids extraction (g/100ml)**
Control	0.0042
4000	0.0109
8500	0.0221
15000	0.0062
20000	0.0129
25000	0.0109
33000	0.0032

The following lipids release trend was observed: between the control and 4,000 rpm, there was 1.85-fold increase for lysozyme alone and 2.17-fold for enzyme combination. At 4,000 rpm to 8,500 rpm, there was 1.58-fold for lysozyme and 1.82-fold for enzyme combination. The highest increase was observed between 8,500 rpm and 15,000 rpm with 3.18-fold for lysozyme and 1.51- fold for enzyme combination. Between 15,000 rpm to 20,000 rpm, the lipids change was 1.33-fold for lysozyme and 1.42-fold for enzyme combination. Moving from 20,000 rpm to 25,000 rpm, there was 1.33-fold for lysozyme and 1.25 for enzyme combination. Lowest increase of lipid extraction was observed from 25,000 to 33,000 rpm at 1.15-fold for lysozyme and 1.08-fold for enzyme combination.

These results further confirm 8,500 rpm to be the critical speed as maximum lipids extraction improvement between homogeniser speeds is consistently observed above this speed and continues to improve in an upward trend.

In terms of dry weight and total lipids for the solvents-enzyme tests, the results were found to be 0.8408 g/100ml and 0.4624 g/100ml respectively.

When calculating the percentage of lipids from broken only cells to total lipids available in the cell at the critical speed of 8,500 rpm, the results were 3.5% and 8.28% for lysozyme and enzyme combination respectively as seen in Figure 4.9b.

Earlier investigations of lipid extraction have shown enzymes to be effective for improving cell disruption during lipids extraction assisting in the release of intracellular lipids (Kumar, 2015). The use of hydrolytic enzymes for improving cell disruption is not uncommon in cell disruption technologies. (Fu et al., 2010) hydrolysed the cell wall of microalgae using immobilised cellulase. The results of the study showed 62% improvement in cell hydrolysis and lipid extraction efficiency also improved to 56% from 32% after enzyme application showing significant cell disruption. (Demuez et al., 2015) identified the advantages of enzymatic cell disruption as being simple reaction parameters from consistent reaction rates, reduced energy requirement, no inhibitory end-products and high reaction selectivity.

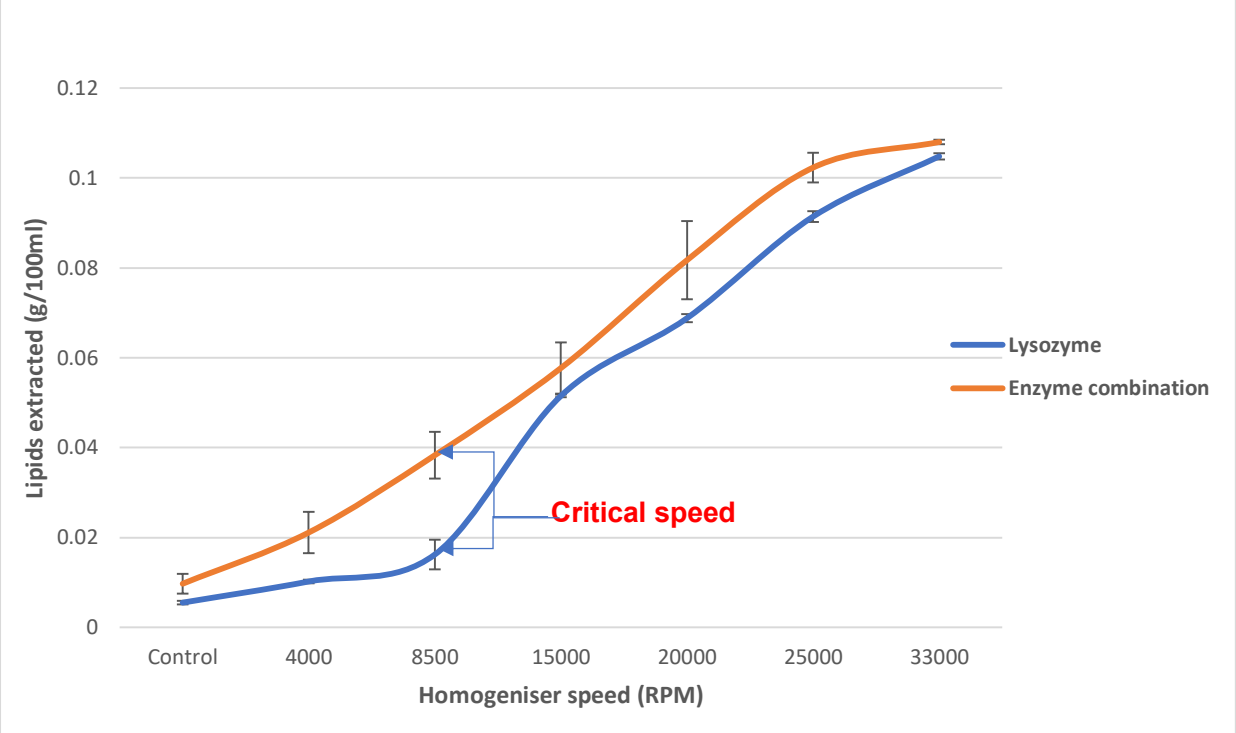
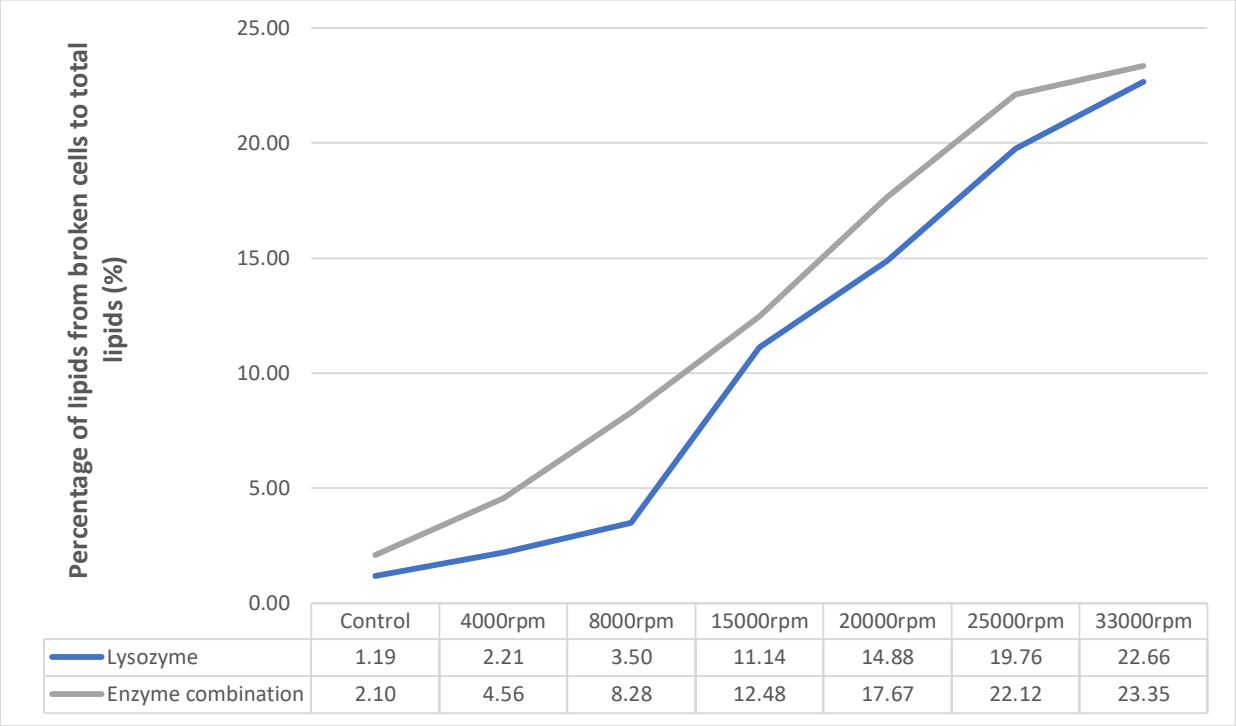


Figure 4.9a: Effect of single and combined enzymes on C.vulgaris cell strength.



	Control	4000rpm	8000rpm	15000rpm	20000rpm	25000rpm	33000rpm
Lysozyme	1.19	2.21	3.50	11.14	14.88	19.76	22.66
Enzyme combination	2.10	4.56	8.28	12.48	17.67	22.12	23.35

Figure 4.9b: Percentage of lipids from broken cells to total available lipids during solvents-enzymes comparison tests (DW=0.8408 g/100ml; total lipids=0.4624 g/100ml).

4.3.6 Validation of Homogeniser Technique using Chlorophyll Analysis

Chlorophyll-a analysis was conducted to further test for a separate compound that can be released if the cell is damaged as *C.vulgaris* cells have been known to contain the pigment. Chlorophyll absorbs light in red and blue regions emitting a green colour with an absorbance of 660 to 665 nm using a spectrophotometer (Hosikian et al., 2010). The pigment is soluble in solvents such as ethanol, methanol, ether, acetone, chloroform, carbon disulphide and benzene. For chlorophyll analysis, 90%v/v ethanol was used as solvent in dissolving chlorophyll a from the algae. Process efficiency was verified from raw chlorophyll with a peak value observed at 664 nm. The trend showed the homogeniser speed was directly proportional to absorbance from chlorophyll a, indicating increase in cell degradation with increased speeds and subsequently lipids extraction. From Figures 4.6a to 4.9b, it can also be seen that there is high increase in lipids extracted starting at 8,500 rpm, which continues to the optimum speed of 33,000 rpm.

Chlorophyll a release shows a similar trend but a much lower slope with lipids concentration using homogeniser as the amount of chlorophyll from homogeniser pretreatment increases steadily as observed in Figure 4.10.

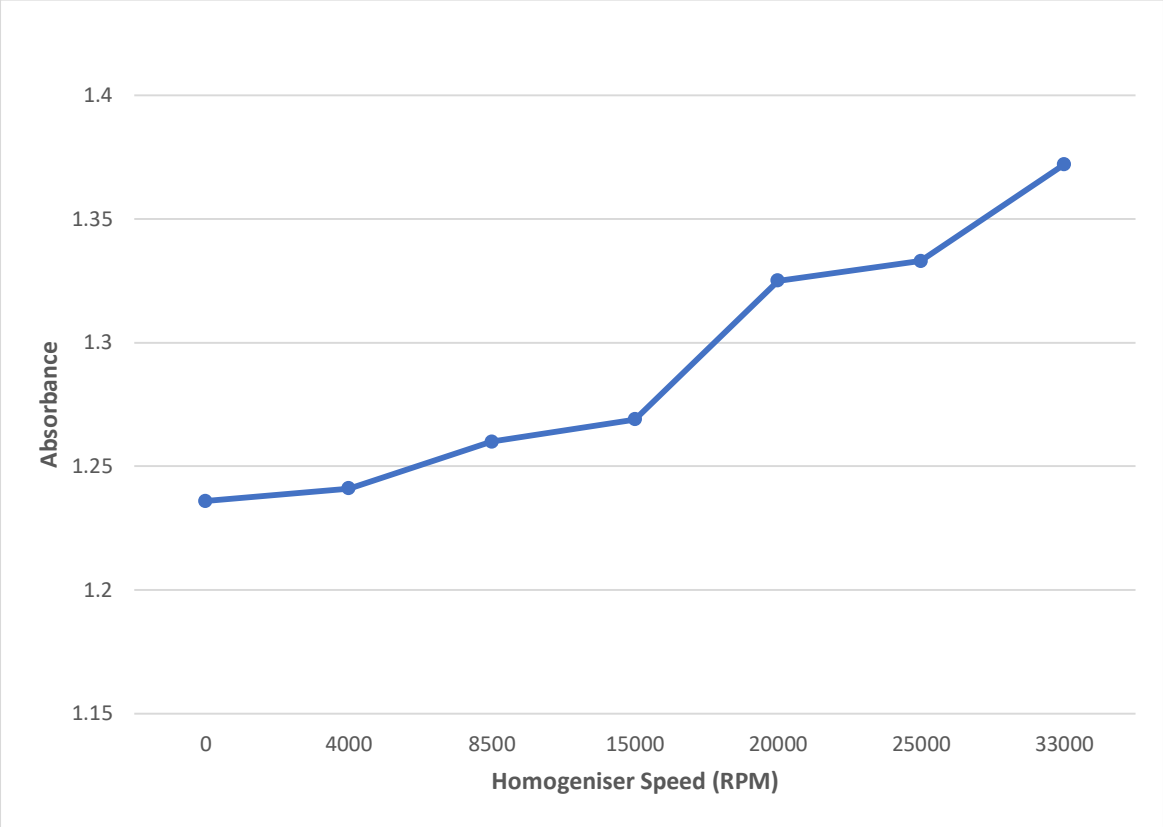


Figure 4.10: Chlorophyll Analysis of Homogenised C.vulgaris at 664 nm

4.3.7 Further Discussion on novel cell strength technique of microalgae

Comparing the novel cell strength technique developed in this chapter to Chapter 3, the results show similar graphical trend with critical speeds observed at 8,500 rpm as seen in Figure 3.4 and Figures 4.6a to 4.8b. At this speed, it can be deduced that hydrodynamic cavitation begins to occur and cavities within the microalgae fluid begin to rapidly collapse. From 8,500 rpm, cell disruption continued to increase with increase in shear rate, which was consistent up to the maximum disruption at 33,000 rpm. Image analysis as a tool for cell disruption is good but time consuming. Hence, using the cell strength technique developed is a more precise method for cell disruption quantification especially for larger volumes of microalgae. The degree of cell disruption may improve the lipids extraction. Comparing disruption rate in Chapter 3 and total lipids produced in this study, 65% of disrupted cells would produce about 50% to 55% of total lipids in the *C.vulgaris* assuming 80% cell disruption as recorded in Chapter 3.

In addition, when observing the effect of solvent type on the lipid extraction from broken *C.vulgaris* cells after the high-speed homogeniser disruption, dichloromethane provided more reliable results than hexane or diethyl ether as seen in Figures 4.7a and 4.8a as it extracted more lipids at higher shear rate. Moreover, the separation of the solvent from the aqueous phase was easier as less water was contained in its solvent phase than diethyl ether or

hexane. These solvent extraction results are also consistent with previous studies that showed dichloromethane has greater lipid extraction efficiency in comparison to other solvents like methanol, diethyl ether, toluene, isopropanol, ethanol and mixtures of chloroform/hexane, chloroform/heptane, hexane/heptane (Byreddy et al., 2015). A study conducted by Jeon et al. (2013) showed dichloromethane extracted almost 20% more lipids than chloroform, 23% more than ethanol, 25% more than acetone, xylene and 1,3-butanediol, and 26% more than ethyl acetate.

4.3.8 Shear Force

The shear force involved in cell disruption can be expressed in terms of shear rate given in equation 4-1.

From the graphs in Figures 4.6a to 4.9b, it can be seen that cell disruption begins occurring from 4,500 rpm owing to mechanical forces such as hydrodynamic cavitation and shear force. As the microalgae passes through the high shear rate zone, the recirculation rate as well as the maximum shear rate increases with increase in speed resulting in increased volume of the fluid with time around that zone.

Between 8,500 to 15,000 rpm in Figures 4.6a to 4.9b, there is a significant increase in lipids extracted. This shows that hydrodynamic cavitation and mechanical shear-force of the cell occurs rapidly at this speed resulting in collapse of cell cavities (Günerken et al., 2015, Lee et al., 2012). This speed can be confirmed as the critical speed (Aarthy A, 2018).

The shear rate trend is consistent with a previous study which evaluated the shear rate using a rotational speed of 500 rpm to be 1079 s^{-1} (Michels et al., 2010).

From the graphs in Figures 4.6a to 4.9b, it may also be seen that lipids extraction were increased at higher speeds with significant and more consistent increase in lipids from 8,500 rpm up to the maximum speed of 33,000 rpm. Also, the change in temperature generated from the homogeniser device discussed in section 4.3.3 and given in Figure 4.6b, may have influenced the shear rate leading to more lipids release as speed increases in Figure 4.11 below.

The shear rate in this chapter was evaluated to get an estimate of shear rate for the use of the cell strength technique developed on an industrial scale versus lipid extraction. A graph of disrupted cells and the shear rate as well as the lipids extracted at the particular homogeniser speeds is given in Figure 4.12 and 4.13. The results of the disrupted cells in Chapter 3 from Figure 4.12 and Figure 4.13 show a similar trend with the lipids extracted. This further confirms that both cell counting method in Chapter 3 and the cell strength technique developed in this chapter are useful for quantifying degree of pretreatments at laboratory (cell counting) and industrial scale (cell strength lipids extraction technique).

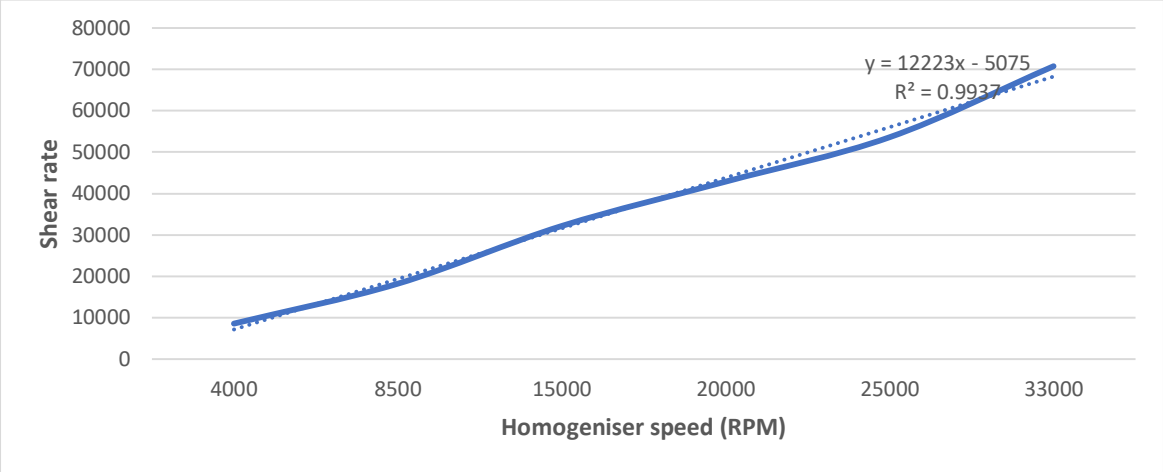


Figure 4.11: Shear rate of C.vulgaris using different homogeniser speeds.

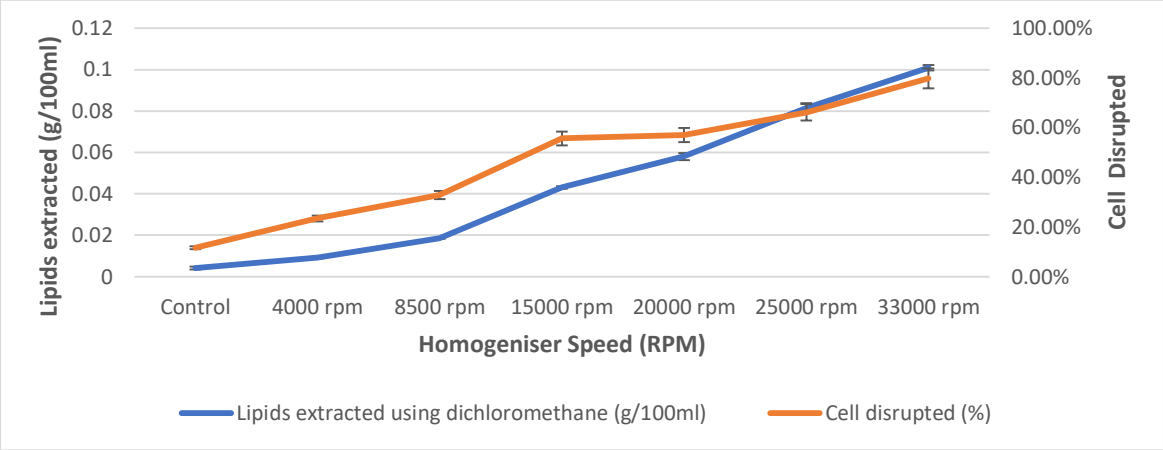


Figure 4.12: Percentage cell disrupted, and lipids extracted at different homogeniser speeds.

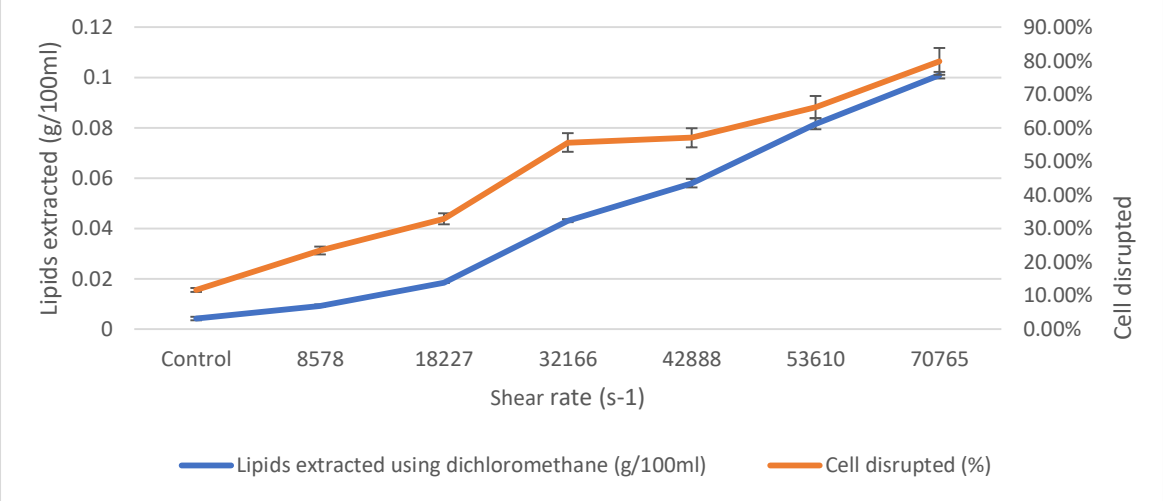


Figure 4.13: Percentage cell disruption and lipids at the shear rate calculated from homogeniser speeds.

4.4 Conclusion

The development of a novel suitable technique to quantify the degree of disrupted microalgae cells irrespective of the type of pretreatment strategy applied was demonstrated. High speed homogenisation is a simple and yet effective means of understanding microalgae cell strength and the impact of pretreatment of algae on their ability to be broken. Until now, little was known about the ideal homogeniser speed required to produce significant lipids release from mechanical shear treatment. Mechanical forces responsible for breaking the walls of microalgae begin to act from around 4,000 rpm with the high-speed homogeniser releasing less lipids at lower speeds. However, from 8,500 rpm, there is a rapid and consistent release of lipids. In addition, microscopic analysis of the cells show disrupted cells with linear increment as homogenisation speed increases up to the maximum homogenisation speed of 33,000 rpm.

It can be concluded more microalgae cells become disrupted with increase in shear rate. 8,500 rpm may also be deduced as the critical speed where significant disruption begins to occur as shear force is increased from this speed eventually breaking the cells and releasing lipids extraction yield.

From the comparison of the different solvents for wet algal lipids extraction to evaluate the cell strength of microalgae, dichloromethane was seen to be more reliable than hexane and diethyl ether for extracting lipids as the results show higher lipids extracted and reduced water content in the aqueous phase making separation easier. Using lysozyme to pre-treat the cells before homogenisation showed a similar linear trend in cell disruption with multiple visible fragments of cells upon visual observation showing visible disruption of the cell structure with cell solubilisation and lipids release even before mechanical shear application.

Comparing the technique developed in this chapter to imaging results from Chapter 3, this technique can be applied in industrial or large-scale microalgae process applications as it is more accurate due to its ability to quantify cell disruption from lipids extracted for greater sample volumes. In addition, the technique provides an indication of the shear rate required for significant lipids extraction. At the critical speed, the shear rate for cell disruption was found to be 18,227s^{-1}.

The technique developed in this study is useful for determination of cell strength and is able to detect changes in microalgae lipids with different pretreatments which can improve the technology of cell disruption prior to anaerobic digestion.

The results from this study have shown that lipid extraction from cells following high-speed homogenisation is a suitable method of estimating the degree of disruption of *C. vulgaris* cells. This is important in commercial biomass applications such as anaerobic digestion, lipids extraction and biodiesel production.

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CHAPTER 5 ANAEROBIC HYDROLYSIS OF *CHLORELLA VULGARIS* USING SELECTIVE MICROBES AND ENZYMES

5.1 Introduction

Anaerobic digestion is an established technology in wastewater treatment to produce energy (Sposob et al., 2020, Chuka-ogwude et al., 2020, Solé-Bundó et al., 2019, Wu et al., 2019, Moheimani et al., 2015a) . Recovered microalgae grown within wastewater systems can be digested anaerobically to produce biogas composing of approximately 60% to 70% v/v biomethane (Ward, 2015, Xiao et al., 2020, Kendir and Ugurlu, 2018, Passos et al., 2013a). The biogas generated can then be utilised for a number of purposes including onsite power generation for the wastewater facilities leading to reduced energy needs and becoming closer to carbon neutral. However, digesting the recovered microalgae can be challenging due to the rigid structure of the cell wall for most microalgae, resulting in low anaerobic digestion rates (Golueke et al., 1957, Sposob et al., 2020, Ward, 2015, Ras et al., 2011). Previous studies have focused on anaerobic digestion of microalgae wastewater using bacteria already present in the system as inoculum (Craggs et al., 2013, De Pauw and Van Vaerenbergh, 1983, Beuckels et al., 2015, Passos et al., 2015b, Solé-Bundó et al., 2019, Chuka-ogwude et al., 2020, Casagli et al., 2020). There have been reports of relatively long retention times of 30 days, 45 days, 64 days and up to 6 months for anaerobic digestion due to challenges involved in digesting the microalgae, which may be attributed to the recalcitrant cell wall components and inappropriate microbial community to act as inoculum for the algae (Solé-Bundó et al., 2018, Mussgnug et al., 2010, Ras et al., 2011, Zhou et al., 2009). To improve microalgae anaerobic digestion, pretreatment of the cells has been established as a key strategy for increasing the permeability of the cells (Ward, 2015, Passos et al., 2015a, Jankowska et al., 2017). Microalgae pretreatment can be energy-intensive, especially mechanical, physical and thermal treatments (Passos et al., 2015a), as the energy used in disruption is sometimes equivalent or more than the overall energy derived from the process (Xiao et al., 2020). Chemical and biological pretreatment are gaining attention due to their lower input energy requirement. Further, chemical pretreatment may introduce impurities in the algae biomass which may result in issues of disposing of the residual digestate. Biological pretreatment involves the use of microbes, such as bacteria and fungi, as well as enzymes to solubilise and disrupt the microalgae cell (Zabed et al., 2019, Carrillo-Reyes et al., 2016, Zheng et al., 2014). The use of purified enzymes alone is cost intensive (Christy et al., 2014). Therefore, investigating a means of utilising microbial pretreatment in a cost-efficient manner is important for adoption of microalgae utilisation in wastewater and the subsequent anaerobic digestion of the harvested algae.

A study of the cell walls of most algae reveals a two or three layered cell wall structure that contains carbohydrates, glycoproteins, and algaenan (Sander and Murthy, 2009). To break down the cell wall structures, enzymes can play an important role in the process. Enzymes from microbes come in form of endo and exo-enzymes depending on their site of action. Exo-enzymes act on the outer cell layer while endo-enzymes operate within the cell membrane (Christy et al., 2014).

Early research viewed anaerobic digestion as a three stage process that included hydrolysis, fermentation and methanogenesis to produce biogas (Parkin and Owen, 1986). Recent studies have broken the fermentation process into hydrolysis, acidogenesis, acetogenesis and methanogenesis making it a four-stage process (Adekunle and Okolie, 2015, Christy et al., 2014, Ward, 2015). Hydrolysis is the rate-limiting step for anaerobic digestion of microalgae as a result of the lignocellulosic nature of the microalgae cell wall causing access difficulty to suitable substrates for hydrolytic microbes (Mata et al., 2010, Varjani et al., 2017). Hydrolysis is usually carried out by facultative bacteria that are capable of surviving with or without oxygen (Chetawan et al., 2020, Fu et al., 2010, Botheju et al., 2010, Cirne et al., 2007a). Hydrolysis can be slow due to microalgae resisting bacterial attack during the reaction process due to the rigid cell wall structure. Incomplete hydrolysis leads to an inefficient anaerobic digestion process and lower biogas yield.

During anaerobic hydrolysis, there needs to be sufficient suitable enzymes available to act on the outer cell wall to soften it and allow other enzymes to break the cell and release the intracellular components required for digestion. Research investigating the use of enzymes to break down the cell walls of microalgae is on-going. In a study that demonstrated the effectiveness of lysozyme enzyme (an anti-bacterium) to degrade *C.vulgaris* for biofuel production, the enzyme degraded the outer cell wall structure after incubating 10µl of enzyme stock on agar containing *C.vulgaris* at 23^{o}C for 5 days (Gerken et al., 2013). This is also consistent with the results obtained so far in this project in chapters 3 and 4 as microscopic evidence showed cell wall degradation of the *C.vulgaris* cells and accessible lipids produced with cells treated with lysozyme were higher than untreated cells.

However, little information is known of the application of enzymatic and bacterial pretreatment for anaerobic hydrolysis and digestion of microalgae, which is worthy of investigation. Also, current anaerobic digestion in wastewater systems occurs using the resident microbial community already acclimatized in the system. There have been reports, however, of low biogas production resulting from the rigidity of microalgae cells using this approach due to suboptimal enzymes being produced by the resident microbial community (Ward, 2015).

Enzymes have proven efficient so far in microalgae degradation, however they are costly, e.g. lysozyme costs are in the region of US$787/50kg (InVitria). Bacteria and fungi produce enzymes that aid in microbial hydrolysis, softening the cells and potentially making digestion

easier. Identifying particular bacteria that secrete the required endo and exo-enzymes needed for anaerobic hydrolysis is key for increasing methane production and improving microalgae anaerobic digestion technology.

The overall objective of this chapter was to identify effective enzymes that would aid in the selection of appropriate microbes if the enzymes produced by the microbes was known. This will subsequently enhance anaerobic digestion of microalgae as additional microbial inoculum will reduce the time for hydrolysis. The specific aims were:

- To identify key enzymes required for efficient microalgae anaerobic hydrolysis.
- To study the impact of lysozyme as a single enzyme and in combination with other enzymes (amylase, pectinase and cellulase) and bacteria (*E. coli, S. thermophilus, L. plantarum)* on microalgae anaerobic hydrolysis and digestion.
- To investigate the effect of selective microbes (*E. coli, S.thermophilus, L.plantarum, A.aceti, A.oryzae)* on *C.vulgaris* hydrolysis.

Process efficiency in this study was measured by analysing the concentration of volatile fatty acids (VFA) produced after various sampling times as well analysing the soluble COD (chemical oxygen demand). VFA production provided an indication hydrolysis rate since the acids produced would subsequently undergo further reactions (acetogenesis and methanogenesis) to produce biogas (Christy et al., 2014, Adekunle and Okolie, 2015, Chuka-ogwude et al., 2020). COD is a measure of chemical oxidation of total organic material present in the water/wastewater sample (Varjani et al., 2017). In anaerobic digestion, the COD is also an indication of the biogas potential of a substrate. In this study, since the focus is on hydrolysis products rather than biomethane production as process efficiency, COD was used as an indicator of changes in organic matter content and to observe potential loss of carbon from biogas production.

5.2 Microbes and enzymes selection

The results from chapters 3 and 4 proved lysozyme was a suitable enzyme for *C.vulgaris* cell wall degradation. Also, a combination of enzymes using amylase, cellulase, pectinase and lysozyme in chapter 4 showed higher lipid extraction than lysozyme as single enzyme alone. Hence, these enzymes were selected as key players and investigated in this chapter for their efficiency for improving anaerobic hydrolysis of *C.vulgaris*. Subsequently, microbes producing these enzymes were further investigated to determine their effect on the hydrolysis. This is to reduce the costs involved in purchasing enzymes and enable commercial applicability of the results obtained in this study. Hence, industrially, it would be best to culture microbes

alongside microalgae for anaerobic digestion as these microbes would produce the desired hydrolytic enzymes saving costs in the process.

Lysozyme is used for hydrolytic cell lysis and has proven effective in disrupting the peptidoglycan of the bacterial cell wall (Ganz, 2006). It is used to disrupt carbohydrates located in certain bacteria cell walls e.g. cocci (Setia, 2010). It works by catalysing the hydrolysis of β-(1→4) glycosidic linkage between N-acetymuramic acid and N-acetylglucosamine in gram-positive bacterial cell walls, acting as an antibacterial agent due to the higher levels of peptidoglycan (Davidson and Zivanovic, 2003). The use of lysozyme in combination with *E.coli,* a gram-negative bacteria, has been investigated in a study showing no inhibition of the enzyme to bacteria growth, due to the low level of peptidoglycan (Kjerstin Ohls, 2008). Here, lysozyme is expected to act as an exo-enzyme, disrupting the outermost structure of the microalgae cell wall, weakening the bonds and paving a way for further bacterial attack on the microalgae cell wall and internal structures. Therefore, investigations of the use of lysozyme alone as well as lysozyme's use with bacteria and other enzymes, such as cellulase, amylase, and pectinase were conducted to confirm its use in microalgae hydrolysis.

Cellulases are enzymes produced by fungi and bacteria that breakdown cellulose into simple monosaccharides (Menon and Watson, 2016). They target the β-1,4-linkages in its polymeric structure to produce glucose (Jayasekara and Ratnayake, 2019). The composition of some microalgae cell wall has been reported to be composed of more than 45% cellulose (Aarthy A, 2018) and cellulose is highly insoluble in water, acting as a defensive barrier for most algae cells from their environment, and resulting in cell wall hydrolysis being challenging. Certain fungi such as *A.oryzae* have been found to secrete cellulase (Pérez et al., 2002, Prajapati et al., 2016, Phutela et al., 2011, Taseli, 2008, Xie et al., 2013). Also, *E.coli* has been found to secrete cellulase as well as other enzymes such as amylase (Amraini et al., 2017, Gao et al., 2015, Pang et al., 2019, Pang et al., 2017). A study carried out by Gupta et al. (2014) stated that the use of cellulase promotes functionality of other hydrolytic enzymes such as proteases, amylase, glucanases, to name a few (Gupta et al., 2014). Herein, cellulase combined with other hydrolytic enzymes such as lysozyme, pectinase, and amylase, were investigated and compared with hydrolytic bacteria during *C.vulgaris* hydrolysis to determine the effect of enzyme mixture for efficient anaerobic hydrolysis, maximising VFA production.

Amylases are a group of hydrolytic enzymes that break down starch into simple sugars such as glucose and maltose (Brahmachari et al., 2017). They are mainly produced by *bacillus* bacteria and *aspergillus* fungi species (Robinson, 2014, Mojsov, 2016). *A oryzae* fungus has specifically been cited to exhibit high amyloytic activity (Kitamoto, 2002). Some *Lactobacillus sp*. such as *Lactobacillus plantarum, L. amylovorus* and *L.manihotivorans* have been isolated for their amylase production (Gopinath et al., 2017, Mehta and Satyanarayana, 2016). This enzyme was selected for use in combination with other hydrolytic enzymes due to its

hydrolytic property to support the catalyses of other enzymes in the anaerobic hydrolysis process. Also, *L.plantarum* has been chosen as a key bacterium due to its secretion of amylase and lysozyme enzymes (Mehta and Satyanarayana, 2016, Saygusheva et al., 2013, Promchai et al., 2020).

Pectinases are hydrolytic enzymes that break down pectin, a complex polysaccharide component of plant cell walls into simpler molecules like galacturonic acid (Verma et al., 2018). They are mainly produced by fungi *Aspergillus* sp. and bacteria *Clostridia* sp (Mojsov, 2016, Sharma et al., 2013b). Other microbes that produce pectinase include *Saccharomyces, Bacillus, Erwinia, Penicillium, Rhizopus and Fusarium* (Verma et al., 2018).

Enzymes such as cellulase, proteases, lipases, esterases and pectinase have been cited for assisting in microalgae cell hydrolysis (Muñoz and Gonzalez-Fernandez, 2017).

Acetobacter aceti (*A. aceti)* is a clostridia bacterium used in the production of acetic acid (Matsushita et al., 2005, Mullins et al., 2008). *A.aceti* is known to secrete dehydrogenase and is responsible for acetate reduction contributing to 70% methane production in anaerobic digestion (Ali Shah et al., 2014, Lynch et al., 2019). Hence, its selection for anaerobic hydrolysis tests in this study.

E.coli are a group of *Enterobacteriaceae* facultative anaerobes that ferment to produce lactate, acetate, formate and CO_2 (Robinson, 2014). It is known to secrete several hydrolytic enzymes including cellulase, (Gao et al., 2015, Pang et al., 2017, Amraini et al., 2017), amylase (Shahhoseini et al., 2003, Rosales-Colunga and Martínez-Antonio, 2014, Lee et al., 2001), and pectinases (Yaqoob et al., 2019, Rebello et al., 2017) which are considered in this study.

S. thermophilus is a fermentative facultive anaerobe bacteria that is used for lactic acid production (Kalyankar et al., 2016, Fuquay et al., 2011). It is used alongside *lactobacillus sp.* in milk fermentation particularly *L. plantarum* at mesophilic ranges between 20ºC to 45ºC (Fuquay et al., 2011).

A study conducted by Turchi et al. (2017) showed enhanced growth of *S.thermophillus* when co-cultured with *L.plantarum*. During this study Turchi et al.(2017), both bacteria were investigated for lysozyme resistance and found to be highly resistant to lysozyme displaying 100% and 96% resistance at a concentration of lysozyme of 6.4mg/ml each for *S.thermophillus* and *L.plantarum* respectively. One of the hydrolysis tests used a mixed hydrolytic bacteria culture in combination with lysozyme to promote hydrolysis. Since *S.thermophillus* and *L.plantarum* have shown lysozyme resistance and *E.coli* has also shown ability to resist the enzyme (Kjerstin Ohls, 2008), it was expected that lysozyme would degrade the outermost structure of the microalgae cell wall, making provision for bacterial hydrolysis.

5.3 Materials and Methods

5.3.1 Microorganisms collection, preparation and harvest

C.vulgaris (Strain obtained from the I.U.C.C. ex Cambridge. CCAP 1988 strain) was obtained from the Australian National Algae Collection, CSIRO. Upon thawing, the algae were first recultured for growth in 250 ml flask, upscaled to a 2 L flask and placed on a Ratek EOM5 model shaker at 120 rpm. The algae were then transferred to a 20L photobioreactor and recultured to grow using bolds basal growth media (CSIROMarineResearch) with continuous stirring and agitation at 120 rpm under artificial fluorescent lights (using two Eziplug grolite 130 watts E-40 base, 220-240 V 50-60 Hz lights) on a 16:8-hour light cycle and at ambient temperature (approx. 20ºC). Air was bubbled into the bioreactor at 200 ml/min. To prepare bolds basal media, the original recipe by (Nichols and Bold, 1965) was used.

E.coli, S. thermophilus, and *L.plantarum* were all obtained from the Victoria University culture collection. The *A.aceti* (American Type Culture Collection, ATCC 15973) was purchased from Cell Biosciences in kwik stick format and was resuscitated using mannitol broth/agar as outlined in the *Acetobacter Beijerinck aceti* product sheet (ATCC, 2017). *A oryzae* fungus was obtained also within the grounds of Victoria University, Werribee campus and re-cultured using the conditions provided in (ATCC, 2017) as seen in Table 5.1 below. All microbes were prepared in the fume cupboard using aseptic techniques and cultured in a shaking incubator using methods and growth conditions as outlined by (ATCC, 2017) and shown in Table 5.1.

Table 5.1: Growth conditions of microbes used in this project obtained from (ATCC, 2017).

Microbe	**Growth media**	**Growth Conditions**
E.coli	Trypticase soy broth	37ºC for 24 hours
S.thermophillus	Trypticase soy broth	37ºC for 24-48 hours
L.plantarum	MRS	37ºC for 24-48 hours
A.aceti	Mannitol broth	26ºC for 48-72 hours
A.oryzae	Potato dextrose	25ºC, 150 rpm 72 hours

C.vulgaris growth rate was monitored over time by measuring optical density scanned at 750 nm using a UV biochrom libra s22 spectrophotometer. The algae were then harvested during the active growth phase when the medium had optical densities of 1.0 to 1.5. The cells were dewatered using an Avanti J26S XP centrifuge (Beckman Coulter) with rotor JLA-16.250 at 3,500 rpm for 5 minutes and a 10% concentration by volume was obtained prior to the anaerobic hydrolysis experiments. Bacteria and fungi used were also harvested in their active growth phase following 1 to 3 days of growth in bioreactors. Dewatering of the cells was again

achieved using a centrifuge at 3,500 rpm for 5 minutes. Bacteria and fungi cells were also scanned at optical density of 1.0 to 1.5 at wavelength of 600 nm. 600 nm is used to ensure the bacteria and fungi remain alive and active, not destroyed by the ultraviolet radiation from the spectrophotometer (Stevenson et al., 2016). The cells were then rinsed and brought back to volume using a phosphate buffer solution obtained from Sigma Aldrich (catalog # SLBH8389V) to help maintain a constant pH in the system. To make phosphate buffer solution from tablet; one tablet was dissolved in 200 ml deionised water to make 0.01M phosphate buffer, 0.0027 M KCl and 0.137M NaCl with a pH of 7.4 at 25^{o}C room temperature.

5.3.2 Chemical Oxygen Demand (COD) Method

COD analysis was conducted using Hach COD digester reagents USEPA approved wastewater analyses (standard method 5220D). High range COD test kits were used for digestion with chromium reduction determined using a Hach DR 5000 spectrophotometer.

The COD values were recorded to determine the variation in organic matter during the anaerobic hydrolysis process. COD measurements were performed using 20-1500 mg/L Hach digestion solution vials (CAT No 2125915).

Algae suspensions were diluted 1:10 prior to the COD measurement by pipetting 1 ml of algae suspension into a 10 mL falcon tube with 9 ml deionised water. Then, 2 mL of the prepared solution was transferred into the Hach reagent vial and the lid tightly closed. A blank vial containing only deionised water was used as a control sample. The Hach digester was set for operation at 150^{o}C and reagents were digested for 2 hours. The mixture was then allowed to cool before measuring COD by spectrophometric analysis using a Hach DR 5000 at 620 nm wavelength. Prior to collecting the readings from the spectrophotometer, the Hach method, 435 COD HR was loaded to determine the amount of soluble organic material present in the sample at the beginning of testing and after 15 days batch test.

5.3.3 GC Standard Preparation method

To analyse the VFA released from anaerobic hydrolysis, a standard was purchased from Sigma Aldrich via Supelco (catalog number: XA26108V) containing a volatile free acid mixture. The mixture contained ten acids namely; formic, acetic, propionic, isobutyric, butyric, isovaleric, valeric, isocaproic, hexanoic and n-Heptanoic of 10mM each in deionised water. From the original standard of 10mM, a 1:100 dilution was made. Then, the sample was further diluted to 1:10, 1:2 and another 1:2 to make the final standard used for comparison in ppm or mg/L. VFA concentrations were analysed using a GC-2010 Shimadzu gas chromatograph with AOC-20i auto injector, flame ionisation detector and AOC-20s auto sampler. To analyse the samples, 1 ml of each digestate was collected and pre-filtered using a 0.45um polytetrafluoroethylene (PTFE) filter.

5.3.4 Enzyme Preparation

Enzymes were purchased from Sigma Aldrich with the following material description: alpha-amylase from porcine pancreas A6255-10MG, pectinase from *aspergillus niger* P4716-5KU, cellulase from *aspergillus niger* C1184-5KU, and lysozyme from chicken egg white L6876-5G. Enzyme stock solutions were prepared to 20 mg/mL using milli Q water. Then, 25 µl of each enzyme stock solution was employed for analysis. To make up the enzyme inoculum, 25 ml of each enzyme solution was added in a Schott bottle to bring the volume to 100 ml.

5.3.5 Algae Concentration and Dry Weight Technique

The biomass concentration and dry weight (as total suspended solids) of the algae was measured using standard methods (Baird, 2017) by passing 100 mL of algae suspension through a pre-weight GF/C glass microfilter (47mm diameter, Whatman GC healthcare life sciences). The filter paper was then dried in an oven at 105^{o}C overnight and allowed to cool in a desiccator to room temperature. The new weight was recorded and the total suspended solids dry weight in g/100mL was calculated.

5.3.6 Experimental Process description

A flow diagram of the steps required for experimental preparation, operation and analysis is shown in Figure 5.1.

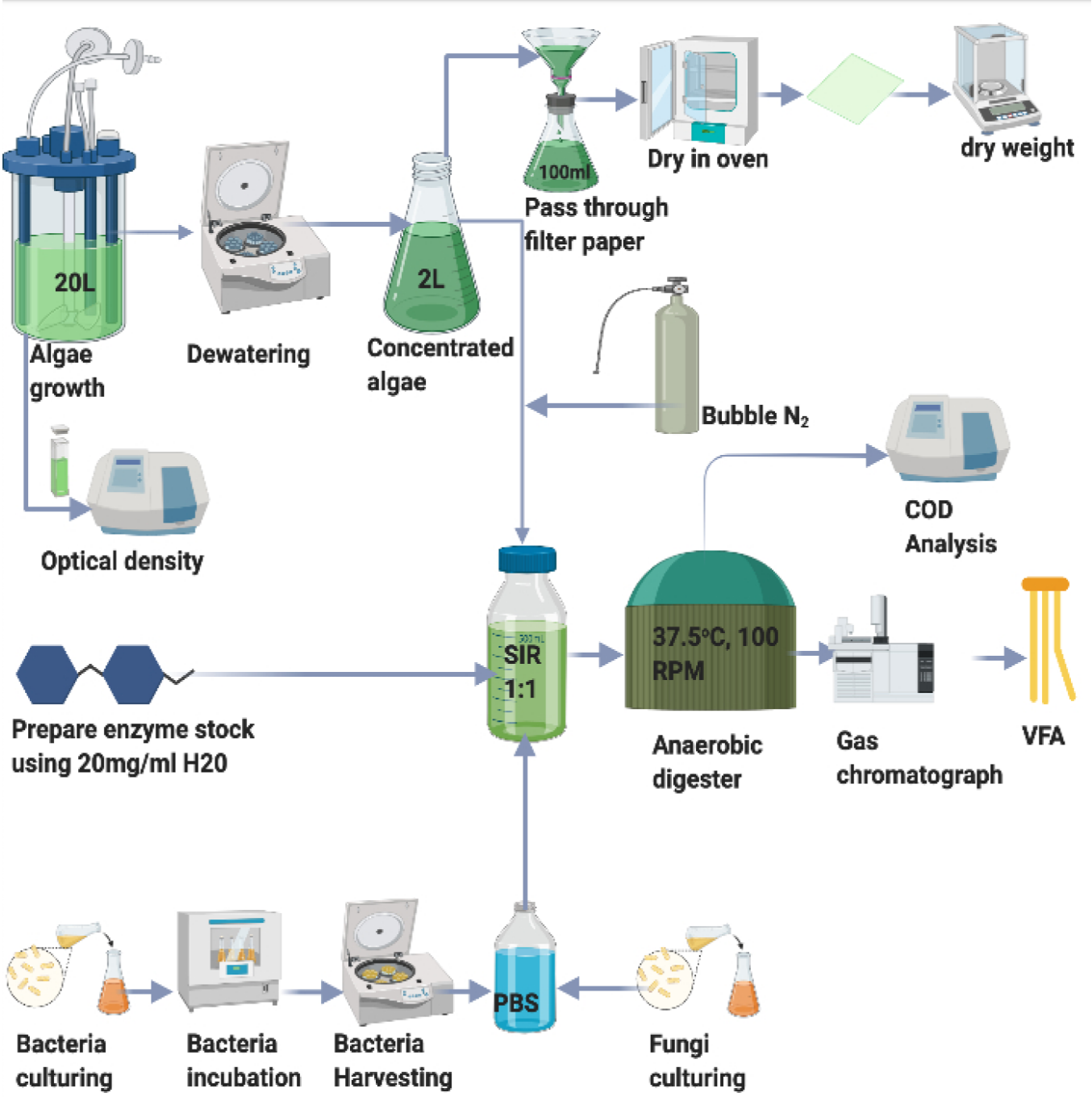

Figure 5.1: Anaerobic Hydrolysis process flowchart.

Two separate experiments were conducted during this study using two batches of *C.vulgaris* as the microalgae substrate with different inoculum in various anaerobic hydrolysis tests. Batch 1 experiments used hydrolysis sampling times of 30, 45 and 60 days with the following sets of duplicate experiments listed below:

i) Control, *C. vulgaris alone,*
ii) *C.vulgaris* with *E.coli, L.plantarum, S.thermophillus*
iii) *C.vulgaris* with *E.coli, L.plantarum, S.thermophillus, & A.aceti*
iv) *C.vulgaris* with Lysozyme, *E.coli, L.plantarum, S.thermophillus*
v) *C.vulgaris* with Lysozyme alone.

Following the results from Batch 1 experiments, 30 days displayed the highest VFA concentrations (see section 4.1). Hence, it was decided to decrease the sampling time for the anaerobic hydrolysis tests. All tests in Batch 1 were repeated in batch 2 except test (Batch 1.iv) which was replaced by a test that used bacteria to produce similar enzymes. Then, a new test was added using *A.oryzae.*
Batch 2 had a maximum of 15 days incubation time using the following set of tests:

i) Control, *C. vulgaris alone*
ii) *C.vulgaris* with *E.coli, L.plantarum, S.thermophillus, A.aceti*
iii) *C.vulgaris* with *E.coli, L.plantarum, S.thermophillus*
iv) *C.vulgaris* with Lysozyme alone
v) *C.vulgaris* with *fungus aspergillus oryzae*
vi) *C.vulgaris* with Lysozyme, amylase, cellulase and pectinase.

A substrate to inoculum (SIR) ratio of 1:1 (200 ml total) was used for algae to inoculum using a 500 mL Schott bottle as an anaerobic digester with a headspace of 300 ml. Before beginning the tests, nitrogen was bubbled into each digester for 15 to 30 seconds to degas oxygen and to maintain the system as anaerobic. The digesters were then sealed by tightening the cap to avoid leakage and placed in a tray to contain any accidental spillage. The tray was placed in an enclosed Innova 4320 incubator shaker (New Brunswick Scientific) to allow continuous mixing with no light to prevent photosynthetic activity and operated at a mesophilic temperature of 37.5^{o}C and 100 rpm to digest anaerobically with no pH control.

5.3.7 Statistical analysis

Experiments were performed in duplicate with statistical data computed and analysed using Microsoft excel including calculating the mean and standard deviation values. The results reported include error bars from the duplicate experiments conducted, and the error bars represent the standard deviation of the duplicate experiments.

5.4 Results and Discussion

5.4.1 Batch 1 Hydrolysis analysis

Initial batch tests had duplicate experiments with sampling times of 30, 45 and 60 days without COD monitoring. The optical density of *C.vulgaris* at time of harvest was 1.063 at 750 nm. For bacteria optical density at 600 nm, absorbance is as follows: 1.385 for *E.coli,* 1.160 for *L.plantarum*, 1.107 for *S.thermophillus* and 1.174 for *A.aceti.*

Microalgae dry weight concentration was measured as 0.182 g/100mL. The total VFA concentration results of the initial tests showed 30 days hydrolysis to result in the highest VFA concentrations compared to 45- and 60-day hydrolysis as seen in Figure 5.2.

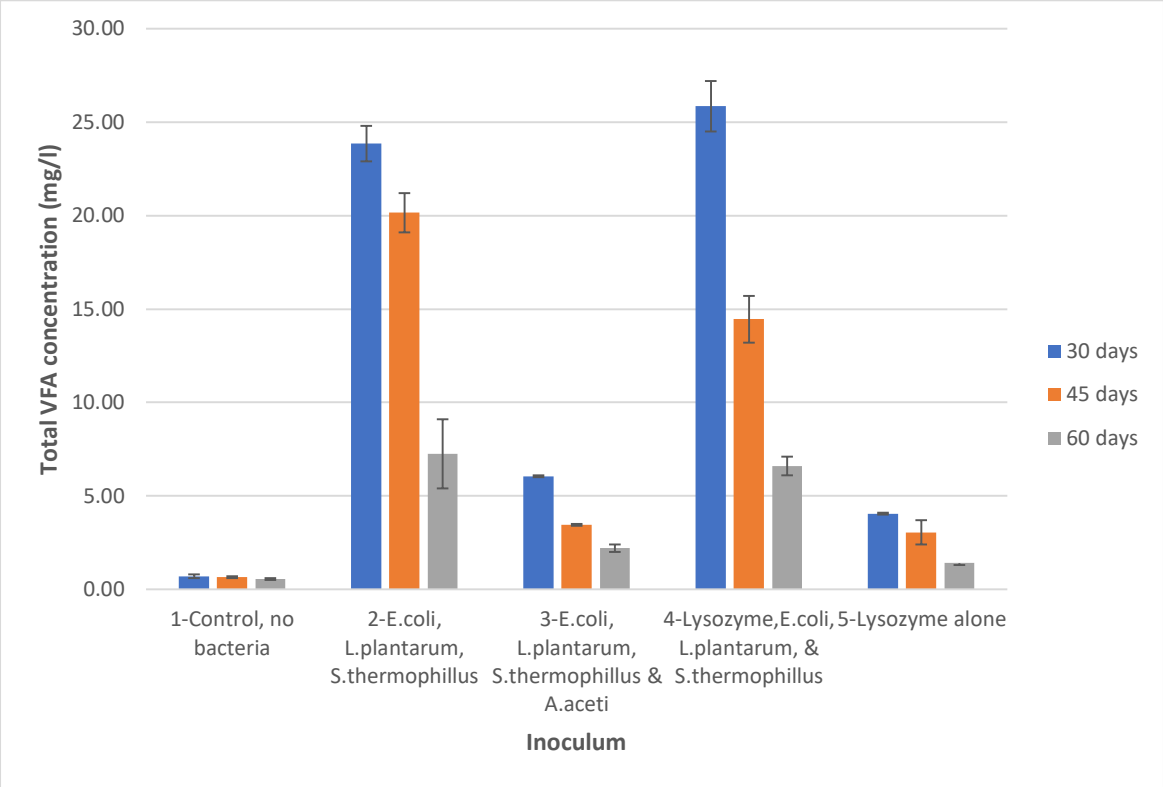

Figure 5.2: Average total volatile fatty acid (VFA) concentration for 30, 45- and 60-days hydrolysis.

The results show the combination of lysozyme, *E.coli, L.plantarum,* and *S. thermophilus* produced the most total VFA at an average of 25.9 mg/L after 30 days, making it a 37-fold increase when compared to the control without microbes. The second highest VFA concentration was the bacterial combination of *E.coli, L.plantarum,* and *S. thermophilus* producing a total average VFA concentration of 23.9 mg/L after 30 days. The bacterial combination of *E.coli, L.plantarum, S. thermophilus and A.aceti* had low VFA production at 6 mg/l. Lysozyme alone also recorded low VFA production at 4 mg/l, a 5.7-fold increase over the control which is quite significant, but not as productive as the lysozyme with the microbes.

The control tests with no addition of microbes or enzymes produced just 0.70 mg/L of VFA after 30 days.

From the results, lysozyme alone was not highly effective in producing hydrolysis products, i.e., VFA production. Instead, lysozyme appears to work synergistically with bacteria to hydrolyse the microalgae. It is postulated that lysozyme may degrade the cell to an extent allowing further bacterial degradation and producing more VFA. This also confirms the results produced by (Gerken et al., 2013) that suggested that lysozyme treats the outermost structure of the cell wall, making the cells more accessible to bacteria attack. Also, lysozyme's antibacterial property had no effect on *E.coli, L.plantarum, S. thermophilus* in agreement with previous studies (Kjerstin Ohls, 2008, Turchi et al., 2017). The resistance of the hydrolytic bacteria to lysozyme allowed lysozyme to sufficiently act on the cell walls permitting the bacteria to hydrolyse the cells further.

Eight volatile fatty acids were detected in the reactor effluent namely: acetic, propionic, isobutyric, butyric, valeric, isovaleric, isocaproic, and hexanoic acids as seen in table 5.2 below:

Table 5.2: Volatile fatty Acids obtained from reactor after hydrolysis at 30, 45 and 60 days.

		1. Acetic Acid			**2. Propanoic Acid**			**3. Iso-Butyric Acid**			**4. Butyric Acid**			**5. Iso-valeric Acid**			**6 A**
Inoculum	**30 days**	**45 days**	**60 days**	**30 days**	**45 days**	**60 days**	**30 days**	**45 days**	**60 days**	**30 days**	**45 days**	**60 days**	**30 days**	**45 days**	**60 days**	**30 days**	**4 d**
1-Control, no bacteria	0.0	0.0	0.0	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0
2-E.coli, L.plantarum, S.thermophillus	8.8	4.9	1.8	2.9	2.8	0.3	4.2	4.2	2.5	2.2	2.2	0.1	3.7	3.7	2.3	1.2	1
3-*E.coli, L.plantarum, S.thermophillus & A.aceti*	4.2	2.0	0.8	0.1	0.1	0.1	0.6	0.5	0.5	0.3	0.2	0.2	0.3	0.3	0.3	0.1	0
4-Lysozyme,*E.coli, L.plantarum, & S.thermophillus*	17.2	6.4	2.3	1.1	0.8	0.3	1.8	1.8	0.4	0.5	0.4	0.0	5.0	4.9	3.6	0.1	0
5-Lysozyme alone	1.9	1.9	0.8	0.5	0.2	0.0	0.3	0.2	0.2	0.3	0.0	0.0	0.9	0.7	0.5	0.1	0

Acetic acid was the highest concentration of VFA in the system produced by lysozyme, *E.coli, L.plantarum,* and *S. thermophilus* at 17.2 mg/L after 30 days. The second highest acetic acid concentration was recorded by the bacterial combination of *E.coli, L.plantarum,* and *S. thermophilus* at 8.8 mg/L after 30 days. The control had no acetic acid production in all sampling tests. All cultures used had different peak acetic acid production times but best overall was observed at 30 days for batch 1 experiments. The acetic acid results for batch 1 are displayed in Figure 5.3.

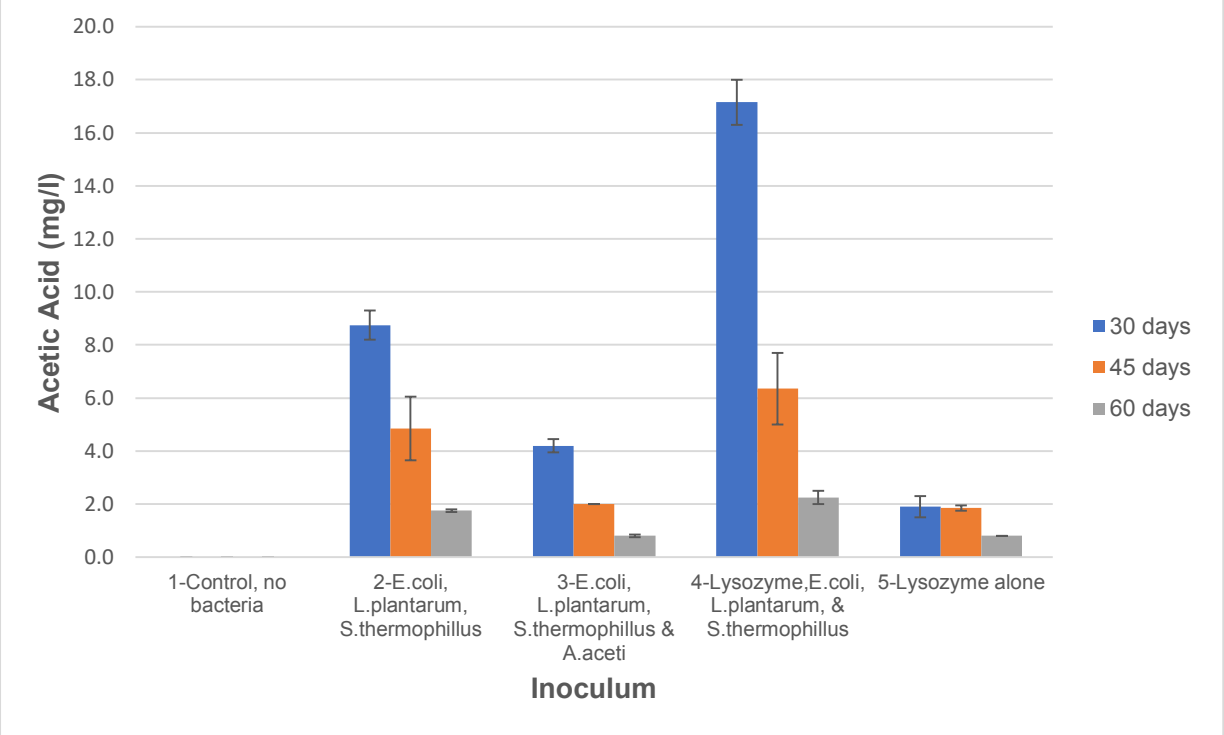

Figure 5.3: Average acetic acid concentration for 30, 45- and 60 days hydrolysis.

5.4.2 Batch 2 Hydrolysis analysis

Following the first experiments, same tests were repeated with a shorter hydrolysis time. The shorter hydrolysis times were chosen to capture the peak in VFA production, as the previous experiment showed decreasing VFA concentrations with time, starting at 30 days. Therefore, the next anaerobic hydrolysis experiments occurred for up to 15 days. The optical density of *C. vulgaris* recorded was 1.457 at 750 nm for batch 2 experiments. The microalgae biomass concentration was 0.327 g/100mL measured by dry weight, which was 1.8 times the concentration of the batch 1 algae feed. For bacteria concentration at 600 nm, absorbance is as follows: 1.419 for *E.coli,* 1.206 for *L.plantarum*, 1.465 for *S.thermophillus* and 1.002 for *A.aceti.* Fungus *A.oryzae* had absorbance of 1.145 at 600 nm.

The aim of this test was to determine if a shorter retention time of 15 days would detect a faster rate of hydrolysis compared to the 30, 45- and 60-days sampling times previously used,

and to confirm the effect of the various bacteria and enzyme additives on hydrolysis of microalgae.

COD was monitored at the beginning and after 15 days hydrolysis to determine process efficiency and understand the removal of organic material by the microbes and enzymes during anaerobic hydrolysis.

The results showed a much higher total concentration of VFA than the previous tests done in batch 1, corresponding to the higher algae concentration in the feed compared to batch 1. The highest recorded total VFA production in batch 2 was obtained by inoculum containing mixed enzymes of lysozyme, cellulase, amylase and pectinase with the concentration being 194.60 mg/L followed by the bacterial combination *of E.coli, L.plantarum* and *S.thermophillus* at 160.95 mg/L as shown in Figure 5.4. There was a 53-fold increase when comparing the enzyme mixture inoculum and the control, which produced only a total of 3.65 mg/L VFA.

Lysozyme alone again had reduced performance compared to its use in combination with other enzymes. This is in line with previous hypothesis that lysozyme degrades the outer cell layer and enables faster hydrolysis but may not promote cell hydrolysis when used alone. Fungi treatment alone also had low production of VFA at 22.70 mg/l. Fungus *A.oryzae* is known to produce cellulase which acts on cellulose.

It is hypothesised that the enzyme combination of lysozyme, cellulase, pectinase and amylase had the highest VFA production because lysozyme had caused initial cell degradation of the outermost cell, allowing cellulase, amylase and pectinase to penetrate the cellulose layers of the cell, resulting in effective cell wall hydrolysis.

Eight volatile fatty acids were also detected as seen in table 5.3.

Table 5.3: Volatile fatty Acids obtained from reactor after hydrolysis at 15 days.

Inoculum	1.Acetic Acid	2.Propanoic Acid	3.Iso-Butyric Acid	4.Butyric Acid	5.Iso-valeric Acid	6.Valeric Acid	7.Isocaproic	8.Hexanoic
1-Control, no bacteria	1.10	0.65	0.60	0.80	0.70	0.10	0.00	0.00
2-*E.coli, L.plantarum, S.thermophillus, & A.aceti*	5.80	2.80	2.20	3.30	2.75	0.40	0.30	0.20
3-*E.coli, L.plantarum & S.thermophillus*	50.25	25.50	23.00	23.25	21.65	15.00	1.60	0.70
4-Lysozyme alone	37.00	10.30	7.70	7.05	2.35	0.40	0.30	0.25
5-fungi alone	16.00	2.80	0.30	2.60	0.60	0.20	0.10	0.10
6-Lysozyme, amylase, cellulase & pectinase	97.15	19.25	20.00	16.35	22.00	17.30	1.60	0.95

Acetic acid was in highest concentration at 97.15 mg/L for mixed enzyme combination, 50.25mg/L for mixed bacteria combination of *E.coli, L.plantarum* and *S.thermophillus* and 37 mg/L for lysozyme alone as seen in figure 5.4.

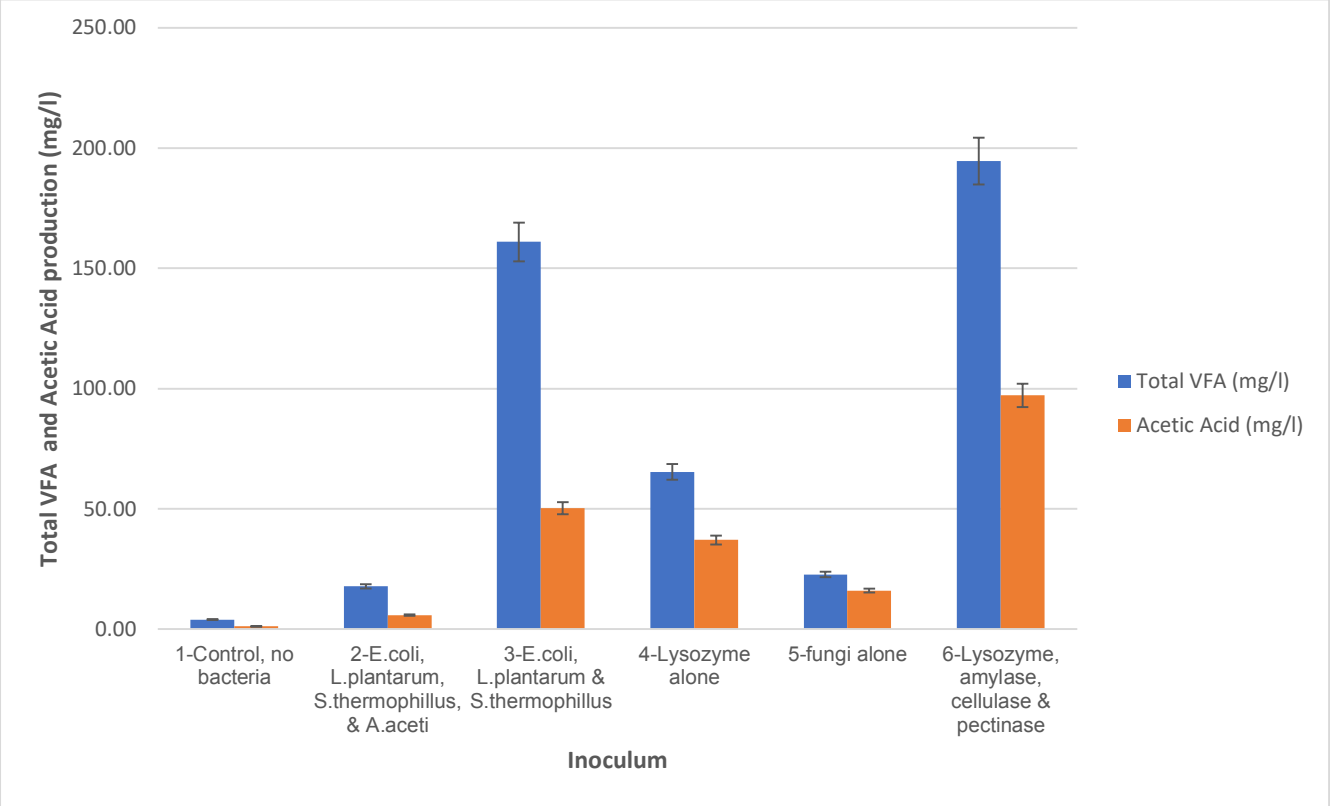

Figure 5.4 Volatile fatty acids (VFA) and acetic acid concentrations for 15-days hydrolysis.

5.4.3 COD Analysis

During the anaerobic hydrolysis process, there was no further addition of algae to the system. The COD was monitored on day-1 and day-15. There was considerable COD loss as was expected during anaerobic hydrolysis.

The results from the COD showed the control had a COD increase of 23%. All other samples dropped in COD concentration with lysozyme-algae mixture having the highest decrease of almost 50%. The fungi inoculum had the lowest COD removal rate of about 10%. The results are displayed in Figure 5.5 below.

The results from the COD analysis shows that the control over time gained more organic material as algae growth would have increased due to the algae still immersed in bolds basal medium supplying nutrients for algae growth. The inoculum containing the mixed enzymes were out of range on the spectrophometric readings.

Following that, lysozyme's COD removal rate of almost 50% indicates its hydrolytic property which were also evident from its high acetic acid production of 37 mg/l, compared to the control

of only 1.1 mg/l of acetic acid production. Acetic acid production is a key indication of efficient anaerobic hydrolysis as 70% of methane in the digestion process is produced from Acetoclastic reaction whilst only 30% of overall methane is generated from hydrogen reduction (Cavinato, 2011, Ali Shah et al., 2014, Ostrem and Themelis, 2004). However, it should also be noted that from table 5.4, even though lysozyme had the highest COD removal rate, it had low VFA production. This suggests that lysozyme's effect may not necessarily lead to biogas production as the enzyme does not appear to degrade to significant VFA production. Nevertheless, lysozyme seems to undergo other reactions due to the high COD removal rate. It is very likely that lysozyme is attacking the cell wall as highlighted earlier and preparing the wall for further bacterial attack by other enzymes or bacteria. This is also demonstrated when lysozyme is used in combination with other bacteria and enzymes.

Mixed hydrolytic bacteria containing *E.coli, S.thermophillus, L.plantarum* also had moderate COD removal rate of 32%. This is also due to the presence of hydrolytic enzymes such as cellulase, amylase, lysozyme being secreted by these bacteria breaking the microalgae. The COD removal rate also corresponds with acetic acid production by this mixed bacteria population as the results obtained showed 25.3 mg/l acetic acid production, next to lysozyme's result.

The lowest COD removal was recorded by the fungus *A.oryzae* inoculum with 10.3%. It is also noted that this fungus produced low acetic acid results of 16 mg/l. *A.oryzae* as stated earlier produces cellulase enzyme (Prajapati et al., 2016, Bhattacharya et al., 2017), a hydrolytic enzyme. However, a study conducted by Kapaun and Reisser(1995) showed *C.vulgaris* species to lack cellulose in their cell walls, hence the possible lack of degradation by the enzyme cellulase from the fungus on the algae. This is also in agreement with work conducted by Gerken et al. (2013) which reports that cellulase had little or no effect on *C.vulgaris* cells. The results of that study was also supportive of previous research which stated that most *C.vulgaris* strains have little or no glucose in the cell wall (Takeda, 1991). Furthermore, a study by Mahdy et al. (2014) using arabanase, cellulase, ß-glucanases, hemicellulose and xylanase showed low methane production. Similarly (Kim et al., 2014) showed that cellulase and amylase had no effect on *C.vulgaris* cell disruption. However, pectinase did have a significant effect on the de-polymerisation of *C.vulgaris* cell wall from their results.

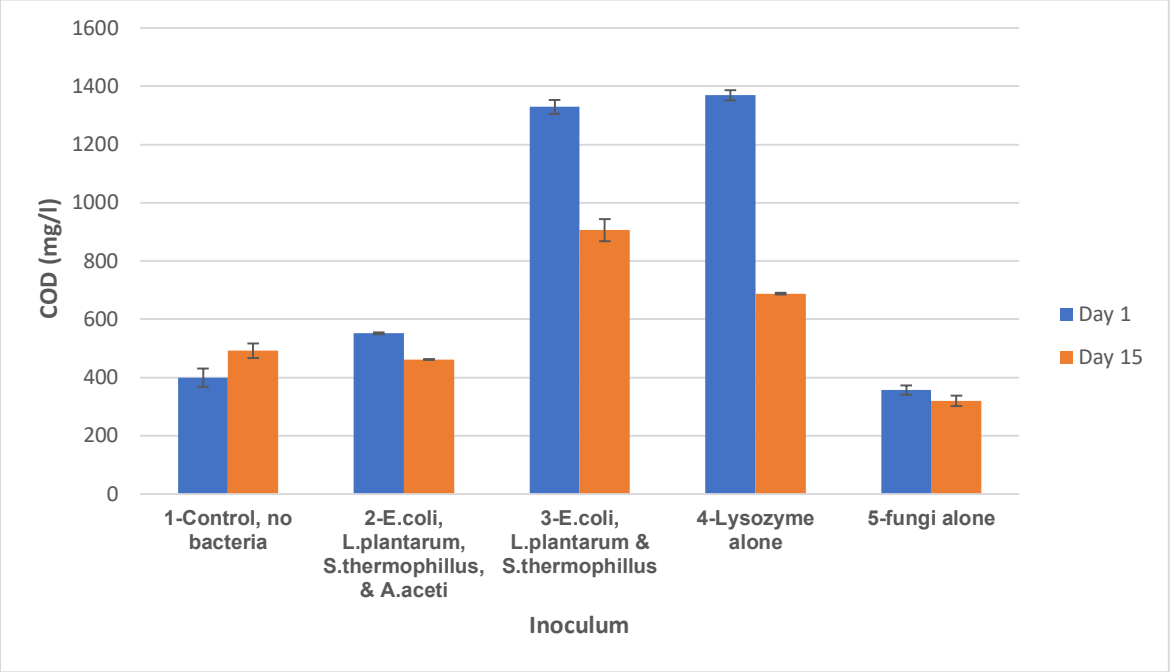

Figure 5.5: COD analysis at day 1 and day 15 hydrolysis

Table 5.4: Total VFA production and COD removal rate at 15-day sampling

Inoculum for 15 day-sampling	Average Total VFA (mg/l)	COD removal (mg/l)
1-Control, no bacteria	4.0	23.15
2-E.coli, L.plantarum, S.thermophillus, & A.aceti	17.8	-16.30
3-E.coli, L.plantarum & S.thermophillus	161.0	-31.83
4-Lysozyme alone	65.4	-49.74
5-fungi alone	22.7	-10.36
6-Lysozyme, amylase, cellulase & pectinase	194.6	Out of range (OFR)

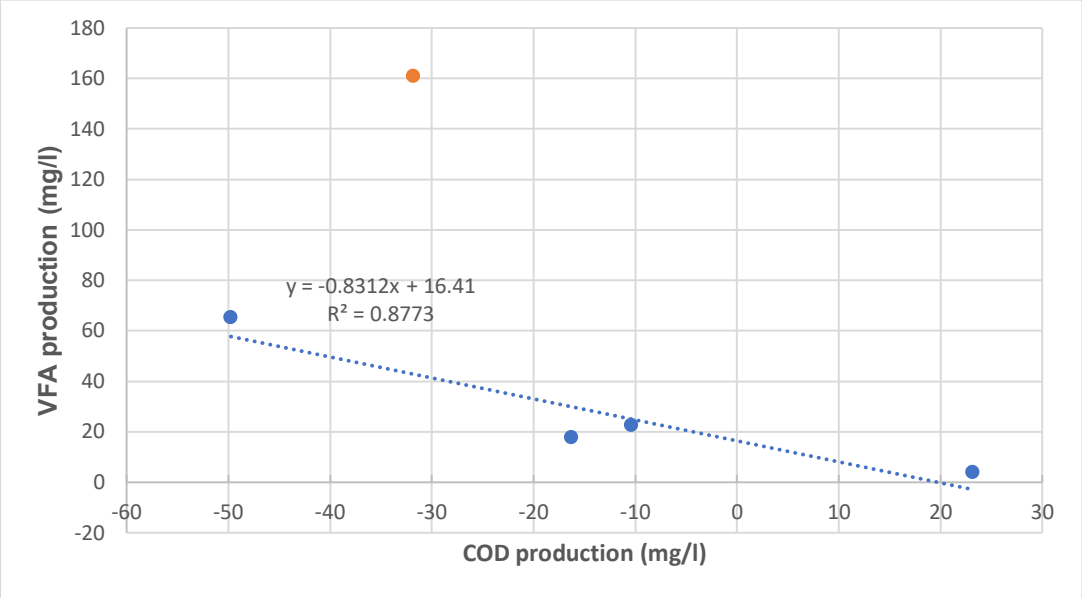

Figure 5.6 COD production versus VFA production. Orange point indicates bacterial combination test.

It is also likely that COD removal may be associated with biogas production. However, since the focus of this study was on hydrolysis, this idea will require further investigation.

5.4.4 Further Results Discussion: Comparison of Batch 1 and Batch 2

Evaluating the research results, there were some key observations detected. Firstly, the results indicate that microalgae hydrolysis is influenced by the algae concentration. Comparing the dry weights of batch 1 and batch 2, there was almost 80% increase in dry weight with batch 1 at 0.182 g/100ml and batch 2 having a dry weight of 0.327 g/100ml. The difference was also observed in the optical density as batch 1 had an OD_{750nm} of 1.063 and batch 2 had an OD_{750nm} of 1.402. In terms of total volatile fatty acids production, batch 2 of 15 days sampling provided higher VFA concentrations compared to all tests of batch 1 including 30-day, 45-day and 60-day sampling. The total volatile fatty acids obtained from the best performing inoculum across both experiments' batches were around 7.5 fold observed for lysozyme, *E.coli, S.thermophillus, and L.plantarum,* mixture at 25.85 mg/l as in batch 1, as well as lysozyme, amylase, cellulase and pectinase at 194.6 mg/l in batch 2.

A.aceti in combination with *E.coli, S.thermophillus, and L.plantarum* in batch 1 and 2 had low VFA production compared to other inoculum. *A.aceti* is a major producer of acetic acid which forms acetates that are reduced to produce 70% methane in the system. Acidogenesis is the fastest reaction in the anaerobic digestion process and occurs almost simultaneously with acetogenesis (Ali Shah et al., 2014). In earlier research, both acidogenesis and acetogenesis were combined to a single step reaction (Parkin and Owen, 1986, Zeeman et al., 1997).

5.5 Conclusion

Based on the findings of this study, mixed specific bacteria (*E. coli, S. thermophilus* and *L. plantarum)* or mixed enzymes (lysozyme, cellulase, pectinase and amylase) are useful as inoculum for anaerobic hydrolysis of microalgae, improving anaerobic digestion. In addition, factors such as algae concentration and retention time influences the extent of anaerobic hydrolysis. The results show that enzymes play a key role in increasing the reaction rate of hydrolysis. However, when considering enzyme cost, utilising bacteria producing the desired enzymes is more beneficial as it more cost effective.

In summary, lysozyme's addition, when used in combination with other hydrolytic enzymes or with specific bacteria that produce specific enzymes, appears to display an ability in degrading the outermost cell layer, causing in cell solubilisation for easy access to bacteria attack. Lysozyme seems to prepare the surface for bacterial attack by bacteria that produce other specific enzymes and that are not impacted by lysozyme. In addition, a combination of *E. coli, S. thermophilus* and *L. plantarum* can produce the desired mixed enzymes; lysozyme, cellulase, pectinase and amylase which were identified as being effective for microalgae degradation by anaerobic hydrolysis.

It can thus be concluded that the effectiveness of enzymes, already effective in anaerobic hydrolysis, can be greatly enhanced when combined with other enzymes or microbial inoculum. Also, the results suggest that the enzyme cellulase may have little effect on the cell wall of *C.vulgaris* owing to the low VFA production from *A.oryzae* which mainly secretes this enzyme. This is in agreement with previous studies that the cell wall of *C.vulgaris* lacks cellulose (Mahdy et al., 2014, Kim et al., 2014, Gerken et al., 2013). It should be noted further from this study that the right enzyme mixture can degrade the microalgae cell quickly and efficiently.

Finally, utilising additional microbes and enzymes to anaerobic hydrolysis can greatly improve anaerobic hydrolysis as the results display more than 50-fold increase in VFA compared to the control that had no added microbes or enzymes. For application of the results of this study, it will be ideal to have a two-stage anaerobic digestion process with seeding of appropriate bacteria applicable for each reaction from hydrolysis to methanogenesis. The first stage will undergo hydrolysis and acid reactions whilst the second stage will undergo acetate breakdown for methane production. Two-stage digestion is known to reduce VFA accumulation which is a challenge in anaerobic digestion. In addition, several studies have been noted that there is increased breakdown of organic matter, increased biogas production and improved control of reactor contents reducing the inhibition of microbial community when a two-stage digestion is employed (Ward et al., 2014, Cirne et al., 2007a, Lunprom et al., 2019).

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CHAPTER 6 EFFECT OF *CHLORELLA VULGARIS* CELL STRENGTH ON ANAEROBIC HYDROLYSIS WITH FUNGUS *ASPERGILLUS ORYZAE*

6.1 Introduction

Fungi has been recommended for improving microalgae harvesting technology by flocculation (Bhattacharya et al., 2017, Prajapati et al., 2016) and is a potential algal harvesting technique as described in Chapter 2. Algae biomass harvesting and dewatering can be challenging due to the small algae size and dilution in solution, hence flocculation has been found to be useful in reducing the difficulty associated with the downstream processing involved in converting microalgae into useful products (Prajapati et al., 2016) such as biomethane, hydrogen, volatile fatty acids, lipids and biodiesel. Several studies have been conducted to flocculate algae with fungi as a means of harvesting and processing the algae (Wrede et al., 2014, Li et al., 2017, Prajapati et al., 2016). Fungal-algae flocculation has also been found to be an effective pretreatment technique for improving methane production through anaerobic digestion technology. (Prajapati et al., 2016) investigated *Chroococcus sp*. pretreated with fungus *Aspergillus lentulus (A.lentulus),* and the results showed almost 100% harvesting of microalgae in 6 hours as well as a 54% increase in anaerobic digestion and a 50% increase in methane production (Prajapati et al., 2016). The study also showed that there was significant cellulase production by the fungi which resulted in soluble sugar release from the algae cells contributing to its digestibility. Also, fungi pretreatment has been employed in lignocellulose biomass for biogas production. There is evidence of fungus *Aspergillus sp*. degrading lignocellulose (Pérez et al., 2002, Phutela et al., 2011, Taseli, 2008). Some authors have observed increased methane production when fungi are used in the pretreatment of lignin agricultural residual biomass (Muthangya et al., 2009, Zheng et al., 2014, Zhao, 2013, Prajapati et al., 2016). In addition, no chemical addition and the low cost of biological pre-treatment is increasing the attractiveness of fungi-assisted algae flocculation technology (Prajapati et al., 2016). Previous work by (Wrede et al., 2014) showed A*spergillus fumigatus* (*A.fumigatus*) fungi cells having a positive effect on biomass production, lipids release and wastewater treatment efficiency by co-cultivating the fungi with microalgae. In addition, (Wrede, 2019b) investigated the capability of fungi to flocculate algae and the results showed *A. oryzae* to be the most effective species with 95% algae removal in monoculture flocculation. When tested on mixed algal communities from wastewater system, the algae removal value was over 70%. This shows *A.oryzae's* potential for pre-treating algae during wastewater treatment. Despite this advancement in microalgae harvesting technology using fungi-algae flocculation, research showing fungi's effect on microalgae cell wall for improving lipids extraction efficiency is yet to be undertaken which is a key focus of this chapter.

Also, pretreatment of the microalgae cell wall has been stated in previous chapters as the key to disrupting the cell wall of microalgae resulting in breakage of the cell, improving lipids extraction, leading to better accessibility of microbes to the interior of the microalgae and improving the anaerobic digestion process. To this effect, several pretreatment strategies have been employed in chapters 3, 4 and 5 including mechanical (high-speed homogeniser), thermal (waterbath, autoclave), enzymatic (single and combined), combination (including waterbath-high speed homogeniser, lysozyme high speed homogeniser) and biological (bacteria and fungi). Most microalgae pretreatments have to balance the cost effectiveness of the process and the energy efficiency. Biological pretreatments are gaining attention for their cost effectiveness and energy efficiency (Carrillo-Reyes et al., 2016, Chun and Peng, 2010). Chapter 3 and 4 conducted experiments on *C.vulgaris* pretreatments by measuring the number of cells disrupted by various pre-treatments and evaluating the lipid extraction efficiency. *A.oryzae* is known to contain lipase and is used for industrial production of the enzyme (Money, 2016, Chang et al., 2014, Machida, 2002). Hence, its use was investigated in this chapter for improving the lipids extraction efficiency of *C.vulgaris.*

Furthermore, the cell wall of most microalgae is known to contain cellulose and hemicellulose adding to the difficulty in breaking down the cell wall (Ward et al., 2014). Some fungi have been known to produce cellulase (which is a key hydrolytic enzyme responsible for degradation of cellulose found in the microalgae cell wall) when flocculated with algae (Xie et al., 2013, Christy et al., 2014, Bhattacharya et al., 2017). *A.oryzae* has been reported to contain amylase, cellulase and lipase (Money, 2016, Chang et al., 2014, Machida, 2002, Horton, 2012). However, previous anaerobic hydrolysis tests conducted in chapter 5 using *A.oryzae* as inoculum to digest *C.vulgaris* with a SIR of 1:1 and a retention time of 15 days, displayed low production of total volatile fatty acids (VFA) and low COD removal rate. There is evidence to suggest that *C.vulgaris* contains little or no cellulose in its cell wall (Kapaun and Reisser, 1995, Gerken et al., 2013, Kim et al., 2014, Mahdy et al., 2014, Takeda, 1991). The results obtained from the tests in Chapter 5 agrees with these findings as fungus *A.oryzae* is known to secrete mainly cellulase (Bhattacharya et al., 2017, Prajapati et al., 2016), so there is no site for the enzyme to act which explains the low VFA production. Nevertheless, to confirm this new information, this project further aimed to determine if *C.vulgaris* cells inoculated with A.oryzae and pretreated with high speed homogeniser, impacting their mechanical stress prior to anaerobic hydrolysis would improve VFA production.

This chapter has three major objectives. Firstly, to investigate the effect of flocculation time on *Chlorella vulgaris (C.vulgaris)* incubated with fungus *Aspergillus oryzae (A.oryzae).* Next, *to* understand study the effect of the fungus *A. oryzae* on *C.vulgaris* cell strength using high-speed homogenisation and lipids extraction efficiency as process verification. Thirdly, to

demonstrate the relationship between *C.vulgaris* cell strength on the rate of anaerobic hydrolysis using *C.vulgaris* incubated with *A.oryzae.*

6.2 Materials and Methods

Microalgae collection, cultivation and harvest were done using same methods as described in chapter 5. The algae used in each experiment was cultured in a 20L photobioreactor with growth monitored and harvested in the active growth phase at wavelength of 750 nm. The experiments were conducted in duplicate. *A. oryzae* fungus used was obtained from Victoria University's culture collection, initially collected from the ground soil of Victoria University Werribee Campus located in Australia. The retrieved fungus was then re-cultured for growth in potato dextrose agar from Sigma Aldrich at 30^{o}C for 72 hours (Wrede, 2019a). Isolated pellets were further collected from the agar with an inoculating loop and added to 2 L conical flask containing 1 L potato dextrose broth on an orbital shaker at agitation speed of 120 rpm, room temperature for 72 hours. After 3 days, the fungi pellets were harvested by spinning at 3500 rpm using an Eppendorf centrifuge for 5 minutes. The pellets were then rinsed twice; firstly, with distilled water and then phosphate buffer solution to rinse off any excess media before experimental use. Chemical oxygen demand (COD), dry weight (total suspended solids) and anaerobic hydrolysis analyses were conducted using the method described in chapter 5.

6.2.1 Experimental process description

6.2.1.1 Fungi-Algae flocculation and cell strength test

Initial tests conducted focused on the effect of time on flocculation efficiency. The *C.vulgaris* were harvested and dewatered to 10% by volume concentration using a high-performance Avanti J26S XP centrifuge by Beckman Coulter at 3,500 rpm for 5 minutes, reducing the volume to 2 L. 250 ml of the rinsed fungi pellets were added to the concentrated 2 L algae culture solution, placed on an orbital shaker at 150 rpm, room temperature and allowed to flocculate for 24-hours and 72-hours respectively. Optical density of the fungi culture used was 0.806 at a wavelength of 600 nm (this wavelength was used to ensure the cells are alive and active, not affected by the ultraviolent rays from the spectrophotometer (Stevenson et al., 2016)). A drop of each of the mixture samples was collected and observed for visual changes in the structure of the microalgae-fungi mixture in solution using the motic BA310 light microscope at 400X magnification. COD of the algae-fungi mixture was monitored after 24 hours and after 72 hours of flocculation. The COD was used to determine the amount of organic matter available in the mixture and to understand the micro-organisms growth condition in the media. Results of COD is provided in the results section 6.3.2.

Then, the fungus *A oryzae*'s effect on *C.vulgaris* cell strength was further investigated. Duplicate cell strength experiments of the fungi-algae mixture flocculated over 24 hours and over 72 hours were conducted. 100 ml each of the fungi-algae mixture was homogenised for 5 minutes using a CAT unidrive X1000 high speed homogeniser at speeds of 4,500 rpm, 8500 rpm, 15,000 rpm, 20,000 rpm, 25,000 rpm and 33,000 rpm respectively. Two controls one containing *C.vulgaris* alone and the other containing fungi-algae mixture without homogenisation was used. Process efficiency was determined from estimating the amount of lipids released after high-speed homogeniser application. Lipid analysis was conducted using dichloromethane as solvent in extracting broken-only cells from the wet fungi-algae mixture. After cell disruption, 25 ml of the dichloromethane solvent was added to each 100 ml homogenised fungi-algae mixture to allow the lipids to dissolve in the solvent. The solvent phase was then collected using a Heidolph rotary evaporator VV2000 at 38ºC and 150rpm to evaporate the solvent and leave behind the lipid's residue. To analyse the amount of lipids released, a calculation of the weight difference in round bottom flask used before and after experiment was quantified using an AND GR-200 series analytical balance. The results obtained from the fungi-algae mixture were compared with cell strength tests conducted using *C. vulgaris* algae alone to determine fungi *A. oryzae's* effect on the *C. vulgaris* cell strength. Then, incubation times of 24-hour and 72-hour were compared to determine the effect of fungus flocculation on *C.vulgaris* algae cell strength.

6.2.1.2 Fungi-Algae anaerobic hydrolysis

From the microscopic observation and cell strength tests, the 72-hour incubation performed better than 24 hours (see discussion in results section). Following this result, an experiment aimed at investigating the effect of cell strength on anaerobic hydrolysis was performed using the flocculation retention time of 72-hours. *C.vulgaris* was inoculated with *A.oryzae* for 72-hours prior to mechanical disruption using a high speed homogeniser before undergoing hydrolysis. The homogeniser speed was varied to assess the cell wall strength of the microalgae as the cells are disrupted from mechanical shear and mixing.

Two controls were also used during the anaerobic hydrolysis tests: control without fungi nor high-speed homogeniser, control with fungi but no high-speed homogeniser. The incubations were conducted in 250 ml Schott bottles using the method outlined in chapter 5. The experiments were conducted in duplicate with sampling every 2 days over a 13-day period. Process efficiency was analysed by determination of VFA concentration of each duplicate test using a GC-2010 Shimadzu gas chromatograph with AOC-20i auto injector, flame ionisation detector and AOC-20s auto sampler. To analyse the VFA concentration, the hydrolysis test method described in chapter 5 was used. However, only three dilutions were utilised as the first three dilutions from chapter 5 earlier was found to be within the calibration points for the

GC to ensure precision in results. From the original standard of 10mM, a 1:100 dilution was made. Then, further dilutions of 1:10, and 1:2 were obtained to make 3 standards (Calculations are contained in appendix). Each acid was further converted from mM to mg/l or ppm. After each sampling, nitrogen was re-bubbled at 50kpa for 15 to 30 seconds into each digester bottle to degas oxygen and keep the system anaerobic. Moreover, 1:10 dilution was used when transferring the fungi-*C.vulgaris* mixture to the vials for VFA analysis.

6.2.2 Statistical analysis

Experiments were performed in duplicate with statistical data computed and analysed using Microsoft excel including calculating the mean and standard deviation values. The results report included the error bar calculations showing standard deviations of the mean.

6.3 Results and Discussion

6.3.1 Microscopic analysis

Light microscopic observation of *C.vulgaris* cells mixed with *A. oryzae* cells showed fungi cells forming an adhesive bond with the algae cells in Figures 6.1B and 6.1C, with the algae and fungi cells sticking together. The new bond formation leads to flocculation and is a form of pretreatment as more cells floc together making algae separation from solution easier.

After inoculation and mixing on the shaker at 150 rpm for 24 hours, algae-fungi cells appeared to form large, but more discrete flocs compared to the control without fungi as seen in Figure 6.1B. Also, after mixing at 150 rpm for 72 hours, microscopic observation showed the microalgae cells were clumped with the fungi and displayed visible attachment of the fungus to the cell wall of algae shown in figure 6.1C. In addition, the images from figure 6.1C shows much larger clumps at 72-hour cultivation than at 24-hour.

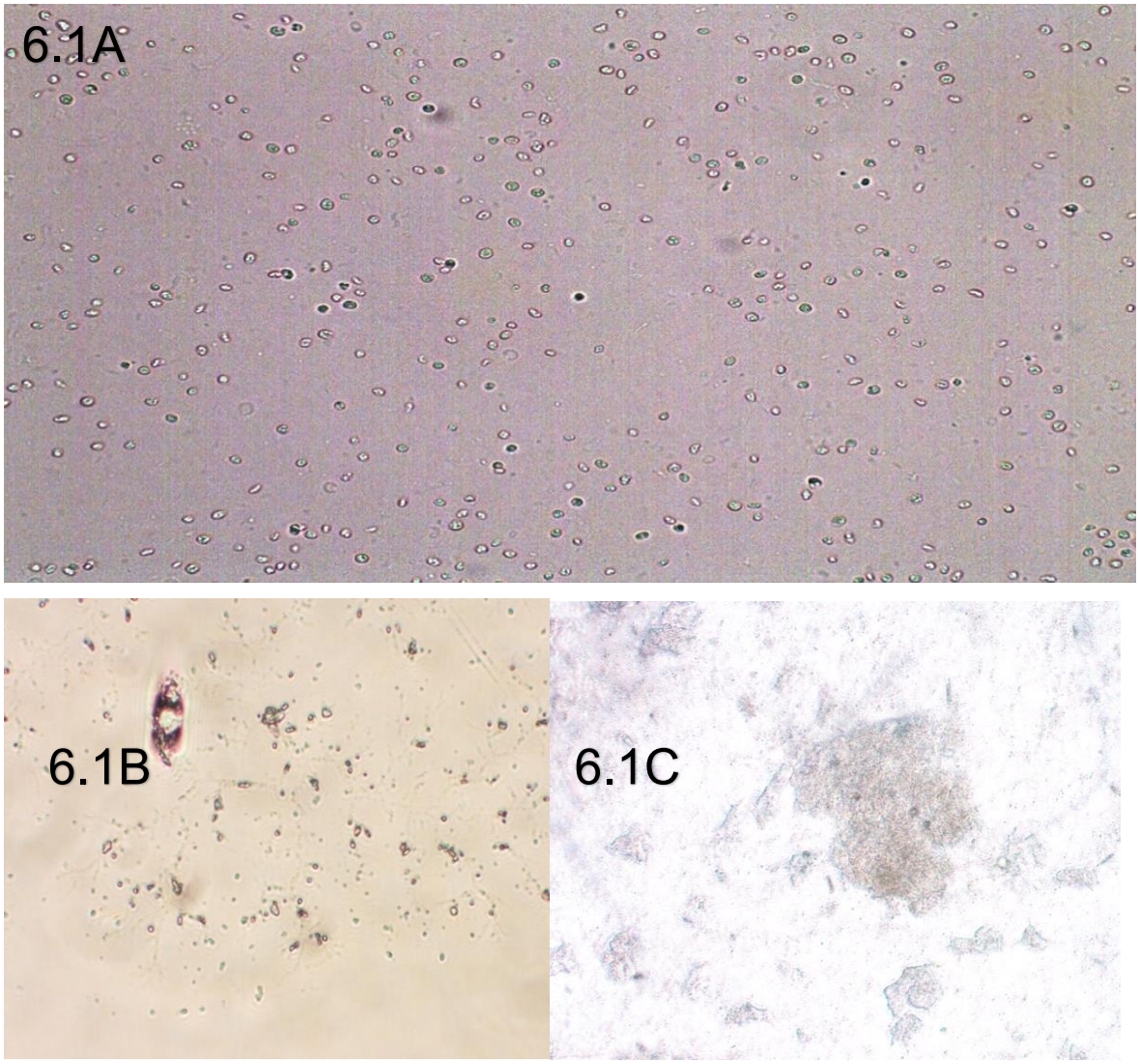

Figure 6.1 Microscopic analysis using 400X magnification showing: A.) C. vulgaris alone. B.) flocculation of C. vulgaris mixed with A. oryzae after 24-hour. C.) flocculation of C. vulgaris mixed with A. oryzae after 72-hour.

6.3.2 Optical Density, Biomass Concentration and COD Analysis

The algae batches used were the 10X concentrated 2 L solutions which had the following ODs. The 24-hour tests had absorbance values of 0.862 at 750 nm. Similarly, the 72-hour *C. vulgaris* test sample had absorbance values of 0.845 at 750 nm. The algae biomass dry weight (total suspended solids) concentrations were 0.1654 g/100ml and 0.1660 g/100ml for 24-hour and 72-hour samples respectively.

Chemical oxygen demand (COD) for 24-hour tests had a reading of 270 mg/l for sample containing algae alone and 448 mg/l for sample containing algae mixed with fungi. The COD readings were similar for 72-hour tests as the algae used for the separate experiments were harvested at a similar OD. For the 72-hour algae batch, the COD readings were obtained on

day 1 (24-hour) and day 3 (72-hour). COD reading on day 1 with readings of 295 mg/l for algae alone and 494 mg/l for algae mixed with fungi.

6.3.3 Effect of time on *A. oryzae's* inoculated with *C.vulgaris* and high-speed homogenisation pretreatment

The effect on fungi flocculated *C.vulgaris* was studied for 24-hour and 72-hour reaction times. Experimental process verification was determined by analysing the amount of lipids released from the *C.vulgaris* microalgae cells after mechanical pretreatment using a high-speed homogeniser at varying speeds. The experiments were done in duplicates and had two separate controls; Controls labelled C1, C2 were samples containing algae alone without high-speed homogeniser pretreatment. Controls labelled C3, C4 had algae mixed with fungi without high-speed homogeniser pretreatment. The remaining duplicate samples labelled 1,2,3,4,5 and 6 were mixed algae-fungi samples pre-treated using high speed homogeniser at 4,000 rpm, 8,500 rpm, 15,000 rpm, 20,000 rpm, 25,000 rpm or 33,000 rpm. The results showed that the lipid extraction improved with increase in homogeniser speeds. When the lipids values for 72-hour were compared to the 24-hour samples, the difference was not significant. However, there is greater lipid release at lower speeds observed in the 72-hour test than at 24-hour. This indicates a weaker cell wall strength at 72-hour than 24-hour treatment. At the maximum cell disruption rate of 33,000 rpm, lipids extraction increased 6% at 72-hour compared to the 24-hour. An average of the repeat experiments was done as well as the standard deviation and plotted in Figure 6.2.

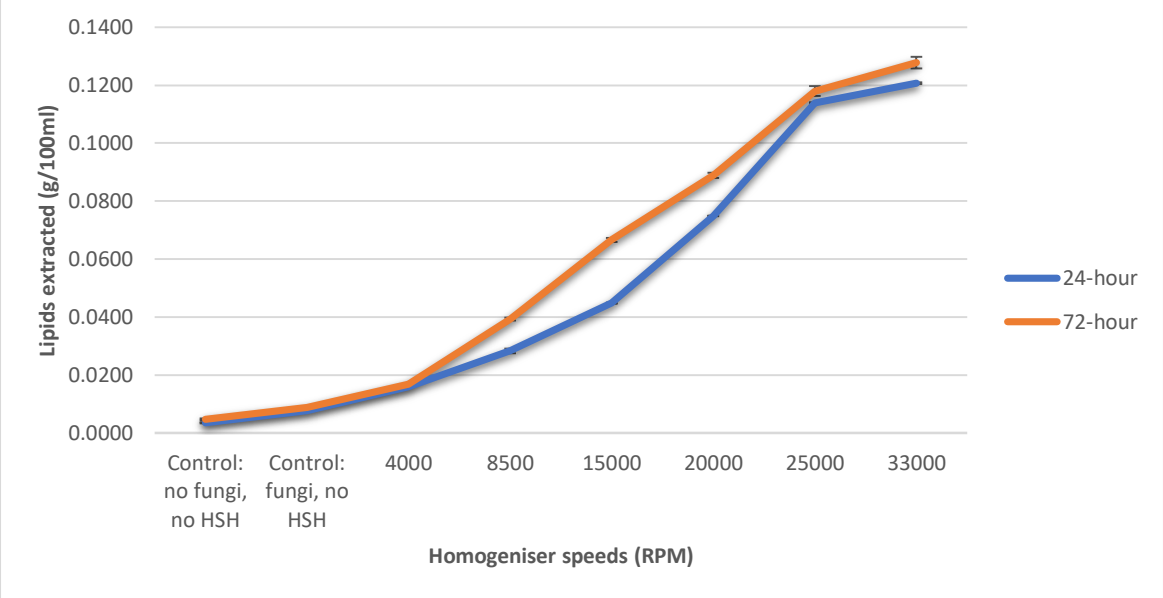

Figure 6.2 Effect of fungi contact time on lipid extraction efficiency from C.vulgaris cells flocculated with fungus aspergillus oryzae with two controls; no fungi with C.vulgaris, no high-speed homogeniser and fungi with C.vulgaris but no high-speed homogeniser.

When the results of fungi's effect on the cell strength of *C.vulgaris* was compared with *C.vulgaris* and dichloromethane solvent alone, the results showed greater lipids released from fungi-algae as shown in Figure 6.3. The results display a 19.62% and 26.66% increase in lipids in the 24-hour and 72-hour fungi-algae flocculation at maximum disruption rate of 33,000 rpm when compared with algae alone, suggesting higher cell degradation with increase in flocculation time. The optical density used for this test was 1.184 at wavelength of 750 nm for *C.vulgaris* and 0.960 at wavelength of 600 nm for *A.oryzae.*

Figures 6.2 and 6.3 show similar trends for lipid release increasing as higher homogeniser speed is applied. Significant disruption was observed at 8,500 rpm, which has earlier been deduced as the critical speed in chapter 4. Comparing the different disruptions at this speed with algae alone as the reference point, there was 52.97% increase in lipids from fungi flocculated for 24-hours, and 112% from fungi flocculated for 72 hours. The increase in lipids release using *A.oryzae* can be attributed to its lipase-producing ability (Money, 2016, Chang et al., 2014, Machida, 2002, Horton, 2012) improving the lipids yield efficiency.

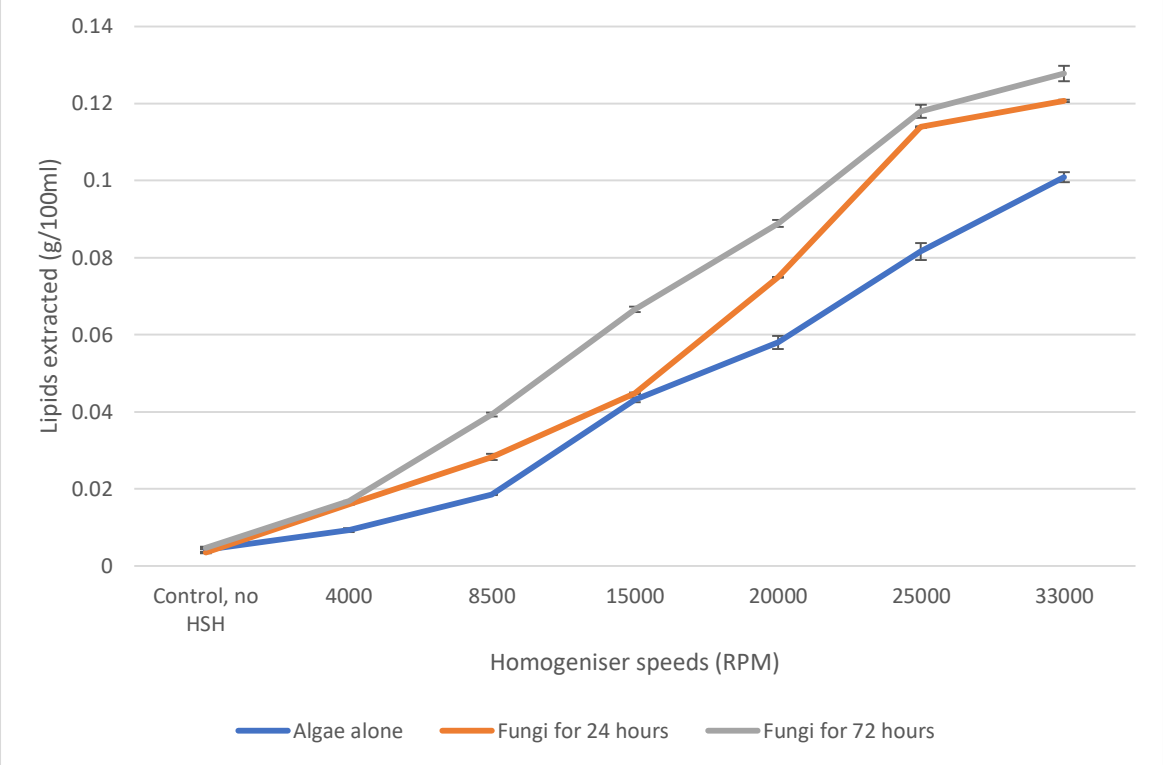

Figure 6.3: C.vulgaris cell strength test comparisons showing lipids extracted.

6.3.4 Effect of the cell strength of *A. oryzae's* inoculated with *C.vulgaris* on anaerobic hydrolysis

For the hydrolysis, the retention time of 72-hours was chosen to flocculate the algae with the fungi prior to the tests since it produced more lipids than the 24-hour incubation.

The results obtained showed better total VFA release between day 3 and day 11. Optimum total VFA were produced on day 5 at 33,000 rpm with concentration of 14.7 mg/l. At day 5, there was a 2.2-fold-increase in VFA released at 33,000 rpm compared to 25,000 rpm. Day 7 also showed good results leading next to day 5 in optimum VFA production with 12.1 mg/l of total VFA released at maximum homogeniser speed of 33,000 rpm.

From day 11 onwards, there was a significant decrease in VFA production as seen in figure 6.4. Eight VFAs were also detected in the reactor effluent including acetic, propionic, isobutyric, butyric, valeric, isovaleric, isocaproic, and hexanoic acids. From the high-speed homogeniser treatment, more VFAs were released from the *C.vulgaris* with increase in homogeniser speeds as seen in Figure 6.4.

Even though VFA production increased with homogeniser speeds, the amount of VFA produced from *C.vulgaris* cells is considerably low compared to earlier results in Chapter 5 with VFA production of 22.7 mg/l for fungi-*C.vulgaris* experiment in figure 5.4 . This confirms the knowledge that *C.vulgaris* cells may indeed lack cellulose in its walls. The difference in VFA production for fungi-algae in chapters 5 and 6 may be due to the concentrations of the algae batches used. *C.vulgaris* used in Chapter 5 had an optical density of 1.457 at 750 nm and the *C.vulgaris* used in this chapter has absorbance value of 0.845 at 750 nm.

The slight increase in VFA as homogeniser speeds increase, may be due to the stress condition of the homogeniser treatment and increase in temperature as homogeniser speed increased.

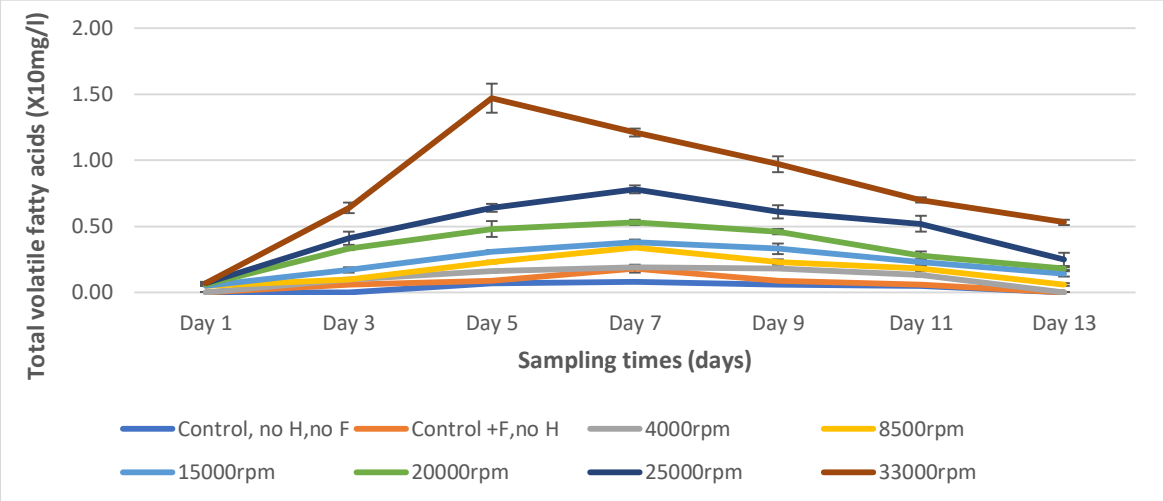

Figure 6.4; Total VFA production from homogenised Fungi-Algae Anaerobic hydrolysis contact time = 72 hours. Dilution of 1:10. Fungi-Algae to milli-Q water.

6.4 Conclusion

In conclusion, 72-hour fungal flocculation is a better and more efficient time to harvest microalgae by flocculation compared to 24-hour as lipids efficiency increase by 6% and more visible clumps were observed from the light microscope.

In addition, the cell strength of microalgae was also shown to be weaker with fungi addition releasing lipids with increase in homogeniser speeds. Further research in characterisation of the bonds formed from fungi-algae association may be beneficial in understanding microalgae harvest using flocculation, leading to improvements' in biofuel downstream technologies.

Also, when considering fungi pretreatment for anaerobic hydrolysis of microalgae, a shorter hydraulic retention between 5 to 7 days may be more useful in enhancement of total VFA and increase in retention time would lower total VFA production and favour methane production.

Inoculating *A.oryzae* with *C.vulgaris* can be used as a solution in improving microalgae harvesting for biofuel processes.

The results of this study will improve the microalgae harvesting processes and maximum lipid extraction efficiency rates from microalgae using fungi, thereby reducing the energy requirement and downstream processes of microalgae biofuel technologies.

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CHAPTER 7 ENERGY CALCULATIONS AND COST BENEFIT ANALYSIS OF PROJECT

7.1 Introduction

Currently, global production capacity of microalgae biomass is 30,000 tonnes annually with a production cost of 5 euros per kg (Pandey et al., 2018). Energy production from microalgae had earlier primarily focused on biodiesel production, however, two major drawbacks identified were the high nutrient requirement for microalgae growth and low energy efficiency of the process (Torres et al., 2013). It was estimated that 85% of the total energy consumption was used in the drying process and a negative energy balance was calculated from biodiesel production using *Chlorella vulgaris (C.vulgaris)* (Lardon et al., 2009). Due to these reasons, anaerobic digestion has been considered a more energy efficient option as some of the downstream processes are eliminated, such as drying (Torres et al., 2013). A less energy-intensive approach is to incorporate anaerobic digestion with microalgae wastewater treatment as the nutrients used for growth are already present in the system. Anaerobic digestion from wastewater systems is beneficial, as it reduces carbon emissions and operational costs from downstream processes (Pandey et al., 2018). In addition, anaerobic digestion offers nutrient recovery as the digestate residue can be re-used as fertiliser (Wainaina et al., 2019) further reducing costs. Furthermore, the intermediates of anaerobic digestion process such as volatile fatty acids (acetic, propanoic, iso-butyric, butyric, isovaleric, valeric, isocaproic and hexanoic acids), carboxylic acids (succinic and lactic acids) and hydrogen are useful across different industries including biofuel production, as bulk chemicals, alcohol production, pharmaceuticals, paints, rubber, bioplastics, biopolymers, textiles, leather and food additives (Wainaina et al., 2019, González-Fernández et al., 2019, Lukitawesa et al., 2020, Magdalena et al., 2019).

Although anaerobic digestion has been deemed a less-energy consuming technology than biodiesel production, there is still some energy use involved in the process. Key steps in the digestion process involves algae cultivation, harvesting, dewatering, lipids extraction and digestion of biomass. These processes are also energy intensive and can lead to inefficiency of the anaerobic digestion process (Benemann, 2013). To improve anaerobic digestion, a pretreatment step which involves disruption of the microalgae cells to release useful intracellular components is necessary, but may also be energy intensive or costly in the case of enzymes (Tran, 2017, Halim et al., 2012).

This thesis began by comparing various microalgae pretreatment techniques in chapter 3 including thermal (waterbath, autoclave), mechanical (high-speed homogenisation), enzymatic (lysozyme) and thermo-mechanical (waterbath-high speed homogenisation). (Aarthy A, 2018) stated that operating costs and energy consumption of pretreatment techniques needed optimization to improve cell disruption and microalgae biofuel processes.

To implement the pretreatment techniques considered in this thesis industrially, an energy calculation of the energy expended during each pretreatment is calculated.
Also, chapter 4 developed a technique to determine the cell strength of *C.vulgaris* microalgae using high-speed homogeniser. In this chapter, the energy used during high-speed homogeniser treatment is estimated. Furthermore, chapters 5 and 6 focused on anaerobic hydrolysis of *C.vulgaris* producing volatile fatty acids (VFA). The results of the various anaerobic hydrolysis experiments conducted showed 15 days retention time using microbes and enzymes produced the maximum VFA concentrations (see chapter 5). Hence, the VFA produced from the 15-day hydrolysis test was used to calculate potential biomethane production and an estimate of the energy efficiency of the proposed process is deduced.

7.2 Energy Calculations for Pretreatment techniques used in Chapter 3: Autoclave and Waterbath

Thermal pretreatment involving waterbath and autoclave were conducted in chapter 3. The energy requirement calculated considered two systems. The first system involves pretreatment without anaerobic digestion. The next focuses on feeding the pretreated microalgae to the anaerobic digester. Energy analysis in this study is conducted to understand the feasibility of the various pretreatments employed and their use at an industrial scale.

7.2.1 Thermal pretreatment energy consumption without energy recovery

Equation (7.1) considers a thermal pretreatment irrespective of methane production using the technique conducted in chapter 3. The energy requirement was calculated using equation 7.1(Holman, 2010). Energy consumption was calculated considering a system where the microalgae was first heated from ambient temperature to the pretreatment temperature which is considered as the key energy consumption in this instance:

$$Ei = \rho \cdot \gamma \cdot (Tp - Tm)/\Phi \qquad (7.1)$$

where *Ei* is the energy input, kJ/L; *Tm* is the heat required to raise the microalgae temperature to the pretreatment temperature, also known as ambient temperature (20ºC); *Tp* is the pretreatment temperature; and φ is heat recovery efficiency from the pretreated biomass (85%). The specific density of microalgae (ρ) and the specific heat (γ) were assumed to be the same as water, 1 kg/L and 4.18 kJ $kg^{-1}\,{}^{\circ}C^{-1}$, respectively.
For this study, *Tp* is 121ºC for autoclave and 100ºC for waterbath respectively.
From equation (7.1), the energy requirement for pretreatment to 121ºC (autoclave) is:
Ei(kJ/L)= 1 × 4.18 × (121–20)/0.85 = 497kJ/L of feed.

The energy requirement to reach 100ºC (waterbath) is:
Ei(kJ/g VS)= 1 × 4.18 × (100–20)/0.85 = 393 kJ/L of feed.

Following the equation (7.1), the energy requirement for 1 litre microalgae using thermal pretreatment including autoclave and waterbath were calculated as 497KJ/L of feed and 393KJ/L of feed respectively considering a process involving only microalgae pretreatment.

7.2.2 Thermal pretreatment with energy recovery

Industrially, thermal energy consumption involves heating the microalgae biomass from ambient temperature, *Tm* to the pretreatment temperature *Tp*, which is then cooled down to the digester temperature *Tdig* prior to anaerobic digestion. This is important, as the heat extracted during the cooling stage can be recovered and used to preheat influent microalgae using a heat exchanger. Typically heat recovery would have an efficiency of 85% (Passos et al., 2013a, Solé-Bundó et al., 2020).
To calculate the energy consumption in a process with heat recovery, equation (7.2) is used.

$$Ei = [\rho \times \gamma \times (Tp - Tm) - \rho \times \gamma \times (Tp - T_{dig})] / \varphi \qquad 7.2)$$

where *Ei* is the energy input, kJ/L; *Tm* is ambient temperature (20ºC); *Tp* is the pretreatment temperature; T*dig* is digester temperature (37.5ºC-mesophilic) and φ is heat recovery efficiency from the pretreated biomass (85%). The specific density of microalgae (ρ) and the specific heat (γ) were assumed to be the same as water, 1 kg/L and 4.18 $kJKg^{-1}{}_{\circ}C^{-1}$, respectively.

Pretreatment temperature = 121 ºC (Autoclave):
Ei(kJ/L)= [1× 4.18 × (121–20) –1× 4.18 × (121–37.5)] /0.85
Ei =86kJ/L of feed

Pretreatment to 100ºC (Waterbath)
Ei(kJ/L)= [1 × 4.18 × (100–20) –1 × 4.18 × (100–37.5)]/0.85
Ei = 86 kJ/L of feed

Following the equation (7.2), the energy required for thermal pretreatment prior to anaerobic digestion for autoclave and waterbath were same value, recorded as 86KJ/L of feed.

7.3 Mechanical Pretreatment Energy Analysis: High-Speed homogeniser

To estimate the amount of energy utilised during the high-speed homogeniser treatment, the shear rate for the high-speed homogeniser pretreatment was evaluated in chapter 4 using equation (4-1) given in section 4.2.3. From the Figures 4.11 to 4.13, the shear rate increased with increase in homogeniser speeds in the order: 4,000 rpm (8578 s^{-1})< 8,500 rpm (18227 s^{-1})< 15,000 rpm (32,166 s^{-1})< 20,000 rpm (42,888 s^{-1}) < 25,000 rpm (53,610 s^{-1})< 33,000 rpm (70,765 s^{-1}). The critical shear rate was identified to be 8,500 rpm which is 18,227 s^{-1}. This is relevant for industrial application of microalgae pretreatment prior to biofuel processing.

The specific energy relies on the shear rate γ and the shear stress τ (Pérez et al., 2006), as the shear rate affects the power consumption, fluid mixing and movement (Kumar, 2010).

To calculate the energy required for each homogeniser speed at the shear rate, a relationship between energy consumed and the shear rate is required. Firstly, the power relationship is given in equations 7.4.1 to 7.4.4 (Pérez et al., 2006, Kumar, 2010).

$$P / V = \tau\gamma \qquad (7.3.1)$$

where P is the power input and V is the volume of the fluid in the tank. When considering Newtonian fluids like the microalgae solution used in this project, the viscosity μ is the ratio of shear stress and shear rate given below.

$$\mu = \tau / \gamma \qquad (7.3.2)$$

Relating equations (7.4.1) and (7.4.2), the power consumption can be written as follows:

$$\frac{P}{V} = \tau\gamma = \tau\gamma\frac{\gamma}{\gamma} = \mu\gamma^2$$

Therefore, power consumption becomes: $P = V \times \mu\gamma^2$ (7.3.3)

Analysing the energy consumption from the power used at each speed, then becomes:

$$Energy\ (J) = Power(W) \times time\ (s) \qquad (7.3.4)$$

Assuming the viscosity μ of the microalgae solution to be that of water, the temperature increases as homogeniser speed increases to determine the true viscosity as viscosity changes with change in temperature (Korson et al., 1969). In addition, the shear rate at the various homogeniser speed is used in calculating the power and energy requirement with varying homogeniser speeds as seen in table 7.1 below:

Table 7.1 Power requirement for Shear rate with increasing homogeniser speeds

Homogeniser speed (rpm)	Temp. change at the Speed (°C)	Viscosity, μ (mPa.s)	Viscosity, μ (Pa.s)	Shear rate, γ (s^{-1})	Shear rate,γ2 (s^{-1})	Power (W)	Energy (J)
4000	24	0.8903	0.0008903	8578	73582084	66	19653
8500	24	0.8903	0.0008903	18227	332223529	296	88734
15000	24	0.8903	0.0008903	32166	1034651556	921	276345
20000	29	0.7975	0.0007975	42888	1839380544	1467	440072
25000	34	0.7195	0.0007195	53610	2874032100	2068	620360
33000	36	0.7195	0.0007195	70765	5007685225	3603	1080909

Therefore, assuming a volume of 1 L, the energy consumption at the critical speed of 8,500 rpm is given as 88,734 J/L feed (88.7 kJ/L of feed).

7.4 Biological Pretreatment-Energy Analysis of Anaerobic hydrolysis using selective microbes and enzymes

Research into energy production in anaerobic digestion processes calculated the total energy yield from 1 kgVS of microalgae to be 28.2 MJ forming 791 L of methane, assuming a cell composition of 30% lipids, 45% proteins and 25% and carbohydrates (Torres et al., 2013). Also, biogas production from microalgae has been identified to be within the range of 0.47 and 0.79 CH_4/g VS (Kendir and Ugurlu, 2018). In addition, the theoretical methane potential of *C.vulgaris* was calculated as 283 ml/gVS assuming 100% conversion of the volatile solids to biogas (Ward et al., 2014).

In chapters 5 and 6, microbes including *E.coli, S.thermophillus, L.plantarum, A.aceti, A.oryzae* and enzymes such as lysozyme, cellulase, pectinase and amylase were used as biological pretreatments for the anaerobic hydrolysis of *C.vulgaris.* A control with only *C.vulgaris* was also used to determine improvement efficiency of the pretreatments. VFA's are known intermediates of the anaerobic digestion process, and can be used as indicative operational parameters to control anaerobic digestion (Lee et al., 2015). The production of biomethane in the system relies on VFA and hydrogen production, as 70% of methane is generated from acetate reduction whilst only 25 to 30% comes from hydrogen reduction of CO_2 (Ali Shah et al., 2014, Moscôso et al., 2019). Also, it should be noted that VFA's contain mainly acetic acid, and they compose approximately 70% of the total VFA composition. Moreover, all VFAs eventually convert into acetate which will further be reduced to produce methane (Wijekoon et al., 2011).

As this project focused on VFA production rather than biomethane generation, to estimate the energy potential, assumptions regarding the conversion of VFA to methane were made.

It was assumed that 1.40g COD/gVFA (Munch, 1998; Rossle et al., 2001). Also, theoretical methane production is evaluated as 1g COD/0.35L CH_4 (Gough et al., 2013; Ahmadi et al., 2017; Joselyn et al., 2020).. Furthermore, the estimated energy generation from the potential biomethane produced was calculated using the assumption that $1m^3$ of methane is equivalent to 36MJ of energy (Suhartini et al., 2019, Barragán-Escandón et al., 2020). Then, the net energy generated was calculated using the difference between the energy produced from each biological pretreatment compared to the control, which had only *C.vulgaris* with no pretreatment. (Suhartini et al., 2019) estimates that $1m^3$ of methane is able to produce 10kWhr of electricity. It was assumed that conversion efficiency of biogas to electricity was 35%. Previous research has estimated methane percentage in biogas to be in the range of 55% to 80% (Barragán-Escandón et al., 2020). This was evident in the work conducted by Passos et al. (2013a), where methane composition in biogas produced was in the range of 68.2% to 69.1% (Passos et al., 2013a).

Potential biomethane calculations at optimum hydrolysis at 15-days HRT are given in the tables below:

Table 7.2: Potential biomethane and energy calculation optimum hydrolysis results obtained at 15 days digester time.

Pre-treatment/Hydrolysis	Lab VFA (mg/L)	Lab VFA (g/L)	Lab gCOD/L	L methane/ L feed	m^3 methane/L feed	Energy produced (J/L feed)	Net energy from Pre-treatment (J/L feed)	Potential Electricity (kWhr)/L feed	$AUD Electricity cost/kWh/Lfeed
1-Baseline Control, no pretreatment	4.0	0.004	0.0056	0.00196	0.00000196	70.56	0	0	0
2-E.coli, L.plantarum, S.thermophillus, & A.aceti	17.8	0.0178	0.02492	0.008722	0.000008722	313.992	243.432	0.000006762	1.33888E-06
3-E.coli, L.plantarum & S.thermophillus	161.0	0.161	0.2254	0.07889	0.00007889	2840.04	2769.48	0.00007693	1.52321E-05
4-Lysozyme alone	65.4	0.0654	0.09156	0.032046	0.000032046	1153.656	1083.096	0.000030086	5.95703E-06
5-fungi alone	22.7	0.0227	0.03178	0.011123	0.000011123	400.428	329.868	0.000009163	1.81427E-06
6-Lysozyme, amylase, cellulase & pectinase	194.6	0.1946	0.27244	0.095354	0.000095354	3432.744	3362.184	0.000093394	1.8492E-05

7.5 Further Discussion and Conclusion

Enzyme pretreatment did not require input operational energy, however, purchasing enzymes are costly. The purchase prices of the enzymes used from Alibaba are as follows: alpha-amylase at $US1-10/kg(Alibaba, 2021a)(Alibaba, 2021a)(Alibaba, 2021a), pectinase at $US7.50/kg (Alibaba, 2021d)(Alibaba, 2021d)(Alibaba, 2021d),cellulase at $US 1.50-2.50/kg (Alibaba, 2021b)(Alibaba, 2021b)(Alibaba, 2021b), Lysozyme at $US5/kg. (Alibaba, 2021c)(Alibaba, 2021c)(Alibaba, 2021c)

Considering cell disruption analysis and energy efficiency of the disruption techniques employed in this study, enzyme pretreatment is the most energy efficient pretreatment technique with no significant energy consumption and 82% cell disruption. High-speed homogeniser has shown good results for cell disruption as well with 80% cell disruption. However, the energy consumption is high, with the applied energy from the shear rates at the various homogeniser speeds ranging from 19.6 KJ/L feed at 4,000 rpm to 1,080 KJ/L feed at 33,000 rpm as seen in table 7.1. At the critical speed, the applied energy was 88.7 kJ/L feed of feed. For thermal pretreatment, two systems were considered. Pretreatment energy consumption with energy recovery and without energy recovery. For thermal treatment without energy recovery, 497 kJ/L of feed was utilised for autoclave and 393 kJ/L was used for waterbath. Moreover, thermal pretreatment involving energy recovery was same value for both autoclave and waterbath at 86 kJ/L which is a lower consumption than without energy recovery.

When thermal energy consumption from the autoclave and water bath are compared to mechanical energy used from the high speed, thermal pretreatment is shown to be less energy intensive. Nevertheless, utilising high speed treatment at the critical speed shows similar energy consumption of 88.7 kJ//L feed compared to thermal with energy recovery at 86 kJ/L feed.

In addition, when analysing the overall energy processes from the various pretreatment energy calculations, biological pretreatments are the most energy savings and feasible. The potential energy that would be derived from potential biomethane yield was evaluated. The results show inoculum containing mixed enzymes (lysozyme, amylase, cellulase and pectinase) to be most energy efficient with a potential net energy production of 3.4 kJ/L of feed. This is followed by mixed bacteria (*E.coli, L.plantarum and S.thermophilus*) at 2.8 kJ/L of feed as seen in table 7.2 above.

Although the cost of the enzymes identified in this research are cheap when purchasing large quantities for commercial use, at an average purchase price of $US6/kg from Alibaba, the constant supply of enzymes for continuous biomethane generation may not be sustainable using enzymes.

Hence, when considering a cost-energy analysis of the application of this research industrially, inoculum containing mixed bacteria is deduced as the most efficient due to their potential net energy production value as well as their ability to secrete the enzymes used for the project.
In conclusion, anaerobic digestion of *C.vulgaris* is worthwhile using additional hydrolytic enzymes including *E.coli, S.thermophilus* and *L.plantarum*, which contributes to increased anaerobic hydrolysis, improving VFA and biomethane production.

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CHAPTER 8 CONCLUSIONS AND RECOMMENDATIONS

8.1 Chapter outcomes and future research

The literature review documented in this project identified gaps on the current information of microalgae use in anaerobic digestion. The objectives of the research from the research questions in Chapter 2 (Section 2.16) included:

- Identifying a suitable pretreatment technique for disrupting *C.vulgaris* cells by comparing various pretreatment methods including mechanical, thermal, thermo-mechanical and enzymatic treatments using visual analysis.
- Evaluation of *C.vulgaris* cell strength measurement by developing a tool to determine effectiveness of microalgae pretreatment.
- Investigating lysozyme's ability to disrupt *C.vulgaris* cells and determine the enzyme's effect on anaerobic hydrolysis efficiency.
- Identifying key enzymes and microbes for *C.vulgaris* microalgae anaerobic hydrolysis.
- Examining fungus *A.oryzae* efficiency in harvesting microalgae using various time-intervals and subsequently determining the effect of the harvested fungus on cell wall strength as well as anaerobic hydrolysis.
- Estimation of the energy requirement of the pretreatment techniques conducted in project.

The majority of the objectives of this research were achieved. The project successfully conducted and compared different pretreatment techniques using a light microscope and staining technique to determine cell disruption under visual examination. The results established enzymatic treatment using lysozyme with 24-hour treatment time and volume of 100µl as most effective with 82% of cell disruption. Similar results were observed with mechanical pretreatment using a high-speed homogeniser with 80% cell disruption, which is not significantly different. Autoclave proved to be least efficient with 42% cell disruption. The experiments were conducted in duplicate and the cell disruption was given as an average of both tests with standard deviation less than 3.6. Lysozyme enzymatic pretreatment was concluded to be most suitable pretreatment technique followed by high-speed homogeniser.

Using the data obtained from chapter 3, chapter 4 successfully developed a reproducible technique to determine the effectiveness of microalgae pretreatments using a high-speed homogeniser. High speed homogeniser was selected as a method to measure pretreatment due to the shear force used in disrupting the cells which was analysed from the power and the rate of shear of the algae at each speed. 8,500 rpm was identified as the critical speed with a shear rate of 18,227 s^{-1} when an increased rate of disruption occurs. From observation, the

forces acting to break the cell wall of *C.vulgaris* become larger with increase in speed particularly from the critical speed, which eventually breaks the cell wall. Understanding the impact of microalgae pretreatments provides information for the energy requirement for cell disruption, enabling better design of microalgae pretreatment systems.

In addition, lysozyme's ability in degrading *C. vulgaris* cell wall was demonstrated in this project. Lysozyme displayed the most disrupted cells in chapter 3 with 82% cell disruption. When tested for use in the high-speed technique developed in chapter 4, lysozyme produced similar graph trend in figure 4.8a, with critical speed also identified at 8,500 rpm. However, when lysozyme is compared as a single enzyme to other enzymes combination comprising of lysozyme, amylase, cellulase and pectinase for lipids production and anaerobic hydrolysis, the lysozyme lipids and VFA are lower than the mixed enzymes. An increase of 2.3-fold in lipids extracted was observed when lysozyme is compared to enzyme combination in chapter 4. In addition, a 3-fold increase in VFA concentration was observed between lysozyme and enzyme combination. It was concluded that lysozyme acts on the cells, disrupting the outer surface and preparing the internal cell component for further degradation by the other enzymes. Hence, the improvement in lipids and VFA concentration with the mixed enzymes than lysozyme alone.

The effectiveness of selective microbes and enzymes for improving anaerobic hydrolysis of *C. vulgaris* was demonstrated. Different inoculum containing various microbes and enzymes were tested for their optimum volatile fatty acid (VFA) release to determine efficient hydrolysis as a means to investigate and predict their efficiency in anaerobic digestion. The microbes employed includes: *Escherichia coli (E.coli), Streptococcus thermophilus (S.thermophilus), Lactobacillus plantarum (L.plantarum), Acetobacter Aceti (A.aceti)* and *fungus Aspergillus oryzae (A.oryzae).* Then, enzymes used were lysozyme, cellulase, pectinase and amylase. The most effective pretreatment inoculum were recorded by mixed enzyme combination of lysozyme, cellulase, pectinase and amylase producing a 53-fold increase in VFA compared to the control at 15-day retention time. This is followed by bacterial combination *of E.coli, L.plantarum* and *S.thermophillus* which had a 43-fold increase compared to the control. The results indicated the usefulness of inoculating the anaerobic digester with the bacteria and enzymes identified in this study, so they become the predominant microbes facilitating the reaction rate of hydrolysis. Future recommendation to this study would be to investigate selective microbes for improvements in biomethane production by conducting laboratory and full-scale anaerobic digestion experiments' and not just hydrolysis.

Following the successful outcomes on the cell wall strength measurement and anaerobic hydrolysis, further experiments to establish a relationship between the cell wall strength and anaerobic hydrolysis were investigated using harvested *C.vulgaris* flocculated with fungus, *A. oryzae* which was the key objective of chapter 6. Initial experiments were focused on investigating fungi flocculation time effect on *C.vulgaris.* This was achieved by comparing *A.oryzae* flocculated with the algae for 24 hour and 72 hour in separate experiment to observe the morphological changes and determine the cell strength from lipids extraction after flocculation using high speed homogeniser at speeds of 4,500 rpm top 33,000 rpm. For the flocculation with fungi, 72-hour treatment time was more efficient than 24-hours in terms of visible flocculation efficiency as the fungi-algae hyphae were close together forming good clumps. This made separation of the clumped fungi-algae cells from solution easier. This is in line with a study by Bhattacharya et al. (2017). For lipid's extraction efficiency, 72-hour produced 6% more lipids than 24-hour flocculation. In addition, 72-hour released 20% more lipids compared to results of cell strength homogeniser pretreatment containing algae alone from chapter 4. The graph trend had a linear curve with lipids extraction proportional to homogeniser speeds as seen in figure 6.2.

Following the positive outcome achieved from the first experiment, the next experiment conducted anaerobic hydrolysis tests using 72-hour fungus flocculated *C.vulgaris* homogenised at speeds from 4,500 rpm to 33,000 rpm. The results from the experiment linking the cell strength and anaerobic hydrolysis of 72-hour flocculated fungus-algae showed optimum VFAs release on day 5. The results indicated that a low HRT of less than 10 days is required for optimum anaerobic hydrolysis when using fungi as inoculum. In summary, fungi addition to microalgae may be useful in harvesting microalgae via flocculation, however when considered for anaerobic hydrolysis, further research is required to understand its effect. It was deduced that the lack of cellulose in the cell walls of *C.vulgaris* may be responsible for the low VFA production during the hydrolysis experiment using the fungus *A.oryzae* as the enzyme mainly secreted was cellulase.

Research recommendations include, combining fungi with bacteria and enzymes conducting anaerobic hydrolysis and digestion using HRT of up to 10 days to understand its role in microalgae anaerobic digestion and effect on biomethane production. In addition, future research can be conducted using a different microalgae species known to be recalcitrant to anaerobic digestion due to the presence of cellulose to determine improvement in cell wall hydrolysis.

The energy requirement of each pretreatment technique employed in this project was calculated and presented in Chapter 7. Then, from the anaerobic hydrolysis experiments conducted, the optimum results at 15 days treatment time were used to determine potential biomethane from the total volatile fatty acids produced. A determination of the energy required

for biological/enzymatic pretreatment as well as the maximum additional energy likely to be obtained following pretreatment was calculated. This is important for industrial application of this project.

The results show enzymatic pretreatment (comprising of lysozyme, cellulose, pectinase and amylase) to be most energy efficient compared to biological, thermal and mechanical pretreatments with a net energy production of 3362 J/L. However, when a cost consideration is drawn, biological pretreatment (containing bacteria mix of *E.coli, S.thermophilus* and *L.plantarum*) was deduced to be the most energy and cost efficient with net energy production value of 2840J/L. The inference is based on the ability of these bacteria to produce the desired enzymes (lysozyme, cellulose, pectinase and amylase) used in this project.

Since, the key focus of this research was on anaerobic hydrolysis, a current and future projection of the intermediate (volatile fatty acids, VFA) of anaerobic hydrolysis is documented below.

8.2 Cost Benefit and future projection of Anaerobic Digestion intermediate-VFA

VFA's are carboxylates with low molecular weights made up of 2 to 6 carbon atoms (Wainaina et al., 2019, González-Fernández et al., 2019). They are traditionally sourced from petroleum and sugar fermentation, however, with the increasing need for renewable fuels and rising cost of commercial sugar, other options for producing these acids is being researched (Wainaina et al., 2019, Magdalena et al., 2019). VFA as highlighted earlier are intermediates of the anaerobic digestion process, produced during the hydrolysis-acidogenic reactions. This project studied the produced VFA's from *C.vulgaris* microalgae anaerobic hydrolysis. The production of VFA from microalgae to be used in other industries is at its pilot stage which is anticipated to improve due to the increasing cost of VFAs globally (Wainaina et al., 2019). Current market prices of VFAs are in the range of US$1500/ton to US$2500/ton which is higher in value than methane, whose price is just around US$400 to US$900/ton (Cerdán et al., 2020). The market value of VFAs increase with increase in the number carbon atoms present. Thus, individual prices of VFAs follow the order:

Butyric acid (US$2163/ton) >propionic acid (US$2000/ton)>acetic acid (US$600/ton) (Zhou et al., 2018). It has been postulated that the added value of VFAs with increasing carbon atoms is due to the difficulty in production of the acids as shorter chain acids are easier to produce than longer chains (Zhou et al., 2018). This was also evident during this project as shorter chain VFAs were released in a shorter time compared to the longer chains. From the hydrolysis reactor, eight VFA's were identified.

In the future, VFAs might become more desirable than methane production due to its higher cost value and increased industrial uses. One of the key benefits of this project is the improved production of VFAs achieved using microbial pretreatment. Hence, for future production of

VFAs and industrial application, the results of this project will be beneficial to increase productivity of VFAs and subsequently biomethane.

8.3 New Knowledge Contribution from Research Outcomes

The findings of this research have provided new information in the following areas:

- Developed a technique to provide a quantitative measure of the mechanical property of *C.vulgaris* cell wall strength so that the effectiveness of operating parameters for microalgae pretreatment technology can be assessed. *C.vulgaris* was used as a model alga as its dominant in wastewater systems in Australia, but technique can be examined across other species in future studies. Also, technique was applied in enzyme pretreatment and worked effectively providing same indication of the shear forces that result in cell disruption.
- Identified the critical speed at 8,500 rpm, which is when significant disruption occurs with a corresponding shear rate of 18,227 s^{-1} for industrial applicability of the novel technique.
- Single (lysozyme) and mixed enzymes (lysozyme, amylase, cellulase and pectinase) effect on *C.vulgaris* cell wall strength as well as on anaerobic hydrolysis was obtained. Lysozyme was found to be effective in initiating cell wall degradation and hydrolysis of *C.vulgaris* cells producing a 18-fold increase than the control having *C.vulgaris* alone. However, mixed enzymes produced increased lipids and better hydrolysis of *C.vulgaris* cells with 53-fold increase than the control.
- Mixed hydrolytic bacteria *(E.coli, S.thermophillus and L.plantarum)* was found to be a suitable additional inoculum mixture for anaerobic hydrolysis, producing 44-fold increase in VFA than the control which contained *C.vulgaris* only, improving potential biomethane production. The improvement in VFA and potential biomethane using the bacteria mixture has been attributed to their capability of producing the enzymes (lysozyme, amylase, cellulase and pectinase).
- *C.vulgaris* cells harvested by flocculation using A.oryzae at 72-hour produced better clumps than C.vulgaris cells harvested at 24-hour flocculation. Also, the 72-hour flocculated algae produced increased lipids than 24-hour by 6% and by 27% using *C.vulgaris* alone. However, using the 72-hour flocculated algae for anaerobic hydrolysis produced lower results than the other inoculum containing hydrolytic enzymes and bacteria. This was attributed to the aerobic property of the fungus as well as the deduction of the possibility of the absence of a cellulose cell wall in *C.vulgaris*
- A positive energy balance of biological pretreatment for *C.vulgaris* hydrolysis and potential anaerobic digestion was obtained.

8.4 Significance of Research

Microalgae digestion is of great significance in anaerobic digestion technology due to its potential to treat wastewater in a low energy manner, thereby approaching carbon neutrality, which leads to the reduction in greenhouse gas emissions and subsequently aids in combating the issue of global warming. Microalgae removes nutrients (i.e. nitrogen and phosphorus) from wastewater for growth such as nitrogen and phosphorus, which are in significant quantities in wastewater (Beuckels et al., 2015) promoting microalgae extraction from wastewater for anaerobic digestion. However, constraints' ranging from effective pre-treatment for biomass solubilisation, cell wall digestibility, effect of microbial community, system imbalance of carbon to nitrogen ratio, lipids concentration, among others have inhibited the use of microalgae for anaerobic digestion (Ward et al., 2014). Investigating and proposing methods of resolving these constraints is important to increase biomethane yields and to facilitate energy recovery. Key research questions from this project successfully provided more insight on the impact of pretreatments on cell permeability which is a major problem in anaerobic digestion. Having an insight into microalgae cell strength enables energy production from microalgae be more energy efficient and leads to improvements in subsequent anaerobic digestion rates. In addition, understanding the role of isolated hydrolytic microbes and enzymes in degrading *C. vulgaris* will enable wastewater companies to evaluate their effectiveness in anaerobic digestion. This would make algal treatment of wastewater more likely in water and waste treatment facilities. The results of this study will enable water utilities and waste management companies with ideas that are more likely to be successful in generating an energy positive process for anaerobic hydrolysis.

8.5 Potential Benefits of research:

- The knowledge of the shear force and critical speed of disruption identified in this research will enable biofuel processing companies to have an indicative value when disruption occurs, saving energy costs.
- This research will enable improved energy efficient integration of algae in wastewater management approaches by enabling VFA production and potential biomethane generation from anaerobic hydrolysis of microalgae.
- The research can also benefit companies producing bulk VFA for use across different industries including biofuels, bioplastics, food, and pharmaceuticals.
- *Benefit to Australia's government;* Growing algae on commercial scale using wastewater or utilizing Australia's arid lands for algae growth in raceway ponds or photo bioreactors will be useful for anaerobic digestion, as biomethane produced can generate energy contributing towards Australia's government renewable energy target leading to 100%

renewable and zero net emissions by 2050 to greenhouse gas mitigation (Teske et al., 2016, 2017).

8.6 Limitations of Research

This project was conducted in the absence of native bacteria from wastewater. The initial *C.vulgaris* culture used in this project was obtained from CSIRO which was a unialgal culture, although not sterile but purer than a typical wastewater system. Future work would include native bacteria acclimatized to wastewater systems as well as the additional microbes identified to improve hydrolysis for anaerobic digestion. This may in turn impact the results of the control sample used. However, the impact will likely be a positive impact as minimal digestion occurs using microbes resident in the wastewater system. Hence, additional microbes and enzymes will only improve the rate of digestion.

Also, during the project development, the initial objective was to isolate microbes used at every stage of anaerobic digestion including hydrolysis, acidogenesis, acetogenesis and methanogenesis. However, current experiments only considered anaerobic hydrolysis and acidogenesis, producing VFAs because of OHS issues with laboratory use of methanogens. Nevertheless, the results of the VFAs produced were used predict potential bio-methane generation. In the future, a demonstration of pretreatment impact on biomethane production rather than VFAs would be a better strategy incorporating a two-stage anaerobic digestion where hydrolysis-acidogenesis is done in one reactor and fed into the next reactor conducting acetogenesis -methanogenesis.

In addition, the project used *C.vulgaris* microalgae. It will, however, be useful to demonstrate the outcomes from this research on other microalgae species.

8.7 Closing Statement

The outcomes of this research will improve the rate of anaerobic hydrolysis and help to maximise commercial production of bio-methane from microalgae in wastewater systems. Evaluating the ability to break the cell wall using mechanical shear will be useful in measuring the effectiveness of pre-treatments to anaerobic hydrolysis and subsequently digestion. In addition, the new knowledge developed from using selective microbes and enzymes as inoculum for anaerobic digestion will facilitate cost effective and energy saving options for the technology encouraging commercial applicability for increased production of bio-methane. Subsequently, the success of this project will promote use of additional microbes and enzymes as seed inoculum for anaerobic digestion start-up in waste and water utilities generating on-site electricity from the biomethane obtained.

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