UTS UNIVERSITY OF TECHNOLOGY SYDNEY

Effective Harvesting and Utilisation of Microalgae with Wastewater Treatment for Biomethane Production

by Phuong Hang Vu

Thesis submitted in fulfilment of the requirements for the degree of

Doctor Philosophy

under the supervision of Professor Long D. Nghiem and Professor Qilin Wang

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CERTIFICATE OF ORIGINAL AUTHORSHIP

I, Phuong Hang Vu declare that this thesis is submitted in fulfilment of the requirements for the award of Doctor of Philosophy, in the School of Civil and Environmental Engineering, Faculty of Engineering and IT at the University of Technology Sydney.

This thesis is wholly my own work unless otherwise referenced or acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

This document has not been submitted for qualifications at any other academic institution.

This research is supported by the Australian Government Research Training Program.

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LIST OF ACHIEVEMENTS

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7. UTS Vice-Chancellor's Conference Fund 2023

LIST OF ABBREVIATIONS

Symbol	Description
ATL	Algaenan trilaminar layer
C	Carbon
Ca	Calcium
$Ca(OH)_2$	Calcium hydroxide
$CaCl_2$	Calcium chloride
$CaCO_3$	Calcium carbonate
CH_4	Methane
CO_2	Carbon dioxide
COD	Chemical oxygen demand
COVID-19	Coronavirus disease
DAF	Dissolved air flotation
DiAF	Dispersed air flotation
DW	Dry weight
EPS	Extracellular polymeric substances
H_3BO_3	Boric acid
IPA	Isopropanol
K_2HPO_4	Dipotassium phosphate
KOH	Potassium hydroxide

MFL	Microfibrillar layer
Mg	Magnesium
$Mg(OH)_2$	Magnesium hydroxide
$MgCO_3$	Magnesium carbonate
$MgSO_4$	Magnesium sulphate
MW	Microwave irradiation
N	Nitrogen
Na_2CO_3	Sodium carbonate
NaCl	Sodium chloride
$NaNO_3$	Sodium nitrate
NaOH	Sodium hydroxide
OD	Oven drying
ODF	Ozonation-dispersed flotation
OLR	Organic loading rate
SF	Synthetic feed
TAG	Triacylglycerol
TS	Total solids
UV	Ultraviolet
VFA	Volatile fatty acid
VS	Volatile solid

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ABSTRACT

Amidst the growing challenge of climate change, microalgae have emerged as a potential platform for producing renewable chemical feedstock and renewable fuel. It is essential to improve the economics of microalgae-derived feedstock and fuel production by lowering the cost of microalgae harvesting and subsequent utilisation. Results from this thesis demonstrated that cationic polymer flocculation was simple, fast, and could achieve 90-99% harvesting efficiency of both freshwater and marine microalgae. Systematic characterisation of the microalgae characteristics over their growth life cycle revealed that microalgae at stationary growth phase were easier to flocculate using less polymer due to the increased extracellular polymeric substances surrounding the cell. The charge neutralisation and bridging mechanisms of cationic polymer allowed the microalgae cells to agglomerate and form a stable polymer-algal matrix without significant damage to the cell membrane. Polymer-algal cell interaction ensures maximum concentration of intracellular organic matter and biochemicals for subsequent processing into biofuel or high-value products. This thesis also showed that microalgae and microalgal biomass residues can be converted to renewable methane by anaerobic digestion. Results here highlighted for the first time the potential of methane production from polymer-harvested microalgae, especially when pretreating the biomass with isopropanol using surplus COVID-19 disinfectant. Up to 230 L CH_4/kg VS of microalgae was produced using dried and isopropanol-pretreated microalgae biomass, which is >210% higher than using wet, non-pretreated biomass. This underscores the possibility of employing outdated/waste hand sanitiser and liquid disinfectant to enhance the methane production of microalgae harvested by polyacrylamide polymer, thus, taking one step closer to energy reliability and sustainability.

CHAPTER 1. INTRODUCTION

1.1 Background

Efforts to develop sustainable and renewable fuel alternatives are spurred by the imminent depletion of fossil fuels and the increasing threat of global warming. Biofuel including biodiesel, bioethanol, and biomethane (CH_4 of organic origin) can be derived from renewable biomass. To date, there have been several generations of biofuel. First-generation biofuel is from the fermentation of starch and transesterification of lipids from food crops (e.g. corn and sugarcane) (Lee & Lavoie, 2013). First-generation biofuel production requires arable land thus it competes with food security. Second-generation biofuel is derived from non-edible lignocellulosic biomass such as agricultural waste, forestry waste, and crop residues. Their conversion to valuable green fuel reduces waste disposal into water streams and landfills. However, they still require extensive pretreatment steps (i.e., additional cost and complexity) to overcome the recalcitrance of lignocellulose to fermentation.

Microalgae are emerging feedstock for third-generation biofuel due to their many advantages (Enamala et al., 2018; Singh et al., 2023). Microalgae are unicellular photosynthetic organisms with diverse structures and metabolic processes, allowing them to thrive in a range of environments (Musa et al., 2019; Vo et al., 2020). Structurally, microalgae cells include cell wall, plasma membrane, cytoplasm, nucleus, chloroplasts, and mitochondria. The cell structure of *Chlorella vulgaris,* a common green microalgae, is provided in Figure 1 as an example. The chloroplasts, containing chlorophyll and other pigments, are essential for photosynthesis, where light energy is converted into chemical energy. Microalgae exhibit autotrophic metabolism, utilising light and carbon dioxide for

photosynthesis. Some species can also grow heterotrophically by metabolising organic carbon in the absence of light. The growth cycle of microalgae involves four phases: lag, exponential, stationary, and death. Initially, cells acclimate to their environment during the lag phase. The initial growth phase is followed by the exponential phase, where rapid cell division occurs. Growth slows in the stationary phase due to nutrient depletion and waste accumulation, balancing cell division and eventually death. In general, microalgae have a rapid growth rate, contain no lignocellulose recalcitrance, and do not compete for arable land. Light, carbon dioxide (CO_2), and some nutrients are required for microalgal growth via photosynthesis to produce pigments, bioactive compounds (e.g. carotenoids, vitamins, phenolics and phycobiliproteins), carbohydrates, proteins, and lipids. These compounds are valuable substrates to produce biodiesel and biogas. By carefully controlling the cultivation conditions and environment of microalgae, their chemical composition can be tailored for specific downstream applications (Musa et al., 2019). For example, microalgae cultivated under nitrogen-depleted conditions achieved higher lipid and fatty acid accumulation (Yaakob et al., 2021; Yodsuwan et al., 2017).

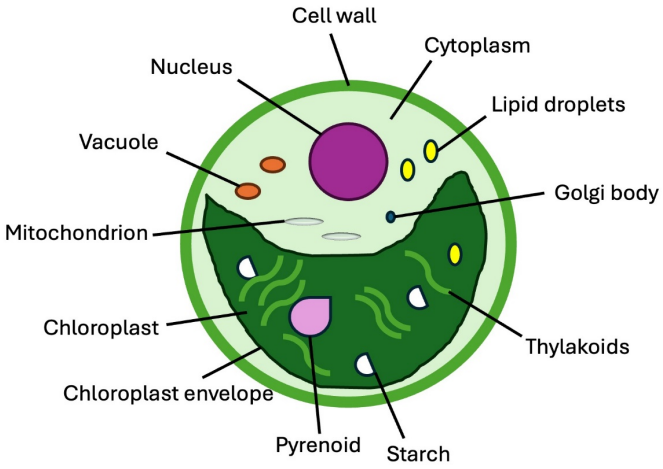

Figure 1: Schematic diagram of *C. vulgaris* cell structure.

In addition, microalgae provide a source of nutrients and high-valued biochemicals to produce human consumables. An extensive range of health supplements and food products from *Spirulina* sp., *Chlorella* and *Dunaliella* is available on the market (Enamala et al., 2018; Milledge, 2011; Spolaore et al., 2006). They are known to improve human health and energy production with their vitamin and antioxidant-rich properties. Microalgae also possess many light-harvesting pigments (e.g. chlorophyll, phycobiliproteins and carotenoids). Many microalgae are useful as natural, non-toxic colourants for food and cosmetic products due to their anti-inflammatory, immunosuppressive, and antioxidant properties (Sosa-Hernández et al., 2019).

Commercial applications of microalgae are currently hindered by the harvesting step. This is a crucial step to achieve a concentrated microalgal slurry (10 to 25 wt. %) for downstream processes (e.g. biochemical extraction). A substantial input of energy and/or chemicals would be required to concentrate microalgal biomass and separate it from the growth medium. The cost of microalgal harvesting step can contribute 20 to 30% of the total cost of microalgal biomass production (Molina Grima et al., 2003; Singh & Patidar, 2018). State-of-the-art harvesting technologies include centrifugation, membrane filtration, flotation, and flocculation (Barros et al., 2015; Singh & Patidar, 2018). Centrifugation and membrane filtration achieve high microalgal recovery efficiency but are energy intensive and necessitate high operation and maintenance costs. Therefore, centrifugation and membrane filtration are not suitable for large-scale production of microalgal biomass for low-valued products (e.g. biofuels) (Vandamme et al., 2013). Flotation embraces compact equipment, short operation time, and low capital cost. However, this method requires surfactants and energy that increase the total cost. Flotation also may not be suitable for harvesting microalgae in seawater cultures with

high ionic strength due to the risk of gas bubble rupture (Barros et al., 2015). Among the techniques, flocculation of microalgae is promising for biofuel application due to its simple operation and minimal equipment (Barros et al., 2015; Okoro et al., 2019). Flocculation with food-grade polymers (e.g. chitosan) also ensures microalgal biomass is safe for pharmaceutical and nutraceutical applications (Van Haver & Nayar, 2017). Polymer properties (e.g. charge density and molecular weight) can be customised during the polymerisation process to optimise flocculation efficiency for specific microalgal species (Nguyen et al., 2022).

Microalgal biomass is also a potential feedstock for anaerobic digestion to produce CH_4 (Aliyu et al., 2021; Singh et al., 2023). CH_4 is a renewable gas, which can be used interchangeably with conventional fossil gas. During anaerobic digestion, anaerobic microbial communities convert carbohydrate, protein, and lipid contents of microalgal biomass into biogas (50-70% CH_4 and 30-50% CO_2). Anaerobic digestion occurs in four stages: hydrolysis, acidogenesis, acetogenesis, and methanogenesis. During hydrolysis, complex organic molecules are broken down into simpler compounds. In acidogenesis, these compounds are converted into volatile fatty acids, alcohols, hydrogen, and carbon dioxide. Subsequently, acetogenesis transforms the products of acidogenesis into acetic acid, carbon dioxide, and hydrogen. Finally, during methanogenesis, methanogenic archaea convert acetic acid and hydrogen into methane and carbon dioxide, producing biogas. This process not only addresses waste management challenges but also provides a sustainable energy source, contributing to renewable energy goals and reducing greenhouse gas emissions.

Researchers have investigated the CH_4 production potential of various microalgae species (Frigon et al., 2013; Ganesh Saratale et al., 2018; Zabed et al., 2019; Zamalloa et al.,

2012). Several challenges associated with CH_4 production from microalgal biomass were identified. Most microalgal species have low digestibility due to thick cell walls that resist bacterial attack (i.e. low CH_4 yield) (Passos et al., 2014c). The high protein content (50 to 60%) of microalgae leads to a low carbon-to-nitrogen (C/N) ratio, which is inhibitory to microbial activity (Parkin & Owen, 1986; Ward et al., 2014). The current approaches to overcome these challenges focus on biomass pretreatment and co-digestion of microalgae with carbon-rich substrates. Pretreatment can improve the digestibility of microalgal biomass and microbial accessibility to intracellular compounds by rupturing the cell walls. Co-digestion of microalgae with carbon-rich substrates helps to balance the overall C/N ratio and enhance process stabilisation.

1.2 Problem statement

There are many available harvesting techniques to separate microalgae from the cultivating medium including centrifugation, flocculation, flotation, and membrane filtration. Conventional harvesting methods may denature the valuable compounds in microalgal biomass. Therefore, it is essential to identify milder harvesting options for algal biomass. Flocculation, in particular, is an easy and flexible method suitable for large-scale operations, yet it has shown highly variable harvesting efficiencies. Understanding the mechanisms behind microalgal flocculation is necessary to improve flocculation efficiency. Additionally, as microalgal biomass is often used for extracting high-value compounds, it is crucial to delineate the impact of different harvesting techniques on cell integrity. Cell damage or lysis during harvesting can lead to biochemical loss, reducing the overall value of the biomass.

The production of CH_4 is crucial for transitioning to sustainable energy sources, reducing greenhouse gas emissions, and mitigating the effects of climate change. However, current CH_4 production faces several challenges: variable feedstock supply and composition, and inefficient anaerobic digestion process. This thesis focuses on microalgae as a promising alternative feedstock for CH_4 production and addresses the current issues associated with CH_4 production from microalgae. Given the diversity in microalgae's biochemical composition and cell structure, identifying suitable species for anaerobic digestion is critical for viable CH_4 production. Understanding the characteristics of microalgae, as well as the impact of harvesting methods and pretreatment on the CH_4 potential of microalgal biomass, is crucial for developing strategies to enhance digestibility and maximise biogas yield. Progress in utilising microalgal biomass for CH_4 production will be a stepping stone towards achieving fuel sustainability.

1.3 Objectives

This thesis intends to gain a holistic understanding of microalgal harvesting via flocculation and explore the potential of CH_4 production from microalgal biomass. The main objectives include:

1. Evaluate the flocculation efficiency of different types of flocculants on a range of freshwater and seawater microalgae;
2. Investigate the impact of various harvesting techniques on microalgal cell integrity and biochemical profile;
3. Establish an understanding of the mechanisms behind polymer flocculation and the characteristics of the microalgal culture that influence flocculation efficiency; and
4. Assess the viability of microalgal biomass as a substrate for CH_4 production, and the impact of polymer harvesting and pretreatment methods.

1.4 Thesis outline

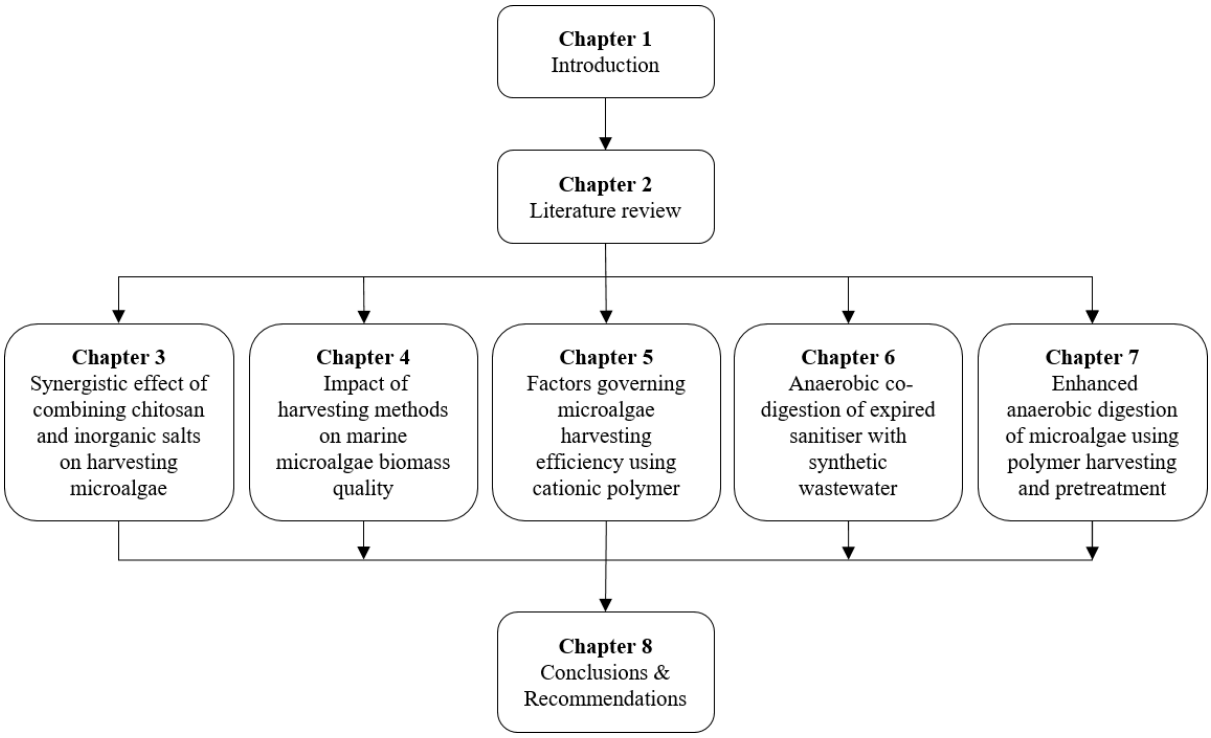

Figure 2. Schematic structure of the thesis

CHAPTER 2. LITERATURE REVIEW

Part of this chapter has been published as the following journal article:

Vu, H.P., Nguyen, L.N., Zdarta, J., Nga, T.T., Nghiem, L.D. 2020. Blue-green algae in surface water: problems and opportunities. *Current pollution reports*, 6, 105-122.

2.1 Characteristics of microalgae

Microalgae are photosynthetic prokaryotic microorganisms. Some microalgae can also be eukaryotic. They are ubiquitous in both marine and freshwater environments. It is estimated that there are between 200,000–800,000 microalgal species, of which only around 50,000 species have been identified and fully characterised (Frigon et al., 2013). Using light as the energy source, microalgae convert CO_2 and nutrients into oxygen and microalgal biomass that is rich in lipids, proteins, and carbohydrates (Frigon et al., 2013). Microalgae contain abundant light-harvesting complexes (e.g. chlorophyll, phycocyanin, and allophycocyanin) with a well-defined nucleus, cell wall, and pigments (Ganesh Saratale et al., 2018).

Microalgal cell walls are complex. The cell wall structure is diverse and has only been revealed for several species including *C. vulgaris* and several other green microalgae. They have rigid cell wall components (glucosamine or glucose-mannose polymer) embedded within a polymeric matrix (Passos et al., 2014c). *C. vulgaris* cell wall contains two layers (Gerken et al., 2013; Nemcová, 2003). The innermost layer consists of cellulose and hemicellulose. The exterior layer is made up of an extracellular polymeric matrix of uronic acids, rhamnose, arabinose, fucose, xylose, mannose, galactose, and glucose (Ward et al., 2014). On the other hand, *Porphyridium purpureum* cell is only encapsulated within a layer of gelatinous polysaccharide matrix (i.e. extracellular

polymeric substances) in the absence of a rigid cell wall (Giordano & Prioretti, 2016; Parkin & Owen, 1986).

Characteristics of the microalgal cell walls can have a significant implication on cultivation, harvesting, and subsequent utilisation and processing of the microalgal biomass. For example, the rigid two-layer cell wall of *C. vulgaris* and several other green algae allows them to resist a certain degree of mechanical or chemical stress and prevent cell lysis. This capability protects the microalgal cells upon chemical addition (e.g. flocculation) or mechanical force (e.g. centrifugation) used to harvest the biomass. On the other hand, the lack of a rigid cell wall increases *P. purpureum'*s susceptibility to cell membrane damage. Compromised cell membranes during harvesting can lead to intracellular leakage and loss of valuable compounds. Thus, it is important to identify the impact of harvesting techniques on targeted microalgae, as the resistance to cell damage varies among species.

2.2 Opportunities from microalgae

The metabolic diversity allows microalgae to possess a range of industrially important biochemicals (e.g. pigments, carbohydrates, lipids, proteins, and polyunsaturated fatty acids). These compounds are valuable feedstocks for the food, health, cosmetic and pigment industries (Figure 3). In addition, the photosynthetic capacity of microalgae makes them among the most promising platforms to produce renewable bioenergy.

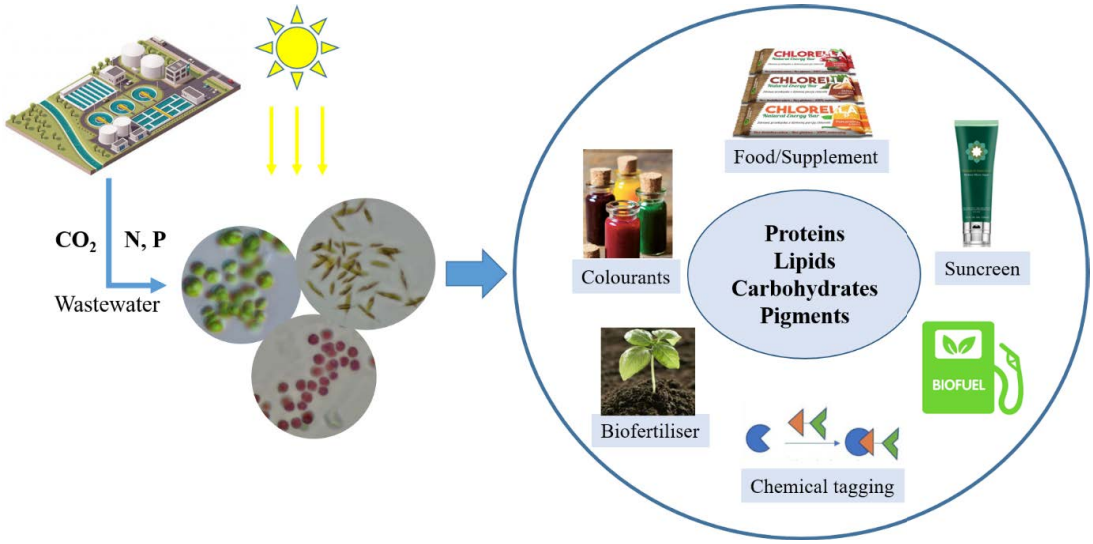

Figure 3. Potential applications of microalgal biomass

2.2.1 Health supplements

Microalgal biomass has been a traditional food source for centuries. *Nostoc* was used to survive famine in China (Spolaore et al., 2006). Kanembu people in central Africa and the indigenous population in Asia and North America consume *Spirulina* as a nutritious food (Chakdar et al., 2012). Microalgae are excellent sources of vitamins (A, B1, B2, etc.) and minerals (potassium, iron, magnesium, etc.) (Becker, 2013; Niccolai et al., 2019). Microalgal-derived proteins have complete Essential Amino Acids profiles, and the protein concentration is noticeably higher in comparison to that of most other natural food such as animal and fish flesh (15-25%), soybean (35%) and eggs (12%) (Koyande et al., 2019). Moreover, the amino acid patterns and digestibility of microalgal protein compare favourably with those of other food such as beans, oats, and wheat (Niccolai et al., 2019; Spolaore et al., 2006). *Chlorella* sp. and *Spirulina* particularly have a high protein content (50-65%) and are often marketed as "superfoods". *Chlorella* contains an active immunostimulator-β-1,3-glucan, exhibiting not only antioxidant properties but also the capability to reduce blood sugar concentration and elevate haemoglobin

concentration (Koyande et al., 2019). Similarly, *Spirulina* with its dense nutritional content has been reported to improve immune function, lower blood pressure and recommended by WHO (World Health organisation) to be added to the diet of NASA (National Aeronautics and Space Administration) astronauts (AlFadhly et al., 2022; Selmi et al., 2011).

Commercial production of microalgae into "nutraceuticals" (food supplements marketed with nutritional and medicinal benefits) has been facilitated over the last few decades. *Spirulina* sp., *Chlorella* and *Dunaliella* are dominating species in commercial microalgal products, with an annual production of several thousand tons of biomass (Enamala et al., 2018; Milledge, 2011; Spolaore et al., 2006). Commercial cultivation and processing of *D. salina* for β-carotene have been reported in several countries such as Australia, China, and Israel (Kim, 2015). *Spirulina* sp. and *Chlorella* sp. have been marketed as dietary supplements in several formulations such as tablets, powder, capsules, and extracts by companies originating from China, Germany, and Japan (Görs et al., 2010; Kim, 2015; Koyande et al., 2019).

2.2.2 Medicine

Beyond the proven health advantages of microalgae-derived bioactive compounds for human consumption, there is a recent exploration of microalgae's role in aiding drug delivery to amplify drug efficacy (Khavari et al., 2021). This extends beyond conventional methods like controlled release systems, films, and hydrogels. *Spirulina platensis* loaded with the chemotherapeutic drug, Doxorubicin, exhibited high drug-loading efficiency and fluorescence imaging capabilities, passively targeting lungs for improved lung metastasis of 4T1 breast cancer (Zhong et al., 2020). Convenient oral

delivery systems using *Spirulina platensis* as a microcarrier of Amifostine and Curcumin, which are tissue radioprotectants with anti-inflammation and anti-cancer properties, have successfully provided effective intestinal radioprotection during radiotherapy of abdominal tumours and colon cancer (Zhang et al., 2022; Zhong et al., 2021).

2.2.3 Cosmetics

Bioactive compounds and pigments extracted from microalgae such as β-Carotene, astaxanthin, and exopolysaccharides, have found diverse applications in cosmetic products. β-Carotene, an orange-yellowish pigment proven to enhance skin elasticity and regeneration, is particularly abundant in *Dunaliella salina*, reaching up to 14% of its dry weight (Ye et al., 2008; Zhuang et al., 2022). Astaxanthin, derived from *Haematococcus pluvialis*, stands out as a potent natural antioxidant, surpassing vitamin E's strength by a factor of 100 (Hamed, 2016). Moreover, chlorophyll, with its odour-masking capabilities, can be easily extracted from microalgae for incorporation into deodorants, toothpaste, and hygiene products. Microalgae like *Nannochloropsis* sp. produce canthaxanthin, a pigment commercially employed in tanning pills (Koller et al., 2014). Additionally, pigments like phycocyanobilin (blue colour) and phycoerythrin (red colour) offer potential applications in decorative cosmetics such as eyeshadow, eyeliner, and lipsticks.

Mycosporine-like amino acids and sporopollenin, harnessed from microalgae, serve as effective natural UV blockers by absorbing UV radiation (Enamala et al., 2018; Kageyama & Waditee-Sirisattha, 2018; Oren & Gunde-Cimerman, 2007). These photoprotective compounds, crucial for shielding microalgae against solar radiation, yield derivatives like tetrahydropyridines that act as sunscreen pigments, offering protection

against UV-induced damage, inflammation suppression, and antioxidant activity (Bhatia et al., 2011; Rastogi et al., 2015; Singh et al., 2017).

Furthermore, exopolysaccharides excreted by blue-green algae, such as *Synechocystis*, exhibit antioxidant properties and show potential as moisturizing agents (De Philippis et al., 2001). Comprising various sugars and uronic acid, these exopolysaccharides demonstrate impressive water adsorption and retention capacities, with sacran from *Aphanothece* sacrum surpassing hyaluronic acid in water absorption efficiency (Morone et al., 2019; Okajima et al., 2008; Okajima et al., 2009). Sacran, therefore, is an economical alternative for moisturising product formulations.

2.2.4 Agriculture

Microalgae are emerging biofertilisers and biocontrol agents in the field of sustainable agriculture. They possess the ability to fix atmospheric nitrogen (N_2) in soil, enhance the solubility of nutrients, and act as a soil conditioner (Chakdar et al., 2012; Pabbi, 2015; Renuka et al., 2018; Singh et al., 2016a). The specialised cells (i.e. heterocysts) of some microalgae have thick cell walls made up of three layers. These layers are impermeable to oxygen but permeable to nitrogen. Heterocysts produce nitrogenase and other proteins that can induce nitrogen fixation. Thus, microalgae in rice fields can contribute to about 20 – 30 kg N/ha (Issa et al., 2014). It reduces the cost of chemical fertilisers without compromising the optimal yield. Nutrient availability (i.e. phosphorus) is also improved as blue-green algae can solubilise and mobilise the insoluble organic phosphates present in the soil (Pathak et al., 2018). The effect of microalgal biofertiliser on crop growth is not spontaneous due to the gradual release of fixed nitrogen into the soil. This enables the

crops to utilise more nutrients available from the soil during the growth stage (Chakdar et al., 2012; Pabbi, 2015).

The beneficial effects of microalgal inoculation in crop fields have been reported for wheat, kale, tomatoes, and willow (Coppens et al., 2016; Gebre et al., 2018; Ghazal et al., 2018; Grzesik et al., 2017). Inoculation of blue-green algae in sandy and calcareous soils improved the soil organic matter, water-holding capacity and soil aggregate stability (Ghazal et al., 2018). This was presumably due to the excretion of several compounds (polysaccharides, peptides, lipids, etc.) from microalgal cells which helps in binding soil particles (Chakdar et al., 2012; Ghazal et al., 2018). Using microalgal (*Klebsormidium* sp., *Nannochloropsis,* and *Ulothrix* sp.) fertilisers, tomatoes with higher sugar and carotenoid content have been reported (Coppens et al., 2016).

2.2.5 Biofuel production

Microalgae, capable of converting nutrients into biomass and high-value cellular compounds at scale, offers a promising avenue for commercial biofuel production, a sustainable alternative to depleting fossil fuels. Natural resources such as sunlight, water, and atmospheric or water-dissolved CO_2 are adequate for microalgal growth, eliminating the competition for arable lands. Under optimal nutrient and growth conditions, microalgae can accumulate up to 70% per dry weight of lipids and up to 65% per dry weight of carbohydrates and proteins, useful for conversion to biodiesel, bioethanol, biomethane and biohydrogen (Jankowska et al., 2017). The most commonly studied strains of microalgae for biofuel production include *Chlorella*, *Scenedesmus*, *Spirulina*, and *Nannochloropsis*. These selective strains share similar competitive advantages in terms of high biomass productivity and organic compound contents, as well as robustness

and resistance to environmental and mechanical stress (Frigon et al., 2013; Yang et al., 2023).

The extraction of lipids from microalgal biomass and conversion to biodiesel has been the most extensively studied biofuel application from microalgae. The composition and properties of lipids from microalgae showed a similarity to plant seed oil and animal fat, making microalgal lipids a potential replacement for crops and animals for biodiesel production (Chen et al., 2018). When considering the circular process concept (cultivation-extraction-transesterification-utilization-cultivation), biodiesel derived from microalgae is responsible for relatively less greenhouse gas emissions, in particular CO_2, than petroleum diesel (Saranya & Ramachandra, 2020). Under adverse environmental conditions (e.g., nutrient starvation, light restriction, and high salinity), microalgae are capable of synthesising high concentrations of triacylglycerols (TAG), essential lipids for biodiesel production via transesterification (Arora et al., 2016; Maltsev et al., 2023). However, achieving an optimal balance between growth, lipid content, and productivity in stress-inducing environments is crucial for cost-effective production. The current microalgae oil extraction method is still in the lab- or pilot- scale using organic solvents such as chloroform, hexane, and methanol, highlighting the need for sustainable and cost-effective techniques (Chen et al., 2018). Additionally, life cycle assessments of biodiesel from microalgae have identified upstream processes i.e., cultivation and harvesting as the most energy intensive processes, urging the development of strategies to enhance techno-economic efficiency and reduce overall production costs (Delrue et al., 2012; Passell et al., 2013; Saranya & Ramachandra, 2020).

The emergence of microalgae as third-generation biofuel feedstock has resulted in advances in genetic tools and metabolic engineering to optimise microalgal bioenergy

productivity. Among the sequenced genomes, *Chlamydomonas reinhardtii* is the most extensively studied, renowned as the best model for lipid research (Brar et al., 2021). Light utilisation of *C. reinhardtii* was improved for both low- and high-light conditions through RNA interference technology, resulting in a faster growth rate and reduced sensitivity to photoinhibition (Mussgnug et al., 2007). The manipulation involving overexpression of DGAT (diacylglycerol acyl transferase) in *C. reinhardtii* and *Phaeodactylum tricornutum* for TAG synthesis pathway also induced a 20–44% increase in the neutral lipids (Niu et al., 2013). Attempts to transform *Synechococcus* sp. with bacterial genes from *Zymomonas mobilis* containing two enzymes, pyruvate decarboxylase and alcohol dehydrogenase, successfully created a catalysed pathway for ethanol synthesis. A significantly higher ethanol yield (0.23 - 5.50 g/L) was achieved by *Synechococcus* sp. PCC 6803 (Deng & Coleman, 1999; Gao et al., 2012).

Microalgae and blue-green algae can also produce molecular hydrogen (H_2), a promising clean fuel for the future. The combustion of H_2 for energy conversion does not result in any air pollution. H_2 has the highest energy per unit weight (142 MJ/kg) among all known fuels (Ali & Basit, 1993; Sarsekeyeva et al., 2015; Singh et al., 2016b). In blue-green algae, nitrogenase enzymes have been reported to produce H_2 most efficiently as a by-product of nitrogen fixation (Allahverdiyeva et al., 2010). However, metabolic and genetic improvement of microalgae strains is necessary to enhance H_2 production. The improved metabolic pathways can minimise the inhibition of hydrogenase activity caused by oxygen via photosynthesis (Anwar et al., 2019). Several engineered microalgal strains have been generated and evaluated for H_2 production (Table 1).

Table 1. Hydrogen production of genetically engineered cyanobacterial strains.

Microalgal strain	**Productivity** (μmol H_2/mg chlorophyll*hour)	**References**
Synechococcus sp. PCC 7002	1	(Srirangan et al., 2011)
Synechocystis sp. PCC 6803	6	(Cournac et al., 2004)
Nostoc sp. PCC 7422	100	(Yoshino et al., 2007)
Nostoc linckia HA-46	93-105	(Mona et al., 2011)
Chlamydomonas reinhardtii CC124	100	(Meuser et al., 2012)

Anaerobic digestion of microalgal biomass is another pragmatic and well-established option for bioenergy production. The whole biomass including cell walls, extracellular biopolymers, and intracellular contents are potential substrates for biogas production. As a result, anaerobic digestion can be applied to either unprocessed microalgal biomass or residual microalgal biomass post-downstream processing to reduce waste (Jankowska et al., 2017). This eliminates additional extraction steps as seen in biodiesel production (i.e. lipid extraction), thus reducing cost. The generated biogas can be used directly for heating or reused as a carbon source for microalgal cultivation. This promotes a holistic biorefinery concept for microalgae biomass (Mussgnug et al., 2010; Ramos-Suárez & Carreras, 2014). Compared to other biofuel applications, biogas production from microalgal biomass is more straightforward and requires no genetic modifications. Besides, anaerobic digestion is a mature technology that has been commercialised in many regions (Mao et al., 2015). Its technology readiness would accelerate the implementation of biogas production from microalgal biomass. This emerges as an advantage over other biofuel production such as biohydrogen, which is still in its infancy. The application of microalgae for biogas production is discussed further in Section 2.4.

2.2.6 Wastewater treatment

Integrating microalgae cultivation with wastewater treatment presents a promising strategy to enhance the feasibility of biofuel production while reducing both CO_2 emissions and costs (Chen et al., 2015; Singh et al., 2023). Traditional microalgae cultivation relies on significant water and nutrient inputs, creating competition for fertilizers and economic challenges for biofuel production. By incorporating microalgal biomass production into wastewater treatment, the process becomes dual-purpose—simultaneously removing water contaminants and generating biomass. Microalgae efficiently uptake nitrogen and phosphorus during their growth, preventing eutrophication in water bodies where treated effluent is discharged (Abdel-Raouf et al., 2012). Widely studied microalgal species for wastewater treatment include *Chlorella* sp., *Arthrospira* sp., *Scenedesmus* sp., and *Nannochloropsis* sp. (Cai et al., 2013; Li et al., 2019; Wollmann et al., 2019). Microalgae utilise resources from wastewater including water, nutrients, and CO_2, to produce biomass that is rich in lipids, proteins, and carbohydrates (Lee et al., 2023b; Tan et al., 2023; Ummalyma et al., 2023; Wang et al., 2023). The resultant biomass serves as valuable substrates for bioenergy and biochemical production, achieving multiple goals of water remediation, energy generation, and environmental protection through a sustainable approach.

2.3 Approaches to effective microalgae harvesting

Microalgal harvesting is an important step in the supply chain of microalgal biotechnology (Figure 4). It is responsible for transforming the diluted microalgal suspension into a concentrated microalgal slurry through intense dewatering. This helps to optimise the working volume and efficiency of downstream processes (e.g. extraction).

However, harvesting is the current bottleneck in microalgal production, accounting for up to 30% of the total processing cost (Singh & Patidar, 2018).

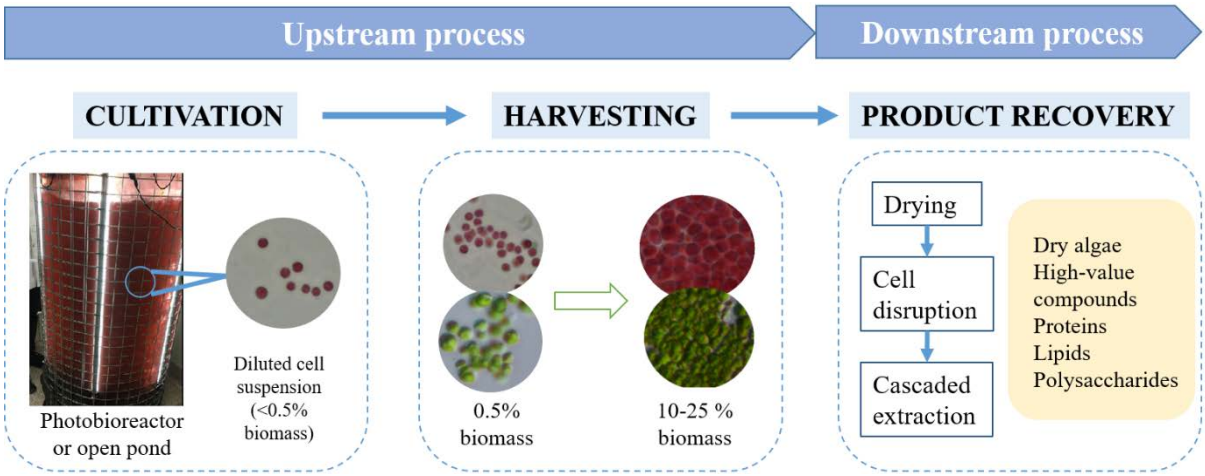

Figure 4. Process flow for biochemical production from microalgae and blue-green algae.

The effectiveness of microalgae harvesting is particularly challenging due to intrinsic cell properties to maintain them in a well-dispersed suspension (Singh & Patidar, 2018). The surface of microalgal cells is negatively charged with zeta potential in the range from −7 to −40 mV (Shaikh et al., 2024). Repulsive electrostatic interaction among individual cells prevents them from aggregation. Besides, microalgae are tiny cells with sizes ranging from 5 to 50 µm (Frigon et al., 2013; Zabed et al., 2019). Microalgal cultures often have low biomass contents of 0.5 to 2 g/L (Zamalloa et al., 2012). A substantial amount of energy and/or chemicals would be required to disrupt the stable microalgal suspension and create an attraction among the cells to form aggregates. This makes harvesting a costly step in the microalgal supply chain. It can account for up to 30% of the total production cost.

A wide range of solid-liquid separation techniques is available for microalgal harvesting with distinct characteristics (Table 2). The selection of harvesting methods depends on:

i) the type of microalgae and their characteristics (e.g. size, growth medium and susceptibility to chemical or mechanical stressors), and

ii) ii) the desired compounds to be extracted from the microalgal biomass.

Table 2. Advantages and disadvantages of common microalgal harvesting techniques.

Techniques	Advantages	Disadvantages	References
Centrifugation	>90% harvesting efficiency Short operation time Applicable to most species	Not suitable for large-scale High capital cost High energy demand Likely cause cell damage	(Barros et al., 2015; Fasaei et al., 2018)
Membrane Filtration	<90% harvesting efficiency Short operation time Low cell damage	Membrane fouling leads to increased O&M cost High energy demand Unfeasible for very small size species	(2014; Singh & Patidar, 2018)
Coagulation/ Flocculation	>90% harvesting efficiency Suitable for large scale Short operation time Low cell damage Low energy demand Applicable to most species	Chemical cost End-product value is limited	(Singh & Patidar, 2018)
Flotation	<90% harvesting efficiency Short operation time Low cell damage Low energy demand Applicable to most species	Surfactant cost pH dependent	(2014; Griffiths et al., 2011; Singh & Patidar, 2018)

2.3.1 Centrifugation

Centrifugation is a mechanical technique, which exerts a centrifugal force on the microalgal cells to separate them from the growth medium. This type of separation depends on the microalgal cell settling characteristics (cell size and density) (Singh & Patidar, 2018). Due to the small size of microalgae, a longer retention time is required to enhance the harvesting efficiency under low flow rates (Barros et al., 2015). However,

despite being a highly effective technique with >90% efficiency, centrifugation requires expensive investment costs for equipment and high energy consumption (Dassey & Theegala, 2013). Furthermore, high gravitational and shear forces during centrifugation may result in microalgal cell damage. This limits its application in the production of high-valued biochemical (e.g. unsaturated fatty acids and pharmaceuticals).

Several centrifuges have been investigated for microalgal harvesting, including disc stack centrifuges, and decanter centrifuges. Disc stack centrifuges are extensively used for high-valued microalgal products with an applied force equal to 4,000–14,000 G (Najjar & Abu-Shamleh, 2020; Singh & Patidar, 2018). Disc stack centrifuges concentrate diluted microalgal suspension (0.02-0.5%) to form a microalgal slurry of 2-15% concentration, but they are highly energy-intensive and mechanically complex (Najjar & Abu-Shamleh, 2020). The energy consumption of a Westfalia HSB400 disc-bowl centrifuge can reach 1.4 kWh/m^3 (Najjar & Abu-Shamleh, 2020). Decanter centrifuges, also known as solid bowl centrifuges, are also commonly used for microalgal harvesting. They are designed to sustain high solid concentration in the feed. Decanter centrifuges can achieve microalgal slurry with a solid content of 15 and 22% (Najjar & Abu-Shamleh, 2020; Sim et al., 1988). Similar to disc stack centrifuges, decanter centrifuges have the disadvantage of high energy consumption.

Combining centrifugation with other harvesting techniques is a potential approach to reduce processing costs. Flocculation and filtration can be used in the first step to pre-concentrate microalgal suspension before centrifugation. The operational energy of centrifugation was significantly reduced from 13.8 to 0.2 MJ/kg DW upon combining bio-flocculation with centrifugation to harvest *C. vulgaris* (Salim et al., 2011). By combining submerged microfiltration with centrifugation, Bilad et al. (Bilad et al., 2012)

showed that the energy consumption can be decreased from 8 to 0.8 kWh/m^3 for *C. vulgaris* and from 8 to 0.9 for *Phaeodactylum tricornutum*.

2.3.2 Membrane filtration

Membrane filtration is an emerging harvesting technology that preserves cell quality and requires no chemical addition. Its operation is dependent on differential pressure on two sides of the membrane to force the movement of the microalgal suspension. The required pressure drop can be achieved through gravity, pressure, vacuum, or magnetic filtration (Barros et al., 2015; Singh & Patidar, 2018). There is a wide range of membrane designs distinguished by their pore size, material, hydrodynamic conditions, and configurations (Mo et al., 2015). Ultrafiltration (0.02–0.2 μm) has been recommended for microalgal harvesting due to better flux and fouling resistance over a prolonged period than microfiltration (0.1–10 μm) (Baerdemaeker et al., 2013; Drexler & Yeh, 2014; Mo et al., 2015). Submerged membrane filtration at lab and pilot-scale showed reduced risks of cell rupture and higher biomass recovery compared to crossflow filtration, which is energy-intensive and prone to fouling (Baerdemaeker et al., 2013; Bilad et al., 2012; Mo et al., 2015).

Membrane fouling is the main disadvantage of filtration, as it demands a high cost of membrane cleaning and reparation. This occurs over time when the accumulation of microalgae and extracellular organic matter on the membrane increases the resistance to flow and decreases the flux upon a constant pressure (Barros et al., 2015). The organic matters secreted by microalgae during their growth include extracellular polymeric substances, algogenic organic matter, and extracellular organic matter (Singh & Patidar, 2018). Qu et al. (2012) observed that ultrafiltration membrane fouling can be caused by

extracellular organic matter through cake layer formation, hydrophobic adhesion or pore plugging. The anti-fouling property can be enhanced by coating the membrane surface with hydrophilic polyvinyl alcohol polymer (Hwang et al., 2013). Nonetheless, further development and testing are vital to overcome the challenge of membrane fouling before it is viable for large-scale microalgal harvesting.

2.3.3 Flotation

Flotation utilises air or gas bubbles to carry the tiny microalgal cells that have been previously mixed with flocculants or surfactants to the water surface. Due to the low density and stable suspension of microalgae, this technique is faster and more effective than sedimentation (Hanotu et al., 2012). As the air or gas bubbles travel up the water column, destabilised microalgal cells attach to the surface of the bubbles. Successful flotation relies on effective bubble-microalgal cell collision and subsequent bubble-microalgal cell adhesion (Qi et al., 2022; Singh & Patidar, 2018). An effective collision between air bubbles and microalgal cells is influenced by bubble size and mixing intensity, while the adhesion of the cells to the bubbles is governed by the microalgal cell surface charge and hydrophobicity (Laamanen et al., 2016). Surfactant/flocculant addition increases the hydrophobicity of microalgal cells and neutralises the negatively charged microalgal cells to facilitate aggregation. This increases the chance of collision and then the adhesion of microalgal cells to the bubbles (Hanotu et al., 2012). Micro-sized bubbles with higher surface area and lower rise velocity lead to more efficient attachment of microalgal cells (Hanotu et al., 2012). Flotation under high ionic strength increases the risk of gas bubble rupture, thus it might not be effective for microalgae in seawater cultures (Barros et al., 2015).

Flotation processes are classified according to bubble size production: dissolved air flotation (DAF, bubble diameter <100 μm), dispersed air flotation (DiAF, bubble diameter 100–1000 μm), electrolytic flotation and ozonation-dispersed flotation (ODF) (Barros et al., 2015; Chen et al., 2011). DAF is the most efficient and widely employed, but very energy intensive. This is due to the need to supersaturate the solution with dissolved air using high pressure to produce dense microbubbles that carry up the microalgal cells (Barros et al., 2015). DiAF consumes less energy by passing the air continuously through a porous material but requires more expensive equipment to create a pressure drop for bubble generation (Chen et al., 2011). Electrolytic flotation utilises electrolysis to form fine hydrogen bubbles. The main disadvantages of this technique are cathode fouling and high power requirement (Chen et al., 2011; Singh & Patidar, 2018). Meanwhile, ODF is a costly process that produces charged bubbles to interact with negatively charged microalgae. Cell lysis or contamination is a concern when using this technique, as it leads to biochemical leakage and compromised cell quality (Cheng et al., 2011).

Microalgae harvesting using flotation has been mostly studied at lab-scale level. Harvesting efficiencies of 60 – 80 % were reported for DAF of *C. vulgaris* using different flotation jar designs and pDMAEMA as the flocculant (Rao et al., 2023). Similarly, DAF of *C. vulgaris* with polyoctyl chitosan as the surfactant also achieved 60% harvesting efficiency (Demir-Yilmaz et al., 2023). The main advantages of flotation for microalgae harvesting are short operation time, compactness, large-scale harvesting, and high flexibility with low initial cost (Barros et al., 2015; Laamanen et al., 2016; Singh & Patidar, 2018). However, flotation requires additional surfactant or flocculant to achieve

high efficiency. Flotation is a promising yet challenging method for harvesting microalgae and is still in the early stages of research.

2.3.4 Flocculation

Flocculation has been proposed as a low-cost and effective technique to harvest a wide range of microalgae at large-scale production. There are three main mechanisms behind microalgal flocculation: charge neutralisation, bridging, and sweeping effect (Vandamme et al., 2013). Depending on the type of flocculants (e.g. chemical or electrolytic process), one or more of these mechanisms can occur to facilitate the agglomeration of microalgal cells. Charge neutralisation is regarded as the major mechanism involved in flocculation. The negative surface charge of microalgal cells can be neutralised by positively charged flocculants, thus reducing the repulsion among the cells to form an agglomerate. Ideal flocculants should be cheap, nontoxic, effective at low doses and sustainable (Singh & Patidar, 2018).

Available chemical flocculants for microalgal harvesting include inorganic salts (iron and aluminium salts), organic polymers (polyacrylamide and polyelectrolyte), and natural polymers (chitosan and cationic starch) (Okoro & Sun, 2019). Cationic inorganic flocculants (e.g. aluminium sulphate, ferric chloride, and ferric sulphate) form polyhydroxy complexes at optimal pH resulting in the neutralization of negative surface charges on microalgal cells (Chen et al., 2011). However, extremely high doses (300 – 2000 mg/g dry microalgal biomass) have been reported for >90% microalgal flocculation using inorganic flocculants (Chatsungnoen & Chisti, 2016; Sanyano et al., 2013; Zhu et al., 2018). These doses are not economical and might cause increased dissolved solids, microalgal biomass contamination and/or discolouration.

Concerns over the quality of the harvested biomass can be avoided by using natural and biodegradable polymers like chitosan. Culture media after chitosan flocculation is reusable and non-toxic, thus contributing to reducing the process cost (Şirin et al., 2012a). Recently, Loganathan et al. (2018) have attempted to combine inorganic flocculants and chitosan to improve the doses and microalgal harvesting efficiency. They reported a reduction of 20 mg flocculants per litre of microalgal suspension was achieved while maintaining the harvesting efficiency of over 95%. The synergistic effect was attributed to the simultaneous charge neutralisation and bridging effect of alum and chitosan (Loganathan et al., 2018).

The flocculating capacity of the organic polymers (i.e. polyelectrolytes) is influenced by charge and functional groups on the surface of microalgae, growth medium pH and density of the microalgal culture (Chen et al., 2011). Only cationic polymers can flocculate microalgae due to them possessing positive charges to facilitate charge neutralisation. Anionic and non-ionic polymers fail to overcome the repulsive forces among negatively charged microalgal cells, and thus cannot flocculate. Besides, the effective doses of cationic polymers decrease with an increase in molecular weight as more polymeric tails and loops bridge the microalgal flocs together. Cationic polyelectrolytes were reported to be 35 times more effective than metal salts (Granados et al., 2012). However, there is a lack of studies on the effect of organic polymer flocculation on microalgae, especially for species like *P. purpureum* with a fragile cell membrane.

Autoflocculation or alkaline flocculation can occur in seawater microalgal cultures, which contain a large amount of alkaline earth metal ions such as magnesium (Mg^{2+}) and calcium (Ca^{2+}). At high pH >9 and under atmospheric conditions, these ions can

precipitate as magnesium hydroxide and calcium carbonate (Besson & Guiraud, 2013; Mayers et al., 2020; Vandamme et al., 2015). The large mass of precipitates, while settling down due to gravitational force, entangles the microalgal cells by a sweeping effect. The sedimentation of the microalgal cells is thus facilitated. The addition of Mg^{2+} and Ca^{2+} to freshwater microalgal suspension at high pH can also lead to alkaline flocculation (Wu et al., 2012). Alkaline flocculation has been reported for seawater and freshwater microalgal cultures such as *Phaeodactylum tricornutum*, *C. vulgaris, Scenedesmus* sp., and *Nannochloropsis oculata* (Vandamme et al., 2015; Wu et al., 2012) and a *Dunaliella salina* hypersaline culture (Besson & Guiraud, 2013). The disadvantage of this technique is the high concentration of precipitates in the microalgal slurry after flocculation. A further step is necessary to remove the solids and obtain microalgal biomass only.

Apart from the choice of flocculants, flocculation efficiency for harvesting microalgae is also influenced by several key operating parameters including mixing speed and time, solution pH, flocculant dose, and settling time. Mixing speed and time are crucial as they determine the formation and stability of flocs. Rapid mixing promotes uniform distribution of the coagulant, while gentle mixing encourages floc growth without breaking them apart (Zhang et al., 2023). Thus, short rapid mixing is often followed by longer gentle mixing in flocculation protocol. The solution pH affects the charge interactions between the microalgae cells and the flocculant, with optimal pH levels enhancing the flocculant's effectiveness in neutralising cell surface charges and promoting aggregation (Li et al., 2020). The flocculant dose must be carefully optimised to balance between sufficient floc formation and the risk of overdosing, which can lead to the re-stabilisation of particles and reduced efficiency (Taghavijeloudar et al., 2023). Finally, settling time allows the flocs to aggregate and settle out of the solution, with

insufficient settling time resulting in incomplete separation and reduced biomass recovery (Hadiyanto et al., 2021). Understanding and optimising these parameters are essential for maximising flocculation efficiency and achieving cost-effective microalgae harvesting.

2.4 Biogas production from microalgae biomass

Anaerobic digestion can convert microalgal biomass to biogas, which is a renewable fuel. This conversion is facilitated by a consortium of microorganisms (hydrolytic and fermentative bacteria, acetogens and methanogens) in the absence of oxygen. Biogas typically consists of 50-70% CH_4 and 30-50% CO_2. Microalgae prevail as attractive substrates for biogas production due to their fast growth rate, high content of biodegradable compounds and minimal requirement for resources (Kröger & Müller-Langer, 2012; Ward et al., 2014). Microalgae can be cultivated in seawater and wastewater, thus avoiding competition with food crops.

2.4.1 Microalgal biomass as feedstock for anaerobic digestion

Biogas production from microalgae is highly strain-specific due to the great diversity in their physiochemical characteristics and cultivating conditions. Carbohydrates, proteins, and lipids are the main constituents of microalgae, but the ratio among these biochemicals varies across strains and species. Seasonal variations and nutrient availability can influence this ratio. For example, a nitrogen-depleted condition usually enhances lipid accumulation in microalgal cells (Yodsuwan et al., 2017). The carbohydrate content of *Arthrospira plantensis* increases under phosphorous stress conditions (Markou et al., 2013).

CH_4 - the most valuable product from biogas can be derived from all three main components of microalgae including carbohydrates, proteins, and lipids. Thus, theoretical CH_4 yield is calculated for each strain based on their biochemical and elementary (C, H, O, and N) composition, as proposed by Buswell and Boruff (1932) (Eq. 1 and Eq. 2).

$$C_aH_bO_cN_d + \left(\frac{4a-b-2c+3d}{4}\right)H_2O \rightarrow \left(\frac{4a+b-2c-3d}{8}\right)CH_4 + \left(\frac{4a-b+2c+3d}{8}\right)CO_2 + dNH_3$$

(Eq. 1)

Where a, b, c, and d equal the carbon, hydrogen, oxygen, and nitrogen content molar composition, respectively.

$$Stoichiometric\ methane\ potential\ (L/g\ VS\ destroyed) = \left(\frac{4a+b-2c-3d}{12a+b+16c+14d}\right)V_m$$

(Eq. 2)

Where V_m denotes the molar volume of methane (e.g. 22.14 L at 0 °C and 1 atm).

Several potential microalgae species have been studied for biogas production, including *Chlorella*, *Nannochloropsis*, *Scenedesmus*, and *Spirulina*. They are robust and easy to cultivate. There are differences in the biochemical composition of these species, but generally, they are very rich in proteins (50 to 70%). The cell structure, particularly the cell wall structure, of listed microalgae is also distinct from one another (Yukesh Kannah et al., 2021). Algaenan trilaminar layer (ATL) and microfibrillar layer (MFL) are the main primary and secondary cell wall layers, respectively. They consist of mainly cellulose, hemicellulose, and extracellular protein. The arrangement of the ATL, MFL, and other layers (e.g. pectic layer and peptidoglycan layer) in the cell wall is diverse among microalgal strains (Yukesh Kannah et al., 2021).

The hemicellulosic and multilayered cell walls of microalgae hinder their biogas production. Microalgal cell walls are thick and rich in carbohydrates. They provide microalgal cells with rigidity and resistance to environmental stresses and enzymes (e.g. cellulases, hemicellulases, and other hydrolases) (Frigon et al., 2013). This reduces the anaerobic digestibility of microalgal biomass. Microalgal cells reportedly resisted the bacterial attack and remained intact in the digestate after 30 to 45 days of anaerobic digestion (Golueke et al., 1957; Qing et al., 2009). Pretreatment of microalgal biomass is a strategy to overcome this constraint and enhance CH_4 yield via cell wall disruption (Section 1.1.1).

The low C/N ratio due to high protein content in microalgal biomass also inhibits the activity of the microbial community in anaerobic digestion. Microalgal species which have been investigated for anaerobic digestion have a C/N ratio varied from 4.16 to 7.82 (Ward et al., 2014). It is significantly less than the optimal C/N ratio of 20 required for the anaerobic bacterial community. The imbalance caused by the low C/N ratio leads to the accumulation of ammonia and volatile fatty acids during digestion. These intermediates are potential inhibitors to methanogenesis when allowed to accumulate (Parkin & Owen, 1986). Co-digestion of microalgal biomass with carbon-rich substrates has been investigated to improve the C/N ratio and efficiency of anaerobic digestion (Section 1.1.1).

Table 3. Biochemical composition of commonly studied microalgae and blue-green species

Microalgae	Carbohydrate (%)	Lipid (%)	Protein (%)	CH_4 potential (L/Kg VS)		Ref
				Theoretical	Experimental	
Chlamydomonas reinhardtii	21	20	61	690	416	(Klassen et al., 2017)
C. vulgaris	26	6	68	479	240	(Mendez et al., 2015)
Isochrysis galbana	30-45	23-30	7-25	494	315	(Roberts et al., 2019)
Nannochloropsis salina	18	42	26	680	240	(Bohutskyi et al., 2015)
Scenedesmus sp.	18-52	16-43	8-18	550	157 – 317	(Saleem et al., 2024)
Scenedesmus obliquus	10-17	12-14	50-56	590-690	370	(Abimbola et al., 2024; Sialve et al., 2009)

2.4.2 Strategies to enhance anaerobic digestibility of microalgae

Pretreatment to disrupt microalgal cell walls is necessary to improve the biodegradability of microalgae for biogas production. Pretreatment can rupture the hemicellulosic cell walls and increase cell permeability (Yukesh Kannah et al., 2021). It also leads to the solubilisation of cellulose and extrapolymeric contents of microalgal cell walls. As a result, both the extracellular and intracellular contents of microalgae (i.e. carbohydrates, proteins, and lipids) are readily available in the aqueous phase for anaerobic digestion. Several reviews have provided comprehensive lists of studies regarding enhanced biogas/CH_4 yield from pretreated microalgal biomass compared to non-pretreated biomass (Jankowska et al., 2017; Passos et al., 2014c; Zabed et al., 2020).

Pretreatment technologies are classified into biological (e.g. bacteria, enzymes, and fungi), chemical (e.g., acid, alkali, ionic-liquid, and oxidation), mechanical (e.g. microwave, sonication, and ultrasound), and thermal (e.g. hydrothermal and steam explosion) (Jankowska et al., 2017). Biological pretreatment is a promising approach due to its eco-friendly and non-energy intensive operation. However, this pretreatment technology is still in its early stages of research and development (Barati et al., 2021). Some challenges associated with biological pretreatment include enzyme production cost, long exposure time and the diversity of microalgal cell walls (i.e. substrate specificity) (Passos et al., 2014c). Chemical pretreatment commonly uses acids or alkalis to solubilise the recalcitrant cell walls. It is often combined with thermal treatment for a greater impact on microalgal digestibility (Mendez et al., 2013; Passos et al., 2014c). Chemical cost and residual chemicals (i.e. potential corrosiveness) in the reactor are major drawbacks (Zabed et al., 2019). Mechanical and thermal pretreatment have been most widely studied

in various microalgae (Zabed et al., 2020). This is because they are less dependent on the characteristics of microalgal species and less susceptible to chemical contamination (Lee et al., 2012). The main disadvantage of these approaches is high energy consumption.

Besides pretreatment, microalgae co-digestion with carbon-rich substrates also offers enhanced CH_4 yield by improving the C/N ratio. A high C/N ratio of 20-25:1 has been achieved for the co-digestion of microalgae with organic waste (Herrmann et al., 2016; Yen & Brune, 2007; Zhong et al., 2013). Improvement in the C/N ratio due to co-digestion supports the stabilisation of anaerobic digestion and minimises inhibitors such as ammonia and volatile fatty acids. CH_4 production from co-digestion of microalgae increased by 20 to 260% compared to the yield of mono-digestion (Zabed et al., 2020). The synergistic effect of microalgal co-digestion is influenced by the type of co-substrate and operating temperature (mesophilic or thermophilic). Other factors such as organic loading rate, inoculum to substrate ratio, and microalgae to co-substrate ratio should also be designed carefully to achieve enhanced methane yield (Ganesh Saratale et al., 2018).

2.5 Summary

Information corroborated in this chapter provides an overview of the key information related to the characteristics of microalgae and the technologies involved in harnessing microalgae's potential as sustainable energy and biochemical feedstock. The chapter highlights the wide range of applications from microalgae biomass, especially in the realm of renewable energy, as well as the challenges the microalgae industry is facing on the road to commercialisation. Microalgae, rich in biodegradable compounds, are the ideal substrate for anaerobic digestion to produce renewable CH_4. Considering harvesting is one of the main bottlenecks in microalgae biorefinery, state-of-the-art technologies for

microalgae harvesting were extensively discussed and flocculation was identified as the most suitable for biofuel production from microalgae. Understanding flocculation mechanisms and their impact on microalgae biomass is critical to optimise the harvesting techno-economic efficiency. Knowledge of microalgae cellular structure also suggests the necessity for strategies such as pretreatment and co-digestion to enhance the CH_4 production from microalgae biomass.

CHAPTER 3. SYNERGISTIC EFFECT OF DUAL FLOCCULATION BETWEEN INORGANIC SALTS AND CHITOSAN ON HARVESTING MICROALGAE *C. VULGARIS*

This chapter has been published as the following journal article:

Vu, H.P., Nguyen, L.N., Lesage, G., Nghiem, L.D. 2020. Synergistic effect of dual flocculation between inorganic salts and chitosan on harvesting microalgae C. vulgaris. *Environmental Technology & Innovation*, 17, 100622.

Summary: The flocculation efficiency of microalgae *C. vulgaris* for subsequent harvesting was investigated using single flocculants of inorganic salts, synthetic polymer, chitosan and dual flocculants of inorganic salts and chitosan. Synthetic polymer (Flopam^{TM}) could achieve over 90% optical density removal (OD_{680} removal) at a low flocculant dose (20 to 40 mg polymer per litre of microalgal suspension) through the bridging mechanism and charge neutralisation. Inorganic salts (i.e. ferric chloride and aluminium sulphate) and chitosan individually resulted in low flocculation efficiency (<90%) despite high doses (i.e. 160 to 200 mg per litre of microalgal suspension). The dual flocculation combining ferric chloride or aluminium sulphate with chitosan induced synergistic effects, resulting in >80% flocculation efficiency, significantly higher than the sum of each flocculation. The improvement in flocculation efficiency was 57 and 24% respectively for ferric chloride/chitosan and aluminium sulphate/chitosan. Charge neutralisation of microalgal cells by ferric chloride or aluminium sulphate combined with bridging by chitosan produced the synergy.

Keywords: Ferric Chloride; Aluminium sulphate; Charge neutralisation; Bridging; Polyacrylamide.

3.1 Introduction

Microalgae are among the most important organisms in the ecological evolution and history of the Earth. They have the potential to shape our future with a wide range of promising applications that tackle worldwide issues. The global fossil fuel supply is depleted and has caused destructive environmental effects over its life cycle. There is growing interest in microalgal biomass as renewable and environmental-friendly feedstock for third-generation biofuel (Vo Hoang Nhat et al., 2018). The nutritive value of microalgal biomass for humans as well as their versatile biochemical features have allowed for the production of health supplements, bioactive compounds, food additives and biotechnology applications, although there are still several hurdles in terms of socio-economic aspects (de la Noue & de Pauw, 1988; Koyande et al., 2019; Rizwan et al., 2018). In particular, harvesting has been a major technical and economic bottleneck in microalgal biomass production due to low cell concentrations in cultures (0.5 to 5 g/L), small cell size (< 30 μm), the stability of cell suspension and variation in culture medium (Edzwald, 1993; Klein-Marcuschamer et al., 2013; Singh & Patidar, 2018; Vandamme et al., 2010). Currently, microalgal harvesting is the most expensive step (i.e. 20-30% of the total cost) in the process of microalgal biomass production (Molina Grima et al., 2003; Singh & Patidar, 2018).

The microalgal harvesting techniques include coagulation, flocculation, flotation, membrane filtration and centrifuge (Barros et al., 2015; Leite et al., 2019; Singh & Patidar, 2018). Amongst them, flocculation has received significant attention for its simple operation and relatively low-cost approach, but efficiency is dependent on the flocculant type (Barros et al., 2015; Okoro et al., 2019; Vandamme et al., 2010). Available chemical flocculants for microalgal harvesting can be grouped into three categories: (i)

inorganic flocculants such as iron and aluminium salts, (ii) synthetic polymers such as polyacrylamide and polyelectrolyte and (iii) natural organic polymers such as chitosan and cationic starch (Okoro et al., 2019; Vandamme et al., 2010). Synthetic polymers often provide high harvesting efficiency at low doses (Nguyen et al., 2019). However, these polymers are expensive. Inorganic flocculants such as ferric chloride and aluminium sulphate are less expensive but require a higher dose. Contamination and/or discolouration of microalgal biomass are possible concerns when using inorganic salts. The presence of these salts in the harvested biomass hinders its applications for biofuel and pigment extraction (Barros et al., 2015). These issues with the quality of the harvested biomass can be avoided by using natural polymers like chitosan. Chitosan is a promising flocculant due to its advantages (e.g. natural product, biodegradation and non-toxic) (Barros et al., 2015; Şirin et al., 2012b). It has been demonstrated that chitosan residual in the culture media (i.e. after biomass harvesting) is non-toxic to microalgae. This feature enhances the reusability of the culture media, which is a potential option to reduce costs (Şirin et al., 2012b). However, the expensive cost of around 20 to 50 USD/kg of chitosan (depending on the purity) sets back its large-scale application (Alibaba.com, 2019; Barros et al., 2015).

Inorganic salts provide flocculation through neutralising microalgal cell charge while chitosan flocculates microalgal biomass through bridging (Barros et al., 2015). Therefore, it is hypothesized that the combination of these two mechanisms can enhance flocculation efficiency or harvesting efficiency. Indeed, a combination of alum and chitosan as flocculant aid induced a synergistic impact on harvesting seawater microalgae (Loganathan et al., 2018). The author indicated that a reduction of 20 mg flocculants per litre of microalgal suspension was achieved while maintaining the harvesting efficiency

of over 95% (Loganathan et al., 2018). However, there have not been any studies on freshwater *C. vulgaris* harvesting using this type of flocculant combination. The most similar approach combining ferric chloride and polyethylene was conducted by Gorin et al. (2015). They reported an increase from 60% to 90% flocculation efficiency of *C. vulgaris* using dual flocculation. However, the dose of ferric chloride was very high at 500 mg/L, which may cause unfavourable effects on microalgal cells. Given the benefits (e.g. biological and pharmaceutical properties, nutrient contents for human health) of microalgae *C. vulgaris* (Sharifah & Eguchi, 2012), effective harvesting of its biomass without compromising the cell quality will be a stepping stone to mass production of microalgal-based products.

This study aims to compare the performance of four types of flocculants including two metal salts ferric chloride and aluminium sulphate, polyacrylamide polymer Flopam^{TM} and organic polymer chitosan on *C. vulgaris* harvesting. From the results of these single flocculation tests, dual flocculation tests using inorganic salt followed by chitosan addition were conducted to determine to what extent this strategy can improve the efficiency and reduce the flocculant dose of the process. Optical density removal, turbidity and zeta potential were measured to evaluate flocculation efficiency and mechanisms. The result from this study is expected to contribute to the greater research on optimising microalgae harvesting, particularly using the flocculation process.

3.2 Materials and Method

3.2.1 Microalgal suspension and materials

Microalgal suspension sample was prepared using the freshwater species *C. vulgaris* (CS-41) (Australian National Algae Culture Collection, CSIRO Microalgae Research, Hobart,

TAS). This species was grown in the MLA medium (Algaboost; Wallaroo, SA, Australia) to its mid-stationary phase following the previous protocol (Nguyen et al., 2019). Its growth phase was monitored daily by measuring the optical density of the solution at wavelengths of 680 nm.

Microalgal suspensions at a mid-stationary growth phase were used for harvesting experiments (Section 3.2.2). The mid-stationary growth phase was selected because of its peak in biomass production. In the microalgal growth cycle, the mid-stationary phase occurs right after their population increases exponentially. At the mid-stationary phase, cell divisions had slowed down significantly due to high cell density thus the decrease in feeding factors (e.g. nutrients, light, pH, and carbon dioxide). Thus, harvesting microalgae at the mid-stationary phase is a common protocol.

Anhydrous ferric chloride powder (>98% purity) was supplied by Chem-Supply (Australia). Aluminium sulphate hydrate (54 – 59% assay) was purchased from Sigma-Aldrich (Australia). Cationic polyacrylamide polymer Flopam^{TM} (model no. FO4808) with very high molecular weight was obtained from SNF Australia. Stock solutions of 2 g/L were prepared for each of these flocculants in 200 mL of Milli-Q water and mixed at 100 rpm for one hour. Cationic polyacrylamide polymer (2 g/L) was used within one hour of preparation to avoid polymer hydrolysis. Chitosan (originated from chitin shells of crustaceans) was purchased from Sigma-Aldrich (Australia). Since chitosan is insoluble in water, 0.4 g of chitosan was dissolved in 10 mL of 0.1% HCl solution, followed by the dilution with 190 mL of Milli-Q water to obtain the desired 2 g/L stock concentration. The stock solutions were stored at room temperature and used within two days of preparation.

3.2.2 Flocculation experiment

A 4G Platypus Jar Tester (Australia Scientific, Kotara NSW) was used in flocculation experiments. Samples of 200 mL microalgal suspension were added to 500 mL beakers. The flocculant was introduced to each beaker to obtain a predetermined dose. The microalgal suspension was rapidly mixed at 200 rpm for one minute followed by 15 minutes of slow mixing at 50 rpm. The flocculated microalgal suspension was allowed to settle for one hour. A supernatant sample of 15 mL was pipetted from the suspension at between one- and two-thirds from the bottom for measurement of the flocculation efficiency.

In the individual flocculation experiments, a dose-response relationship protocol was used to define the optimal flocculant dose. Ferric chloride and aluminium sulphate were dosed at a concentration of 40 to 180 g per litre of microalgal suspension. This corresponds to 112 to 504 mg flocculant/g dry biomass. Flopam^{TM} was dosed at 10 to 100 mg per litre of microalgal suspension (i.e. 28 to 280 mg polymer/g dry biomass). While chitosan dose was 40 to 200 mg per litre of microalgal suspension equivalent to 112 to 560 mg chitosan/g dry biomass.

In the dual flocculation experiments, ferric chloride or aluminium sulphate was added at a fixed 40 mg per litre microalgal suspension during the rapid mixing stage (200 rpm). This concentration was selected as it was the lowest dose tested in the single flocculation experiments, thus emphasise the purposes of dual flocculation i.e. limiting the number of metal salts in harvested biomass and minimising potential contamination of microalgal cells. Chitosan was then added at doses of 0 to 80 mg per litre of microalgal suspension (i.e. 0 to 224 mg/g dry biomass) during the slow mixing period (50 rpm).

3.2.3 Analytical methods

The optical density of *C. vulgaris* solution before and after flocculation was measured at a wavelength of 680 nm using the UV spectrophotometer (UV 6000 Shimadzu; Japan). The flocculation efficiency was then calculated using the values as below:

$$\text{Flocculation efficiency (\%)} = \left(\frac{OD_{i-OD_f}}{OD_i}\right) \times 100 \qquad \text{Eq. (3)}$$

Where OD_i and OD_f are the optical density of the culture before and after flocculant addition. Each flocculant was repeated three times for individual and dual flocculation experiments.

A volume of 150 mL of microalgae cell suspension was filtered through a 1.1 μm pre-weighed glass fibre filter paper. The biomass concentration of the microalgae culture was then obtained gravimetrically by drying the sample on the filter paper overnight at 60 ^{o}C to a constant weight. The weight of the final filter paper was used to determine the dry microalgal biomass.

The Zetasizer nano instrument (Nano ZS Zen 3600; Malvern, UK) was used to measure the zeta potential of the microalgae solutions using the 15 mL aliquots taken before and after flocculation.

The solution pH was measured using a pH/conductivity meter (Orion 4-Star Plus Thermo Scientific; Waltham, MA, USA). Turbidity of the microalgae solution before and after flocculation was measured using a portable turbidity meter kit (Apera TN400; Colombus, OH, USA) with accuracy ±1% or 0.02 NTU. Statistical analysis, including calculations of means and standard deviations of replicate samples, was performed in Microsoft Excel.

3.3 Results and discussion

3.3.1 Optimal doses for ferric chloride and aluminium sulphate flocculants

A dose-response relationship can be observed when ferric chloride and aluminium sulphate were used individually as the flocculant (Figure 5). The flocculation efficiency was less than 40% OD_{680} removal at 120 mg flocculant per litre of microalgal suspension (i.e. 336 mg flocculant/g dry biomass), after which the flocculation efficiency steadily increased (Figure 5). A higher flocculation efficiency was achieved as 86% and 77% at 160 mg ferric chloride per litre of microalgal suspension (i.e. 448 mg/g dry biomass) and 180 mg aluminium sulphate per litre of microalgal suspension (i.e. 504 mg/g dry biomass) respectively.

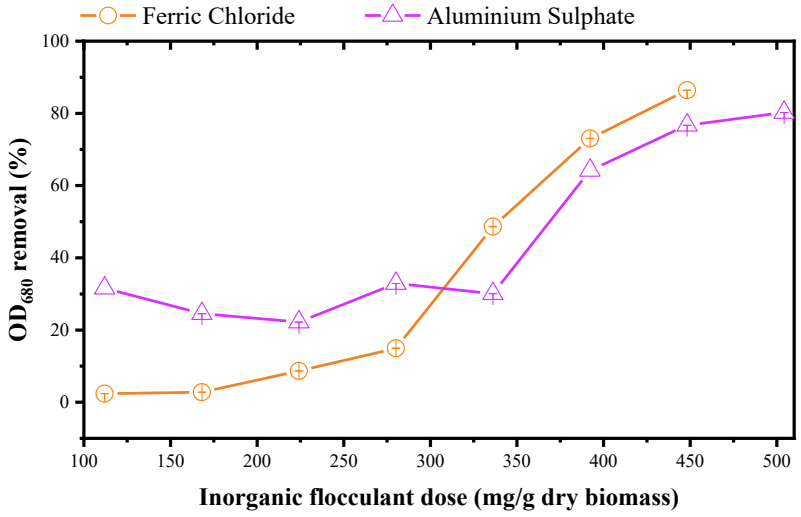

Figure 5. The *C. vulgaris* flocculation efficiency indicated by OD removal at λ = 680nm for inorganic flocculants (a) ferric chloride and (b) aluminium sulphate at different doses. Value and error bars represent the mean and standard deviation (n = 3).

Charge neutralisation is the main flocculation mechanism by inorganic flocculants (Barros et al., 2015; Singh & Patidar, 2018). Small microalgae cells are very stable in

suspension due to the repulsive force caused by their negatively charged surface (- 20.2 mV for *C. vulgaris* in this study). Thus, positively charged ferric or alum ions are required for charge neutralisation to overcome this electrostatic stabilisation by neutralising the charge of microalgae cells (Wyatt et al., 2012). This was demonstrated by the plateau region below 350 mg flocculant/g dry biomass (Figure 5) where the OD_{680} removal value remained quite low, < 35% for ferric chloride and < 20% for aluminium sulphate. Although the optimal flocculation efficiency was acceptable, it was achieved at very high doses of ferric chloride and aluminium sulphate. This finding aligns with the literature results, which show that achieving high flocculation performance (> 90%) with inorganic flocculants like ferric chloride and aluminium sulphate requires high doses (Table 4). The variation in the microalgal culture and growth conditions might be accountable for the difference in optimal doses among these studies.

Table 4: Summary of literature on the flocculation of *Chlorella* genus using aluminium sulphate ($Al_2(SO_4)_3$) and ferric chloride ($FeCl_3$ compared to the results from this study.

Microalgae ($g_{dry\ biomass}/L$)	**Flocculant**	**Optimal dose ($mg/g_{dry\ biomass}$)**	**Efficiency (%)**	**References**
C. vulgaris (0.36)	$Al_2(SO_4)_3$	504	77	This study
	$FeCl_3$	448	86	
C. vulgaris (1.2)	$Al_2(SO_4)_3$	2083	> 90	(Zhu et al., 2018)
	Chitosan	208		
Chlorella sp. (0.12)	$Al_2(SO_4)_3$	1266	> 90	(Sanyano et al., 2013)
	$FeCl_3$	1191		
C. vulgaris (1.0)	$Al_2(SO_4)_3$	350	> 95	(Chatsungnoen & Chisti, 2016)
	$FeCl_3$	300		
C. vulgaris (0.25)	$Al_2(SO_4)_3$	600	> 95	(Vandamme et al., 2012b)

3.3.2 Flocculation performance by organic polymers

3.3.2.1 Synthetic polyacrylamide polymers

Synthetic cationic polymer Flopam™ showed the highest OD_{680} removal of 96% at 20 mg polymer per litre of microalgal suspension (i.e. 56 mg polymer/g dry biomass) (Figure 6). A further increase in its dose up to 100 mg per litre of microalgal suspension (i.e. 280 mg/g dry biomass) caused the flocculation performance to decrease gradually. Results in Figure 6 suggest that polymer over-dosing can be counterproductive. This observation is in good agreement with the literature (Nguyen et al., 2019).

Flopam™ is a high molecule weight and highly charged cationic polymer. Thus, charge neutralisation is the first step of flocculation, followed by entanglement and bridging of microalgal cells and the polymer (Biggs et al., 2000; Pugazhendhi et al., 2019). As this process continues, more microalgae cells are bridged or connected to each other, forming bigger flocs. A combination of mechanisms performed by synthetic cation polymer enhances its flocculation efficiency.

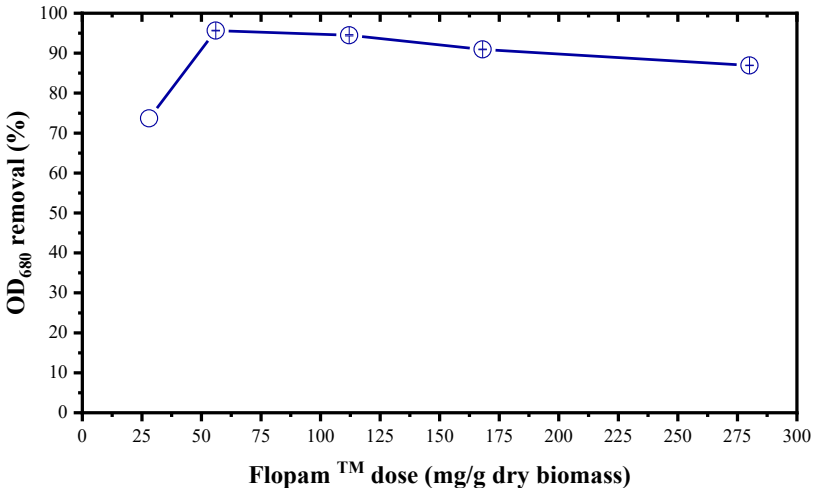

Figure 6. The flocculation performance of Flopam™ indicated by its OD removal efficiency at λ = 680 nm. Value and error bars are the mean and standard deviation (n = 3).

3.3.2.2 Natural polymer Chitosan

In the flocculation of *C. vulgaris* using natural polymer chitosan, the value of OD_{680} removal improved with the increasing doses (Figure 7), suggesting a proportional relationship between flocculation efficiency and chitosan dose. At the lowest dose of 40 mg chitosan per litre of microalgal suspension (i.e. 112 mg chitosan/g dry biomass), the OD_{680} removal was 20%. This was increased to 62% when using 200 mg chitosan per litre of microalgal suspension (i.e. 560 mg chitosan/g dry biomass). The flocculation efficiency of chitosan in this study is not only much lower, but it also required a dose twenty times that of the synthetic cationic polymer Flopam™ to achieve the same OD_{680} removal of around 60%.

Flocculation using chitosan works based on a small degree of charge neutralisation and mostly bridging mechanism, similar to the synthetic cationic polymers made from polyacrylamide in section 3.3.2.1 (Chen et al., 2003; Divakaran & Sivasankara Pillai,

2002). pH plays a key role in the efficiency of chitosan flocculation since at both acidic and very alkaline conditions, the performance is decreased (Divakaran & Sivasankara Pillai, 2002; Harith et al., 2009). In an acidic environment, chitosan exists as a linear chain and remains dispersed due to the repulsive forces between closely placed -NH_2 groups and -NH^{3+} group carrying positive charge (Gualtieri et al., 1988). This prevents chitosan from effectively flocculating the microalgae cells. With an alkaline pH, the positive charge of chitosan is gradually neutralised, thus charge neutralisation of microalgae cells becomes less efficient (Harith et al., 2009). Optimal flocculation using chitosan is obtained within a narrow pH range of 6 to 8 (Divakaran & Sivasankara Pillai, 2002). In this experiment, the pH of the microalgal solution after the addition of chitosan was 8.05. However, the removal efficiency reported was relatively low with high dosage, leading to the subsequent study of dual flocculation using inorganic flocculants and chitosan.

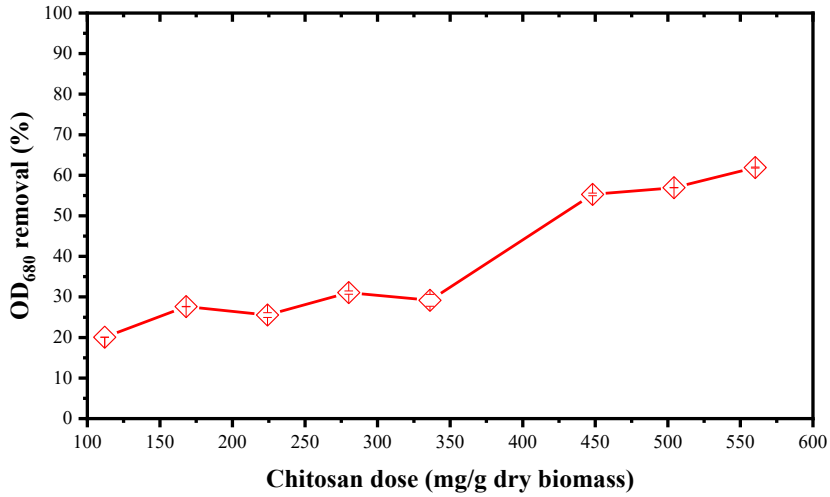

Figure 7. The effect on *C. vulgaris* flocculation using Chitosan, based on its OD removal efficiency at λ = 680 nm. Value and error bars are the mean and standard deviation (n=3).

3.3.3 Synergistic effect of dual flocculation

3.3.3.1 Improved flocculation using a combination of inorganic flocculants and chitosan

Significantly better OD_{680} removal efficiency was observed for dual flocculation combining inorganic salts with chitosan, compared to that achieved by individual flocculation (Figure 8). Dual flocculation using ferric chloride and chitosan achieved an OD_{680} removal of 81% at 80 mg chitosan per litre of microalgal suspension (i.e. 224 mg chitosan/g dry biomass). Likewise, aluminium sulphate (40 mg/L) and chitosan (80 g/L per litre of microalgal suspension or 224 mg chitosan/g dry biomass) achieved 89% efficiency (Figure 8). In comparison with individual flocculation, an additional 57 and 24% harvesting efficiency was achieved by dual flocculation between ferric chloride/chitosan and aluminium sulphate/chitosan, respectively. A synergistic effect in dual flocculation using inorganic flocculants and chitosan, therefore, was present. It increased the flocculation efficiency by approximately two to four times, depending on the type of inorganic salts. This synergistic effect presumably was the result of multiple flocculation mechanisms (e.g. charge neutralisation and bridging) used by inorganic flocculants and chitosan interacting with and assisting each other. These results from the dual flocculation experiments suggest that by combining low doses of inorganic flocculant and chitosan, it is possible to harvest microalgae biomass at an improved efficiency with minimised cell contamination and a cheaper cost.

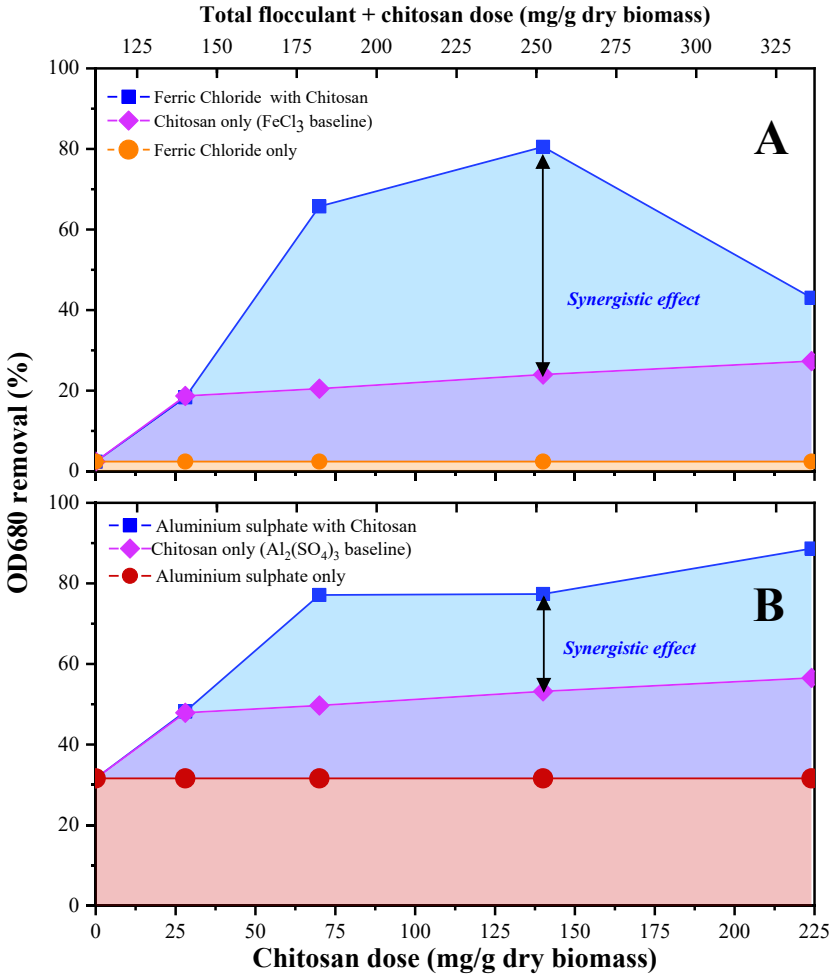

Figure 8. The synergistic effect of combining inorganic flocculant (a) ferric chloride and (b) aluminium sulphate with organic polymer Chitosan in flocculating *C. vulgaris*, indicated by the OD removal efficiency at λ = 680 nm.

3.3.3.2 Synergistic mechanisms of enhanced performance mechanisms

The combination of charge neutralization and bridging is the main reason for the observed synergy. By adding ferric chloride or aluminium sulphate as a primary flocculant in the rapid mixing step, negatively charged *C. vulgaris* cells were neutralised to higher zeta potential and no longer remained stable in suspension (Figure 9). Collision among cells was initiated leading to the formation of small flocs. When chitosan was slowly mixed in at this stage, particle entrapment and bridging took place [16]. Chitosan chains attached

to existing microalgal-alum/ferric flocs and further agglomerated them into bigger masses. These combined mechanisms increased the flocculation efficiency of the dual experiment to above 80%, much greater than that achieved by solely ferric or aluminium flocculation (Section 3.3.1).

At high doses of chitosan (>70 mg/g dry biomass for ferric chloride/chitosan and >140 mg/g dry biomass for aluminium sulphate/chitosan), a synergistic effect is observed for charge neutralisation of the microalgae cells (Figure 9). Flocculation using positively charged ferric chloride, aluminium sulphate and chitosan primarily works based on neutralising negatively charged microalgal cells to destabilise cells in suspension (Barros et al., 2015; Singh & Patidar, 2018). Although the main mechanism of chitosan flocculation is bridging, the addition of chitosan at a higher dose in the dual flocculation still significantly increased the charge neutralisation compared to single ferric chloride or aluminium sulphate flocculation. At optimal chitosan dose, charge neutralisation was 13.8 mV for ferric chloride/chitosan flocculation and 17.2 mV for aluminium sulphate/chitosan flocculation (Figure 9). A lower dose of chitosan (< 70 mg/g dry biomass) did not induce any synergistic effect because chitosan was working mostly on the bridging mechanism and charge neutralisation had a negligible effect on the dual flocculation performance.

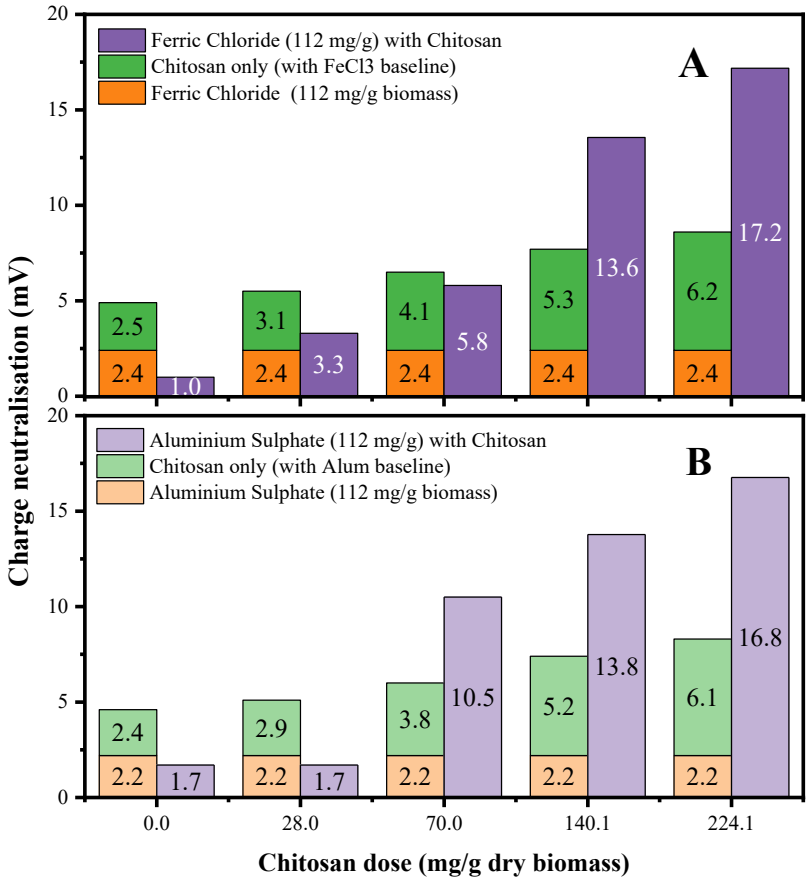

Figure 9. The synergistic effect of dual flocculation using (a) ferric chloride with chitosan and (b) aluminium sulphate with chitosan on the zeta potential of particles in *C. vulgaris* solution, demonstrated by the change in charge neutralisation.

3.3.4 Comparison of flocculants

An indicative cost analysis was conducted for each individual and dual flocculation to obtain an overview of the large-scale feasibility (Table 5). Flopam^{TM} performed excellent flocculation of *C. vulgaris* cells, however, the cost per ton of dry *C. vulgaris* biomass is estimated at 120 USD (Table 5). This value is more than the cost per ton of dry biomass for aluminium sulphate (105 USD) but less than that of ferric chloride (364 USD). Chitosan is the most expensive (i.e. 20-50 USD/kg) among all the flocculants investigated

in this study. The cost to achieve >90% flocculation efficiency per ton of dry *C. vulgaris* biomass using chitosan is approximately 7280 USD (Table 5).

For dual flocculation, the combination of aluminium sulphate and chitosan would cost 4920 USD per ton of dry *C. vulgaris* biomass, while it is 7925 USD for ferric chloride and chitosan combination. This suggests that by combining aluminium sulphate and chitosan, the cost could be reduced significantly by approximately 30%. With further research into the optimisation of dual flocculation for microalgae using inorganic flocculant and chitosan, there is potential for prospective applications of this method in a large-scale environment.

Table 5. Cost comparison for types of flocculants or polymers used in this study based on their current market value.

Flocculant/Polymer (s)	**Indicative cost, US$/ton[a]**	**Cost (US$) per ton dry *C. vulgaris* biomass[b]**
Single flocculation		
Flopam™ (FO 4808)[c]	2 000 – 2 300	120
Chitosan	20 000 – 50 000	7280[d]
Aluminium Sulphate	150 – 200	105
Ferric Chloride	455 – 1 000	364
Dual Flocculation		
Aluminium sulphate + Chitosan	--	4920
Ferric chloride + Chitosan	--	7925

a Prices are collected from Alibaba.com

b Average value from indicative cost is used for calculation

c Price is reported by SNF Australia

d Based on the optimal dose of chitosan for C. vulgaris flocculation reported by Vandamme et al. (2012b)

3.4 Conclusions

A preliminary assessment of microalgal *C. vulgaris* flocculation efficiency was reported in this study. It was shown that the combination of inorganic flocculants (ferric chloride or aluminium sulphate) and chitosan resulted in a synergistic effect. Additional 57 and 24% of harvesting efficiency was achieved with ferric chloride/chitosan and aluminium sulphate/chitosan, respectively. Individually, inorganic flocculants and chitosan required a high dose to achieve 90% harvesting efficiency. The synergistic effect resulted from the interaction between charge neutralisation and bridging mechanisms. The cost is reduced when compared to chitosan-only flocculation while the microalgal cell contamination from inorganic salts can be mitigated. Further research in dual flocculation optimisation can provide a solution to the bottleneck of microalgal biomass production. High-quality products are useful for applications such as biofuels and health supplements.

CHAPTER 4. HARVESTING *PORPHYRIDIUM PURPUREUM* USING POLYACRYLAMIDE POLYMERS AND ALKALINE BASES AND THEIR IMPACT ON BIOMASS QUALITY

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Summary: This study aims to examine the flocculation efficiency of *P. purpureum,* a red marine microalga with a high content of pigments and fatty acids, grown in seawater medium using polyacrylamide polymers and alkaline flocculation. Polymers Flopam™ and FO3801 achieved the highest flocculation efficiency of over 99% at the optimal dose of 21 mg per g of dry biomass through charge neutralisation and bridging mechanism. The addition of sodium hydroxide, potassium hydroxide, and sodium carbonate also achieved flocculation efficiency of 98 and 91%, respectively, but high doses were required (i.e. > 500 mg per g of dry biomass). Calcium hydroxide was not as effective and could only achieve 75% flocculation efficiency. Precipitation of magnesium hydroxide was identified as the major cause of hydroxide-induced flocculation. On the other hand, sodium carbonate addition induced flocculation via both magnesium and calcium carbonate co-precipitation. The large mass of precipitates caused a sweeping effect and enmeshed the microalgal cells to trigger sedimentation. Cell membrane integrity analysis of flocculated *P. purpureum* indicated that polyacrylamide polymers led to significantly compromised cells (i.e. 96%), compared to the alkaline bases (70-96% compromised cells). These results appear to be the first to demonstrate the high efficiency

of polyacrylamide polymer and alkaline flocculation of *P. purpureum* but at the expense of the biomass quality.

Keywords: *Porphyridium purpureum*; Flopam; Alkaline flocculation; Cell membrane integrity; Algae harvesting.

4.1 Introduction

Microalgae have emerged as a promising platform to produce renewable feedstock for biorefinery applications (Kumar et al., 2020a), remove nutrients from wastewater (Hom-Diaz et al., 2017; Nguyen et al., 2020; Tolboom et al., 2019), and sequester CO_2 from flue gas (Cheng et al., 2019; Yadav et al., 2019). Replacing fossil fuels with microalgal biomass for biorefinery applications will reduce greenhouse gas emissions and global warming. *P. purpureum* is a red marine microalga notable for its high content of valuable biochemicals such as red pigments (e.g. phycoerythrin), phycobiliproteins, polyunsaturated fatty acids, and exopolysaccharides (Di Lena et al., 2020; Gaignard et al., 2019; Kavitha et al., 2016). This species is particularly high in phycoerythrin, a water-soluble bioactive compound with anti-inflammatory, immunosuppressive, and antioxidant properties (Sosa-Hernández et al., 2019). The cultivation of *P. purpureum* is well understood and can be easily performed in seawater medium, thus eliminating the need for arable lands. However, microalgal biomass harvesting at large-scale remains a challenge to the overall economic viability of *P. purpureum* cultivation. *P. purpureum* cells are about 12 μm in diameter and have almost the same density as water. At the stationary phase, a *P. purpureum* culture has a biomass content of 0.5 to 2 g/L, therefore intense dewatering is needed to harvest the biomass (Aizdaicher et al., 2014; Oh et al., 2009; Singh & Patidar, 2018).

Microalgal harvesting is an important step in the supply chain of microalgal biotechnology. It accounts for up to 30% of the total processing cost (Singh & Patidar, 2018). Common harvesting methods include centrifugation, membrane filtration, flocculation, and flotation (Kumar et al., 2020b; Singh & Patidar, 2018). Centrifugation can recover high (>90%) microalgal biomass concentration, but significant energy consumption is a drawback (Singh & Patidar, 2018). Membrane filtration is an emerging technology that still needs to overcome the issue of membrane fouling and high maintenance costs (Singh & Patidar, 2018). Among these methods, flocculation has proven to be an energy efficient, environmentally-friendly, and effective approach to harvest a wide range of microalgae (Fasaei et al., 2018; Nguyen et al., 2019). Nonetheless, the selection of harvesting methods is dependent on i) the type of microalga and its characteristics (e.g. size and growth medium), and ii) the desired compounds to be extracted from the microalgal biomass.

Monitoring cell membrane integrity during harvesting is important as valuable intracellular compounds (e.g. pigments and fatty acids) can be lost if the cell membrane is damaged during the harvesting process. Due to the absence of a rigid cell wall, *P. purpureum* is likely to be susceptible to cell membrane damage (Heaney-Kieras & Chapman, 1976; Kendir Çakmak & Ugurlu, 2020). This particular species is encapsulated within a layer of gelatinous polysaccharide matrix called extracellular polymeric substances (EPS) (Geresh et al., 2002; Heaney-Kieras & Chapman, 1976). This EPS layer contains proteins, sulphate, xylose, galactose, glucose, and glucuronic acids (Kendir Çakmak & Ugurlu, 2020). During growth in aerated cultures and the harvesting process, it is expected that the EPS will partially dissolve into the medium (Heaney-Kieras & Chapman, 1976). Harvesting methods may introduce hydraulic forces (e.g. differential

pressure on two sides of membrane filtration or radial centrifugal forces exerted on biomass during centrifugation) and chemical bonding or bridging (i.e. flocculation) to the cells that can potentially damage the cell membrane. Compromised (i.e. damaged) cell membrane could lead to intracellular leakage. The effects of harvesting methods on *P. purpureum* cell membrane are still largely unknown.

P. purpureum biomass is cultivated in a seawater culture medium that contains a large amount of alkaline earth metal ions such as Mg^{2+} and Ca^{2+}. At high pH >9 and under atmospheric conditions, these ions can precipitate as magnesium hydroxide and calcium carbonate (Besson & Guiraud, 2013; Mayers et al., 2020; Vandamme et al., 2015). The large mass of precipitates, while settling down due to gravitational force, entangles the microalgal cells by a sweeping effect. The sedimentation of the microalgal cells is thus facilitated. Alkaline flocculation has been reported for seawater and freshwater microalgal cultures (e.g. *P. tricornutum*, *C. vulgaris, Scenedesmus* sp., and *N. oculata*) (Vandamme et al., 2015; Wu et al., 2012) and a *D. salina* hypersaline culture (Besson & Guiraud, 2013). However, it has not been studied in a *P. purpureum* culture.

Recent studies have also demonstrated the effectiveness of cationic polyacrylamide polymers as flocculants for microalgae harvesting from freshwater and seawater cultures (Nguyen et al., 2019; Vu et al., 2020a). These previous studies have not examined the effectiveness of polyacrylamide polymer for *P. purpureum* harvesting. Due to the specific composition and structure of the cell membrane of *P. purpureum*, it is necessary to elucidate the effect of flocculation on cell integrity to assess the practicality of this harvesting method for *P. purpureum*.

This study aims to investigate the harvesting performance of *P. purpureum* in seawater medium using: (a) polyacrylamide polymers Flopam^{TM} and FO3801, and (b) alkaline flocculation at high pH through the addition of common bases (i.e. sodium hydroxide, potassium hydroxide, calcium hydroxide and sodium carbonate). Flocculation experiments are further conducted in saltwater medium lacking Mg^{2+} and Ca^{2+} to determine the influence of these cations on the harvesting efficiency of *P. purpureum*. Cell membrane integrity analysis was performed to examine the impact of polyacrylamide polymers and the alkaline bases on the quality of the microalgal cells after flocculation. The new understanding of the floc harvesting of *P. purpureum* in this study will contribute to the process optimisation of biorefinery for a wider range of microalgal species.

4.2 Materials and Method

4.2.1 Microalgae strains and growth conditions

The marine red microalgae *P. purpureum* was obtained from the Australian National Algae Collection at CSIRO Microalgae Research (Hobart, Tasmania, Australia). It was maintained in marine f/2 media (Guillard, 1975) using 0.22 μm filtered autoclaved seawater collected from Sydney Harbour (salinity of 33-35 g/L). The chemical composition of the seawater medium was analysed using Microwave Plasma-Atomic Emission Spectrometry (Agilent) (Section 4.2.4). Stock cultures were maintained at the Climate Change Cluster (C3, University of Technology Sydney).

The *P. purpureum* culture for flocculation experiments was scaled-up from a 1 L Schott's bottle to a 350 L bag following the procedure described in previous studies (Labeeuw et al., 2021; Nguyen et al., 2019). The bag bioreactor was bubbled with air through air lines

on either side of the bioreactor and maintained at 23 °C and 400 µmol photons/m^2/s light in a 16:8-hour light:dark cycle. The seawater medium for the large-scale bioreactor was first sterilized by the addition of 100 mL of 12% sodium hypochlorite, followed by 100 mL of 2 M sodium thiosulphate. Filter sterilized stock components of f/2 media for marine water were then added. The pH of the microalgal culture was checked twice a day and maintained below 9.3 by CO_2 sparging. This cultivation protocol was developed at the Climate Change Cluster facility (University of Technology Sydney, Australia). Microalgal suspension at the mid-stationary growth phase (e.g. approximately 20 days of cultivation) was used for flocculation experiments as it was previously determined to be the best growth phase for harvesting other species (Labeeuw et al., 2021).

4.2.2 P. purpureum flocculation

4.2.2.1 Experimental setup

The flocculation experiments were conducted using a 4G Platypus Jar Tester (Australia Scientific, Kotara NSW Australia). Samples of 500 mL *P. purpureum* suspension were added to 2 L beakers. The jar test was carried out following the procedure from Vu et al. (2020a). The microalgal suspension was rapidly mixed at 200 rpm for one minute followed by slow mixing at 50 rpm for 15 min. The flocculated microalgal biomass was allowed to settle for one hour. To measure the flocculation efficiency, 15 mL of the supernatant was pipetted from the suspension at between one- and two-thirds from the bottom. The optimal flocculant dose was determined by a dose-response relationship protocol (Section 4.2.4). All experiments were conducted in three technical replicates using one biological replicate of the microalga.

4.2.2.2 Flocculants and chemicals preparation

Two cationic polyacrylamide flocculants (FO3801 and Flopam^{TM}) with high-charge (>80% charge), high-molecular weight (>15 Megadalton) (SNF Pty Ltd; Corio, VIC, Australia) were used in the first set of flocculation experiments. A stock solution of each polymer (2 g/L) was prepared in accordance with Vu et al. (2020a) and used within one day of preparation. FO3801 and Flopam^{TM} were dosed at a concentration of 5 to 20 mg/L microalgal suspension (i.e. 7 to 36 mg polymer/g dry biomass), followed by the jar test. The flocculation efficiency was determined using optical density measurement as described in section 4.2.4.

Solutions of 0.1 M sodium hydroxide (NaOH), potassium hydroxide (KOH), calcium hydroxide ($Ca(OH)_2$) and sodium carbonate (Na_2CO_3) were prepared for alkaline flocculation experiments. These chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). The pH of the *P. purpureum* suspensions was adjusted to 9.5, 10 and 10.5 using the alkaline solutions, followed by the jar tests. The volume of 0.1 M stock solution required to raise the pH to the desired level was recorded for each alkali. The flocculation efficiency was calculated as described in section 4.2.4.

4.2.3 Effect of cations on P. purpureum flocculation

The mechanisms governing the flocculation of marine *P. purpureum* in seawater culture through pH adjustment using 0.1 M NaOH and Na_2CO_3 were investigated. These bases represent widely available and effective approaches to increase the pH of the solution i.e. NaOH releases hydroxide ions while Na_2CO_3 removes hydrogen atoms from the suspension. Since Mg^{2+} and Ca^{2+} are dominant elements in seawater medium, their relative importance to the alkaline flocculation of *P. purpureum* using two different bases

was evaluated. The cation Na^+, Mg^{2+}, Ca^{2+}, and K^+ concentrations in the seawater medium were 10.55, 1.36, 0.44, and 0.46 g/L, respectively. These were measured using Microwave Plasma-Atomic Emission Spectrometry (Section 4.2.4).

P. purpureum suspensions of 35 mL volume were centrifuged at 4000g for 10 min to separate the biomass from the initial medium. The resultant biomass was rinsed gently with Milli-Q water to remove the residual medium and resuspended in a new medium of 35 mL containing 38 g/L sodium chloride (NaCl) to maintain the equivalent Na^+ level (10.55 g/L) as in the initial medium. Likewise, magnesium sulphate ($MgSO_4$) was added to the new medium (i.e. containing only NaCl) to maintain a Mg^{2+} concentration of 1.36 g per litre of microalgal suspension. This experiment was to investigate the role of magnesium in alkaline flocculation. In another new NaCl medium containing microalgal biomass, calcium chloride ($CaCl_2$) was dosed at 0.44 g Ca^{2+} per litre of microalgal suspension to study the role of calcium in alkaline flocculation. These concentrations of Mg^{2+} and Ca^{2+} correspond to their concentration in the initial microalgal seawater medium. The alkaline flocculation at pH 10.5 using NaOH and Na_2CO_3 was carried out as described in section 4.2.2. The initial microalgal suspension was used as the control. The description of the samples is provided in Table 6.

Table 6. Samples of 35 mL (including 2 technical replicates) for studying the influence of calcium and magnesium in *P. purpureum* alkaline flocculation

Assay	Sample name	Description	Dosage (g/g dry biomass)
1	Control	Initial microalgal suspension without chemical addition	Nil
2	1 (NaOH)	Microalgal suspension subjected to NaOH induced flocculation	0.57 (NaOH)
3	2 (Na_2CO_3)	Microalgal suspension subjected Na_2CO_3 induced flocculation	4.5 (Na_2CO_3)
4	Mg Control	Suspended microalgal biomass in a $MgSO_4$ + NaCl medium	9.6 ($MgSO_4$) 38.3 (NaCl)
5	Mg (NaOH)	Suspended microalgal biomass in $MgSO_4$ + NaCl medium subjected to NaOH flocculation	
6	Mg (Na_2CO_3)	Suspended microalgal biomass in $MgSO_4$ + NaCl medium subjected to Na_2CO_3 flocculation	
7	Ca Control	Suspended microalgal biomass in a $CaCl_2$ + NaCl medium	1.7 ($CaCl_2$) 38.3 (NaOH)
8	Ca (NaOH)	Suspended microalgal biomass in $CaCl_2$ + NaCl medium subjected to NaOH flocculation	
9	Ca (Na_2CO_3)	Suspended microalgal biomass in $CaCl_2$ + NaCl medium subjected to Na_2CO_3 flocculation	

4.2.4 Analytical methods

The optical density of the microalgae medium before and after flocculation was measured by a spectrophotometer (UV 6000 Shimadzu, Japan) at the wavelength of 730 nm.

The flocculation efficiency was calculated based on the change in the optical density of the suspension before and after flocculation occurs, as shown in the following equation.

$$\text{Flocculation efficiency (\%)} = \left(\frac{OD_i - OD_f}{OD_i}\right) \times 100 \qquad \text{(Eq. 3)}$$

Where OD_i and OD_f are the optical density of the culture before and after flocculant addition.

The *P. purpureum* biomass concentration was determined gravimetrically. A 150 mL sample of microalgae suspension was filtered through a 1.1 µm pre-weighed glass fibre filter paper. The weight of the final filter paper after 12 h drying at 60 °C was used to calculate the dry microalgal biomass.

The solution pH was measured using a pH/conductivity meter (Orion 4-Star Plus Thermo Scientific; Waltham, MA, USA).

Statistical analysis of flocculation efficiency and biomass quality measurements was performed in Microsoft Excel using Student's unpaired *t*-Test, with a two-tailed distribution.

The chemical analysis (Mg, Ca, K, Na) was conducted using Microwave Plasma Atomic Emission Spectrometry (MP-AES, Agilent, USA). The sample was diluted 1000 times (i.e. 50 µL stock into 49.95 mL Milli-Q water) before the analysis.

Cell membrane integrity of the flocculated *P. purpureum* biomass under conditions described in Section 4.2.2 was determined in an endpoint assay using Celltox Green kit (Promega; Madison, WI, USA) and CytExpert v2.4 (flow cytometer, Becton, Dickinson and Company). This assay measures the loss of cell membrane integrity using a non-toxic dye that can enter a damaged cell membrane to bind to the DNA. Intact microalgal cells have a lower amount of fluorescence as the dye cannot enter the cells. Damaged and intact cells are then counted by flow cytometry.

4.3 Results and discussion

4.3.1 *P. purpureum* characteristics and floc formation

The biomass concentration of the *P. purpureum* suspension used in this study was determined gravimetrically to be 0.7 g/L. The initial optical density at the wavelength of 730 nm was 0.601. The microalgal suspension had a pH of 8.9.

Differences in the morphology of the flocs from polyacrylamide polymers and alkaline flocculation at optimal doses were visually observed (Figure 10). Polyacrylamide polymers (Flopam^{TM} and FO3801) flocculated the microalgal cells into a large clump. The clump settled to the bottom of the beaker and the clear supernatant was observed (Figure 10b). It is relatively easy to collect the floc formed by polyacrylamide polymer from the solution through a strainer. On the other hand, alkaline chemicals (i.e. NaOH, $Ca(OH)_2$, and Na_2CO_3) induced a foamy and powdery layer of flocculated biomass at the bottom of the beakers (Figure 10b). This layer can be easily disintegrated, making microalgal biomass recovery difficult. The appearance of the supernatant also varied among different types of alkaline flocculation. Dosing *P. purpureum* suspension with NaOH achieved a much clearer supernatant than when using $Ca(OH)_2$ and Na_2CO_3 (Figure 10b). The supernatant from $Ca(OH)_2$ flocculation still contained an amount of *P. purpureum* microalgal cells as suggested by its red colour and low flocculation efficiency (75%) at the optimal pH of 10.5 (Figure 10b). Na_2CO_3 induced flocculation caused the supernatant to become cloudy due to the precipitation of calcium carbonate (Figure 10b).

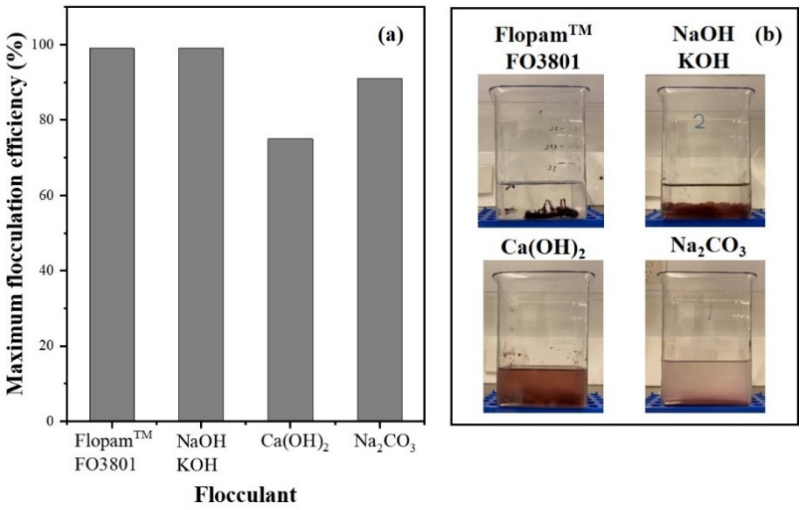

Figure 10. (a) The maximum *P. purpureum* flocculation efficiency achieved by dosing polyacrylamide polymers (21 mg/g dry biomass), NaOH (571 mg/g dry biomass), $Ca(OH)_2$ (875 mg/g dry biomass), and Na_2CO_3 (4,542 mg/g dry biomass) and (b) their corresponding floc formations observed visually.

4.3.2 *P. purpureum* flocculation using polyacrylamide polymers

Both polyacrylamide polymers in this study show high *P. purpureum* flocculation efficiency (Figure 11). Flocculation efficiency of 80 and 97% was observed for low doses of 7 and 14 mg polymer per g dry biomass, respectively. Flocculation efficiency was over 99% for both FO3801 and Flopam™ at the optimal dose of 21 mg polymer per g dry biomass. The further increase in polymer doses did not improve the flocculation efficiency of the *P. purpureum* suspension (Figure 11). Overdosing polymers may cause a counteractive effect (i.e. reduced flocculation efficiency and increased polymer residual in the medium) on the flocculation efficiency (Nguyen et al., 2019; Vu et al., 2020a).

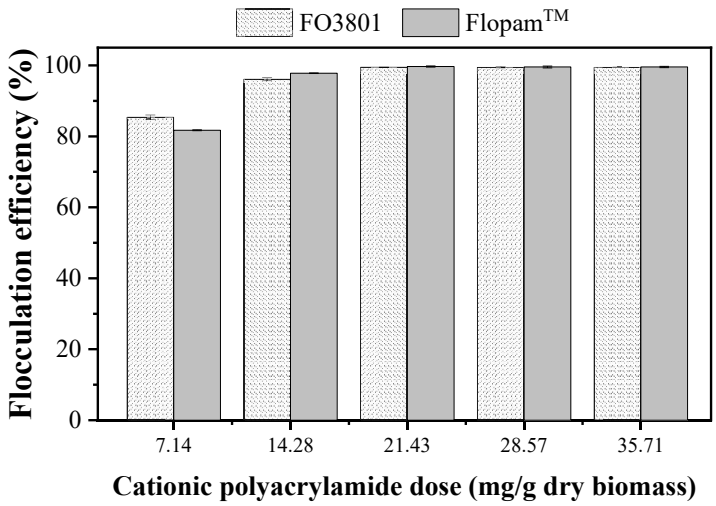

Figure 11. Flocculation efficiency of *P. purpureum* using cationic polyacrylamide polymers Flopam™ and FO3801. Value and error bars represent the mean and standard deviation (*n = 3 technical replicates*).

Polyacrylamide polymers neutralise the negatively charged microalgal cells due to their highly charged and cationic characteristic. When microalgal cells lose their negative surface charge, the electrostatic repulsion force between the cells diminishes and flocculation can occur (Nguyen et al., 2019). These polyacrylamide polymers are also high molecular weight polymers (MW = 15 MDa) to facilitate entanglement and bridging with the microalgal cells to form large and stable flocs. The excellent performance of FO3801 and Flopam™ for other microalgae species has been reported in the literature (Nguyen et al., 2019; Vu et al., 2020a). The optimal doses to achieve 90-99% flocculation efficiency for *C. vulgaris* and *Phaeodactylum tricornutum* were 18.9 and 13.7 mg polymer/g dry biomass, respectively (Nguyen et al., 2019). In this study, similar doses (14-21 mg polymer/g dry biomass) were required to obtain 97-99% flocculation efficiency for *P. purpureum.* This demonstrated that marine *P. purpureum* can be effectively harvested from seawater culture using polyacrylamide polymers at a low dose.

4.3.3 Alkaline flocculation of *P. purpureum*

The alkaline flocculation of marine *P. purpureum* was pH-dependent. The flocculation efficiency was low (i.e. 11%) at pH 9 for all samples using NaOH, KOH, $Ca(OH)_2$ and Na_2CO_3 (Figure 12). This is because the initial pH of the microalgal suspension was pH 8.9. When the pH was increased to 9.5, 10, and 10.5 using NaOH or KOH, a significant improvement in the harvesting performance was observed (Figure 12). The flocculation efficiency was 51.5, 65.3, and 97.5% for pH 9.5, 10, and 10.5, respectively. Thus, the optimal pH to obtain the highest efficiency for NaOH and KOH-induced flocculation was pH >10.5. $Ca(OH)_2$ was not as effective at inducing microalgal agglomeration. The flocculation efficiency was 20 and 50% when the pH was raised to 9.5 and 10, respectively, using $Ca(OH)_2$. The maximum flocculation efficiency obtained was 75% at pH 10.5. In terms of Na_2CO_3, the alkaline flocculation remained low (11%) at pH 9 and 9.5. It started to significantly increase when more Na_2CO_3 was added to reach pH >10. The highest flocculation efficiency of 91% was recorded at pH 10.5.

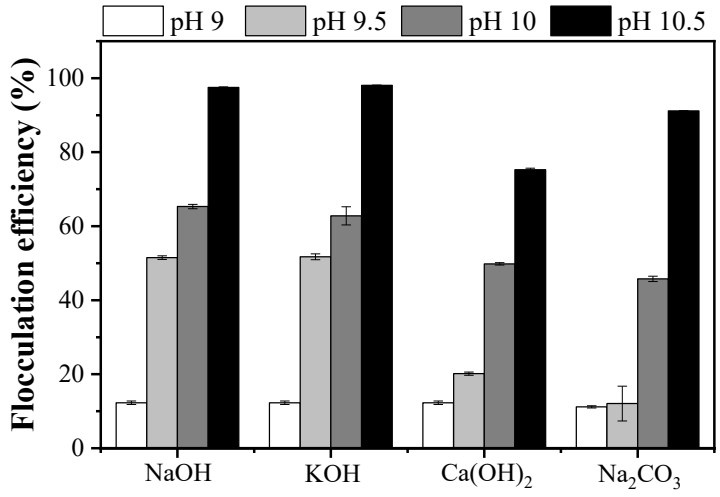

Figure 12. *P. purpureum* alkaline flocculation efficiency using NaOH, KOH, $Ca(OH)_2$, and Na_2CO_3. Value and error bars represent the mean and standard deviation (*n = 3* technical replicates).

The dose of alkaline bases necessary to increase the pH and induce effective microalgal flocculation is an important factor in considering large-scale applications. NaOH and KOH required the lowest doses (i.e., 571 and 800 mg chemical/g dry biomass, respectively) to achieve the highest flocculation efficiency of 98% at pH 10.5 (Table 7). Na_2CO_3 showed effective performance (i.e., 91% flocculation efficiency) at pH 10.5 but required a very high dose of 4,542 mg chemical/g of microalgal suspension.

Table 7. The concentration of bases required to adjust the pH to desired values.

pH	**Alkaline**	**Concentration (mg chemical/g dry microalgal biomass)**
9.5	NaOH	194
	KOH	271
	Na_2CO_3	861
10	NaOH	308
	KOH	431
	Na_2CO_3	2482
10.5	NaOH	571
	KOH	800
	Na_2CO_3	4542

The bases (i.e., NaOH, KOH, and $Ca(OH)_2$) studied in this paper were chosen because of their accessibility and common use as pH-adjusting agents (Vandamme et al., 2012a). However, they are hazardous chemicals that can impact the quality of the harvested microalgae biomass (i.e., loss of lipid and fatty acid contents), thus limiting its industrial applications (Borges et al., 2016). Na_2CO_3 was investigated in this study as a widely available, and less hazardous alternative to sodium hydroxide. Since Na_2CO_3 also provides an inorganic carbon source for microalgal growth (Duan et al., 2020), there is the potential for recycling the supernatant as culture media.

4.3.4 The role of cations in *P. purpureum* flocculation

The results showed 26% of Mg^{2+} and 50% of Ca^{2+} reduction in the medium after NaOH and Na_2CO_3 flocculation occurred at pH 10.5 (Figure 13). The flocculation efficiency was >99% and 78% using NaOH and Na_2CO_3, respectively. This observation indicates that magnesium and calcium salt precipitation at high pH can lead to good alkaline flocculation of *P. purpureum* in a seawater medium. The large mass of metal precipitate rapidly forming and settling induced the sweeping flocculation of the microalgae cells (Besson & Guiraud, 2013; Vandamme et al., 2012a). When the sweeping phenomena occurred, microalgal cells were enmeshed in the precipitate and settled. This explains the layer of powdery flocculated *P. purpureum* observed on the bottom of the beaker after pH adjustment. In the medium containing the same concentration of Na^2, $Mg^{2+,}$ and Ca^{2+} but without microalgal biomass, the precipitation and sedimentation of magnesium and calcium salts were also observed.

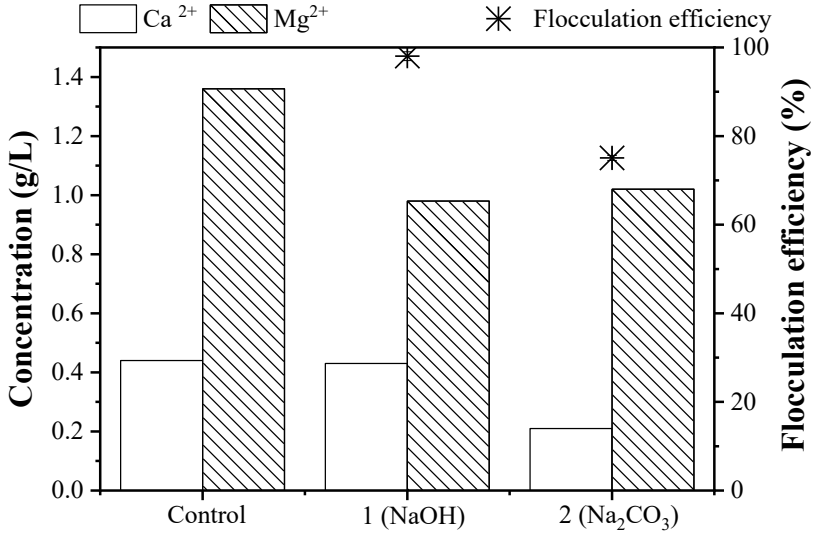

Figure 13. The change in Mg^{2+} and Ca^{2+} concentration in the microalgal solution (supernatant) due to alkaline flocculation. Values represent the mean (*n = 2,* technical replicates).

The role of Mg^{2+} and Ca^{2+} precipitation in microalgal flocculation depends on the alkaline base (Figure 14). In the new medium containing Na^{+} (10.55 g/L) and Mg^{2+} (1.36 g/L), NaOH was able to achieve effective flocculation (i.e., 94%) (Fig. 5a). A 47% reduction in Mg^{2+} concentration in the medium was recorded. On the other hand, flocculation did not occur when NaOH was added to the medium containing only Ca^{2+} (Figure 14b). The reduction in Ca^{2+} concentration in the medium was also minimal (i.e., 5%). This indicates that the main cause for the flocculation of *P. purpureum* by NaOH is the precipitation of magnesium hydroxide ($Mg(OH)_2$). Meanwhile, Na_2CO_3 caused magnesium carbonate ($MgCO_3$) precipitation in a medium containing only Mg^{2+}, thus a 39% of Mg^{2+} reduction (Figure 14a). However, $MgCO_3$ is a white solid that can affect the light absorbance measurement. This caused the flocculation efficiency induced by Na_2CO_3 to be significantly lower (i.e., 60%) than that of NaOH (i.e. 94%). In the medium containing Na^{+} (10.55 g/L) and Ca^{2+} (0.44 g/L), a flocculation efficiency of 75% was observed for Na_2CO_3. This is due to the 51% reduction in Ca^{2+} concentration, which had precipitated out of the medium in the form of calcium carbonate ($CaCO_3$) (Figure 14b). Carbonate precipitates caused the supernatant to be cloudy. Thus, both $MgCO_3$ and $CaCO_3$ are involved in Na_2CO_3-induced flocculation of marine *P. purpureum.*

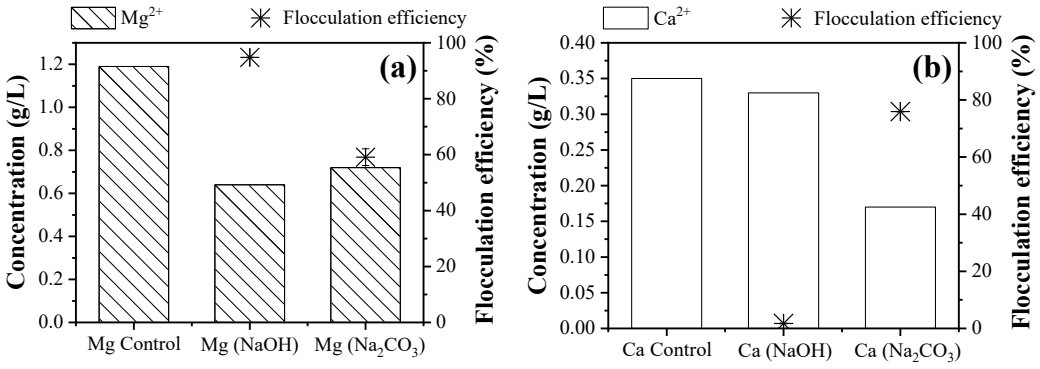

Figure 14. Alkaline flocculation efficiency of *P. purpureum* for (a) medium containing NaCl and MgSO-4 only, and (b) medium containing NaCl and $Ca(OH)_2$ only. Values represent the mean (*n = 2,* technical replicates).

4.3.5 Biomass quality after flocculation

Polyacrylamide polymers and alkaline agents could potentially damage the cell wall of *P. purpureum*. The proportion of compromised cells was 96% and 68-96% for polyacrylamide polymer and alkaline bases, respectively (Figure 15). This suggests that polyacrylamide polymers induced the highest degree of damage to the *P. purpureum* cell membrane despite being the most efficient flocculants (> 99% flocculation efficiency). NaOH and Na_2CO_3 caused high cell membrane damage at 68-70% but had the least impact on *P. purpureum* among the chemicals tested. A comparable observation was reported in a *Euglena gracilis* harvesting study where the NaOH-induced flocculation at pH >10 caused the microalgal cells to be completely ruptured (Wu et al., 2020). On the other hand, Sales et al. (2019) revealed that *Nannochloropsis oculata* cells, a green marine microalga, were intact after being subjected to a three-step harvesting process: (1) pH-induced flocculation using sodium hydroxide, (2) Flopam FO4800 (1 mg/L), and (3) 6000*g* centrifugation. Harvested *N. oculata* showed 99-100% cell viability (i.e. 0-1% damaged cells compared to the fresh microalgal culture). Sales et al. (2019) results suggest that polymer and alkaline flocculation have a negligible effect on the microalgal

cell membrane, which disagrees with our results. This is attributed to the different morphologies of *P. purpureum* and *N. oculata* (e.g. *N. oculata* has a thick cell wall resistant to shock while *P. purpureum* has no rigid cell wall) and the chemical doses used. The effect of polyacrylamide polymers and alkaline bases on cell membrane integrity is, therefore, dependent on the microalgal species, its cell wall characteristics, and operational parameters.

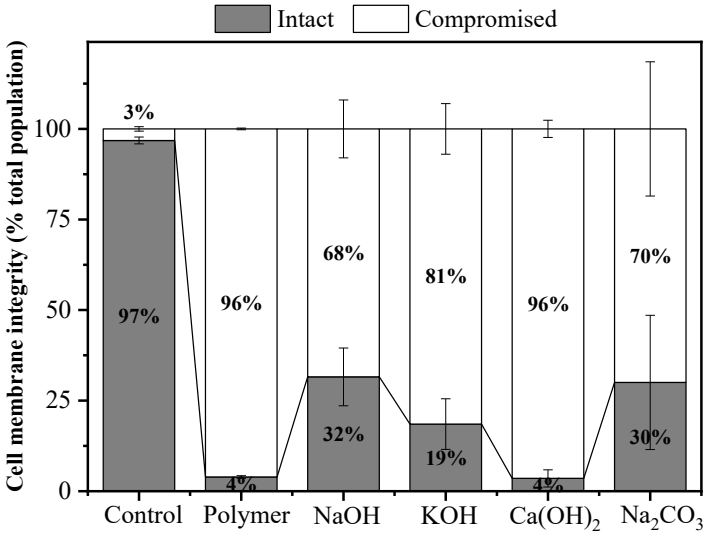

Figure 15. *P. purpureum* cell membrane integrity after flocculation (intact vs compromised). Value and error bars represent the mean and standard deviation ($n = 3$, technical replicates).

Polyacrylamide polymers (e.g. Flopam^{TM} and FO3801) have high molecular weight and positive surface charge, thus strongly attracting the negatively charged EPS cell wall of *P. purpureum*. When the EPS is attached to the tails and loops of the polyacrylamide polymer, the structural arrangement of EPS on the microalgal cell wall is likely to be modified. This could lead to severe damage to the cell walls (e.g. the EPS gel no longer covers and protects the cells, and the intracellular matter can leak through the cell wall). Meanwhile, after alkaline addition, extremely high pH (10-11) causes the EPS proteins to lose their natural shapes and the gelatinous EPS layer to be solubilised. These

disruptions are facilitated through chemical degradation and ionisation of the hydroxyl group (Wingender et al., 1999). The subsequent swelling and solubilisation of the EPS cell wall could cause the microalgal cell to lose its viability as it can no longer maintain an appropriate turgor pressure (i.e. the hydrostatic pressure within the cell against the cell wall) and disrupts the cell membrane (Neyens et al., 2004). The cell cytoplasm and nucleus are exposed to the environment, thus releasing intracellular components (e.g. organic matter and pigments) into the medium (Du et al., 2020).

Maintaining the integrity of microalgal cells during harvesting is crucial to preserving valuable intracellular compounds such as proteins, lipids, and carbohydrates for downstream applications (Singh & Patidar, 2018). Cell damage or lysis during the harvesting process can lead to the release and subsequent degradation of these compounds, significantly reducing the biomass's economic value and functional quality (Singh & Patidar, 2018; Yang et al., 2021). Disrupted cells may complicate the downstream extraction process and lower the efficiency of bioconversion pathways.

In this study, polyacrylamide polymers induced *P. purpureum* floc formation that is dense and easy to remove (section 4.3.1). Their impact on the cell membrane was, however, the most severe with only 4% of the population remaining entirely intact after flocculation. Alkaline flocculation not only caused significant microalgal cell damage, but its flocs also contained a high concentration of salt precipitates and were difficult to collect from the medium (i.e. the settled biomass was highly disintegrated). The composition of the biomass before and after flocculation, together with the extent of intracellular compound leakage will be a topic of interest for future study to further investigate the impact of these flocculants on biomass quality and potential end-products.

4.4 Conclusions

Polyacrylamide polymers effectively neutralise charge and bridge microalgal cells to flocculate over 99% of red marine *P. purpureum* at a low dose (i.e. 21 mg/g dry biomass). The floc formation was dense and easy to remove from the supernatant. However, cell membrane integrity results showed that polymers compromised the membrane of 96% of microalgal cells (i.e. the most negative impact on *P. purpureum* cells among the chemicals). Alkaline flocculation achieved up to 98% flocculation efficiency, but the alkaline doses were high at around 500 to 4,500 mg/g dry biomass. These high doses of alkali are not cost-effective and practical. In addition, the microalgal flocs induced by alkaline flocculation were powdery and disintegrated, making it harder for subsequent microalgal biomass recovery compared to polyacrylamide polymer flocs. Around 70% of microalgal cells were compromised after NaOH or Na_2CO_3 addition, lower than that caused by polymers (96%), KOH (81%), and $Ca(OH)_2$ (96%).

CHAPTER 5. FACTORS GOVERNING MICROALGAE HARVESTING EFFICIENCY BY FLOCCULATION USING CATIONIC POLYMERS

This chapter has been published as the following journal article:

Vu, H.P., Nguyen, L.N., Emmerton, B., Wang, Q., Ralph, P.J., Nghiem, L.D. 2021. Factors governing microalgae harvesting efficiency by flocculation using cationic polymers. *Bioresource Technology*, 340, 125669.

Summary: This study aims to elucidate the mechanisms governing the harvesting efficiency of *C. vulgaris* by flocculation using a cationic polymer. Flocculation efficiency increased as microalgae culture matured i.e. 35–45, 75, and >97% efficiency at early, late exponential, and stationary phases, respectively. Unlike the negative impact of phosphate on flocculation in traditional wastewater treatment; here, phosphorous residue did not influence the flocculation efficiency of *C. vulgaris*. The observed dependency of flocculation efficiency on the growth phase was driven by changes in microalgal cell properties. Microalgal extracellular polymeric substances (EPS) in both bound and free forms at the stationary phase were two and three times higher than those at the late exponential and early phases, respectively. Microalgae cells also became more negatively charged as they matured. Negatively charged and high EPS content together with the addition of high molecular weight and positively charged polymer could facilitate effective flocculation via charge neutralisation and bridging.

Keywords: microalgal extracellular polymeric substances; *C. vulgaris*; Growth phase; Phosphorous; Zeta potential.

5.1 Introduction

Microalgae are an emerging feedstock for third-generation biofuel, which can address the imminent depletion of fossil fuel and the increasing threat of global warming (Nagarajan et al., 2020; Rajesh Banu et al., 2020). The first-generation (i.e. food crops) and second-generation (i.e. lignocellulosic biomass) biofuel are more environmentally friendly than fossil fuel, but they also have inherent drawbacks especially as they compete with food security and have low conversion efficiency (Nagarajan et al., 2020; Rajesh Banu et al., 2020). As phytoplankton, microalgae are fast-growing photosynthesizing microscopic organisms that can be cultivated without any requirement for arable land and with minimal input of resources. Large-scale microalgae production has been demonstrated in the desert or even on the ocean surface. Microalgae are rich in carbohydrates, proteins, and lipids. These compounds are valuable substrates for the production of renewable fuels such as biodiesel, biomethane, and green hydrogen (Rajesh Banu et al., 2020).

The harvesting process remains a major challenge in the microalgae supply chain. The current high cost of harvesting reduces the competitiveness of large-scale biofuel production from microalgae (Khoo et al., 2020; Yin et al., 2020). Microalgae harvesting is the process of recovering a concentrated microalgal slurry (10 – 25% dry biomass) from the diluted microalgal suspension (0.02 – 0.05% dry biomass) and reusing the cultivation solution for subsequent microalgae production. In the current microalgae industry, the harvesting process accounts for 20 to 30% of the total microalgal biomass production cost (Singh & Patidar, 2018). Current microalgae harvesting methods include centrifugation, filtration, flocculation, flotation, electrocoagulation, bioflocculation, and magnetic separation (Ananthi et al., 2021; Yin et al., 2020). Comprehensive reviews of the pros and cons of these methods have highlighted flocculation as the most promising

technology for low-cost harvesting of microalgae biomass for biofuel production (Ummalyma et al., 2017; Yin et al., 2020).

Microalgae flocculation using synthetic cationic polymer is a promising technique to overcome the current constraints of microalgal harvesting. It has been shown to effectively flocculate over 90% of freshwater and seawater microalgae at low doses with a simple and fast operation (Gerchman et al., 2017; Nguyen et al., 2019; Udom et al., 2013; Vu et al., 2020a; Vu et al., 2021b). Charge neutralisation and bridging effects have been shown to be the mechanisms behind cationic polymer flocculation, although there are still questions as to how these mechanisms and the flocculation efficiency may be influenced under different microalgal culture conditions. Labeeuw et al. (2021) reported that the growth phases (i.e. early exponential, late exponential, and stationary) of microalgae influenced the flocculation efficiency using a highly charged cationic polymer. Three microalgal species (cyanobacteria *Synechocystis* sp., freshwater *C. vulgaris*, and marine diatom *Phaeodactylum tricornutum*) showed different responses to polymer flocculation at three growth phases. Flocculation by cationic polymer addition was 98% effective at flocculating *Synechocystis sp.* regardless of the growth phase, whereas it was 50% less effective for *C. vulgaris* and *P. tricornutum* at the early stationary phase (Labeeuw et al., 2021). This variation may be attributed to differences in biomass concentration and microalgal biochemical composition at each growth phase. Thus, it is necessary to delineate the factors that may affect the polymer flocculation of microalgae at different growth phases. This will help to gain further knowledge of microalgal flocculation and identify the strategies to optimise microalgal harvesting using cationic polymer.

Phosphorous is an essential nutrient for microalgal growth, especially for the synthesis of biomolecules such as phospholipids, adenosine triphosphate, and deoxyribonucleic acids in the microalgal cells. Phosphorus is present in the microalgal medium as orthophosphates and decreases in concentration gradually with culture age. Residual phosphorous has been reported to impact coagulation and flocculation in wastewater treatment (Liu & Liss, 2007; Morgan, 1958; Park et al., 2016). The presence of phosphorous as phosphates (PO_4^{3-}) in wastewater hindered the flocculation and sedimentation processes (Morgan, 1958). A higher flocculant dose and a longer settling time were required to overcome the interference caused by PO_4^{3-} compounds (Morgan, 1958; Park et al., 2016). Conversely, the gravitational settling velocity of sludge flocs was enhanced for wastewater with a reduced phosphorous concentration (Liu & Liss, 2007). This improvement was attributed to larger and more compact flocs formed under phosphorous-limited conditions. In other words, a lower phosphorous concentration can lead to a higher flocculation efficiency in a wastewater matrix. However, it is still unknown if these findings are also applicable to a microalgal culture with a very different matrix compared to wastewater.

As microalgae grow, they also secrete metabolites (e.g. carbohydrates and proteins) that surround the cells, known as microalgal extracellular organic matter, or extracellular polymeric substances (EPS). EPS can influence the surface properties of microalgal cells as well as promote or inhibit floc formation (Henderson et al., 2010; Roselet et al., 2017; Sano et al., 2011; Vandamme et al., 2012b; Zhang et al., 2012). Microalgal EPS can act as a polymer aid at low concentrations, as it frequently contains biopolymers that can bridge the cells and/or with hydroxide precipitates to form large flocs (Gonzalez-Torres et al., 2017). On the other hand, EPS can decrease the efficiency of the coagulation-

floatation process as EPS can form complexes with the coagulant thereby increasing the required coagulant dose to floc microalgal cells (Bernhardt et al., 1985; Roselet et al., 2017; Vandamme et al., 2012b). Given these effects of EPS on microalgal harvesting, the concentration and composition of microalgal EPS would likely influence the flocculation efficiency using cationic polymer at different growth phases.

This study aims to elucidate the underlying factors affecting the flocculation efficiency of *C. vulgaris* at different growth phases (i.e. early exponential, late exponential and stationary phase). Factors including residual phosphorus concentration, surface charge of microalgal cells, and cell EPS content are examined at each growth phase. The results presented here are useful for further optimisation of microalgae harvesting by flocculation using organic polymers.

5.2 Materials and Method

5.2.1 *C. vulgaris* cultivation

The freshwater microalgae *C. vulgaris* (CS-41) was obtained from the Australian National Algae Culture Collection at CSIRO Microalgae Research (Hobart, TAS, Australia). The stock culture was maintained in 0.22 μm filtered autoclaved freshwater MLA medium (Algaboost; Wallaroo, SA, Australia). The main nutrient composition of this MLA medium includes approximately 49, 170, 35, and 2 mg/L of $MgSO_4.7H_2O$, $NaNO_3$, K_2HPO_4, and H_3BO_3, respectively (Bolch & Blackburn, 1996).

C. vulgaris culture was prepared in three steps from 1 L bottle to 350 L photobioreactor. The stock culture was first cultivated in a 1 L bottle, then transferred to a 10 L bottle for further cultivation until the early stationary phase. Finally, the 10 L culture was used to

inoculate two identical 350 L photobioreactors (i.e. two biological replicates). The 350 L photobioreactors were maintained at 25 °C, 100-400 μmol photons/m^2/s light in a 16:8 light:dark cycle, and air supply through air lines. These photobioreactors were also sparged with 100% CO_2 for 1 min/day to provide carbon and maintain the pH below 9.3. Microalgal growth was monitored daily by optical density measurement. Microalgae suspensions from these two photobioreactors were extracted at the same time of the day for flocculation and determination of EPS.

5.2.2 Microalgae flocculation

5.2.2.1 Materials

A cationic polyacrylamide polymer (FO3801) was purchased from SNF (SNF Pty Ltd; Corio, VIC, Australia). The polymer is highly charged (75 mV by zeta potential) with a high molecular weight (over 15 MDa) and a charge density of 80%. A stock polymer solution (2 g/L) was prepared in Milli-Q water under mixing for 60 minutes using a magnetic stirrer. The stock solution was used for the flocculation experiment within one day to avoid any polymer hydrolysis during long-term storage.

C. vulgaris suspension (10 L) at early exponential, late exponential and stationary phases were collected from the two 350 L photobioreactors (section 5.2.1) for the flocculation experiment.

To investigate the impact of residual phosphorous in the microalgal culture on flocculation efficiency, dipotassium phosphate (K_2HPO_4) was added to *C. vulgaris* suspensions at stationary phase to achieve the PO_4^{3-} concentration of 10, 20, 30, and 40 mg/L. *C. vulgaris* suspension at the stationary phase without K_2HPO_4 addition was used

as the control. The PO_4^{3-} concentration of this control suspension was 3.7 mg/L. After K_2HPO_4 was completely dissolved in the suspensions, algae flocculation was performed with FO 3801 at 35 mg polymer/g dry biomass.

5.2.2.2 Experimental protocols

The flocculation test took place in 250 mL glass beakers containing 100 mL of microalgal culture. The polymer solution was dosed at 35 mg/g dry microalgal biomass. This dose was the optimal dose for *C. vulgaris* flocculation as reported in a previous study (Labeeuw et al., 2021). After polymer dosing, the microalgae suspensions were rapidly mixed for 1 minute at 200 rpm, followed by slow mixing for 5 minutes at 50 rpm. The suspensions were allowed to settle for 10 minutes. Then 10 mL aliquot was pipetted at a height between one- and two-thirds from the bottom of the beaker. The optical density of this aliquot sample was measured to determine the flocculation efficiency.

5.2.3 Analytical methods

5.2.3.1 Microalgae growth analysis

Two samples of 100 mL (i.e. two technical replicates) are taken from each of the two 350 L photobioreactors every second day for measurements of dry weight, optical density, pH, residual phosphorous concentration, and zeta potential.

The dry weight of *C. vulgaris* culture (i.e. dry biomass concentration) was determined gravimetrically by filtering 100 mL solution through a 1.1 μm pre-weighed glass fibre filter paper. After 12 h of oven drying at 60 °C, the weight of the filter paper with retained biomass was used to calculate the dry microalgal biomass concentration.

The optical density of the microalgal culture was measured by a spectrophotometer (Shimadzu UV 6000) at a wavelength of 680 nm. The residual phosphorous concentration in the microalgal culture was determined using Phosphorous TNTplus Vial Test high range (1.5-15.0 mg/L PO_4^{3-}) and a spectrometer (DR3900, Hach Pacific, Australia). Samples of the microalgal culture were filtered through 0.45 μm Nylon syringe filters to remove microalgal cells before applying the vial test to the supernatant. The zeta potential of the microalgal culture was measured using the zeta instrument (Zetasizer Nano ZS Zen 3600, Malvern, UK).

5.2.3.2 Flocculation efficiency

The optical density and zeta potential of the microalgal culture before and after polymer flocculation were measured as outlined in Section 5.2.3.1. The flocculation efficiency was determined using:

$$Flocculation\ efficiency\ (\%) = \left(\frac{OD_i - OD_f}{OD_i}\right) \times 100 \qquad \text{(Eq. 3)}$$

where OD_i and OD_f imply the optical density of the microalgal culture before and after flocculation.

5.2.3.3 EPS extraction and determination

EPS consists of soluble EPS and bound EPS. Microalgal suspension of 35 mL was centrifuged at 3,500 *g* and 4 °C for 30 min. The supernatant was then filtered through a 0.45 μm Nylon syringe filter to obtain soluble EPS. The microalgal pellet was re-suspended to a volume of 35 mL in a PO_4^{3-} buffer solution (10 mM NaCl, 1.2 mM KH_2PO_4, and 6 mM Na_2HPO_4). The re-suspended microalgal suspension was subjected to low-strength sonication for 40 s. The sample was centrifuged again at 9,000 *g* and 4

°C for 15 min. Filtered supernatant contained bound EPS. Carbohydrate and protein concentrations of the soluble and bound EPS were determined using the phenol-sulfuric acid method (Nielsen, 2010) and Lowry method (Lowry et al., 1951), respectively.

5.2.3.4 Statistical analysis

Statistical analysis of flocculation efficiency and biomass quality measurements was performed using Student's *t*-test (OriginPro 2019). Appropriate assumptions (i.e. data sets are normally distributed and have equal variances) were checked before statistical analysis.

5.3 Results and discussion

5.3.1 Biomass production and nutrient profile in pilot-scale photobioreactors

Batch autotrophic cultivation of *C. vulgaris* in the 350 L pilot-scale photobioreactor showed a typical S-shape growth curve with three distinctive phases (Figure 16) similar to that reported in the literature (Do et al., 2020; dos Santos et al., 2016; Klin et al., 2020). The duration of each microalgal growth phase in this study is similar to the growth of *C. vulgaris* in a previous study under the same condition (Labeeuw et al., 2021). In early exponential growth phase (day zero to six), cells were adapting to the new 350L environment and grew slowly. Once fully adapted, microalgal cells started to rapidly multiply. The culture entered the exponential phase on day seven. At the end of the exponential phase (day 18), cell growth reached its limit as defined by the availability of nutrients, light, and carbon sources. The culture entered the stationary phase when the production of new cells was gradually offset by cell death. Lag phase was not observed in this cultivation as the microalgal cells had already been acclimatised to the culture

medium by the three-step cultivation process as described in 5.2.1. Samples were taken on day seven (i.e. early exponential), day 18 (i.e. late exponential), and day 28 (i.e. stationary) for subsequent flocculation experiments.

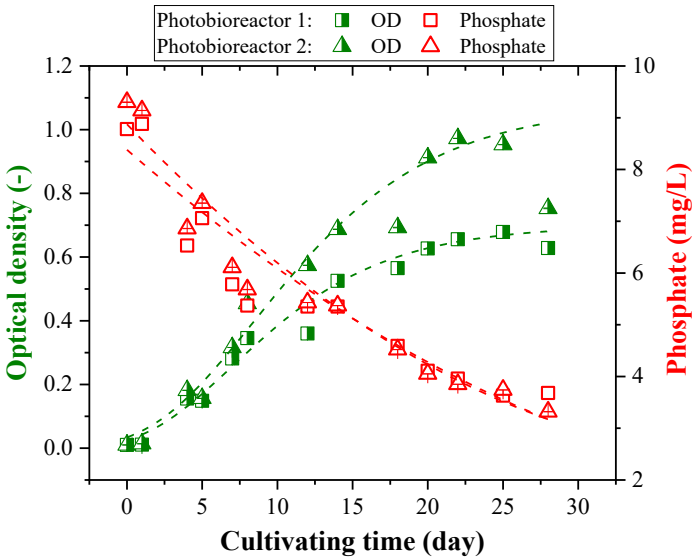

Figure 16. Change in optical density and phosphate concentration of *C. vulgaris* culture during 28-day cultivation. Values and error bars represent the mean and standard deviation from two technical replicate measurements (n = 2), respectively.

As microalgal biomass was produced (i.e. increase in optical density and dry weight), phosphorous content in the culture decreased over time (Figure 17). Microalgal cells uptake nutrients for the growth and synthesis of intracellular proteins, lipids, and carbohydrates (Anto et al., 2019; Chu et al., 2013). Over 28 days of cultivation, the phosphorous concentration decreased from 9.0 mg/L PO_4^{3-} (day zero) to 5.8, 4.6, and 3.7 mg/L PO_4^{3-} for the early exponential, late exponential and stationary phase, respectively. This represents a final 60% reduction in phosphorous availability during *C. vulgaris* growth (Figure 16). The phosphorous reduction is low in this study compared to previous studies whose aim is to remove phosphorous from wastewater (Vu et al., 2020b). However, our data (optical density, dry microalgal biomass concentration, PO_4^{3-}

depletion, and zeta potential) are consistent between the two biological replicates (i.e. two photobioreactors) (t-test, $p > 0.05$), indicating the experimental reproducibility. The low phosphorous uptake was probably due to both light and carbon source limitations in our photobioreactor cultivation system. Nevertheless, the change in residual phosphorous will facilitate the investigation regarding its impact on microalgal harvesting.

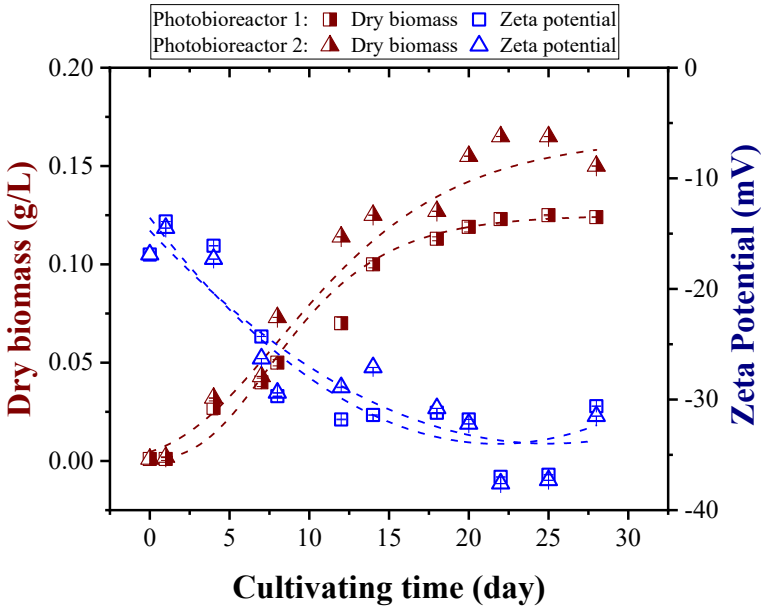

Figure 17. Change in dry biomass concentration and zeta potential of *C. vulgaris* culture during 28-day cultivation. The culture pH was fluctuating within the range of pH 8 to 9. Value and error bars represent the mean and standard deviation from two technical replicate measurements ($n = 2$), respectively.

The surface charge of microalgal cells became more negative as the culture solution matured over time (Figure 17). Microalgae cells are negatively charged so that they repel one another by electrostatic interaction to stay dispersed in suspension. This maximises access to sunlight for photosynthesis by individual microalgal cells. The net negative charge of the cell surface is derived from the carboxylic groups on the cell membrane (Vandamme et al., 2013). In this study, the microalgal culture pH was slightly basic at pH 8-9, thus, these carboxylic groups dissociated to attain a negative charge for each

microalgal cell. The increase in surface charge was significant within the early exponential growth phase (day zero to six) (Figure 17). Changes in surface charge from the early exponential growth phase to the stationary phase were discernible but not statistically significant. Increasing surface charge leads to stronger electrostatic repulsion to prevent the agglomeration of microalgal cells (Zheng et al., 2019). Thus, it is useful to examine if changes in cell surface charge would affect flocculation efficiency.

5.3.2 Flocculation efficiency at different growth phases

The flocculation efficiency of FO3801 was dependent upon the growth phase of *C. vulgaris* (Figure 18). This observation agrees with previous studies (Labeeuw et al., 2021; Zhang et al., 2018). There is some variation in flocculation efficiency as well as the zeta potential of the initial and post-flocculation microalgae at the three growth phases between the two biological replicates (i.e. two independent photobioreactors) as can be seen in Figure 18. These differences could have been due to the random biological variation of the two photobioreactors, despite the efforts to operate them in the same conditions. However, the overall pattern is the same and the difference in absolute value is also small.

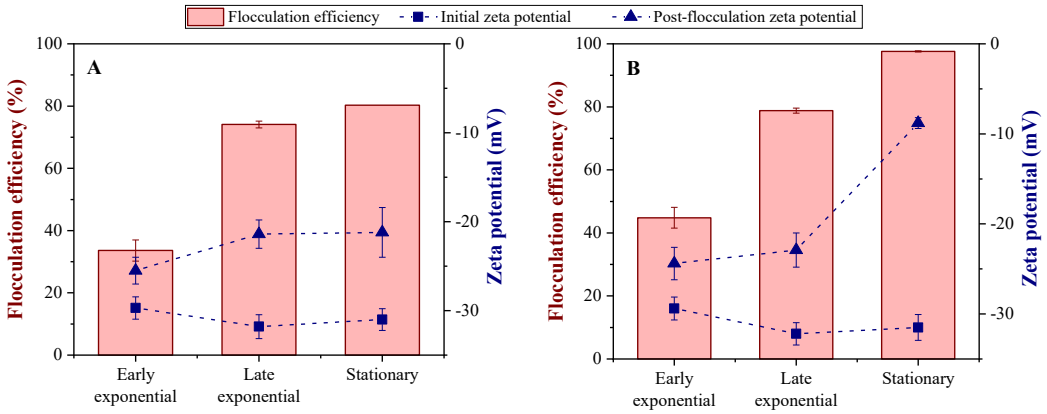

Figure 18. Flocculation efficiency and the increase in zeta potential of *C. vulgaris* culture at 35 mg polymer/g dry biomass of two biological replicates: (a) photobioreactor 1 and (b) photobioreactor 2. Flocculation was conducted at three different growth phases: early exponential, late exponential, and stationary. Values and error bars represent the mean and standard deviation from two technical replicate measurements (n = 2), respectively.

Charge neutralisation is an important flocculation mechanism and can partially, but not fully explain the increase in flocculation efficiency as the microalgae culture progressed from the early exponential to the stationary growth phase (Figure 18). Results in Figure 18 show that the highly charged cationic polymer FO3801 could significantly reduce the cell surface charge. However, complete charge neutralisation did not occur even at the stationary phase when the highest flocculation efficiency of 97% was achieved. Previous studies have suggested that complete charge neutralisation is not necessary to achieve high (>95%) flocculation efficiency (Nguyen et al., 2019). It is noteworthy that in Figure 18 the same polymer dose was applied to all flocculation experiments and that the differences in the initial zeta potential between the late exponential and stationary growth phase were negligible (t-test, $p > 0.05$). Thus, the initial surface charge is not the only factor governing the dependency of flocculation efficiency on the growth phase. As the microalgae culture continued to mature, the composition of the media and physiochemical properties of the microalgal cells also changed. The possible influence of

media matrix (in terms of PO_4^{3-} content) and cell properties on the effectiveness of polymer flocculation at different growth phases will be elucidated in subsequent sections.

5.3.3 Impact of phosphorous residue on flocculation

In this study, residual phosphorous in the microalgal media did not show any influence on flocculation (Figure 19). The variation in flocculation efficiency of the control sample and the samples with added K_2HPO_4 (i.e. 10 to 40 mg/L PO_4^{3-}) was negligible (*t*-test, $p > 0.05$). In addition, there was no observed correlation between microalgal cell zeta potential after flocculation and phosphorous concentration. Charge neutralisation of the cultures with 10 to 40 mg/L PO_4^{3-} was comparable to that of the control culture for both photobioreactors (*t*-test, $p > 0.05$). These observations conclusively affirm that the variation in flocculation efficiency of *C. vulgaris* at different growth phases was not induced by residual phosphorous in the culture media.

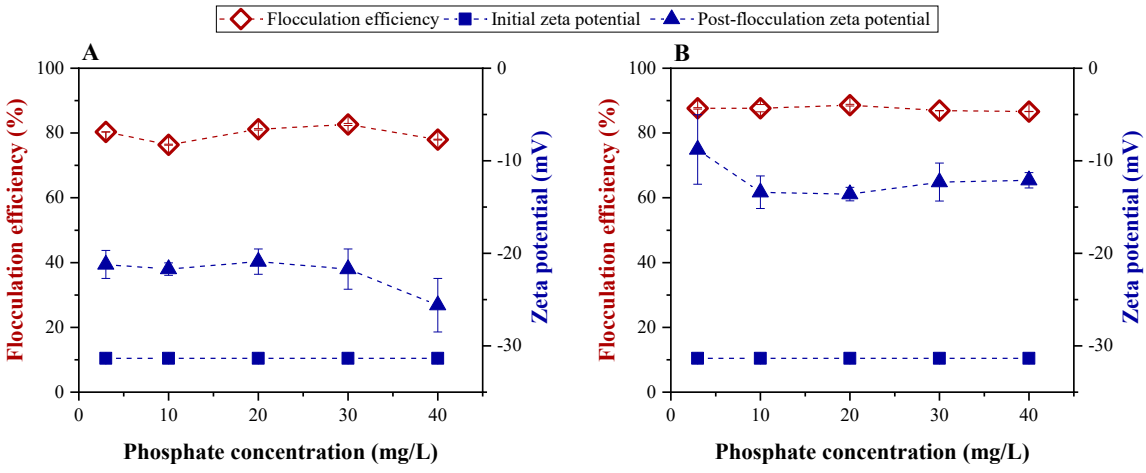

Figure 19. Flocculation efficiency and charge neutralisation of C. vulgaris culture at various concentrations of phosphate in the media during the stationary phase for (a) photobioreactor 1 and (b) photobioreactor 2. The polymer dose was 35 mg FO 3801/g dry biomass. Value and error bars represent the mean and standard deviation from two technical replicate measurements (n = 2), respectively.

5.3.4 EPS content and impact on flocculation efficiency

The soluble and bound EPS content of *C. vulgaris* increased as the microalgal culture sequentially transited through the three growth phases (Figure 20). The concentration of soluble EPS in terms of both carbohydrate and protein reached the highest value at the stationary phase (Figure 20a, c). This value is approximately two and three times the total soluble EPS of the late exponential and early exponential phases, respectively. Likewise, microalgal culture media at the stationary phase had the highest bound EPS content (6.5 mg/L), followed by late exponential (3.9 mg/L) and early exponential (2.5 mg/L) phase (Figure 20b, d).

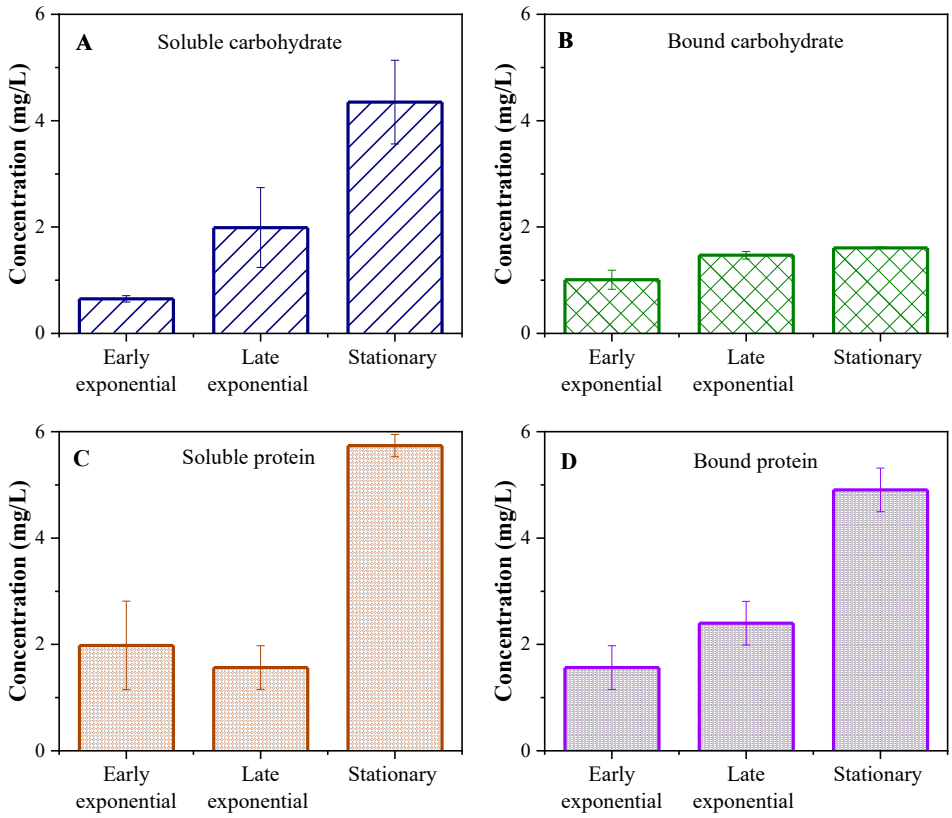

Figure 20. Accumulation of EPS in terms of (a) soluble carbohydrate, (b) bound carbohydrate, (c) soluble protein, and (d) bound protein in the *C. vulgaris* culture media at the early exponential, late exponential and stationary growth phases. Value and error

bars represent the mean and standard deviation from two biological replicate measurements (n = 2), respectively.

A similar trend of increasing EPS content as the microalgal culture continued to mature has been reported for other microalga species such as *Asterionella formosa*, *C. vulgaris*, *Microcystis aeruginosa,* and *Ettlia texensis* (Henderson et al., 2008; Salim et al., 2013; Zhang et al., 2018). Microalgal cells were actively dividing and excreting metabolites (i.e. carbohydrates and proteins) during the exponential growth phase. Thus, a higher EPS content was observed at the late exponential phase than at the early exponential phase. Although growth in the stationary phase ceased, microalgal cells continued releasing metabolites, partly due to cell autolysis. As a result, the EPS components at the stationary phase were influenced by the microalgal intracellular contents (i.e. the biochemical composition). This explains the significant increase in protein content of soluble and bound EPS at the stationary phase (Figure 20) as *C. vulgaris* is a protein-rich microalgae. The solubilisation of the bound EPS fraction formed during the exponential phase also contributed to the increase in soluble EPS content of microalgal culture media at the stationary phase (Henderson et al., 2008). Therefore, the increase in EPS content with culture age appears to be a key factor influencing *C. vulgaris* flocculation and can elucidate its growth-phase-dependent flocculation efficiency.

Microalgal EPS is dominated by hydrophobic proteins and hydrophilic carbohydrates (Henderson et al., 2008). These biopolymers can contribute to the bridging mechanism, which facilitates flocculation. In other words, these biopolymers from the microalgal cells and the added cationic polymer can form EPS-cell-polymer networks via electrostatic interaction to induce the flocculation process (Rao et al., 2021). As discussed in section 5.3.2, electrostatic attraction is expected between the negatively charged microalgal cell

surface and the positively charged cationic FO3801 polymer to form large EPS-cell-polymer networks (i.e. flocs) via a combination of charge neutralisation and bridging effects (Gonzalez-Torres et al., 2017; Rao et al., 2021). Results from Figure 20 are consistent with the dependence of flocculation efficiency on growth phase.

Previous studies have established the dependency of flocculation efficiency on culture maturity. Results from this study reveal for the first time the underlying mechanisms governing the relationship between flocculation efficiency and culture maturity. The results are significant for optimising microalgae cultivation and harvesting. Microalgal EPS production is species-dependent. Thus, further work is necessary to corroborate findings from this study with other microalgae species.

5.4 Conclusions

Flocculation efficiency of *C. vulgaris* using cationic polymer increased with microalgae culture maturity. The highest flocculation efficiency (>97%) was achieved with the microalgal culture at the stationary phase. Phosphorous residue in the culture did not affect *C. vulgaris* flocculation and cell surface charge, contrary to its negative impacts on flocculation in wastewater treatment applications. The dependency of flocculation efficiency on the growth phase was induced by changes in cell properties (e.g. EPS and surface charge). High EPS content with negative charges surrounding microalgal cells interacted with cationic polymer to form polymer-EPS-cell networks via charge neutralisation and bridging, thus promoting the flocculation process.

CHAPTER 6. ANAEROBIC CO-DIGESTION OF EXPIRED ALCOHOL-BASED HAND SANITISER WITH SYNTHETIC WASTEWATER FOR BIOGAS PRODUCTION

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Vu, H.P., Cai, Z., Tra, V.-T., Wang, Q., Nghiem, L.D. 2023. Anaerobic co-digestion of expired alcohol-based hand sanitiser with synthetic wastewater for biogas production. *Environmental Technology & Innovation*, 103319.

Summary: In this work, anaerobic co-digestion is demonstrated as a promising viable method for utilising expired alcohol-based hand sanitiser from COVID-19 response for additional biogas production. Co-digestion experiments were conducted using three parallel continuous-flow anaerobic digesters. The results highlight the importance of acclimatisation to avoid process instability. Process instability was observed when co-digesting ethanol-based sanitiser (at 0.3% v/v) with sewage sludge without acclimatisation. However, the digester was fully recoverable and a gradual increase in the addition of ethanol-based sanitiser showed stable and good performance even at 0.6% v/v. The specific biogas production was in the range of 295 – 304 mL/gCOD.day and the COD removal efficiency was 76%. When the isopropanol-based sanitiser was used as a co-substrate, the digester previously acclimatised with ethanol-based sanitiser showed 20% higher biogas production than the digester that had not been acclimatised. These results highlight the need for an acclimatisation period so that the microbial community can adjust to the new substrate. Furthermore, due to the easily degradable nature of alcohol-based sanitiser and synthetic wastewater in this study, there was a threshold of organic loading rate of 3.5 – 4 gCOD/L.day. This threshold can be explained by the accumulation of volatile fatty acids that could inhibit the methanogenesis process. Results

in this study demonstrate anaerobic co-digestion as a sustainable option for valorising expired alcohol-based hand sanitiser.

Keywords: anaerobic co-digestion; biogas; alcohol-based sanitisers; COVID-19; sewage sludge.

6.1 Introduction

As the world begins to transition to a new normal post-COVID-19, some environmental consequences of this pandemic have only begun to emerge. Indeed, COVID-19 has resulted in a tsunami of biomedical waste such as single-use face masks and single-use medical gowns (Nghiem et al., 2021; Sangkham, 2020; Tripathi et al., 2020). These are mostly plastic waste that authorities around the world, including those in developed countries, have not developed plans to manage (Nghiem et al., 2021; Ray et al., 2022). A hidden but not less significant issue is the surplus of medical equipment and chemicals that have been stockpiled by the authorities to control COVID-19. In particular, an excessive stockpile of hand sanitiser or liquid disinfectant has begun to emerge.

According to the industry standard, liquid sanitiser can be stored for up to 3 years before it must be discarded. This is because volatile components of liquid sanitiser gradually evaporate over time, making it less effective. Expired alcohol-based hand sanitisers from the COVID-19 pandemic pose a significant waste management issue for authorities around the world (Karimi et al., 2024). Using such alcohol-based materials for personal, environmental, and medical disinfection was one of the main COVID-19 preventive measures implemented globally since the outbreak in early 2020. During the peak of COVID-19, in many regions of the world, hand sanitisers were among the trend of "panic-buying" or "panic stock-up" products in supermarkets as a response to real or perceived

lack of resources due to the unprecedented pandemic (Chen et al., 2022). This has led to increased manufacturing of hand sanitiser globally to meet the high demands from the public and private sectors. Although the excessive stockpiling of medical goods to combat COVID-19 is not well documented in peer-reviewed literature, there have been numerous pieces of evidence from reliable sources. For instance, according to the online news agency Politico, at the beginning of the COVID-19 pandemic, New York Governor Andrew Cuomo ordered New York prison labour to make liquid sanitisers. As of May 2022, New York City has over 700,000 gallons of expired alcohol-based hand sanitiser from this production line that according to Politico, it "cannot get rid of". Without treatment, the discharge of expired sanitisers can lead to a range of environmental consequences. These chemicals can disrupt aquatic ecosystems and contaminate the food chain (Dhama et al., 2021; Rai et al., 2020). The biological degradation of liquid sanitisers in the natural environment can also contribute to fugitive methane release and, thus, greenhouse gas emissions. Thus, promoting sustainable disposal methods for expired sanitisers is essential to reduce pollution and protect the ecosystems.

Anaerobic digestion – a well-established technology to convert organic matter to collectable methane (CH_4) can be a potential treatment for expired alcohol-based sanitiser disposal. In the absence of oxygen, a consortium of anaerobic microorganisms (hydrolytic and fermentative bacteria, acetogens, and methanogens) break down organics to produce volatile fatty acids (Feng et al., 2022) and/or biogas which is a mixture of CH_4 (50-75%), CO_2 (25-50%) and some trace gases (Vu et al., 2022; Wickham et al., 2018). The produced biogas can be further purified into CH_4 to replace natural gas for industrial applications or directly used for generating heat and electricity (Córdova et al., 2022; Nguyen et al., 2021). A broad range of organic wastes including manure, animal effluent,

sewage sludge, and agriculture biomass residue can be used as feedstocks for the anaerobic digestion process. It is also common to combine two or more feedstocks to improve the balance between organic carbon content and nutrients, and to reduce the concentration of inhibitory contaminants in the waste materials. This practice, known as co-digestion, has been extensively investigated in the literature (for example: (Battista et al., 2020; Gautam et al., 2022; Le et al., 2022; Nghiem et al., 2017)).

The main components of many common hospital-grade sanitisers on the market are 60 – 99% ethanol or isopropanol (IPA) (TGA, 2023). At this concentration, ethanol- and IPA-based sanitisers are effective in inactivating many enveloped and nonenveloped viruses, including the coronavirus (Itiki & Roy Chowdhury, 2020; Lee et al., 2023a; WHO, 2009). Due to their potent antimicrobial property, these sanitisers are classified as hazardous waste that can cause harmful effects to the ecosystems if disposed of in regular waterways. However, as organic solvents, ethanol, and isopropanol can provide carbon and hydrogen as substrates to facilitate methanogenesis in anaerobic digestion (García-Gen et al., 2013; Vermorel et al., 2017). Since biogas and CH_4 are increasingly regarded as more sustainable energy alternatives to fossil fuel (Nguyen et al., 2021), disposing of expired alcohol-based sanitisers via anaerobic digestion can accomplish two goals with one effort: reducing waste and producing sustainable energy

Anaerobic digestion of sanitiser products has rarely been discussed in the literature, although inhibition of alcohols on the pure culture of methanogenic bacteria has been reported. Isopropanol was inhibitory for methanogens at concentrations higher than 0.2 M at 36 °C (Widdel, 1986). The addition of isopropanol as a co-substrate with acetate at a concentration > 4 g COD/L also suppressed the CH_4 production of non-acclimatised methanogen culture at 35 °C (Chou et al., 1978). This inhibition could be due to the

process imbalance between acetogens, and methanogens caused by the accumulation of VFA via rapid alcohol degradation. Anaerobic co-digestion of alcohols with another substrate to provide better chemical balance for anaerobic digestion is a feasible option to overcome the aforementioned inhibition (Arhoun et al., 2019; Prasertsan et al., 2021).

This study aims to investigate the feasibility of using expired alcohol-based hand sanitiser as a co-substrate for anaerobic co-digestion to produce biogas. In addition, the impact of an acclimatising period on sanitiser exposure to biogas production will be studied. This approach to handling expired alcohol-based sanitisers presents a promising solution for environmentally friendly waste disposal that is necessary in the post-pandemic era.

6.2 Materials and Method

6.2.1 Chemicals

Digestate was collected from a full-scale anaerobic digestion plant in Sydney (Australia) to inoculate the digesters in this study. Digestate inoculum was stored at 4 °C and used within 3 days. Ethanol (absolute), D-(+)-Glucose, peptone (bacteriological), sodium acetate, urea, potassium phosphate monobasic (KH_2PO_4), magnesium sulphate monohydrate ($MgSO_4.H_2O$) and iron (III) sulphate hydrated ($FeSO_4$) were obtained as analytical reagents from Sigma-Aldrich and Chem-Supply. Rubbing alcohol (Isocol) sanitiser was purchased from a local pharmacy. This liquid sanitiser contained 64% v/v isopropanol and was used without any further purification.

6.2.2 Anaerobic digestion

Three identical laboratory-scale anaerobic digesters were operated in parallel. Each digester consisted of a 1 L jacketed glass reactor with a mixing paddle operating

intermittently at 100 rpm (15 min on, 15 min off), a rubber head plate with seven ports for sample collection, and pH/ORP probe insert (Moubio Fermentor Company, Taiwan), a temperature control unit (Thermoline Scientific, Australia), a peristaltic hose pump (Masterflex L/S, USA), and a biogas counter (Ritter Company, MilliGascounter). Intermittent agitation at a lower speed (80 to 120 rpm) has proved to be favourable for anaerobic digesters by promoting a homogeneous environment and stable microbial community at a lower power consumption (Babaei & Shayegan, 2019; Kaparaju et al., 2008; Singh et al., 2022). Excessive continuous mixing distorts the granule structure that is required for bacteria growth, causing digester instability (Singh et al., 2020). Thus, for a lab-scale 1 L reactor, intermittent agitation at 100 rpm is deemed suitable.

The working volume of each digester was maintained at 900 mL. Three digesters were fed daily by first withdrawing 90 mL of digestate and then replacing it with 90 mL of the feed, resulting in 10 days of hydraulic/solid retention time (HRT/SRT). The temperature and oxidation–reduction potential (ORP) of all digesters were maintained at 38.0 ± 0.1 °C and below −480 mV, respectively.

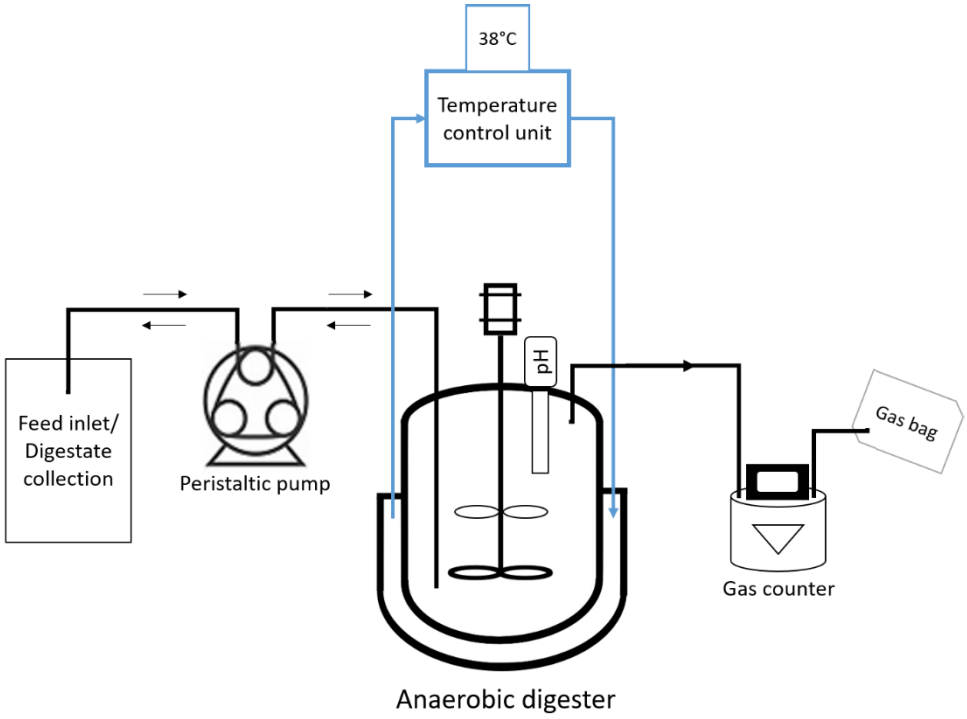

Figure 21. Schematic diagram of anaerobic digester setup.

6.2.3 Anaerobic co-digestion of solvents with synthetic wastewater

This study was conducted in five distinctive phases (Table 8) to study the anaerobic co-digestion of solvents ethanol and isopropanol with synthetic wastewater. In all phases, the three anaerobic digesters were operated with the same conditions (i.e. temperature, HRT/SRT, and mixing setting) as previously described. The ability to degrade solvents was investigated via different feed concentrations and compositions in each phase. Three anaerobic digesters were denoted as R1, R2, and R3. R1 operated as the control with the same operational parameters and feed composition throughout the experiment. Operating the digesters in five phases with increasing organic loading rate (OLR) and change in co-digestion substrates allows for stepwise microbial adaption. Thus, abrupt disturbance to the digester performance is avoided, minimising process failure.

Table 8. Experimental plan overview for anaerobic co-digestion of ethanol and Isopropanol (IPA) with synthetic feed (SF).

Phase	Day	Digesters	OLR (gCOD/L.day)	Feed composition
1	1-6	R1	2.5 ± 1.3	SF
		R2	3.3 ± 0.0	SF+Glucose
		R3	3.3 ± 0.0	SF+Ethanol
2	7-14	R1	2.5 ± 1.3	SF
		R2	3.3 ± 0.0	SF+Glucose
		R3	2.5 ± 1.3	SF
3	15-23	R1	2.6 ± 0.1	SF
		R2	3.3 ± 0.0	SF+Glucose
		R3	3.5 ± 0.4	SF+Ethanol
4	23-67	R1	2.7 ± 0.1	SF
		R2	3.8 ± 0.2	SF+Glucose
		R3	3.8 ± 0.2	SF+Ethanol
5	67-87	R1	2.7 ± 0.1	SF
		R2	3.7 ± 0.0	SF+IPA
		R3	3.7 ± 0.0	SF+IPA

Before this study, all three digesters were already subjected to an acclimatising period of three months during which they were fed using identical SF containing the following per litre: glucose (16 g), peptone (3 g), urea (0.7 g), KH_2PO_4 (0.7 g), MgSO4 (1.015 g), $FeSO_4$ (0.7 g), and sodium acetate (9 g). Chemical oxygen demand (COD) and total nitrogen of the feed were 25.3 ± 1.3 and 1.6 ± 0.1 g/L, respectively. The organic loading rate (OLR) for phase 1 is 2.5 ± 1.3 gCOD/L.day.

Once the three anaerobic digesters showed stability and consistent daily biogas production, a baseline experiment with analytical ethanol as co-substrate was conducted to check the digesters' ability to degrade ethanol (Phase 1 to 3). The OLRs of R2 and R3

were increased to 3.3 gCOD/L.day using SF + 5 g glucose/L and SF + 3.2 mL ethanol/L, respectively (Table 8). Once R2 and R3 have shown stable performance at 3.3 gCOD/L.day after day 23, the OLRs of R2 and R3 were further increased to 3.8 ± 0.2 gCOD/L.day in phase 4 using SF + 10 g glucose/L and SF + 6.4 mL ethanol/L, respectively. Phase 4 was prolonged from day 23 to 67 (i.e. 44 days).

In phase 5, Isopropanol (IPA) at 64% v/v replaced ethanol as the co-substrate for R2 and R3 when both digesters have adapted to a high OLR and R3 has acclimatised to digest ethanol. The OLRs of R2 and R3 were maintained at 3.7 gCOD/L.day using a mixture of SF + 0.6 mL IPA/L. Phase 5 lasted from day 67 to 87 (i.e. 20 days), after which the experiment was concluded.

6.2.4 Analytical methods

pH and ORP of the anaerobic digesters were monitored using a portable pH/ORP probe and meter (Hach, Australia). Digestate samples from three digesters are collected every Monday and Thursday for total solids (TS), volatile solids (VS), and COD measurements. TS and VS were determined according to Standard Methods 1684. COD concentrations were measured by using digestion vials (Hach, Australia) and Hach DR3900 spectrophotometer program number 435 COD HR following the US-EPA Standard Method 5220D.

6.2.5 Statistical analysis

Statistical analysis of biogas production and COD removal efficiencies was performed using paired *t*-test (Excel 2016 Analysis Toolpak). The variations are considered statistically significant at a confidence interval of $p<0.05$.

6.3 Results and discussion

6.3.1 Anaerobic co-digestion of ethanol and synthetic feed

At the beginning of this study, all three digesters had reached similar daily biogas production of 760, 730, and 735 (mL/day) for R1, R2, and R3, respectively using the same SF (section 6.2.3). During the three months of the acclimatising period, three digesters maintained 60-80% COD removal efficiency, pH 7.2-7.8, and around 1.1% TS. Throughout 87 days of the experiment, control reactor R1 maintained a stable daily biogas production of 789 ± 77 mL/day at COD loading of 2.6 ± 0.1 g/L.day (i.e. 292 ± 31 mL/gCOD.day) (Figure 23). The COD removal efficiency was 68 ± 6 % and pH remained at 7.6 ± 0.2.

Daily biogas production of R2 increased by 24% to 907 ± 52 mL/day (i.e. 271 ± 19 mL/gCOD.day) in phases 1-3 after the COD loading was increased by 30% to 3.3 g COD/L.day using additional 5 g glucose/L feed (Figure 22). The performance of the reactor was relatively stable during this period with COD removal was 70.7 ± 0.1%. The performance of R2 started to show instability in Phase 4 where COD loading was further increased by 20% to 3.8 g COD/L.d (by adding 10 g glucose/L feed) compared to phase 1-3 SF, and 50% compared to control SF used for R1. During phase 4, the pH of R2 dropped to as low as pH 7. This result is consistent with observations by Mercado et al. (2023) in which methane yield and pH decreased with higher organic load for sludge acclimatised to a simple substrate. The organic load of R2's feed pre-experiment and phases 1-4 mainly comes from glucose, which is a simple substrate. Acclimatisation of the microbiome to a simple substrate can reduce microbial diversity and impede the sludge's resilience to organic load fluctuations due to the loss of important microbial

groups (Mercado et al., 2023). Furthermore, anaerobic digestion of easily degradable substrates such as glucose invigorates the rapid production of VFA while methanogenesis remains the rate-limiting step (Khan et al., 2016). This imbalance can cause serious disturbance to the operation of a single-stage anaerobic digester.

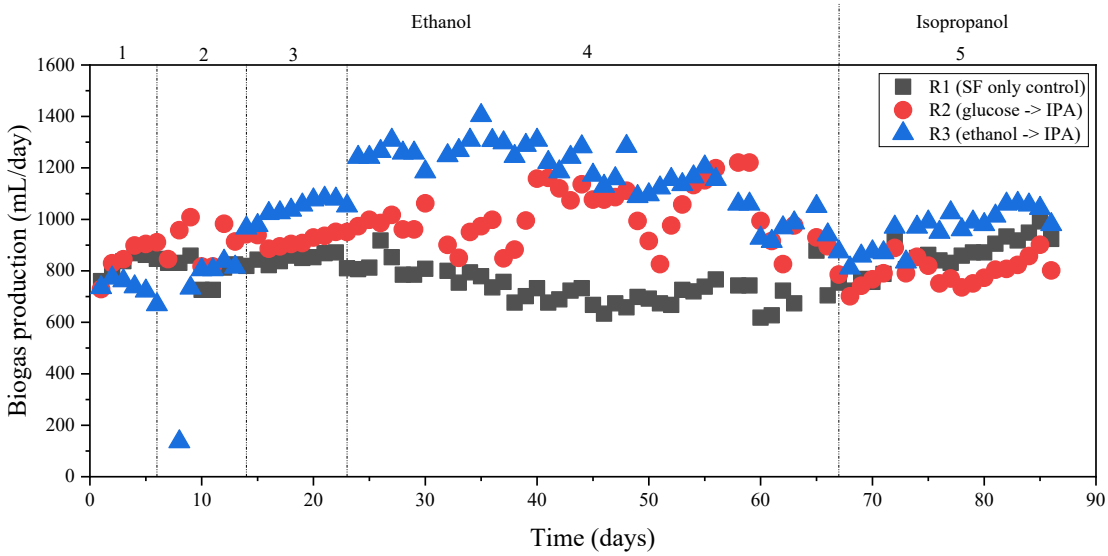

Figure 22. Daily biogas production of anaerobic digesters R1, R2, and R3 as a function of time.

On the other hand, co-digestion of ethanol and SF in R3 showed a reversed reaction where the performance was destabilised and then acclimatised over time. When absolute ethanol was added as a co-substrate at 0.3% v/v for the first time in phase 1, R3 was disturbed, and the daily biogas production quickly dropped by over 70% to < 200 mL/day after 6 days. Ethanol feeding was immediately stopped and replaced by control SF to restabilise R3 from day 7-14 (i.e. phase 2). This allowed the daily biogas production of R3 to gradually recover. COD removal efficiency during phase 2 was <60%, lower than that in phase 1. This reflects the poorer performance of the microbial community after the ethanol shock. However, the quick recovery of R3 biogas production in phase 2 from <200 mL/day on day 7 to >800 mL/day on day 10 suggests that some microbes are more

resilient to ethanol exposure and still capable of anaerobic digestion. TS and VS/TS ratio of R3 did not show any significant fluctuations during phases 2 and 3.

After R3 was restabilised, ethanol was added again as a co-substrate at 0.3% v/v in phase 3 to confirm if ethanol is destructive to anaerobic digestion. However, R3 showed increasing daily biogas production and COD removal efficiency (Figure 22). R3 had the higher daily biogas production of 1037 mL/day compared to R2 of 924 mL/day at the same COD loading of 3.3 g/L.day (i.e. 304 ± 21 mL/gCOD.day for R3 versus 279 ± 7 mL/gCOD.day for R2). This suggests that after the first exposure of R3 sludge to ethanol, the microbial community was able to acclimatise and avoid performance shock when ethanol was added the second time. Microbial acclimatisation plays a crucial role in anaerobic digestion, particularly when introducing new substrates. Microorganisms in anaerobic digestion possess the ability to adjust their metabolic capabilities and population dynamics to adapt to new substrate or enriched environment (e.g. high ammonia, high sodium, and high lipid) (Hu et al., 2020; Nakasaki et al., 2019; Yan et al., 2019). This adaptation ensures the establishment of an efficient microbial consortium that can degrade the new substrates and minimise process destabilization. Biogas production from anaerobic digestion is thus enhanced.

Ethanol concentration in feed was doubled in phase 4 (0.6% v/v). The COD loading in phase 4 was 3.8 g/L.day for both R2 and R3, but R3 showed more stable and better performance in biogas production and COD removal efficiency (Figure 22 and Figure 23). During phase 4, R3's co-digestion with ethanol achieved the best COD conversion efficiency to biogas at 295 ± 29 mL/gCOD.day compared to 273 ± 26 mL/gCOD.day and 255 ± 28 mL/gCOD.day for R1 and R2, respectively. This was reflected in the COD removal efficiency of three digesters in phase 4 in which R3 had the highest efficiency

(76%), followed by R1 (70%) and R2 (68%). The R3 microbial community had acclimatised to ethanol substrate steadily and was able to produce biogas effectively. The performance of R3 in phases 3 and 4 indicates anaerobic digestion can convert ethanol-based sanitiser as a co-substrate to biogas.

In recent years, direct interspecies electron transfer (DIET) has been investigated as an approach to enhance anaerobic digestion. In DIET, ethanol is metabolised to acetate, which simultaneously releases protons and electrons that can be consumed by electrotrophic methanogens to reduce carbon dioxide to methane (Eq. 4-6) (Feng et al., 2021). Several studies have suggested that ethanol significantly stimulated methane production during anaerobic digestion (Morita et al., 2011; Zhao et al., 2020). Meanwhile, the glucose digestion pathway is significantly influenced by H_2 concentration for electron transfer, known as indirect interspecies electron transfer (Kalyuzhnyi & Davlyatshina, 1997; Li et al., 2021). Glucose as the sole substrate for digestion is easily acidified and can inhibit the activity of methanogens due to VFA accumulation (Ma et al., 2019). This may explain the fluctuating biogas production of R2 when additional glucose was used as the substrate in phase 4, in comparison to the relatively stable performance of R3 with ethanol as the co-substrate (Figure 22).

Ethanol digestion:

$$C_2H_5OH + H_2O \rightarrow CH_3COOH + 2H_2 \quad \text{(Eq. 4)}$$

$$CH_3COOH \rightarrow CH_4 + CO_2 \quad \text{(Eq. 5)}$$

$$2C_2H_5OH + CO_2 \rightarrow CH_4 + 2CH_3COOH \quad \text{(Eq. 6)}$$

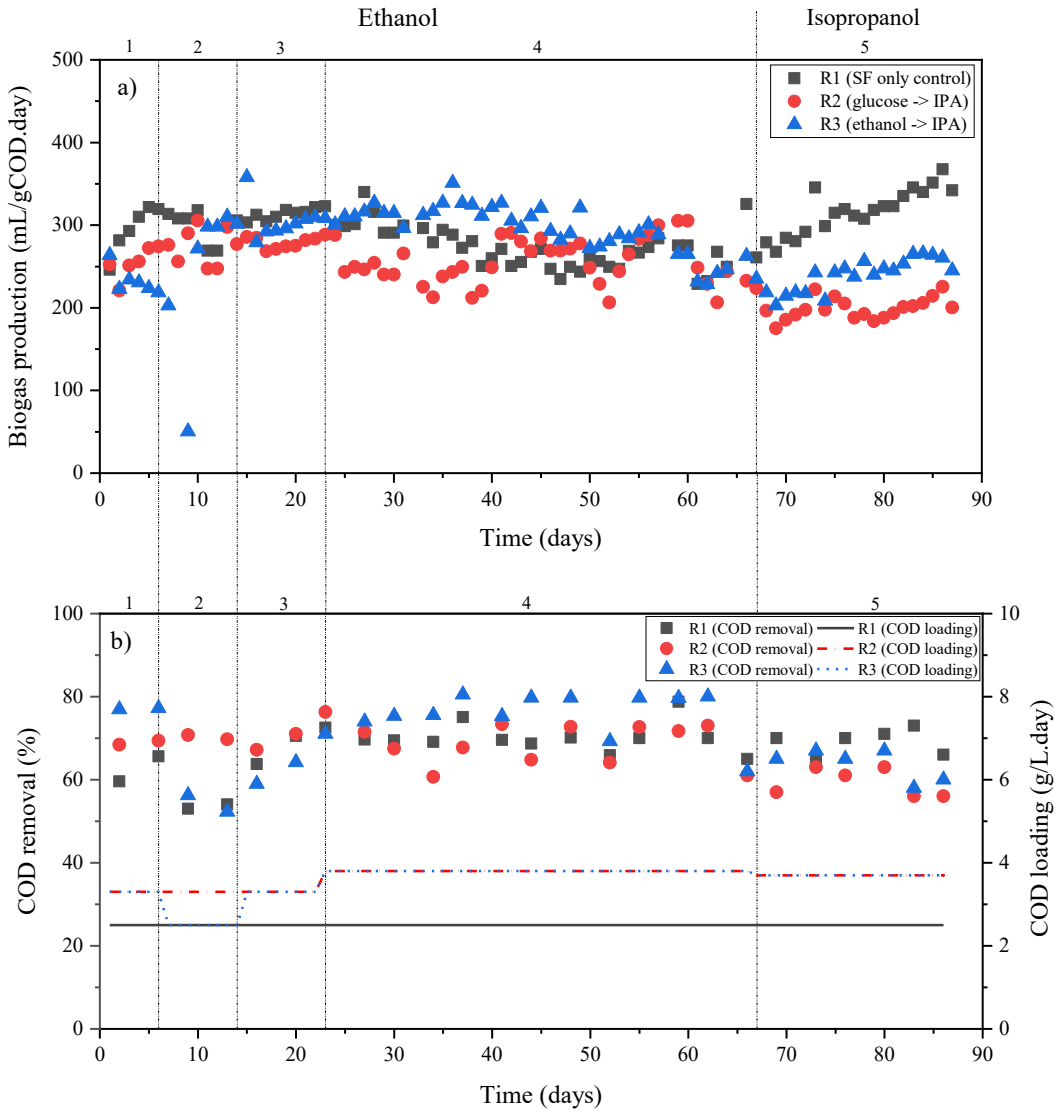

Figure 23. (a) Specific biogas production per gram of influent COD of R1, R2, and R3 and (b) COD loading and COD removal efficiency as a function of time.

6.3.2 Anaerobic co-digestion of isopropanol and synthetic feed

In phase 5, glucose and ethanol co-substrates in the feed of R2 and R3, respectively, were replaced by 0.6 mL 64% v/v Isopropanol/L while maintaining the same COD loading as phase 4 (Figure 23b, Table 8). The daily biogas production of R2 in phase 5 was 20% lower than in phase 4 (paired *t*-test, $p < 0.05$). The average COD removal efficiency of R2 was also the lowest among the three digesters, at 59% compared to 69% and 64% for

R1 and R3, respectively. On the other hand, there was no significant effect on the daily biogas production of R3 in phase 5 using isopropanol co-substrate compared to phase 4 using ethanol (paired *t*-test, $p > 0.05$). However, COD removal efficiency was significantly lower (paired *t*-test, $p < 0.05$) at 64 ± 4% compared to 76 ± 5% in phase 4.

Adaptation of the microbial community in the anaerobic digester to a new substrate is a complex chemical, enzymic and bacteriological process. The adaption can occur over a long period with significant changes to the biochemical properties of the inoculum (Hattingh et al., 1967). The starting microbial community is an important factor in determining the digester's response to fluctuations in organic load and the type of substrate (Steinberg & Regan, 2011). In this experiment, the inoculum has been acclimatised to glucose substrate for over 2 months. The sudden change from high-loading glucose substrate to glucose-isopropanol co-substrates to R2 slightly destabilises the system and requires its microbial community to slowly adapt. Whereas in R3, the microbial community has been acclimatised to ethanol co-substrate in phases 3-4 so when a molecularly similar sanitiser (i.e. isopropanol) is added as the co-substrate, R3 was able to adapt quicker and maintain a stable biogas production (Figure 23a).

Table 9. Biogas composition of three digesters in phases 5 and 6.

Gas	**R1**		**R2**		**R3**	
	Phase 5	*Phase 6*	*Phase 5*	*Phase 6*	*Phase 5*	*Phase 6*
CH_4 (%)	47	49	44	46	49	52
CO_2 (%)	37	41	39	33	44	43
H_2 (ppm)	36	42	39	48	108	44
H_2S (ppm)	803	1242	1060	1048	1485	1498
Residual N (%)	10	7	14	18	6	3

In terms of biogas composition, the CH_4 and CO_2 percentages are relatively similar between R1, R2, and R3. The CH_4 percentage did not change significantly when glucose or ethanol co-substrate of R2 and R3 was changed to isopropanol in phases 5 and 6 (

Table 9). However, lower N_2 residue was observed in the biogas from R3 (3-6%) compared to R1 (7-10%) and R2 (14-18). At the same time, R3 has a much higher H_2S concentration (ca. 1500 ppm) than R1 (ca. 800 – 1250 ppm) and R2 (ca. 1050 ppm). This could be due to the higher acetic acid concentration in R3 via the ethanol digestion pathway which is a favourable environment for SO_4^{2-} reduction to H_2S by sulphate-reducing bacteria (Vu et al., 2022).

6.4 Conclusions

Co-digestion of expired alcohol-based hand sanitiser with sewage sludge was reported in this study. The biogas production and COD removal efficiency were enhanced by 15% and 8%, respectively using 0.6% v/v ethanol sanitiser as the co-substrate. Similar results were observed with isopropanol co-digestion. The addition of isopropanol-based sanitiser as the co-substrate did not affect the culture that had been previously acclimatised to ethanol-based sanitiser possibly due to their chemical similarity, but it destabilised the non-acclimatised culture. This affirms the necessity for metabolic adjustment to a new substrate to avoid process disturbance. Anaerobic digestion using expired alcohol-based hand sanitiser as a co-substrate also allowed for higher COD loading tolerance compared to mono-substrate digestion.

CHAPTER 7. ENHANCED BIOMETHANE PRODUCTION FROM SCENEDESMUS SP. USING POLYMER HARVESTING AND REDUNDANT COVID-19 DISINFECTANT AS PRETREATMENT

Summary: Expired isopropanol (IPA) COVID-19 disinfectant (~70% w/w) was evaluated for pretreating microalgal biomass to enhance CH_4 yield and reduce waste. The impact of harvesting methods (centrifugation and polymer flocculation) and microwave pretreatment on CH_4 production from *Scenedesmus* sp. microalgal biomass were also investigated. Harvesting methods had minimal impact on the overall CH_4 yield from *Scenedesmus* sp. Wet centrifuged and polymer-harvested biomass exhibited comparable and low CH_4 production at 66 and 74 L/kg VS, respectively. Microalgae drying showed significantly higher CH_4 yield compared to wet biomass, attributed to cell shrinkage, and enhanced digestibility. Consequently, the efficiencies of microwave and IPA pretreatment were improved when applied to dried microalgae, yielding a 135% and 212% increase in CH_4 production, respectively, compared to non-pretreated wet biomass. These findings highlight the significantly higher CH_4 yield of dried *Scenedesmus* sp. over wet biomass and emphasize the enhanced effect of combining oven drying with IPA treatment to enhance CH_4 production from microalgae whilst reducing COVID-19 waste.

Keywords: Microalgae; Biomethane production; Isopropanol; COVID-19 waste; Pretreatment.

7.1 Introduction

Biomethane (CH_4) production from biomass through anaerobic digestion is a key solution to the global climate change challenge (Bertasini et al., 2023; Gray et al., 2022). CH_4 is a reliable renewable fuel where electrification is not possible. In the natural environment, anaerobic decomposition is responsible for fugitive CH_4 greenhouse gas emissions from

waste biomass and organic waste, accounting for about 20% of global climate warming (Mar et al., 2022). The anaerobic decomposition process can also be engineered into purposely built digesters to produce biogas which is a mixture of CH_4 (50-75%) and CO_2 (25-50%) and nutrient-rich digestate that can be utilised as fertilizers (Shi et al., 2018; Wickham et al., 2018). The produced biogas can be used for heating and electricity production or further refined into biomethane (with CH_4 content of over >95%) for many high-value applications such as injection into the natural gas grid, transport fuel, and raw feedstock for the industry (Nguyen et al., 2021). CH_4 production from organic waste and biomass not only mitigates the environmental impact but also contributes to the circular economy by efficiently converting organic waste streams into clean and sustainable energy. Given the urgent need to reduce greenhouse gas emissions, exploring different substrates for anaerobic digestion and optimizing the CH_4 production process are central to the transition towards a low-carbon future.

Microalgae have recently emerged as a potential platform for producing renewable chemical feedstock and fuel. Microalgae are fast-growing photosynthetic microorganisms that can potentially be cultivated at scale (Nguyen et al., 2023; Sung et al., 2022). They are rich in biopolymers, lipids, and a range of biochemical molecules that can be harvested to replace feedstock from fossil fuels (Khanra et al., 2022; Rahpeyma & Raheb, 2019). Microalgal biomass is also rich in sugars, hemicellulose, and other carbohydrates that can be anaerobically digested to produce renewable CH_4 (Kumari et al., 2021; Zhang et al., 2019).

Microalgae (either after the extraction of valuable biomolecules or in whole) can potentially be an important substrate for anaerobic digestion. Common microalgae species such as *Chlorella*, *Scenedesmus*, *Spirulina* and *Dunaliella* are rich in

carbohydrates, proteins, and lipids. Carbohydrate, protein, and lipid contents as fractions of all organic molecules of these microalgae can be up to 69, 84, and 63%, respectively (Xia et al., 2015). Microalgal biomass can be the primary substrate to support anaerobic microbes. Previous studies have demonstrated CH_4 production from whole microalgae, typically ranging from 0.09 to 0.44 L CH_4/g $VS_{microalgae}$ (Barreiro-Vescovo et al., 2018; de Oliveira et al., 2022; Ganesh Saratale et al., 2018). However, most of these studies employed conventional sedimentation and/or centrifugation processes for microalgal biomass harvesting, which are time-consuming and energy intensive (Singh & Patidar, 2018). Harvesting remains a critical bottleneck for large-scale microalgae biorefinery, making it essential to explore cost-effective harvesting methods to enhance the feasibility of CH_4 production from microalgae.

Microalgae harvesting using cationic polymers has proven effective. Recent studies have reported microalgae harvesting efficiency of 90 to 99% from both freshwater and seawater matrices using low polymer doses (Nguyen et al., 2019; Vu et al., 2021a; Vu et al., 2021b). These cationic polymers work by neutralizing the negative surface charge of individual microalgae and inducing cell aggregation via the bridging effects induced by their large molecular weight (Vu et al., 2021a). The aggregated microalgae can then be separated from the water phase by a simple low-pressure filtration process or sedimentation. Polymer harvesting has a limited impact on cell membrane integrity, resulting in a higher number of intact cells post-harvesting (Labeeuw et al., 2021; Wu et al., 2015). This factor can significantly influence the biodegradability of microalgae biomass during anaerobic digestion, as anaerobic bacteria require access to intracellular organic materials to convert them to CH_4. Despite the research into microalgae harvesting via polymer flocculation, there is limited exploration of the CH_4 potential of polymer-

harvested microalgae. Understanding this aspect could pave the way for strategies to optimise the anaerobic digestion of microalgae.

Another challenge hindering large-scale microalgal-based CH_4 production is the recalcitrant cell walls, particularly among green algae such as *Chlorella* and *Scenedesmus* (Yukesh Kannah et al., 2021). Several pretreatment methods have been studied to enhance cell release for biofuel production and extraction of valuable biomolecules from microalgae (de Oliveira et al., 2022; Ganesh Saratale et al., 2018; Rana & Prajapati, 2021). For thermal pretreatment, harvested microalgae are subjected to a moderate temperature of up to 100 ^oC. Widely used at large scale, thermal pretreatment can dewater and solubilize cellulosic cell walls, thus increasing access to intracellular organic materials (de Oliveira et al., 2022). Mechanical pretreatment by microwave and ultrasonication have also been studied but mostly at bench scale. These methods induce internal structural modification of the cell wall, leading to cell membrane rupture (Passos et al., 2014a). Microwave and ultrasonication can effectively release cell content but they are also energy intensive (Passos et al., 2014a; Passos et al., 2013). Chemical pretreatment, involving acids and alkalines, has been demonstrated for biomass solubilisation to produce CH_4 (Jankowska et al., 2017). Often, these chemicals are coupled with thermal pretreatment (50-100 ^oC) to improve efficiency and reduce chemical costs. However, drawbacks include the inhibition of methanogenic microorganisms, as toxic by-products like furfural and 5-hydroxymethylfurfural can accumulate during acid-alkaline pretreatments.

Recently, a novel cell disruption method using alcohol (i.e. ethanol and isopropanol) has been explored for lipid extraction from microalgae (Zhou et al., 2022). These chemicals are safe, show no deteriorating effect on extract compounds, and may not require

additional drying or heating (Huang et al., 2015). As a low-cost industrial alcohol, isopropanol (IPA) possesses the capability to penetrate microalgae cells, dissolving and releasing intracellular compounds (Miazek et al., 2017). During the COVID-19 pandemic, there was a large stockpile of expired alcohol-based hand sanitisers and disinfectants. Most of these hand sanitisers contain IPA at 64% as the active ingredient. This presents an opportunity to explore IPA-based disinfectant as a chemical pretreatment to enhance microalgae biodegradability for biomethane production while offering a dual benefit of waste reduction and green energy production.

This study aims to investigate the biological methane potential (BMP) of polymer-harvested *Scenedesmus* sp. via batch-test anaerobic digestion. The CH_4 production of wet and dried biomass was also compared. Furthermore, the impact of two pretreatment methods, namely microwave irradiation and IPA treatment, on the BMP of polymer-harvested *Scenedesmus* sp. will be studied. The results presented here will contribute to the optimisation of microalgae applications in biofuel production.

7.2 Materials and Method

7.2.1 Microalgae culture and digestate inoculum

The green microalgae strain *Scenedesmus* sp. (UTS-LD) was cultivated, harvested, and used as feedstock for biomethane production. This *Scenedesmus* sp. was isolated by the University of Technology Sydney from environmental water in Australia. A 300 L pilot-scale open cascade system was used for microalgae cultivation. At the stationary growth phase, the fresh *Scenedemus* sp. suspension was collected for characterisation in terms of pH, optical density, dry weight, volatile solid (VS), and chemical oxygen demand (COD)

(Table 10). Different techniques to harvest and pretreat the *Scenedesmus* sp. biomass were evaluated in terms of biomethane production.

Table 10. Characteristics of *Scenedesmus* sp. suspension at the stationary growth phase

	***Scenedesmus* sp. suspension**
pH	9.52
Optical density (at λ = 680 nm)	1.92
Dry weight or TS (g/L)	1.62
VS (g/g dry weight)	0.70
COD (g/g dry weight)	0.70

Anaerobic digestate from a full-scale anaerobic digestion plant in Sydney (Australia) was used as the inoculum for BMP experiments. The digestate was stored at 4 °C after collection and characterised for pH, COD, total solids (TS) and VS. It had a pH of 6.68, 43.01 g/L TS, 24.52 g/L VS, and 46.18 g/L COD.

7.2.2 BMP experiment

7.2.2.1 BMP apparatus and protocol

The BMP experiment was conducted using an Automatic Methane Potential Test System II (AMPTS® II) from BPC Instrument (Sweden). The AMPTS II system consisted of three interconnected units: thermostatic water bath (unit A), CO_2 absorption tray (unit B) and gas volume measuring device (unit C) (Figure 24). Unit A had 15 glass bottles (500 mL) as reactors in an 18 L thermostatic water bath. Each reactor was equipped with a plastic cap, a motor-controlled agitator, and two tubing ports. Unit B had 15 glass bottles (100 mL) each with a plastic cap, and two tubing ports, containing 3 M NaOH solution for CO_2 quenching. The gas outlet of reactors in unit A was connected with the inlet of unit B bottle via a plastic tube. Unit C consisted of a covered water bath with 15 flow cells and a power adapter. The gas measuring device in unit C worked according to the

principle of liquid displacement and buoyancy. A laptop computer was used for recording CH_4 production data from unit C and controlling the time and speed of the agitator on unit A.

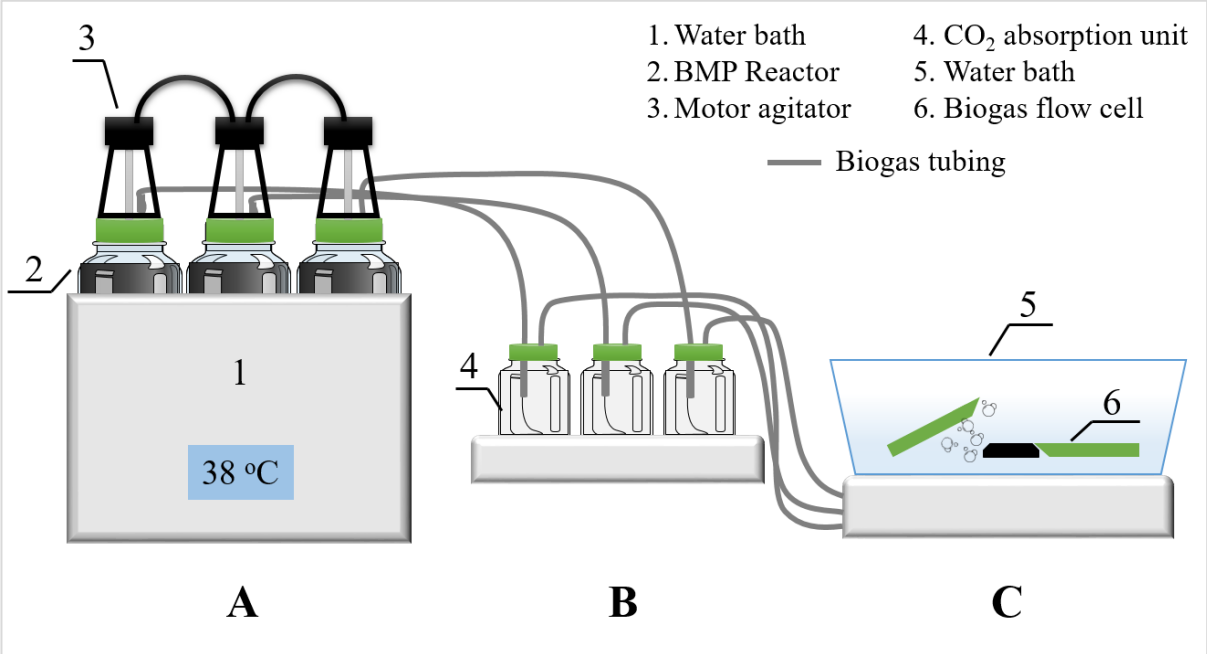

Figure 24. Schematic AMPTS® II set up for BMP investigation

In Unit A, each reactor received 190 mL of inoculum. For all BMP tests involving microalgae, a constant substrate-to-inoculum ratio ($VS_{substrate}/VS_{inoculum}$) of 0.2 was maintained. Consequently, each BMP reactor contained 190 mL of inoculum (i.e. 3.5 g of VS), along with 1 g of microalgae biomass by dry weight (i.e. 0.7 g of VS). The total working volume was adjusted to 400 mL by adding Milli-Q water. Blank BMP samples were created using only 190 mL of inoculum and 210 mL of Milli-Q water. The reactors were submerged in a water bath at 38 °C. The agitators operated intermittently at 10-minute intervals, at a speed of 60 rpm. Before the experiment, the reactors were purged with 100% N_2 gas for one minute to establish anaerobic conditions in the headspace. All BMP experiments were repeated three times.

All outlets, tubing and flow cells were checked for blockages or leakage before the experiment. The BMP experiment was operating for 15 days or until no further increase in accumulated CH_4 was recorded.

7.2.2.2 Microalgae harvesting and pretreatment

Wet biomass paste was recovered from the microalgae suspension by either centrifugation or polymer addition. Centrifugation was performed using an Allegra V-15R Centrifuge (Beckman Coulter) at 3000 rpm and 21°C for 10 min. The cationic polyacrylamide polymer FO3801 (SNF Pty Ltd; Corio, VIC, Australia) was used for polymer harvesting. A stock 2 g/L polymer solution was prepared and used immediately to avoid any polymer hydrolysis. FO3801 was dosed at the optimal dose of 50 mg polymer/g dry microalgal biomass determined in a previous study (Labeeuw et al., 2021). After polymer addition, the microalgae aggregate was separated from the water phase and recovered following the protocol outlined in a previous study (Vu et al., 2021a). Microalgal biomass recovery efficiencies by centrifugation and polymer addition were 95% and 90%, respectively.

Wet microalgal biomass paste obtained after centrifugation and polymer harvesting was stored at 4 °C for BMP evaluation within 24 hours. Wet biomass paste was dried in the oven at 60 °C for 12 hours to obtain dry microalgal biomass for further experiments. This drying process also served as a thermal pretreatment step. The dried microalgae biomass was ground to powder using a Nutribullet blender and stored in airtight containers.

Two other pretreatment methods namely microwave irradiation and IPA extraction were investigated in this study. Microwave treatment entails subjecting 1 g dry weight of harvested microalgae biomass at a concentration of 200 g/L to 800 W radiation for 30

seconds. This is equivalent to a specific energy of 24 MJ/kg of microalgae biomass. For IPA pretreatment, 1 g dry weight of microalgae biomass was mixed with 5 mL of 64% IPA for 1 min. Because IPA is a source of carbon for biomethane production, IPA residue after pretreatment was removed by evaporation at 80 °C in an oven for 15 min. This allows for a systematic comparison to microwave irradiation.

7.2.2.3 Experimental protocol

To investigate the impact of polymer harvesting on methane production from microalgae, the BMP tests were conducted with wet microalgae biomass harvested by either centrifugation or polymer addition, denoted as (C) and (P), respectively.

To investigate the impact of pretreatment methods after harvesting on methane production from microalgae, the BMP tests were conducted with five types of biomass: a) polymer-harvested and oven-dried microalgae (P + OD), b) polymer-harvested and microwaved microalgae (P + MW), c) polymer-harvested and isopropanol-treated microalgae (P + IPA), d) polymer-harvested, oven-dried and microwaved microalgae (P + OD + MW) and e) polymer-harvested, oven-dried and isopropanol-treated microalgae (P + OD + IPA). The protocols for microwave and isopropanol pretreatment have been described above.

All biomass samples were added to BMP reactors at a predetermined weight (i.e. equivalent to 1 g dry weight) to obtain 0.2 $VS_{substrate}/VS_{inoculum}$.

7.2.2.4 Biomethane production calculation

In this study, N_2 was used to flush the headspace to create an anaerobic environment. Because N_2 is an inert gas and cannot be removed by the quenching NaOH solution, it is

necessary to correct for the initial volume of N_2 in the headspace (Koch et al., 2016). Correction for N_2 in the headspace was calculated as (Strömberg et al., 2014):

$$V_{corr,i} = V_{exp,i} - V_{overestimated,i} \quad \text{(Eq. 7)}$$

$$\text{Where } V_{overestimated,i} = V_{headspace} \times (X_{biogas,CO_2} - X_{flush,CO_2}) \times \left(\frac{V_{exp,i} - V_{exp,i-1}}{V_{headspace}}\right) \quad \text{(Eq. 8)}$$

$V_{corr,I}$ is the corrected CH_4 volume at the measurement point i (i≥1).

$V_{exp,I}$ is the recorded volume by the BMP system at point i.

$V_{overestimated,I}$ is the overestimated volume of CH_4 at point i. If the volume is underestimated this value will be negative.

$V_{headspace}$ is the total volume of headspace including tubing. It was estimated to be 225 mL.

$X_{biogas, CO2}$ is the CO_2 fraction in produced biogas. It was assumed to be 35%

$X_{flush, CO2}$ is the CO_2 fraction in the flush gas. It was 0% in this experiment since 100% N_2 was used to flush the headspace.

The CH_4 production of the blank sample was subtracted from the final accumulated CH_4 production of microalgae samples to obtain CH_4 production solely from microalgae feed.

7.2.3 Analytical analysis

pH of the BMP samples was measured using a portable pH probe and meter (Hach, Australia). The optical density of the microalgal culture was measured using a spectrophotometer (Shimadzu UV 6000) at a wavelength of 680 nm.

The fresh microalgal culture was centrifuged at 300 rpm for 10 min, separated from the supernatant, rinsed, and resuspended in milli-Q water for COD measurement of microalgal biomass. COD concentrations were measured using digestion vials (Hach, Australia) and Hach DR3900 spectrophotometer program number 435 COD HR following the US-EPA Standard Method 5220D. Total solids (TS) and volatile solids (VS) of the BMP samples before and after the experiment were determined according to Standard Methods 1684.

The algae samples were prepared to be observed under an optical microscope by smearing a very little algae solution on top of a glass slide. The glass slide was placed under the lens of the optical microscope (Leica DM 2500 M). The lens of required magnification was chosen, and the focus was adjusted until a clear image was obtained. The image was then captured and saved.

Statistical analysis of replicate samples was performed using a one-way ANOVA test (OriginPro 2019) with a significant level of 0.05.

7.3 Results and discussion

7.3.1 Impact of harvesting methods on biogas production

Centrifuged and polymer-harvested *Scenedesmus* sp. biomass exhibited rapid CH_4 production in the first few days and a gradual increase until about day 12 of the BMP test (Figure 25). For each type of biomass (C, P, and P + OD), the three biological replicates showed relatively consistent CH_4 production (ANOVA, $p>0.05$). The inoculum (blank) samples also yielded a cumulative CH_4 production value of 156.6 ± 2.1 L CH_4/kg VS. Results in Figure 25 confirmed the healthy state of the inoculum's microbial community

for the BMP test. For simplicity, in all subsequent discussions, only CH_4 production from microalgal biomass was reported by subtracting the contribution from the inoculum.

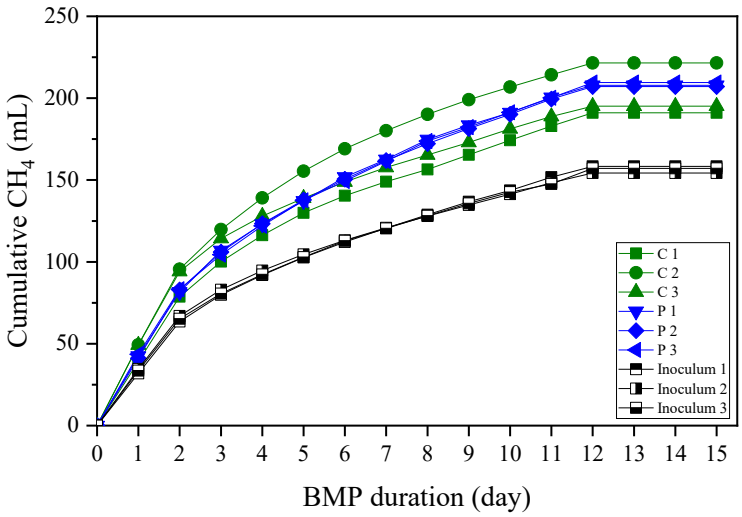

Figure 25. Cumulative CH_4 production over 15 days from anaerobic digestion of wet microalgae samples subject to different harvesting methods including the blank samples containing only the inoculum (1, 2 and 3 represent biological replicates for each treatment). C = centrifuge. P = polymer.

Daily CH_4 production was highest on day 2 of the BMP test across all microalgae samples (Figure 26a). Centrifuged biomass achieved a peak daily CH_4 yield of 18.0 ± 8.5 L/d.kg VS, which was 38% higher than that of polymer-harvested biomass at 13.0 ± 1.7 L/d.kg VS. From day 3, daily gas production of both centrifuged and polymer-harvested samples declined steadily and dropped to 1.3 ± 1.3 L/d.kg VS and 2.6 ± 1.5 L/d.kg VS by day 12. This observed difference in daily gas production in the early days between centrifugation and polymer harvesting can be attributed to the impact of harvesting methods on the algae membrane cell wall integrity. *Scenedesmus* sp. has a rigid cell wall that is recalcitrant to anaerobic digestion. The cell wall of *Scenedesmus* sp. consists of a trilaminar structure composed of cellulose, algaenan and glucosamine-containing biopolymers and glycoproteins (Inglesby et al., 2015). Polymer harvesting has been shown to have a lower

impact on cell membrane integrity compared to other methods, resulting in a higher number of intact cells post-harvesting (Labeeuw et al., 2021; Wu et al., 2015). In contrast, the shear stress induced by centrifugation causes more damage to green algae compared to flocculation, leading to a higher number of compromised algae cells and a reduced intracellular concentration of several metabolites such as amino acids, sugars, and polyols (Kuzhiumparambil et al., 2022). The disruption of microalgae cells enhances the digestibility of microalgae biomass since the recalcitrant cell wall is broken down. This explains the higher daily CH_4 production of centrifuged biomass at the beginning of the BMP experiment, as more biodegradable cellular components (e.g. carbohydrates and proteins) were readily available compared to polymer-harvested samples.

It is noteworthy that despite a slower initial digestion rate, polymer-harvested biomass achieved a cumulative CH_4 yield of 74.1 ± 1.7 L CH_4/kg VS, which is slightly higher than that (66.1 L ± 23.6 CH_4/kg VS) from centrifuged biomass (Figure 26b). These results show that the impact of harvesting methods on the overall cumulative CH_4 production from *Scenedesmus* sp. microalgae is negligible. The polymer used for microalgae harvesting can hydrolyse and become a substrate for CH_4 (Dai et al., 2014; Xiong et al., 2018). However, the polymer dosage is very small (<50 mg/g dry microalgae) in this study. Thus, the difference in CH_4 production observed here is mostly due to the harvesting method rather than the influence of the polymer itself.

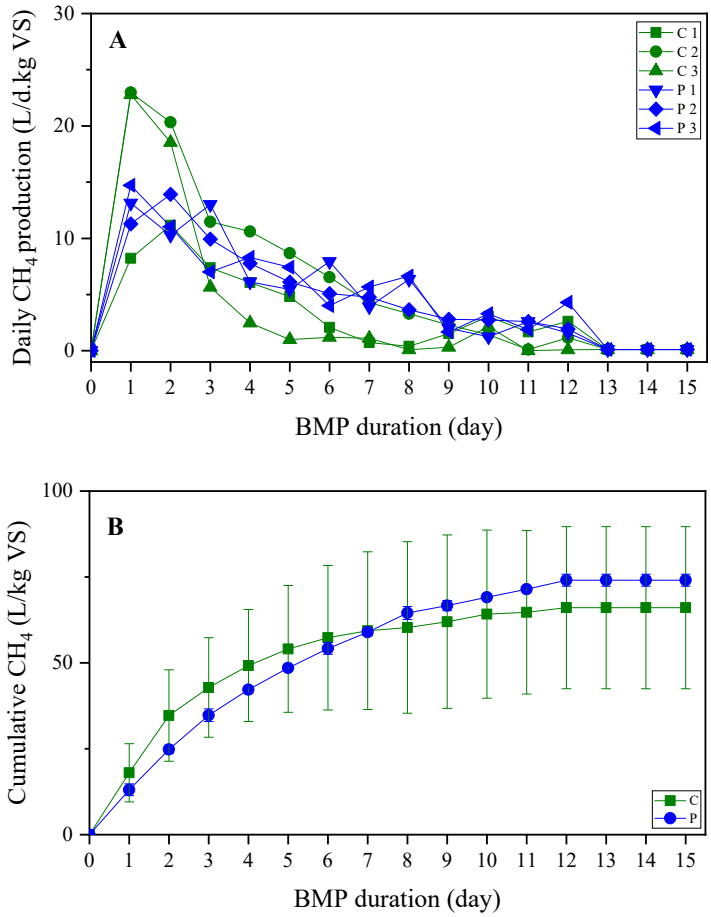

Figure 26. (a) Daily CH_4 production (1, 2 and 3 represent biological replicates) and (b) average cumulative CH_4 production of *Scenedesmus* sp. biomass subjected to different harvesting methods. C = centrifuge. P = polymer. Blank values had been deducted. Value and error bars represent the mean and standard deviation of three replicate experiments.

7.3.2 Pretreatment of wet and dry microalgal biomass

7.3.2.1 Pretreatment of wet microalgal biomass

When utilized as a pretreatment for wet microalgal biomass (P), oven drying (P + OD) demonstrated the most significant enhancement in CH_4 production, surpassing both IPA treatment (P + IPA) and microwave irradiation (P + MV). Oven-dried *Scenedesmus* sp. yielded cumulatively 134.9 ± 25.8 L CH_4/kg VS, marking an 82% increase compared to wet polymer-harvested biomass (Figure 27b). Pretreatment with IPA on wet polymer-

harvested biomass improved the CH_4 production by 23% to 90.7 ± 6.7 L/kg VS (Figure 27b). Microwave pretreatment, however, exhibited no enhancement in CH_4 yield for wet *Scenedesmus* sp. After microwave irradiation, the cumulative CH_4 production amounted to 49.8 ± 4.4 L/kg VS (Figure 27b), reflecting a 33% decrease compared to non-pretreated, wet biomass.

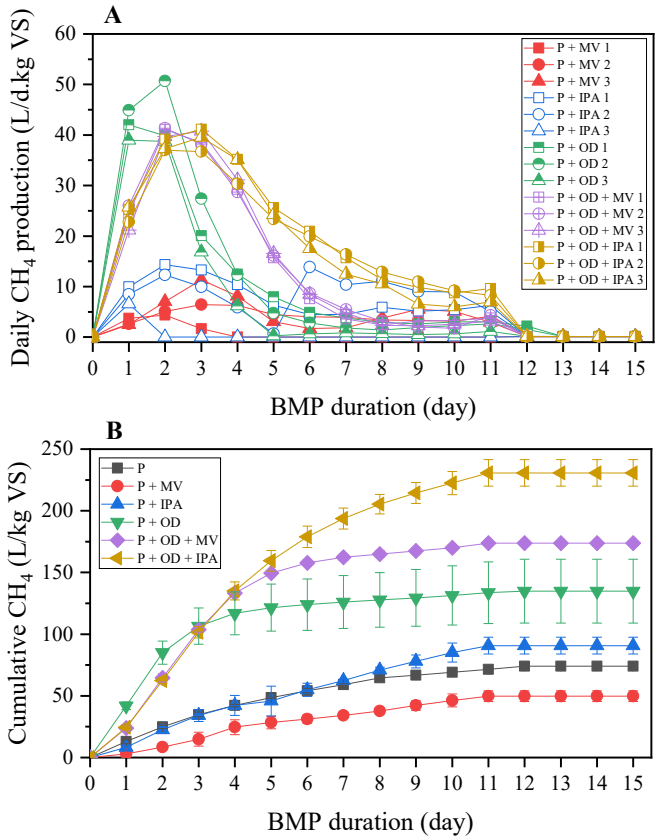

Figure 27. (a) Daily CH_4 production (1, 2 and 3 represent biological replicates) and (a) average cumulative CH_4 production of polymer harvested *Scenedesmus* sp. biomass subjected to different pretreatment methods. P = polymer. OD = oven drying. MV = microwave. IPA = isopropanol. Value and error bars represent the mean and standard deviation of three replicate experiments.

Oven drying stands out among various techniques, such as sun drying, freeze drying, and spray drying, used to eliminate bound water content in microalgae post-harvesting. This process, relying on diffusion, effectively removes trapped moisture within cell structures,

inducing potential cell shrinkage and membrane disruption (Hosseinizand et al., 2018). While conventional methods like sun drying have minimal energy and capital requirements, they are weather-dependent and prone to contamination (Aljabri et al., 2023). Freeze drying and spray drying, known for retaining crucial intracellular byproducts, entail high operational and maintenance costs, making them more suitable for high-value applications. However, for cost-effective biofuel applications, oven drying, with its lower energy and capital demands, emerges as a more fitting choice. Notably, in addition to effective cell membrane disruption, oven drying at 60–80°C could preserve more lipid and carbohydrate content of microalgae, crucial substrates for anaerobic digestion (Amin et al., 2021; Hosseinizand et al., 2018). In this study, oven drying the polymer harvested *Scenedesmus* sp. biomass before the BMP test led to more efficient decomposition of microalgal organic materials, evident in the majority of CH_4 being produced within the initial 5 days, followed by a gradual decline of 1-3 L CH_4/d.kg VS (Figure 27a).

Both microwave irradiation and IPA have been extensively studied for microalgal cell disruption and extraction (Huang et al., 2015; Lage et al., 2021; Wahidin et al., 2014). IPA is capable of penetrating microalgae biomass to dissolve and release intracellular compounds but typically requires longer reaction times, ranging from 30 minutes to several hours (Miazek et al., 2017). A pretreatment step to disrupt the cells also often preceded IPA exposure to enhance the extraction yield (Yao et al., 2013). In this study, IPA alone was not effective enough to break down the cell walls of *Scenedesmus* sp., which was tangled in the polymer matrix, resulting in only a minor improvement.

Microwave treatment is known for its ability to generate rapid localized heating due to intense dipole reactions and molecular collisions, exerting high pressure on cell walls and

causing cell disintegration (Rana & Prajapati, 2021; Wahidin et al., 2014). In this study, microwave pretreatment did not enhance CH_4 yield from polymer-harvested biomass. By contrast, when applying microwave pretreatment at high energy intensity, Passos et al. (2015) achieved a 21% increase in CH_4 yield at a specific energy of 34.3 MJ/kg using a microalgal biomass concentration of 32 g/L. Passos et al. (2013) reported a 12-78% higher CH_4 yield with a higher microwave-specific energy (21.8-65.4 MJ/kg) applied to 16.5 g/L microalgae biomass. Another study by the same author group demonstrated a 60% increase in CH_4 yield by pretreating mixed culture microalgae using microwave irradiation at 35 MJ/kg (Passos et al., 2014b). The observed discrepancy between this study and the literature may also arise from differences in microalgae species, harvesting methods (polymer versus settling), and harvested biomass concentration (200 g/L vs 15-20 g/L). Further investigation is necessary to determine if the polymer matrix from polymer harvesting impacts the efficiency of microwave pretreatment for CH_4 production.

7.3.2.2 Pretreatment of dry microalgal biomass

The effectiveness of microwave and IPA pretreatment exhibited a notable improvement when applied to oven-dried *Scenedesmus* sp. (P + OD). Both microwave-treated (P + OD + MV) and IPA-treated (P + OD + IPA) samples achieved their peak daily CH_4 production on days 2 and 3 of the experiment, reaching 41.4 ± 2.3L CH_4/d.kg VS (Figure 27a). However, IPA-treated dried biomass continued to yield 6-10 L CH_4/d.kg VS daily until day 12, whereas the daily production of microwaved biomass significantly declined to 2-4 L CH_4/d.kg VS after day 7. By the end of the test, the cumulative CH_4 production for dried, IPA-treated *Scenedesmus* sp. (P + OD + IPA) reached 230.7 ± 10.7 L/kg VS,

representing a 33% increase compared to dried, microwaved biomass (P + OD + MV) (Figure 26b).

7.3.3 Optimal CH_4 yield from *Scenedesmus* sp. Biomass

7.3.3.1 Optimal pretreatment method

Overall, the combination of oven drying, and IPA pretreatment could significantly enhance CH_4 production from polymer harvested *Scenedesmus* sp. biomass. A remarkable increase in BMP of 212% was observed in drying-IPA combined pretreatment, followed by oven drying and microwave combination, which saw a 135% enhancement (Figure 28). This enhanced effect can be primarily attributed to oven drying, which acted as an additional cell disruption mechanism, thereby improving the effectiveness of subsequent pretreatment.

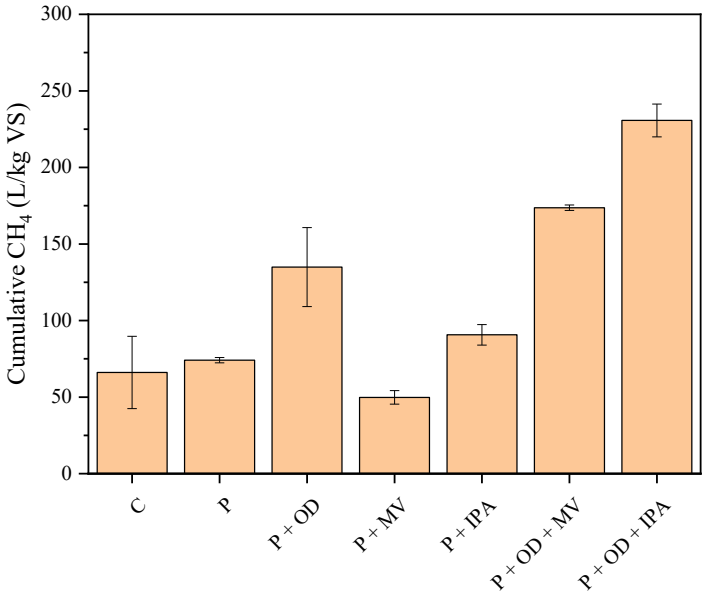

Figure 28. Methane production from anaerobic digestion of microalgae samples subjected to different harvesting and pretreatment methods. C = centrifuge. P = polymer. OD =

oven drying. MV = microwave. IPA = isopropanol. Value and error bars represent the mean and standard deviation of three replicate experiments.

The capacity to break down the robust cell walls of *Scenedesmus* sp., entangled in the polyacrylamide polymer matrix, appeared to be compromised for both IPA and microwave pretreatments, especially when conducted without the preceding oven-drying step. Microscopic images of microwaved and IPA-pretreated wet biomass displayed nearly intact cells, similar to non-pretreated *Scenedesmus* sp. (Figure 29). The *Scenedesmus* sp. in this study exists in coenobia of four cells attached (Aditya et al., 2023). Microscopic images of wet *Scenedesmus* sp. after harvesting and pretreatment showed mostly individual cells and a few clusters of two or three cells (Figure 29). Polymer harvesting has caused colonised cells to detach but not break down, thus inducing minimal enhancement in CH_4 production from *Scenedesmus* sp. Conversely, the microscopic image of oven-dried biomass showed severely deformed *Scenedesmus* cells and dispersed cell fragments (Figure 29). This is consistent with previous observations from Aljabri et al. (2023) and Behera and Balasubramanian (2021), who have reported that the diffusion of water molecules out of microalgal cells due to oven drying led to cell shrinkage and eventually cell collapse. Consequently, the microscopic image of pretreated, dried biomass (P + OD + MW and P + OD + IPA) also showed significantly deformed cells, leading to better digestibility and higher CH_4 production.

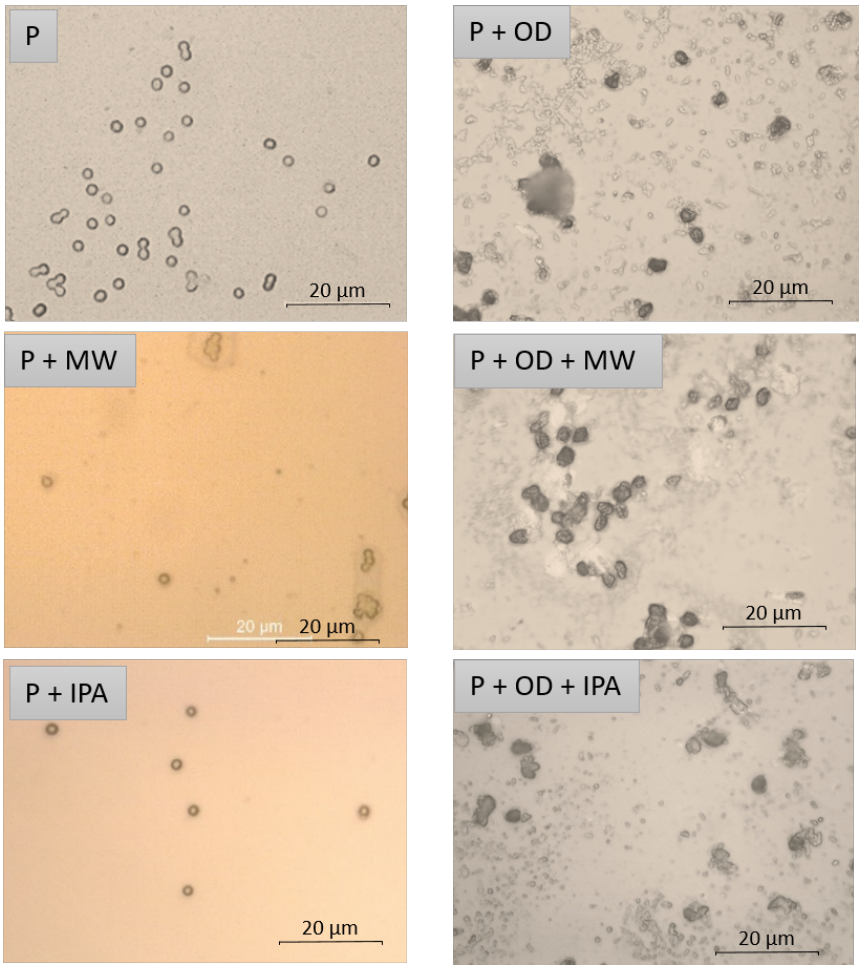

Figure 29. Microscopic images of *Scenedesmus* sp. subjected to polymer harvesting, drying and pretreatment. P = polymer. OD = oven drying. MV = microwave. IPA = isopropanol.

7.3.3.2 Comparison with literature values

Results in this study are consistent with the BMP of *Scenedesmus* sp. observed in the literature with comparable experimental protocols (Table 11). Higher BMP values reported in other studies for non-pretreated biomass are likely to stem from variations in harvesting methods, substrate composition and sludge retention time. However, the BMP of non-pretreated *Scenedesmus* remains relatively low compared to other substrates such as lignocellulosic biomass (200-350 L CH_4/kg VS) (Thomsen et al., 2014). To enhance the economic viability of microalgae in biofuel production, it is imperative to improve

the digestibility of microalgae cells without significantly increasing costs. A combination of multiple harvesting methods (e.g., flotation, flocculation, and centrifugation) has been explored to improve microalgae's biomethane potential (Candia-Lomeli et al., 2021; González-González et al., 2018), but it may substantially raise overall costs. This study presents a promising solution to this challenge through the application of oven drying and IPA pretreatment before anaerobic digestion of microalgae. Polymer-harvested *Scenedesmus* biomass subjected to oven drying and IPA or microwave pretreatment showed significant improvement in CH_4 production, reaching 230.7 ± 10.7 L CH_4/kg VS. This value surpasses all previously reported figures for both non-pretreated and pretreated *Scenedesmus* sp. and is comparable to CH_4 potential observed in lignocellulosic biomass (Table 11).

Table 11. Comparison of BMP values for *Scenedesmus* from literature and this study

Substrate(s)	**Harvesting method**	**Pretreatment**	**CH_4 yield (L/kg VS)**	**Conditions**	**Reference**
Scenedesmus sp.	Centrifugation	No	66	38 °C 15 days	This study
Scenedesmus sp.	Polymer	No	74	38 °C 15 days	This study
Scenedesmus sp.	Polymer	IPA	91	38 °C 15 days	This study
Scenedesmus sp.	Polymer	Oven drying	135	38 °C 15 days	This study
Scenedesmus sp.	Polymer	Oven drying + Microwave	174	38 °C 15 days	This study
Scenedesmus sp.	Polymer	Oven drying + IPA	231	38 °C 15 days	This study
Scenedesmus sp.	N/A	No	54	37 ± 1 °C 10 days	(Inglesby et al., 2015)

Scenedesmus obliquus	Centrifugation	No	67	35 °C 15 days	(Mai et al., 2022)
Scenedesmus sp. and *Chlorella* sp.	Centrifugation	No	82	35 °C 30 days	(Barreiro-Vescovo et al., 2018)
Scenedesmus sp. and *Chlorella* sp.	Sedimentation	No	107	35 ± 1 °C 40 days	(Zhen et al., 2016)
Scenedesmus sp.	Centrifugation	No	154	35 °C 46 days	(Kinnunen & Rintala, 2016)
Scenedesmus obtusiusculus	Flocculation and centrifugation	Oven drying	155	35 °C 30 days	(Candia-Lomeli et al., 2021)
Scenedesmus sp.	N/A	Bead milling	97	37 ± 1 °C 10 days	(Inglesby et al., 2015)
Scenedesmus sp.	Sedimentation	Ultrasound	154	35 °C 34 days	(Gonzalez-Fernandez et al., 2013)
Scenedesmus sp.	N/A	Thermoalkaline	160	35 °C 38 days	(Mahdy et al., 2014)

7.4 Conclusions

This study provided new insight into the potential of polymer-harvested *Scenedesmus* sp. as a sustainable feedstock for CH_4 production through anaerobic digestion. The 15-day BMP test of polymer-harvested *Scenedesmus* revealed that oven-dried biomass produces significantly higher CH_4 than wet biomass. CH_4 yield was further increased by a total of 135-212% when subjecting dried biomass to pretreatments (microwave or IPA), contrary to 0-22% enhancement induced by pretreatments on wet biomass. IPA pretreatment of *Scenedesmus* sp. particularly resulted in higher CH_4 production than microwave irradiation. This underscores the possibility of employing outdated/waste hand sanitiser

and liquid disinfectant to enhance the CH_4 production of microalgae harvested by the cationic polymer.

CHAPTER 8. CONCLUSIONS AND RECOMMENDATIONS

8.1 Conclusions

New data and knowledge from the thesis have contributed to improving microalgae harvesting and utilization for the production of valuable biomolecules and biomethane. The investigation into flocculation efficiency, using various flocculants and combinations thereof, revealed crucial insights into optimizing microalgae harvesting, thus reducing cost. This thesis also demonstrated the potential of using whole microalgae biomass as a substrate for anaerobic digestion to produce biomethane.

The investigation into the flocculation efficiency of microalga *C. vulgaris* revealed notable insights for effective harvesting (Chapter 3). Individual flocculants, including synthetic polymer (FlopamTM) and inorganic salts (ferric chloride and aluminium sulphate), along with chitosan, demonstrated varying efficiency levels. While synthetic polymer achieved over 90% optical density removal at a low dose through bridging and charge neutralization mechanisms, individual inorganic salts and chitosan exhibited lower efficiency despite higher doses. The combination of inorganic flocculants and chitosan, however, produced a synergistic effect, yielding >80% flocculation efficiency—57% and 24% higher than the sum of individual ferric chloride/chitosan and aluminium sulphate/chitosan flocculation, respectively. This synergy resulted from the interplay between charge neutralization by inorganic salts and the bridging mechanism by chitosan. The dual flocculation approach not only enhances harvesting efficiency but can also reduce the costs compared to chitosan-only flocculation and mitigate microalgal cell contamination from inorganic salts.

Chapter 4 examined the harvesting efficiency of *P. purpureum*, a red marine microalga with high pigment and fatty acid content, by polymer flocculation. Polyacrylamide polymers, specifically Flopam^{TM} and FO3801, demonstrated superior flocculation efficiency exceeding 99% at an optimal dose of 21 mg per g of dry for *P. purpureum*. Conversely, alkaline flocculation (sodium hydroxide, potassium hydroxide, and sodium carbonate) achieved up to 98% efficiency but required impractical and ineffective doses of over 500 mg/g dry biomass. Calcium hydroxide proved less effective, achieving only 75% flocculation efficiency, attributed to the precipitation of magnesium hydroxide causing hydroxide-induced flocculation. Sodium carbonate-induced flocculation operated through co-precipitation of magnesium and calcium carbonate, resulting in a sweeping effect that enmeshed microalgal cells, triggering sedimentation. Cell membrane integrity analysis revealed that polyacrylamide polymers compromised the membrane of 96% of microalgal cells, representing the most significant negative impact on *P. purpureum* cells among the chemicals tested. This susceptibility was attributed to the unique cell characteristics of *P. purpureum*, which lacks a rigid cell wall, rendering it more prone to damage. Consequently, the impact of polyacrylamide polymers on the harvested biomass quality is contingent on the specific microalga species, its cell wall characteristics, and operational parameters.

As microalgae progress through growth phases – early exponential, late exponential, and stationary – their biochemical properties and culture media composition change, particularly a drop in phosphorus. This thesis revealed a significant increase in flocculation efficiency as the microalgae culture matured (Chapter 5). Notably, unlike the detrimental impact of phosphate on traditional wastewater treatment flocculation, phosphorous residue exhibited no influence on *C. vulgaris* flocculation efficiency. The

observed dependency of flocculation efficiency on the growth phase was attributed to changes in microalgal cell properties. At the stationary phase, microalgal extracellular polymeric substances (EPS), in both bound and free forms, were two and three times higher than those at late exponential and early phases, respectively. Additionally, microalgae cells became more negatively charged as they matured. The combination of negatively charged and high EPS content, along with the addition of a high molecular weight and positively charged polymer, facilitated effective flocculation through charge neutralization and bridging mechanisms. The dependency of flocculation efficiency on the growth phase, driven by changes in cell properties, underscores the importance of understanding microalgal biology for optimizing harvesting strategies in industrial applications.

This thesis explored for the first-time possible application of expired COVID–19 alcohol-based hand sanitiser in anaerobic co-digestion for enhanced biogas production (Chapter 6). The experiments, conducted in three parallel continuous flow anaerobic digesters, underscore the importance of acclimatization to prevent process instability. Process instability was initially observed when co-digesting ethanol-based sanitiser at 0.3% v/v with sewage sludge without acclimatization, but recoverability was achieved, and stable performance was maintained even at 0.6% v/v ethanol concentration. The digester previously acclimatized to ethanol-based sanitiser exhibited a 20% higher biogas production compared to a non-acclimatized digester. The study also revealed a threshold of organic loading rate (3.5–4 gCOD/L.day) due to the accumulation of volatile fatty acids, potentially inhibiting the methanogenesis process.

Another application of expired COVID–19 disinfectant/sanitiser as a pretreatment for *Scenedesmus* sp. to enhance biomethane production from whole biomass was investigated

(Chapter 7). Biochemical methane potential test over 15 days revealed harvesting methods (centrifugation versus polymer harvesting) minimally affected overall CH_4 yield. Drying the microalgae significantly increased CH_4 yield compared to wet biomass, attributed to cell shrinkage, and enhanced digestibility. CH_4 yield was further increased by 135-212% when subjecting dried biomass to pretreatments (microwave or IPA), highlighting the effectiveness of these treatments on dried biomass compared to wet biomass (0-22% enhancement). Notably, IPA pretreatment resulted in higher CH_4 production than microwave irradiation, emphasizing the possibility of using outdated or waste hand sanitiser and liquid disinfectant to enhance CH_4 production from microalgae harvested by the cationic polymer.

In summary, this thesis presents a comprehensive and integrated approach to harnessing the potential of microalgae for biomethane production, addressing key challenges, and providing innovative solutions. The findings contribute significantly to the broader field of renewable energy and underscore the viability of microalgae-based biomethane as a sustainable and economically feasible energy source.

8.2 Recommendations for future work

This thesis has effectively showcased the prowess of polymer harvesting in microalgae biomass recovery for biomethane production. However, there are other avenues for further exploration within microalgae characteristics and biomass processing to optimize the feasibility of harvesting.

Chapters 3 and 4 highlighted the outstanding harvesting efficiency achieved with commercial polyacrylamide polymers for both freshwater and marine microalgae. Building on the insights into polymer flocculation mechanisms from Chapter 5, there is

an opportunity to delve into the production of tunable polymers at lower costs. Investigating optimal monomers and polymerization techniques to achieve varying molecular weights and charge densities could significantly enhance the techno-economic viability of microalgae harvesting across diverse microalgae species.

The feasibility of using polymer-harvested microalgae biomass for biomethane production at the lab scale was demonstrated in Chapter 7. However, expanding this research to include further exploration of pretreatment methods and conducting investigations on larger scales is imperative to garner a comprehensive understanding of the process and its commercial viability.

To further enhance the economic efficiency of biomethane production from microalgae, the possibility of recycling waste streams from the harvesting process (i.e., water) and anaerobic digestion (e.g., nutrient-rich digestate) should be explored. This approach establishes a dynamic loop of sustainable bioenergy and biochemical production from waste, aligning with the principles of a circular economy derived from microalgae. Such investigations will contribute substantially to the advancement of sustainable practices in microalgae-based biomethane production.

Upon a better understanding of the technical aspects surrounding microalgae production and CH_4 generation, it is suggested that a comprehensive cost analysis or life cycle assessment be conducted. This comprehensive evaluation is crucial to affirm the commercial viability of the research concept. By delving into the economic and environmental factors associated with the entire lifecycle of microalgae production and CH_4 generation, this assessment will provide insights into the feasibility of translating the research findings into a commercially sustainable endeavour. Such a rigorous analysis

serves as a pragmatic approach to ensure that the promising research concept in this thesis and further studies aligns with practical and economic considerations, laying the groundwork for successful integration into real-world applications.

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