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The Effect of Steam Explosion on Lipids Extraction From Microalgae and Derivation of Pectin Films from Waste Culture

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ABSTRACT

The objective of this study was to investigate the properties of sustainable replacements for plastics and diesel fuel, derived from microalgae (*Chlorella vulgaris*) subjected to steam explosion. During the process, oven temperatures of up to 500 °C were reached, with the experiments left for different times after reaching their maximum internal temperature. Lipids were extracted from algal cultures using a modified Folch method. The waste microalgae were combined with pectin and glycerol to form biodegradable films, and their solubilities and tensile strengths were measured. The highest yield was 124 mg lipids/g microalgae from 400 °C steam explosion for 45 minutes after reaching maximum conditions. The highest tensile strength of the pectin films was 10.8 MPa, from 400 °C steam exploded microalgae. Overall, steam explosion can improve lipid accessibility and tensile properties of pectin films, and a major recommendation was to perform a life cycle analysis on the entire process to determine energy balances and its impact on the environment.

Keywords: steam explosion, microalgae, algal biodiesel, pectin films, lipid extraction

SUMMARY FOR LAY AUDIENCE

The use of plastic and diesel made from crude oil has severely affected the health of people around the world, as well as the health of the water and land surrounding them. Alternatives from microalgae are promising, as they greatly reduce land use and hence reduce the burden on the environment. Examples of these products are biodiesel and bioplastics, both of which were researched in this study, and are completely biodegradable. To produce biodiesel, molecules known as lipids must be reacted with an alcohol. However, in *Chlorella vulgaris*, a type of microalgae, these lipids are protected by a thick cell wall which makes it difficult to extract them. Steaming the microalgae under high temperature and pressure, then quickly decreasing the pressure broke the cell walls and allowed more of the lipids to be extracted. This technique is known as steam explosion. After lipids were extracted from steam exploded microalgae, the remaining debris was then combined with glycerol and pectin to produce bioplastic films.

ACKNOWLEDGEMENTS

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CHAPTER 1: INTRODUCTION

1.1 Motivation for Research

While plastics and gasoline have their use and are widespread across the globe, the main issue with them are their impacts on human and environmental health. Plastic production in particular has grown more than 20 times in the last 50 years, and the total amount of plastic produced is around 9,200 megatons (Mt), of which a staggering 6,900 Mt is sent to landfills and contributes to environmental pollution. In 2019 alone, 368 Mt of plastic was produced, and this amount is expected to increase twofold in the next 20 years [1]. Conversely, due to a boom in industrialisation in the 19th century and its resulting search for fossil fuels to use in machinery [2], as well as an increasing global energy demand and limited fossil fuel reserves [3], petroleum fuels are becoming scarcer as time passes. As of 2008, the global consumption of fossil fuel was 85 million barrels of oil and 260 billion cubic feet of natural gas per day, with reserves amounting to 40 and 64 more years of oil and natural gas respectively [4]. Currently, the global oil consumption is predicted to be around 100 million barrels per day [5]. Not only is there concern for the scarcity of fossil fuels, but its consumption also generates a significant amount of greenhouse gases (GHGs). As of 2016, 38.2 gigatons (Gt) of carbon dioxide (CO_2) were emitted, and it has been projected that the emissions will rise 2% each year. 90% of these emissions are from fossil fuel and industry use [6], and since plastics comprise of 6% of global oil consumption [1], it is estimated that around 2.06 Gt of CO_2 were emitted solely from the processes involved in plastic production in 2016. The effects of an increased level of GHGs are well documented and contribute towards a climate tipping point: that is, the level in the climate beyond which drastic changes continue to occur without further forcing. An example of one such effect is the melting of the ice in the Arctic [7]. Besides climate change, there are a myriad of

detrimental health and environmental concerns associated with the consumption of fossil fuel and the production of petroleum plastics. Acute exposure to diesel exhausts has shown to cause pulmonary inflammation, whereas long-term exposure presents a higher risk of cough, phlegm and chronic bronchitis. Additionally, exposure to diesel particulate matter has shown to exacerbate pre-existing lung conditions and cause respiratory infections and cancer [8]. Similar health effects have been shown for inhalation of airborne microplastics [9]. Moreover, besides human health, plastics and microplastics have an adverse effect on the environmental as well. With microplastics able to alter the temperature of a marine ecosystem, research has shown this affects turtle hatcheries, distorting to male to female ratios in these populations. Coral species have also gone extinct due to the changes caused by marine plastic pollution. The effects of plastic pollution are widespread beyond oceanic environments and are effectively summarised by S. Sharma et. al:

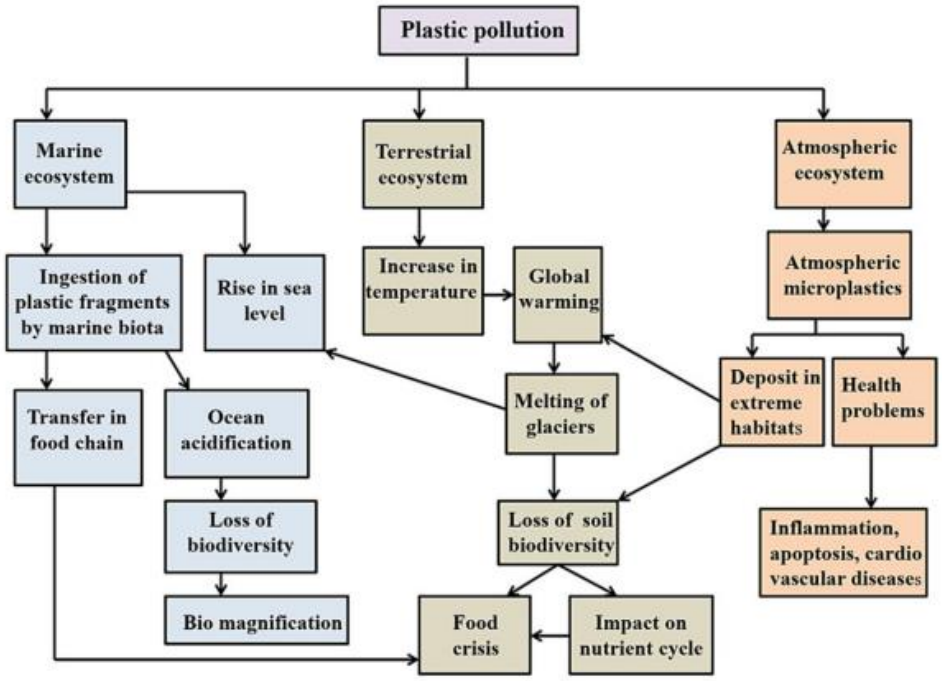


Figure 1-1: A summary of the effects of plastic pollution, by Sharma et. al [10]

Diesel pollution has similar effects to the environment; it does damage to marine, atmospheric, and terrestrial systems. Besides GHG emissions, the primary atmospheric pollution attributed to diesel is a reduction of visibility from particles generated from conversion of nitrogen oxides (NO_x), sulphur dioxide (SO_2), ammonia and hydrocarbons. Nitric acid and sulphuric acid rain from deposition of NO_x and SO_2 in the atmosphere affects the health of agricultural crops, large bodies of water and forests. It was also shown that particle deposition was the major source of chemical contaminants in the Great Lakes. Diesel fuel spills in water bodies have increased mortality of aquatic organisms, impaired their reproduction, decreased their growth rates and increased their susceptibility of diseases. Finally, the deposition of particles can cause aesthetic pollution to structures and culturally important articles. Soot promotes corrosion of metal, and the costs associated with soiling are significant [11]. To summarise, the effects of petroleum and plastic pollution are detrimental and widespread, and replacements must be found urgently. Such replacements are biodiesel and bioplastics or biocomposites, and these may be sourced from microalgae.

1.2 Objectives

The overall objective of this research was to investigate the effect of temperature and duration of steam explosion on the amount of lipids accessible from cultures of *Chlorella vulgaris*. Three specific objectives were to:

- Determine the optimum condition for steam explosion and investigate the use of enzymes and combined enzymatic and steam explosion treatment on lipid accessibility.
- Produce algal biodiesel from the optimum treatment.

- Make pectin films from waste microalgae once their lipids have been extracted and determine the effect of steam explosion on the tensile strength and water solubility of these films.

1.3 Novel Contributions

As far as we are aware, this is the first study which simultaneously increases the yield of lipids from microalgae via steam explosion and produces pectin films from the process waste. Searches for '"steam explosion" "pectin film" "microalgae"' yields no results in the university library, nor any similar studies from Google. Changing "pectin film" to "bioplastic" shows no identical studies from either source. This research also adds to the repertoire of studies which include microalgae in pectin films and bioplastics, and could provide information for further studies in pilot-level waste-to-value algal plants.

1.4 Structure of Thesis

The current chapter has included the motivation and significance of the research, as well as the objectives of this study. The second chapter will provide background knowledge on the concepts related to this research, summarise recent literature and identify a few examples of current technology, as well as knowledge gaps in the field of lipid extraction and algal biodiesel. The third chapter will briefly go over the equipment and methods used in the study. The fourth chapter will discuss the results of the study and provide possible explanations for them. Lastly, the fifth chapter will draw conclusions from the results and make recommendations for further research. The bibliography and appendices, which contains supplementary information not included in the principal study, will follow.

CHAPTER 2: LITERATURE REVIEW

2.1 Background

2.1.1 Microalgae and *Chlorella vulgaris*

Microalgae are a group of microorganisms that are either unicellular or simple multicellular. They are photosynthetic and exist in all ecosystems across the globe [12]. They are advantageous over microbes, which require a reduced carbon source to meet their energy needs [13], as they only require sunlight (or ultraviolet light, described in later sections) and simple nutrients such as sulphur, nitrogen, phosphorus and CO_2, for their growth [12]. A particular species of microalgae, *Chlorella vulgaris*, is one of the most studied and many of these species have numerous benefits. The fatty acids of *Chlorella* can be used as feedstock for fuels and their proteins as food supplements. The dietary intake of their pigments has shown health-boosting benefits, and their carbohydrates can be converted to bio-ethanol or other value added molecules. However, a major challenge in extracting the beneficial compounds of *C. vulgaris* is the disruption of its cell wall. Though numerous studies have been conducted on the biochemical composition of *C. vulgaris*' cell walls, their structure, development and composition are not yet fully understood. Though, it is known that at the beginning of their growth phase, a microfibrillar layer is developed. As the cells grow, the wall develops into a three-layer structure, shown as follows [14]:

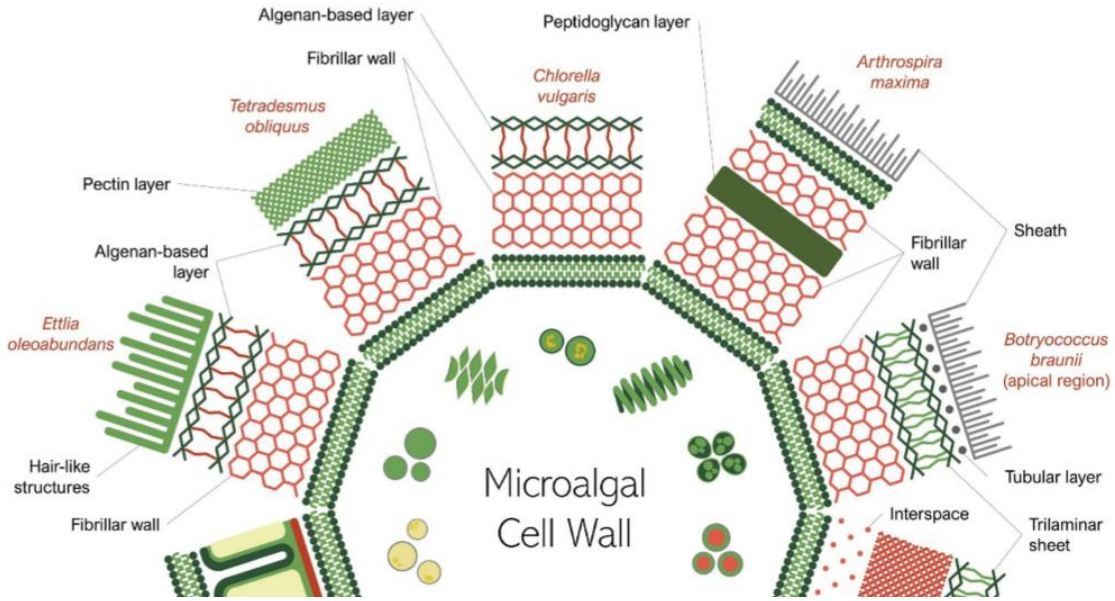


Figure 2-1: Chemical structure and composition of various species of microalgae [15]

The first layer, described in this section as the bottom-most layer, is a simple phospholipid bilayer [16]. The second layer, directly on top, is more rigid, consisting of polysaccharides similar to chitin and chitosan, galactose and rhamnose. It also consists of 20-25% neutral sugars, 15-20% uronic acids, 7-17% glucosamine and 6-10%, while the rest is unknown [14]. The polysaccharides in the second layer are not to be confused with extracellular polysaccharides (EPS), which are secreted by microalgae into their surrounding environment. They help create a polymer network for cells to interact with each other and assist with their adhesion to surfaces. The EPSs of *C. vulgaris* are mainly polymers of glucose, xylose and glucuronic acid [17]. Finally, the outermost layer is made of a resistant, hydrocarbonaceous, non-hydrolysable biopolymer known as algenan or sporopollenin. This polymer consists of thirty- to forty-carbon mono- or di-unsaturated omega-hydroxy fatty acids linked by ester, ether or glycosidic bonds. The presence of this durable polymer reduces the susceptibility of *C. vulgaris* to degradation via mechanical, chemical or enzymatic methods [18].

2.1.2 Steam Explosion

Steam explosion is a relatively new technology, and the first instance of it found in literature dates back to 1983, where it was known as auto-hydrolysis explosion and used in the pre-treatment of sugarcane bagasse [19]. It is one of the most promising methods of pretreatment due to its low environmental impact and reduced energy consumption. Saturated steam at high pressure is injected into a batch or continuous reactor with the required biomass for a short duration. There are two main steps to the process: steam boiling, which uses high temperatures to promote hydrolytic breakdown of a lignocellulosic matrix, and an explosion phase, where thermal energy is converted into mechanical energy. During the latter phase, the sudden pressure drop within the vessel leads to vapour expansion within the fibers of the cell wall, which leads to its break down [20]. Currently, it is mostly used for pre-treatment of lignocellulosic biomass, such as wood waste, fruit bunches and cotton stalks [21] The process can be visualised as follows:

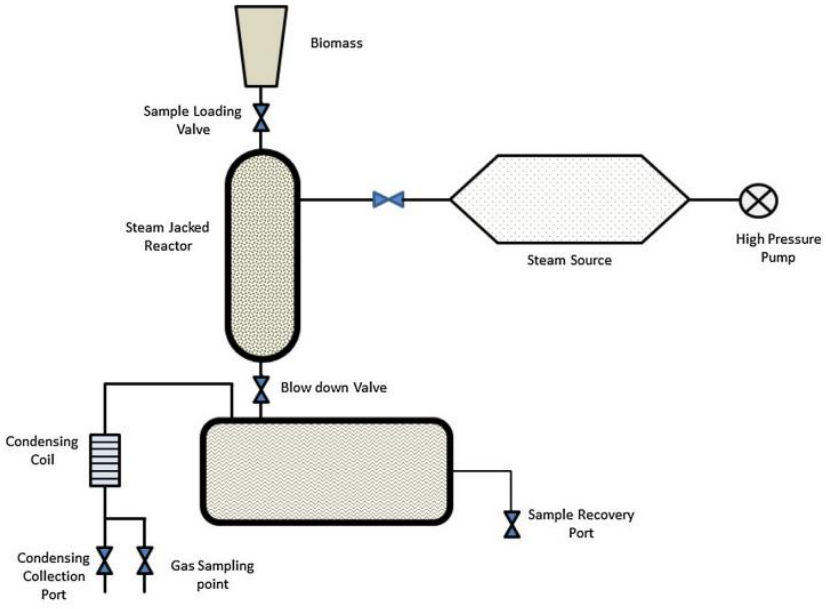


Figure 2-2: The basic components of a steam explosion process [22]

The process has also been used for pretreatment of microalgae, where its main advantages are the accessibility of lipids without release of hazardous wastes and the relatively low cost maintained during its operation. Additionally, it avoids the high costs of chemicals and enzymes, and the high energy demands of mechanical and ultra-sonic pretreatment. Its major technical challenge is that its efficiency depends on the species of microalgae [21].

2.1.3 Biodiesel

Early on in history, it became apparent that alternative fuels were needed and even during World War II, the search of “alternate biogenic fuels” was underway. Research only intensified during the energy crisis of the 1970s. The earlier use of vegetable oils in diesel engines were problematic due to their high viscosity, and it became ‘academically necessary’ to split of the triglycerides from the oils and run on the residual fatty acid. Further research in Belgium during 1938 produced what is the first documented instance of biodiesel by swapping the glycerol for ethanol and generating ethyl esters of palm oil via a transesterification reaction. This influenced further research and in the 1980s, South African researchers produced methyl esters of vegetable oil which remedied the issue of high viscosity seen prior. Today, biodiesel is generally known as a mono-alkyl ester of tri-acylglycerol-containing feedstocks. The reversible reaction can be visualised as follows [2]:

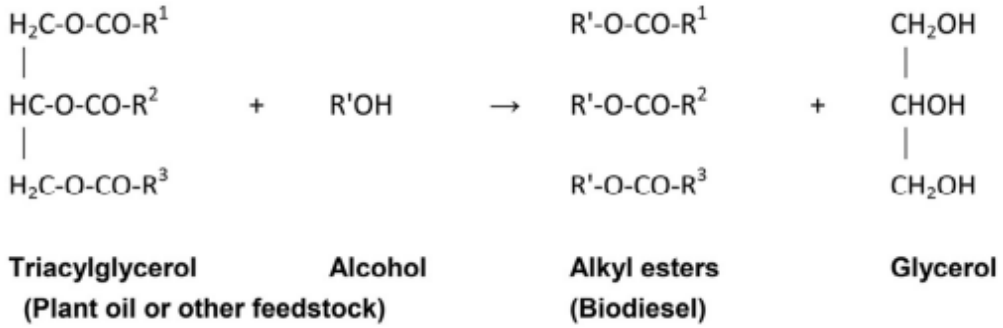


Figure 2-3: Chemical structure of components involved in transesterification [2]

Typically, the reaction also involves the addition of a strong acid or strong base catalyst, though base catalysts typically give a faster reaction. The optimal conditions are a molar ratio of 6:1 of alcohol to oil, 60 °C, 1 hour reaction time and either 1% sodium hydroxide or 0.5% sodium methoxide as a catalyst for reactions involving methanol and vegetable oils as feedstocks. The biodiesel also typically requires washing with water after the reaction is complete, and on an industrial scale, the methanol and glycerol may be recovered with separation columns [2]. The main advantages of biodiesel directly counter the issues brought up in the previous section: emissions from prototype vehicles using biodiesel showed a 94% reduction in particulate matter, decreases dependency on oil reserves while increasing the markets for the local farm economy, and degrades four times faster than petroleum-based diesel thus reducing its persistence in the environment. Other advantages include its ease of transport and storage, and its higher lubricity which extends engine life and reduces maintenance costs [3]. However, first-generation and second-generation biodiesels, fuels created from edible (e.g., palm, coconut, soybean) and non-edible oils (e.g., Jatropha, Neem, rubber seed) respectively, come with their challenges. First-generation biodiesels limit a food supply and create a choice between food or fuel for the population, and their feedstocks have restricted areas of cultivation and adaptability to climatic conditions [23]. Additionally, the presence of water or free fatty acids (FFAs) in feedstocks could

lead to saponification, which is why a second step of esterification with an acid-catalyst may be needed to reduce FFA content [3]. Second-generation biodiesels have the issue of lower yield and, with first-generation fuels, may require fertiliser which increases NO_x emissions. More general challenges are low volatility and energy content, poor spray characteristics and high cloud points. To combat these challenges, engines are modified and additives are used in fuels, but the feedstock itself could also be varied. This is where third-generation biodiesels come in, and the sources for the oil used in its production are not from crops. Waste oils, such as those from restaurants, soap factories or fish farms, can significantly reduce the cost of feedstocks [23]. Oils may also come from microbes such as yeast, fungi and bacteria, or they can come from microalgae [3]. Microalgae in particular have the advantage of low culturing cost, adaptability to harsh climates and high lipid yields of up to 70% dry weight, and 90% in specific conditions [23].

2.1.4 Pectin Films and Bioplastics

At around the same time biodiesel research was underway, bioplastic research began in the 1960s. Researchers had already identified the need to reduce the impact of conventional plastics on the environment and had begun the search for replacements. Plastics that were sourced from renewable resources, such as plants, animals and microorganisms, and made from any biological material other than fossil fuels were being developed, and these became known as bioplastics. However, it is important to differentiate between biodegradable plastics and bioplastics, as the latter may include plastics that come from natural resources but are not biodegradable, such as bio-polyethylene-terephthalate. The converse is also true, where plastics may be biodegradable but not sourced from natural materials, such as polybutylene-adipate-terephthalate [24]. This being said, a major advantage of bioplastics is their biodegradability, which limits its carbon

footprint and alleviates the issues associated with it (discussed in Chapter 1). They may also be reclaimed at the end of their product life cycle and energy may be recovered from them (e.g., by use in the generation of syngas), making them a potential renewable resource [25]. The main challenges of bioplastics are their limitations in mechanical and thermal properties compared to traditional plastics, and their high cost of production [24]. For example, as of 2017, the cost of conventional plastics was 1.3-1.7 USD/kg, whereas poly(3-hydroxybutyrate) cost 5.5 USD/kg and polylactic acid filaments were priced at 21 USD/kg [25]. One polymer to combat these challenges is pectin: it is abundant in citrus waste, which is a cheap feedstock [26], and progress has been made to improve the toughness of pectin films [27], giving them more applications. Pectin is usually available as a powder, and possesses a self-binding ability: under certain temperatures, it denatures and dissociates, which leads to the formation of new bonds and the aggregation of the polymer to new forms [26]. This can be seen in the following diagram:

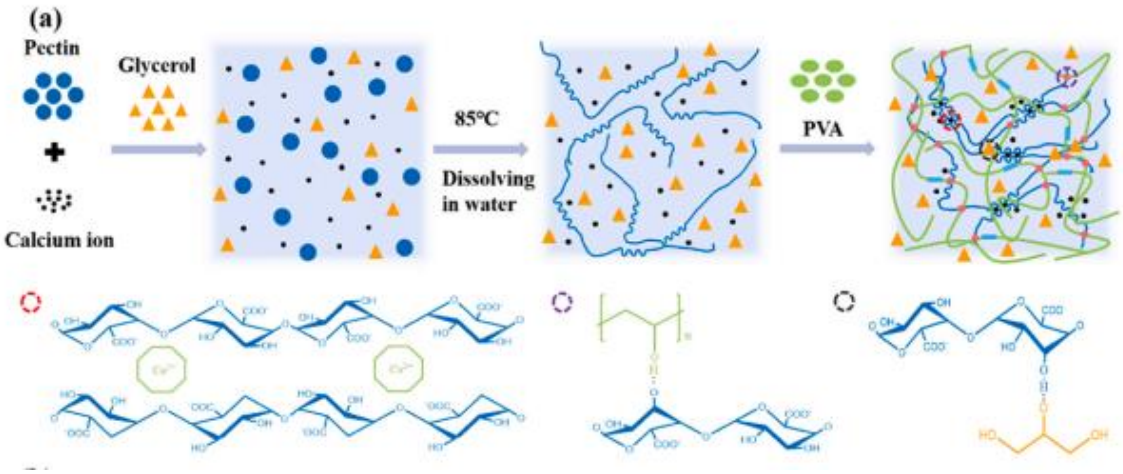


Figure 2-4: Structural and chemical changes involved in the formation of pectin films [27]

To improve the processability of the films, glycerol is added as a plasticizer due to its tendency to absorb water. It also causes a structural disruption when interacting with the polymer matrix, reducing tensile strength and increasing the elongation of break [28]. Specifically, it achieves this

by reducing interactions between the carboxyl groups of pectin chains, seen in the bottom right of Figure 2-4. Other polymers may be blended in to improve the mechanical properties of the film, and in this research, the addition of microalgae is investigated.

2.2 Current Research

2.2.1 Lipid Extraction from Microalgae

Lipid extraction from microalgae has been extensively studied in literature, and there were a plethora of reviews and new experiments performed within recent years. The traditional methods of extraction are the Bligh-Dyer, Folch and Soxhlet methods. Each of these are described in a review by Zhou et. al. [29] and come with their own challenges, shown in Table 2-1:

Table 2-1: Advantages and disadvantages of traditional lipid extraction methods [29]

Method	Advantages	Disadvantages
Bligh-Dyer	High yield, simple operation and low cost	Long reaction time and large energy consumption
Folch	Gentle process, large throughput, quick	Toxic reagents used; harmful to health and environment
Soxhlet	Extraction and separation achieved simultaneously	High cost, selective reagents which are toxic

The use of chloroform and methanol in food industries was not favourable, so the Hara and Radin method was developed to combat this challenge, which uses isopropanol and n-hexane (2:3 in a volumetric ratio). The method is described in a very recent review from A.M. Husin et. al. [30]. Prior to extraction, pretreatment methods may also be employed, and are generally classified into mechanical and non-mechanical methods. In the review by Husin, the mechanical methods are shear forces, electrical forces, waves and temperature shocks; while the non-

chemical methods are chemical based, osmotic pressure and biological based. There were 16 techniques across these methods. In another review by Ying Lee et al. [31], there are more techniques that are not mentioned in the previous reference, namely: rotor-stator homogenisation, freeze drying and grinding, hydrodynamic cavitation, explosive decompression, and the use of surfactants. Table 2-2 shows the advantages and challenges of a few of these methods, while Figure 2-5 shows their energy requirements.

Table 2-2: Advantages and disadvantages of pretreatment methods [30,31]

Method	Advantages	Disadvantages
Pulsed electric fields	High throughput, simple, pollution free	Control of field strength: too high of a strength will have a negative effect
Ultrasonic waves	Low energy requirements, high extraction rate in short time	Control of intensity and time of ultrasound
Microwaves	Reduced extraction time and energy consumption	Polarity of solvent can impact extraction, not suited for heat-sensitive applications
Enzymes	Selective to substrate, room temperature and pressure reduces energy requirement	Enzyme prices are high, must optimise conditions to achieve maximal result
Organic solvent	Simple and quick extraction, easy solvent recovery	High toxicity and flammability, need to dry biomass for maximum yield
Supercritical solution	Rapid extraction, low toxicity, avoid degradation of thermally labile compounds, tunable selectivity, can be used with wet biomass	High equipment cost, need a polar cosolvent for polar lipids
Switchable solvent	Interaction of solvent with biomass in different forms for efficient extraction, easy solvent recovery with gas bubbling	High sensitivity to water, technical viability has not been investigated past the laboratory scale

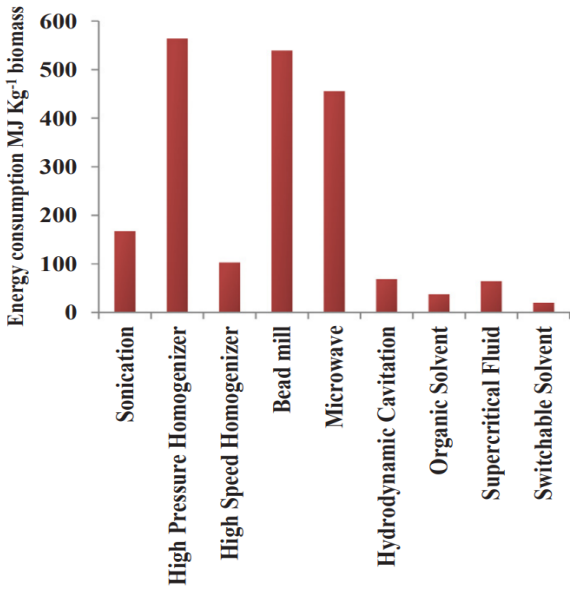


Figure 2-5: Energy consumption of various pretreatment methods for lipid extraction [32]

To fully understand if a certain method is worth considering, the section will also examine four different techniques employed recently. To ensure a fair comparison, only studies on *Chlorella vulgaris* will be considered. A summary of yields and technologies can be found in Table 2-3:

Table 2-3: Lipid yields and extraction techniques on C. vulgaris from various studies

Study	**Strain/Location**	**Technology and Conditions**	**Yield (g lipid / g dry biomass)**
[33]	Algae Research & Supply (USA)	Switchable solvent (DMCHA): 360 W & 45 s microwave pretreatment, 22 °C and 1,000 rpm mixing. Total time of ~ 40 minutes	0.6385
[34]	University of Warmia (Poland)	Microwave pretreatment: 2.45 GHz, 400 W & 60 s, 140 °C	0.4131

[35]	Algae Research & Supply (USA)	Ultrasonic waves: 20 kHz, 20 mins, room temperature	0.1154
[36]	UTEX 259 / University of Austin (USA)	Combined bead milling and enzymes: 2,039 rpm & 22 °C, lipase at 37 °C, total time of 24 h	~ 0.5

Considering yield, time taken and the energy consumption of each technique, it would appear as though the method using switchable solvents is the best option, providing the highest yield within a decent time. Though, its energy requirements are still high, considering it uses a microwave pretreatment. However, assuming 1 kg of biomass is treated, and the energy consumed is directly proportional to the time taken for the experiment, the process still uses the second-least amount of energy. Interestingly, the same culture of microalgae provided the lowest yield when subjected to ultrasonic waves. Russel and Rodrigez provide a possible explanation, which is a technical challenge of extracting lipids from microalgae: the culture conditions and extraction method can cause a discrepancy in lipid yields, seen when one study reported a 25% lipid content, and another reported a 60-68% content in *Chlorella vulgaris* [33]. In this case, Russel and Rodrigez used a black box reactor designed to keep conditions constant (at pH 7), harvesting the microalgae at day 28. Conversely, Krishnamoorthy et. al. kept the culture at pH 9 and harvested at day 21, when a greater number of algal cells began to die [35]. Both studies were performed in the same location during the same year, implying that the effect of growth conditions and the extraction method had an impact on the amount of lipids extracted. Microwave pretreatment and the combination of shearing and enzymes are also decent methods of extraction but have the disadvantage of highest energy consumption and longest reaction time, respectively. However, while solvents appear to be the best solution due to their low energy consumption, innovative methods such as deep eutectic solvents, which offer the advantage of

biodegradability, have yet to be investigated past the laboratory scale. This is also true of ionic liquids, but the added challenge of these solvents is the high toxicity towards aquatic life [31]. If energy costs are to be reduced, Nagappan et. al. found that an alternate route of skipping the cell lysis step in physical and mechanical methods needs to be found [32]. Two years later, Ying-Lee et. al. [31] made progress on this challenge and discovered that integrating unit operations in downstream processing reduced power costs. Moreover, the yield of lipid extraction is increased when the cell disruption step is merged with the extraction step [31]. Though the techniques of lipid extraction are expanding, a major knowledge gap is the structure and features of the cell wall of different microalgal species, as accessing valuable compounds depends on its disruption [30,31].

2.2.2 Algal Biodiesel

From the lipids of microalgae, researchers can produce biodiesel through three main methods: conventional, two-step and in-situ transesterification. There is also a fourth method which involves producing soap from the lipids, then extracting the esters from it [37]. The processes can be visualised as follows:

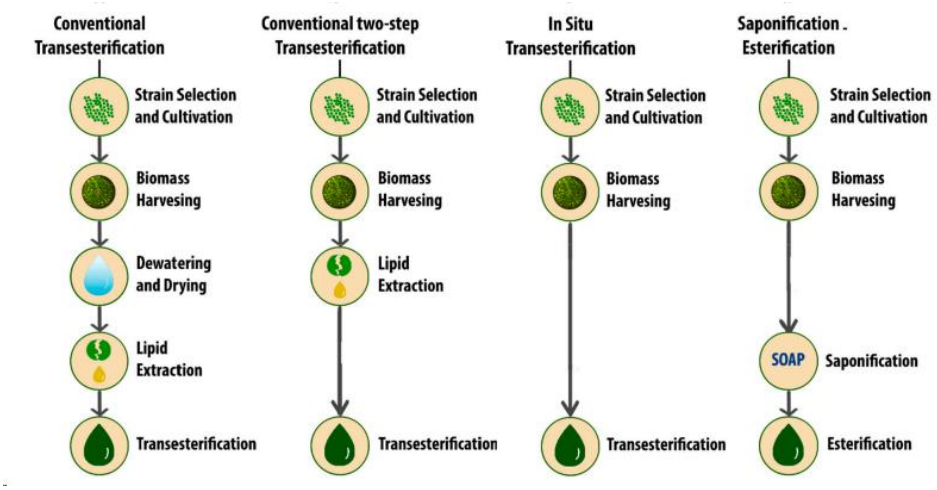


Figure 2-6: Summary of four processes involved in biodiesel synthesis [37]

The conventional method of producing biodiesel typically uses cultures of microalgae at low concentrations, and there are numerous techniques studied which increase the settling rate, such as flocculation, centrifugation and electrocoagulation. However, on the industrial scale, the major disadvantage of using this method is the high cost and energy consumption associated with it: the drying and dewatering step accounts for 70-75% of costs and 89% of the energy used [37], whereas dewatering alone accounts for around 30% of the total cost [38]. The energy distribution of various reactors is summarised in Figure 2-7, and additionally shows which system is the least energy intensive:

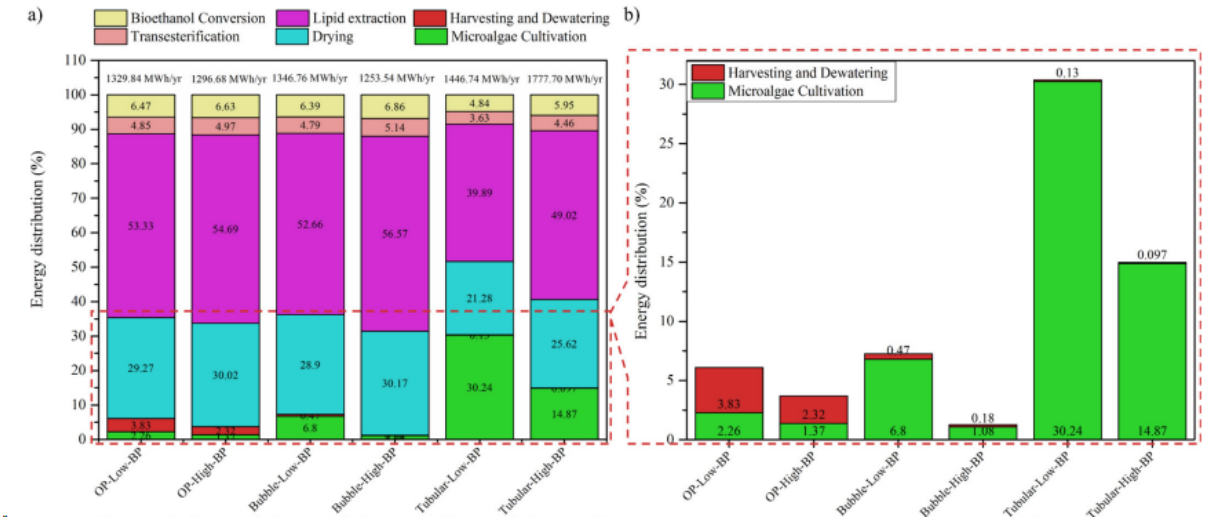


Figure 2-7: Energy distribution of each step of various cultivation systems. OP = open pond, BP = biomass productivity [39]

Removing the dewatering stage would cut energy demands and cost significantly, though it introduces the problem of lower biodiesel yields due to the limited mass transfer of lipids from inside cells to the surrounding water [37]. Lipid extraction is also a major factor affecting the cost of biodiesel production; up to 90% of expenses could be used on this process [38]. The best method to mitigate these costs is the in-situ transesterification method, which directly converts

the lipids in microalgae to biodiesel without extraction or dewatering. Progress has already been made to reduce costs of biodiesel, as seen in one study by Silva et. al. which estimates the cost of producing 1 kg of biodiesel with base catalyst transesterification as $1.2, which is close to the price of petroleum-derived diesel: $0.73 per liter. However, the cost largely depends on the method, as Heo et. al. had produced a kg of biodiesel using enzymes for $12.53 [37]. The methods of in-situ transesterification can be split into two main groups: conventional and advanced. Conventional methods include the use of acid, base and biological catalysts whereas the more advanced methods are ultrasonic assistance, microwave assistance, co-solvents and supercritical fluids. Each method comes with its advantages and disadvantages, shown in the following table:

Table 2-4: Advantages and disadvantages of transesterification methods [38]

Method	**Advantage**	**Disadvantage**
Alkali catalyst	Shorter reaction time, reaction happens at milder conditions	Can only be used for low FFA (<2%), excessive catalyst leads to soap formation and decreases yield
Acid catalyst	Suitable for high FFA with no saponification	Corrosion of equipment, longer reaction time, high ratio of alcohol to oil needed
Biocatalyst	Simple purification, low ratio of alcohol to oil can be used	Very long reaction time, excessive methanol can deactivate catalyst
Microwaves	Greatly reduced reaction time and energy consumption	Difficult to scale up; rapid, non-uniform heating causes hot spots

Four studies using different methods will be analysed to see if a certain method is worth considering. An equal number of studies using the conventional approach and the advanced approach will be examined:

Table 2-5: Studies comparing the yields of various methods used to obtain biodiesel from microalgae. Biodiesel and lipid yield is based on g of lipids or ester per 100 g of dry microalgae

Study	**Conditions**	**Lipid Yield (%)**	**Biodiesel Yield (%)**
[40]	Electrochemical: 20:1 methanol to oil molar ratio, tetrahydrofuran (THF) to methanol molar ratio of 0.25:1, 2 h, voltage of 20 V/cm, water content of 2 wt.%, H_2SO_4 loading of 15 wt.%.	5	4.75
[41]	Subcritical conditions: nitrogen purged, 220 °C, 2 h, 8 ml methanol per gram of wet biomass	-	52.24
[42]	Three phase partitioning: 1:1 ethyl acetate to ethanol, 1:1 solvent to dipotassium phosphate, 60 °C, 3 h	15.9	0.19
[43]	Homogenous acid catalyst and microwave irradiation: 450 W power, 70 mins, 30% H_2SO_4,15:1 methanol to biomass ratio	-	31.56

In terms of energy consumption, yield and reaction time, the study by Felix et. al. [41] shows the highest yield and second-best reaction time of all the studies in Table 2-5. Additionally, the technique they used can bypass the limitation of mass transfer between water and lipids and uses a non-toxic pretreatment: subcritical water. The only concern of this method is the use of methanol, but overall, it has an excellent fatty acid methyl ester (FAME) yield of 74.6%.

Additionally, the feedstock used in the study was microalgae with a moisture content of 80%. The second highest yield is from a study by Kalsum et. al. [43] and is another in-situ method. However, since it uses microwave irradiation – which has a high energy consumption (Fig 2.5) – and uses a relatively high amount of sulphuric acid, it is not suitable for industrial use. Moreover, the study had used dry algae, hence the yield may be lower when moisture is introduced. A study by Moradi and Saidi [40], shows a low yield of biodiesel to algae, but is the second-best in terms of energy consumption, due to the use of potassium chloride instead of sodium chloride in the electrochemical cell. Interestingly, this procedure has the highest FAME yield of 95%. The reason for its lower overall yield is because the lipid content of 5% was an estimate, hence the procedure may be more beneficial for algal species with higher lipid contents. The least energy-intensive technique of the four was studied by Asfrafum Alam et. al. [42], and it used microalgae with a high water content. The procedure was reported to extract twice the lipid amount than the Bligh and Dyer method. Considering the study also used microalgae whose cells were not broken by pre-treatment methods, and therefore is limited in accessibility to lipids, the FAME yield of 12.09% was satisfactory. Though each method has its advantages and challenges, the path forward is using in-situ transesterification methods to significantly increase biodiesel yield from microalgae. However, since these procedures are so recent, it is a knowledge gap in the field of algal biodiesel and must be studied further [44]. Other key steps which must be taken in the future are emission assessments, energy and economic balances, optimisation of reaction parameters and further innovation [45].

CHAPTER 3: MATERIALS AND METHODS

3.1 Materials

The following were used: modified Bold's Basal Media (BBM) [0.25 g/L $NaNO_3$, 0.025 g/L $CaCl_2.2H_2O$, 0.075 g/L $MgSO_4.7H_2O$, 0.075 g/L K_2HPO_4, 0.175 g/L KH_2PO_4, 0.025 g/L NaCl, 63.9 mg/L Na_2EDTA, 4.98 mg/L $FeSO_4.7H_2O$, 11.42 mg/L H_3BO_3, 8.82 mg/L $ZnSO_4.7H_2O$, 1.44 mg/L $MnCl_2.4H_2O$, 1.57 mg/L $CuSO_4.5H_2O$] for culturing *Chlorella vulgaris* and acetate buffer pH 5.5 [7.355 g/L CH_3COONa, 0.6207 g/L CH_3COOH, adjusted to desired pH with HCl] to dissolve enzymes. Pectin from orange peel from VWR International (Toronto, Canada) and pure glycerol from Research Products International Corp (Illinois, USA) were used for the formation of pectin films. Cellulase (Onozuka R-10) and macerozyme R-10 were also purchased from Research Products International Corp, and lysozyme was sourced from Bio Basic Canada Inc. (Markham, Canada). *Chlorella vulgaris* was cultured in the lab at the university, with the original culture from the Canadian Phycological Culture Centre at the University of Waterloo (Waterloo, Canada).

3.2 Methodology

3.2.1 Culturing Microalgae

Chlorella vulgaris was initially grown in 2 different 4 L Erlenmeyer flasks with a starting inoculum amount of 1,000 mL from stock culture (25% v/v of the flask) under a fume hood. The flasks were then filled with modified BBM until 4 L. They were fitted with stoppers, syringes and air filters, then taken out of the fume hood and attached to an aquarium air pump or the laboratory air line. Optical density readings were taken once every 2-3 days at a wavelength of 680 nm. For this initial batch ('Batch A'), microalgae were harvested by day 28 of culturing, as limiting nutrients increases lipid accumulation in algal species [46]. Once day 28 was reached,

all the culture in the flasks were mixed and centrifuged to give a total concentration of 1 g/L, with 6 L of the supernatant (spent modified BBM) discarded. The centrifuged microalgae was then used for experiments and part of the culture was diluted with BBM and re-grown under aeration. About four months later, three 4 L flasks were filled with 1 L of concentrated *Chlorella vulgaris* from different cultures and 3 L of autoclaved Bold's Basal Media (BBM) under a fume hood, then grown similarly to Batch A. For this second batch, 'Batch B', microalgae were harvested at day 17 and the average concentration of the culture after mixing and centrifuging was 4 g/L. All cultures were subjected to constant fluorescent light and aerated using a Top Fin Air-8000 aquarium pump.

3.2.2 Enzyme Tests

For the enzyme tests, 120 mL of microalgae from either batch were extracted into a 250 mL flask and either 50% wt of microalgae (60 mg) for Batch A or 40% wt (192 mg) for Batch B of enzymes were added. These enzymes were cellulase (Onozuka R-10), macerozyme R-10 or lysozyme in any combination. Figure 4-1 and Figure 4-6 show the exact combinations of enzymes used for Batch A and Batch B respectively. The flask was then covered with aluminium foil and placed in a shaker at 40 °C. The suspension was left to shake at 150 RPM for 8 hours.

3.2.2 Steam Explosion

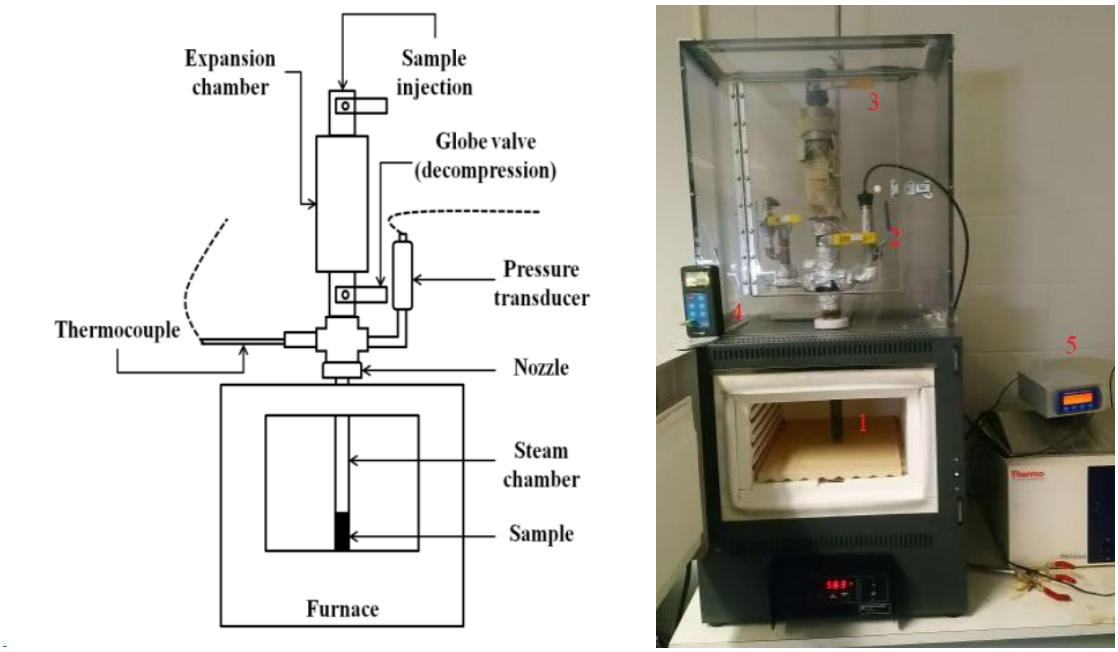


Figure 3-1: Steam explosion apparatus used: 1 = reaction chamber, 2 = blowout valve, 3 = loading valve, 4 = thermocouple, 5 = pressure reader connected to transducer

The procedure followed the same method described by Aguirre [47], with a few modifications. First, the furnace was pre-heated to either 400, 500 or 600 °C and once the temperature was reached, the steam device was slotted on top. 40 mL of microalgae from either batch was poured in from the sample injection port and all the valves sealed once the suspension settled to the bottom. The internal temperature and pressure were read every 10-30 minutes, and once both parameters reached a stationary point, the microalgae were left to steam for an additional 15 or 45 minutes. Afterwards, the device was placed in a bucket of cool water and left until the internal temperature was around 28 °C. The device was then taken out and inverted to retrieve the steam exploded algae. The procedure was repeated twice more before cleaning. To clean the device, it was filled with about 100 mL of tap water and rinsed by sealing the valves and inverting it upside-down and upright three times. It was then rinsed with distilled water similarly, and the

process was repeated one more time for a total of four rinses for Batch A. Steam exploded microalgae from this batch was then stored in a 5 °C fridge for up to 7 days. For Batch B microalgae, the procedure was the same except for the device cleaning. In this trial, the device was rinsed twice with distilled water, then three times with 20 mL of chloroform to collect residual lipids, followed by a final rinse with distilled water. The cleaning was done after three trials were completed. The water and chloroform (containing residual solids and liquids respectively) were placed in separate beakers in an incubator at 50 °C. They were then transferred to pre-weighed aluminium boats once enough liquid had evaporated. Once all liquid was evaporated from the boats, the amount of residual solids and lipids were determined gravimetrically, by taking the difference of masses of the boats after evaporation and before wash water or wash chloroform were placed in them. The microalgae from this trial were stored outside at room temperature, in front of a fluorescent lamp for up to 4 days.

3.2.3 Lipid Tests

The procedure followed that from Axelsson and Gentili [48] as it provides lipid extraction comparable to the traditional methods and is easier and quicker to perform. For the initial experiment (Batch A), microalgae from either the control (microalgae not pretreated with steam explosion or enzymes), enzyme tests or steam explosion were divided into 3-6 25 mL tubes and centrifuged for 20 minutes at 10,000 RPM. The supernatant from one tube was placed in another tube, while the solids were kept in the original tube. To each of the tubes, 4 mL of 2:1 chloroform/ethanol was added then the tubes were shaken vigorously for a few seconds. 1 mL of 0.73% sodium chloride solution was also added to the tubes containing solid material, and no solution was added to the supernatant. All the tubes were then shaken again for a few seconds and vortexed to mix the contents evenly. Afterwards, the tubes were centrifuged again at the

same RPM for 10 minutes. Once centrifuged, a pipette was used to extract the bottom layer of each tube, which contains lipids dissolved in chloroform. The organic solution was dripped into pre-weighed aluminium boats and if there were visible solid particles in the solution, the liquid was vacuum-filtered using a 20 μm pore filter and poured back into the boat and left overnight to dry in an incubator at 50 °C. The lipid amount was then determined gravimetrically. In the second experiment (Batch B), 40 mL of microalgae were placed straight into a pre-weighed aluminium boats. The boat was dried overnight at 50 °C, then the solids were transferred to a centrifuge tube. 8 mL of 2:1 chloroform/ethanol was added to the tube, shaken, then 2 mL of 0.73% sodium chloride solution was added. The centrifuging and extracting procedures were the same as before. To calculate lipid yields, the lipid amount (mg) was divided by the estimated amount of microalgae (g) in the control for Batch A. In Batch B, the measured lipid amount (mg) was divided by the measured solids amount (g) for each data set. The mass of the solids was obtained gravimetrically by taking the difference between the mass of the empty aluminium boat and the mass of the boat with dried solids, after all the liquid had evaporated. A similar calculation was performed for the residual lipids. Furthermore, the total lipid amount in Batch B includes the residual lipid amount. This was calculated by dividing the residual lipid amount from cleaning the device by 3 and adding it to the lipid amount obtained by extraction.

3.2.4 Biodiesel

200 mL of microalgae from a different culture (concentration ~ 5 g/L) was centrifuged until only the solids remained. The lipids were extracted according to the Batch B method as described above and the chloroform phase was placed into a 50 mL Erlenmeyer flask. The flask was heated on a hot plate at 90 °C until all the chloroform was evaporated. The lipids were determined by taking the difference of the mass of the flask after chloroform was evaporated and the mass of

the flask before chloroform was added. 0.05 g of sodium hydroxide was dissolved in 10 mL of methanol and ethanol, which was then placed into the heated flask. The flask was covered with aluminium foil and stirred at 300 RPM for an hour at 90 °C before 2 drops of glycerol were placed in. The reaction was then stirred for another hour at the same temperature, and the solution was left to settle and cool for about 2 hours. Once the solution was settled, a pipette was used to extract the top layer containing the esters, then placed into pre-weighed aluminium boats and dried overnight at 50 °C. The biodiesel mass was then determined gravimetrically.

3.2.5 Production of Films

120 mL of microalgae suspension after lipid testing, 120 mL of distilled water, 13.5 g pectin and 13.5 g glycerol (approximate mass ratio of 90% suspension, 5% pectin and 5% glycerol) were mixed on a magnetic stirrer at up to 1,000 RPM and heated to a temperature of up to 80 °C for 10 minutes until the suspension thickened. After this, the suspension was stirred manually using a laboratory spatula until all the pectin dissolved. Any excess pectin was filtered using a tea strainer (8 cm filter diameter, 0.5 to 1 mm pore size). The control film suspension was prepared similarly, but used 240 mL of distilled water, 13.3 g pectin and 13.3 g glycerol (approximate mass ratio of 90% water, 5% pectin and 5% glycerol). The suspension was poured into a 200 mL vacuum flask, which was then placed on the hot plate and connected to the laboratory's vacuum line. A stopper was placed on top of the flask and the vacuum was started until the suspension was just on the verge of frothing into the vacuum line, at which point the vacuum was turned off. The process was repeated until there were no bubbles left in the suspension. The suspension was then emptied onto a 20 cm by 50 cm tray and dried in an incubator at 50 °C overnight. The film was then extracted using tweezers and set aside in the open.

3.2.6 Thermal and Mechanical Properties

Several strips of dimension 1 by10 in were cut from the films and the tensile tests were performed using the TTS-series machines from Adelaide Testing Machines Inc. (Toronto, Canada). The machines were fitted with screw-action grips with wave jaws that had longitudinal grooves. Films were loaded into the jaws and the grips tightened with a socket wrench. The grip length for each test was about 1 in on both ends of the film. TC-100 Tensile/Compression software was used to execute the tests. Once the test was started on the software, the grips would vertically pull the sample a distance of 10 mm. If the sample did not break, the grips would pull the sample another 10 mm. This carried on until the sample broke, noticeable by the sudden drop in loading force. Once the test was complete, vertical displacement of the grip (mm) and tensile load (N) at each point was stored by the software, and then converted into an Excel sheet. This procedure was repeated twice more on different strips from the same film, giving a total of three repetitions for each film. Stress was calculated by dividing the tensile load at each point by the cross-sectional area of each film (m^2). Each strip's thickness (mm) was measured at the ends and middle using vernier calipers before testing. An average thickness was then taken. Strain was calculated by dividing the grip displacement by the original length of the film (254 mm). Once the stress vs strain curve was plotted, Young's Modulus was calculated by taking the gradient of the most linear portion of the curves, before the slopes noticeably change.

3.2.7 Water Solubility Tests

After tensile testing, 1 by 2 in sections from the strips were cut and weighed. The sections were then placed into 200 mL of distilled water for 60 minutes without any mixing. Afterwards, the

water was drained and any solids that remained in the beaker were dissolved in about 10 mL of distilled water. The suspension was poured into 6 cm diameter Petri dishes and left in an incubator at 50 °C overnight. The resulting films were pried from the dishes and weighed again. The solubility percentage was determined by dividing the difference in mass between the original section and the redissolved film by the mass of the original film.

3.3 Experimental Design and Data Analysis

All experiments were performed in triplicates, except for biodiesel synthesis experiments, which were performed in duplicates. The experiments for steam explosion were designed using a simple factorial design. Specifically, in the case of Batch A, there were three levels of temperature (400, 500 and 600 °C) and two levels of time (15 and 45 mins) to be tested, so the number of experiments to perform was $2 \times 3 = 6$ sets of data. For Batch B, two levels of temperature and two levels of time resulted in 4 experiments (2×2). Error bars were calculated by dividing the standard deviation of each data set by the square root of number of trials within that data set (3) [49]. ANOVA and t-tests were performed in Excel, using a one-tail test, as this would check if there was specifically an increase or decrease of the mean of the data sets, rather than a non-directional change.

CHAPTER 4: RESULTS AND DISCUSSION

4.1 Lipid Testing on Enzyme and Steam Explosion Studies

Figure 4-1 shows the results of the lipid extraction experiments on the control and enzyme tests for Batch A. Without any treatment, the yield is 95.41 ± 0.48 mg lipids/g microalgae. The use of enzymes clearly increases the yield, with the highest yield of 136.86 ± 3.41 mg/g resulting from the use of cellulase and macerozyme. Interestingly, the two-enzyme mix outperformed the three-enzyme mix, and this may be explained by the fact that has been theorised to cause an increased settling rate [50], hence some microalgae may not have been mixed thoroughly when the enzyme was present. It is important to note that mixes which involved macerozyme provided higher yields than those which did not include this enzyme. This could be due to macerozyme being a mixture of cellulase, hemicellulase and pectinase [51], and therefore provides additional cellulase. Furthermore, hemicellulose is present in small concentrations in the cell walls of *C. vulgaris* [52], so macerozyme – specifically the hemicellulase – would break it down and allow further access of lipids.

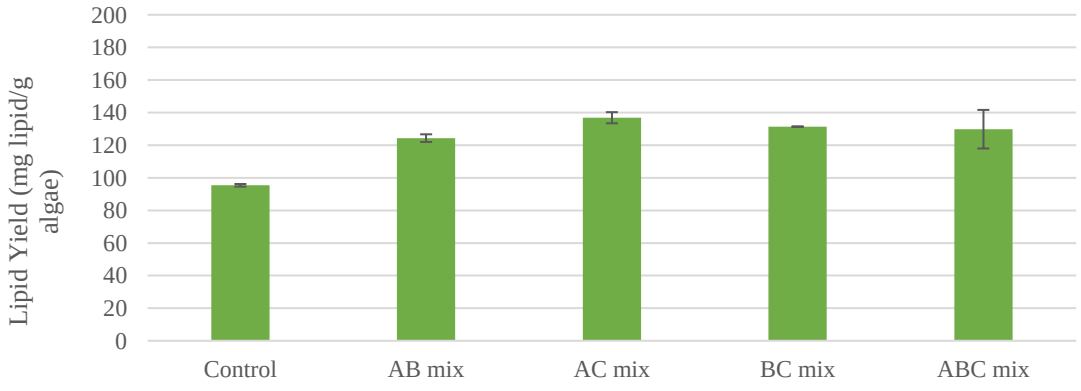


Figure 4-1: Yields of total lipids from microalgae after various enzyme treatments. A = cellulase, B = lysozyme, C = macerozyme

Tables 4-1 and 4-2 show the results of the ANOVA test and t-test on the values of yields from the enzyme experiments. Table 4-1 shows that the F value is much lower than the critical F value, hence the ANOVA test concludes that there is no significant difference between using one enzyme mix over the other. This may be explained by the use of an excessive amount of enzyme. Leaving the suspensions for 8 hours ensured that the maximum amount of substrate was used by each enzyme, and since macerozyme and cellulase share some enzymes in, this means that approximately the same amount of the same substrate was being degraded in cultures that used either of these two enzymes. This leads to approximately equal amounts of lipids being released. Table 4-2 shows that there is a significant difference between lipid yields on the control (unbroken cell walls due to no enzymatic treatment) and those on microalgae with enzymatic treatments, as all t-tests show a p-value below 0.05. This makes sense as each enzyme will act on their respective substrates found in the cell wall of *C. vulgaris*. The breaking of the cell wall can be seen in Figure 4-8.

Table 4-1: ANOVA test performed on the lipid yields of enzyme treatments for Batch A

Source of Variation	**SS**	**Df**	**MS**	**F**	**P-value**	**F crit**
Between Groups	238.5338	3	79.5113	0.6744	0.5915	4.0662
Within Groups	943.1259	8	117.8907			

Table 4-2: t-tests comparing the lipid yields of various enzyme treatments for Batch A

Comparison	**p-value**	**Significant (p<0.05)?**
Control with ABC	0.0220	Yes
Control with AB	0.0002	Yes

Control with BC	6.6792E-07	Yes
Control with AC	0.0001	Yes

The changes of internal temperature and pressure of the device were measured over time. Data for 600 °C was obtained using Batch A, whereas the data for 500 and 400 °C was acquired using Batch B. The data was similar over both batches and for both times (15 and 45 minutes), hence only the graphs for 45 minutes are shown in Figure 4-2. The temperatures and pressures in these figures closely match with each other, and this makes sense as pressure is proportional to temperature in the Van der Waal relation, which models a real gas. The highest internal temperature and pressure reached are 160 °C and 400 psi for an oven temperature of 600 °C, and the lowest are 80 °C and 32 psi for an oven temperature of 400 °C. Higher temperatures and pressures are reached with higher oven temperatures, as more heat energy is available to flow into the device and affect the water molecules However, temperature and pressure both decrease after the first run. Aguirre [47] observed this as well, and this can be attributed to the fact that in the first run, heat travels through the air via convection, whereas in subsequent runs, water vapour is present, and the heat must travel via conduction through the water molecules, which absorbs some of the heat. This also explains why temperature and pressure take longer to stabilise. Interestingly, there is a spike in internal temperature on the first run of 400 °C, which goes beyond the highest internal temperature of 500 °C. This may be explained by the fact that there was a significant time period between running Batch A and Batch B experiments, so the device had over 8 weeks to completely dry, hence heat transfer via convection was at its maximum, with no water molecules at all to intercept.

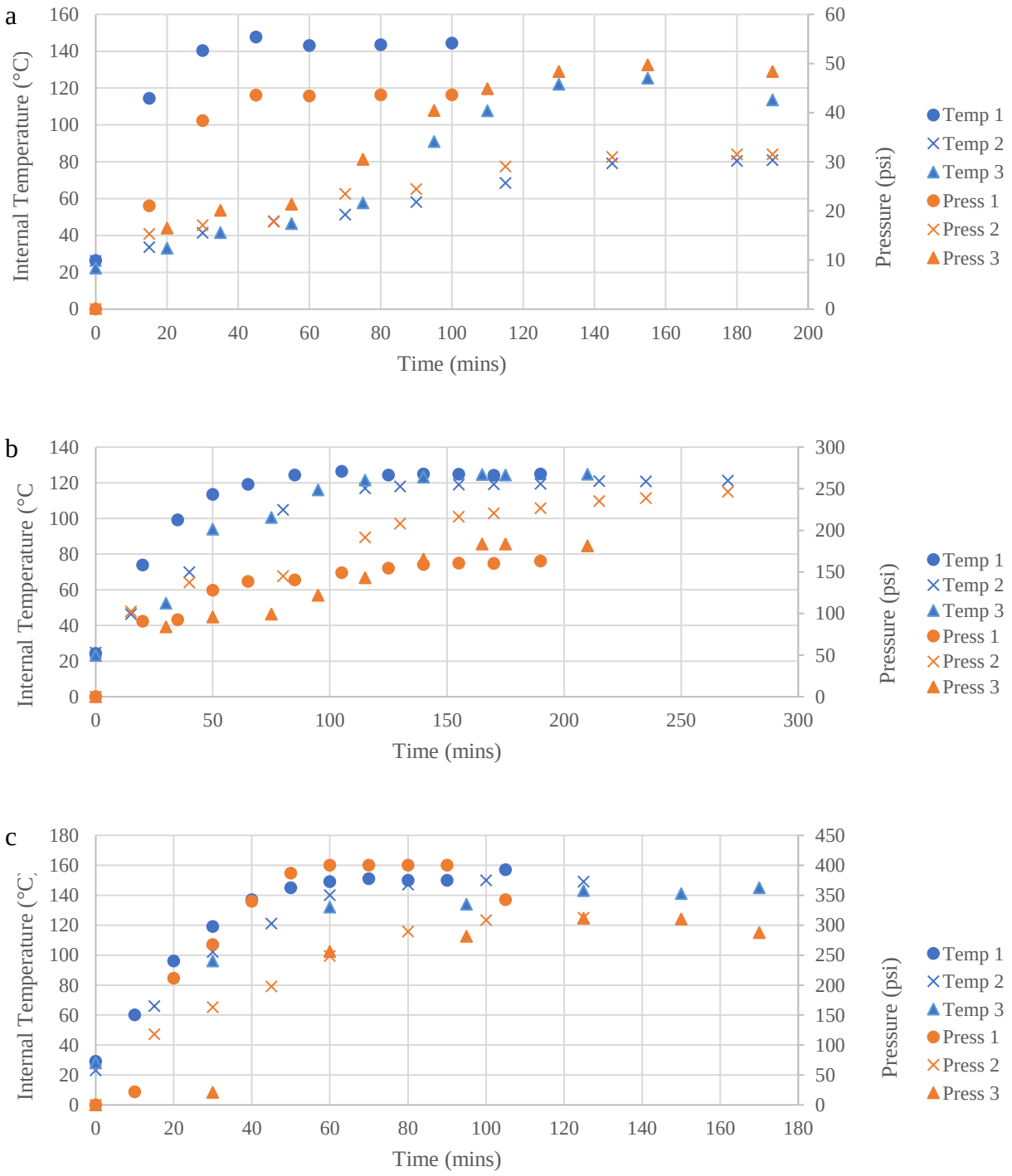
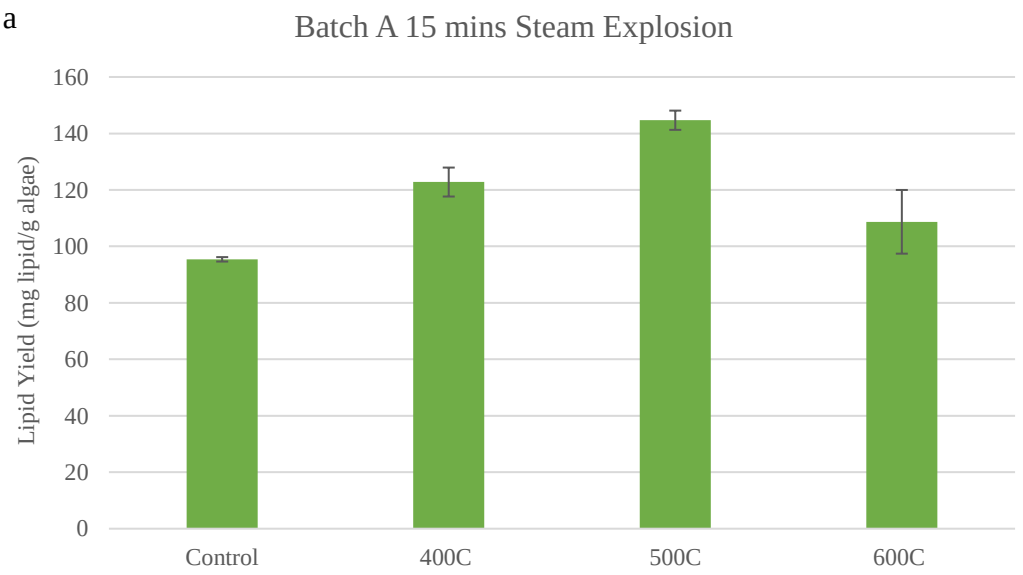


Figure 4-2a, b & c: Internal pressure and temperature change over time for oven temperatures of 400 (a), 500 (b) and 600 °C (c). Temp = temperature, press = pressure

Figure 4-3 shows the lipid yields of microalgae that were subject to steam explosion for 15 mins and 45 mins after reaching the maximum internal temperature and pressure. The highest yield of 144.68 ± 3.41 mg/g was achieved from steam explosion at 500 °C for an additional 15 minutes after reaching the maximum conditions. The second highest yield was 125.91 ± 9.22 mg/g from steam explosion at 600 °C for an additional 45 minutes. The trend for 45 minutes of additional steam explosion follows the same observed by Aguirre [47]: lipid yield increases with temperature as higher temperatures tend to cause more damage to cellulosic structures, and hence improve accessibility to the cell [53]. However, the result from 600 °C and an additional 15 minutes contradicts Aguirre's study, as the yield decreases with an increased temperature. Since the temperature of degradation for lipids is around 200 °C [54], this cannot be the result of thermal degradation.

a



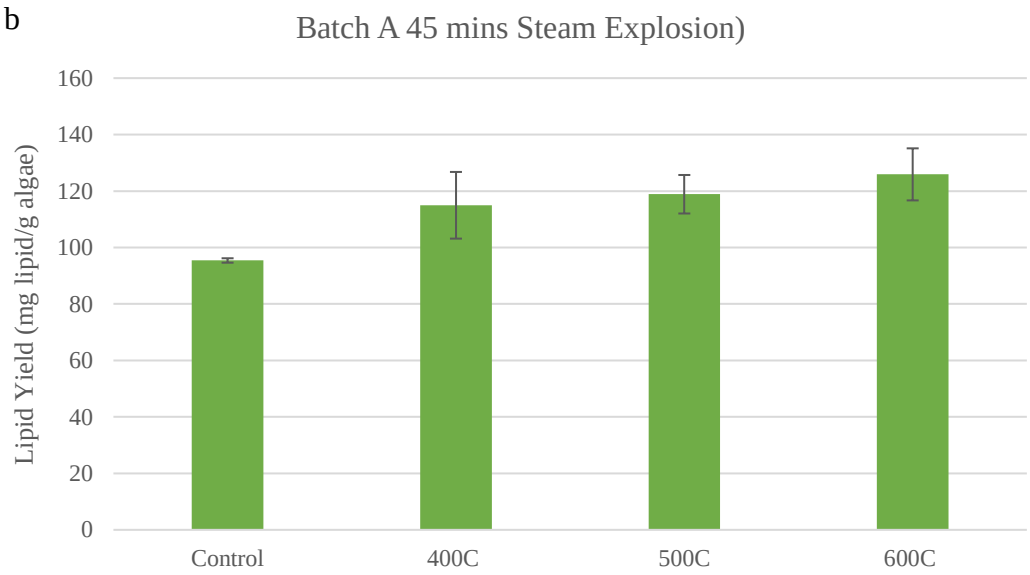


Figure 4-3a & b: Yields of total lipids from microalgae after steam explosion treatments of 15 (a) and 45 minutes (b) additional time

Tables 4-3 and 4-4 show the results of the ANOVA and t-tests on the lipid yields of steam exploded microalgae. Table 4-3 shows there is no significant difference between time and temperature treatments for Batch A as the critical F value is higher than the F value obtained. However, if the confidence level is decreased from 95% to 90%, the interaction between temperature and time becomes significant ($p < 0.1$). This implies that at this confidence level, the time spent in steam explosion enhances the temperature's effect, and vice versa. The former statement could be demonstrated by the highest yield being microalgae from 500 °C and 15 minutes of steaming time surpassing the yield from 600 °C and 45 minutes. However, this case falls in line with one study by Szabo et. al. [55] and contradicts another study by Song et. al. [56]. Szabo poses that prolonged heating of oils can change its composition, and that this should be avoided. This would explain why the yield at 600 °C and an additional 45 minutes would be lower than that from 500 °C and an additional 15 minutes. However, it does not explain the decrease in yield when comparing yields at 600 °C: Song shows that increasing the steaming time breaks down the cell wall more effectively, so lipids should become more available.

However, a study by Kong et. al. [57] may explain why yields decrease from 15 minutes to 45 minutes for 400 and 500 °C. They propose that lipids form complexes with amylose in their study, and since polymers of xylose and glucose are present in the EPS's of *C. vulgaris*, these may have formed complexes with the lipids over time. The t-tests in Table 4-4 show that steam explosion mostly causes a significant lipid yield increase compared to using control microalgae. Increasing the confidence level from 95% to 90% would make the lipid yield from the 400 °C and 45 minute run more significant, but the test at 600 °C and 15 minutes is only significant at an 80% confidence level ($p < 0.2$), and needs to be repeated to determine if the contradictory results hold true.

Table 4-3: ANOVA test performed on the lipid yields of steam exploded microalgae from Batch A

Source of Variation	**SS**	**df**	**MS**	**F**	**P-value**	**F crit**
Time	134.8633	1	134.8633	0.618513	0.4469	4.7472
Temperature	756.5803	2	378.2901	1.734923	0.2179	3.8853
Interaction	1400.01	2	700.0049	3.210379	0.0764	3.8853
Within	2616.532	12	218.0444			

Table 4-4: t-tests comparing the lipid yields of steam exploded microalgae from Batch A

Comparison	**p value**	**Significant (p<0.05)?**
Control with 400°C, 15 mins	0.0031	Yes
Control with 400°C, 45 mins	0.0869	No
Control with 500°C, 15 mins	7.3683E-05	Yes

Control with 500°C, 45 mins	0.0134	Yes
Control with 600°C, 15 mins	0.1524	No
Control with 600°C, 45 mins	0.0150	Yes

To see if more lipids could be drawn out, a combination of the best enzymatic treatment and best steam explosion conditions was used. Figure 4-4 shows the resulting suspension from these tests. A key takeaway of this result is that the suspension of microalgae had a particular grassy, sweet smell after underdoing the combined treatment. Additionally, the colour of the suspension was a deeper brown than that of regular steam explosion trials. This is possibly due to both pheophytin and pheophorbide being generated. The formation of both compounds is explained further in section 4.1.1.

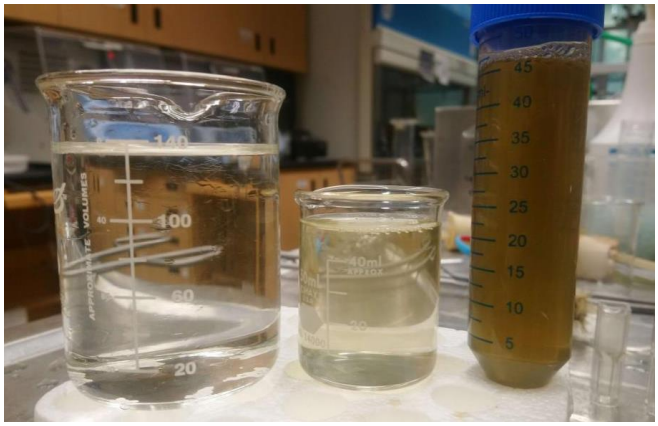


Figure 4-4: Resulting suspension of combined enzymatic treatment and steam explosion at 500 °C for 15 mins (right)

The second experiment involving enzymes and steam explosions used a batch of microalgae with a higher concentration, with experiments being performed within a shorter time. Figure 4-5 shows the results of enzymatic treatment on *Chlorella vulgaris* from Batch B. The first major difference is the decrease in yield from 95.41 mg/g in Batch A to 15.16 ± 1.52 mg/g in Batch B.

This sharp decrease can be explained by two factors: firstly, in Batch A, the lipid test was performed on the solid and liquid portion of the microalgae culture, then added together, whereas it was only performed on the dried solid part in Batch B. Secondly, the method used to calculate the lipid yield in both batches were different. This is apparent in the lipid yields for enzyme tests, where the average lipid amount was 2.83 mg and the average solid amount was 186.67 mg. For the enzyme tests, the average lipid amount was 10.63 mg for cultures subjected to a mixture of cellulase, lysozyme and macerozyme. However, the average solid amount was 394.47 mg. The additional solid mass comes from residual enzymes in the suspension, and the calculation included this additional mass, resulting in the highest yield of 26.47 ± 6.82 mg/g.

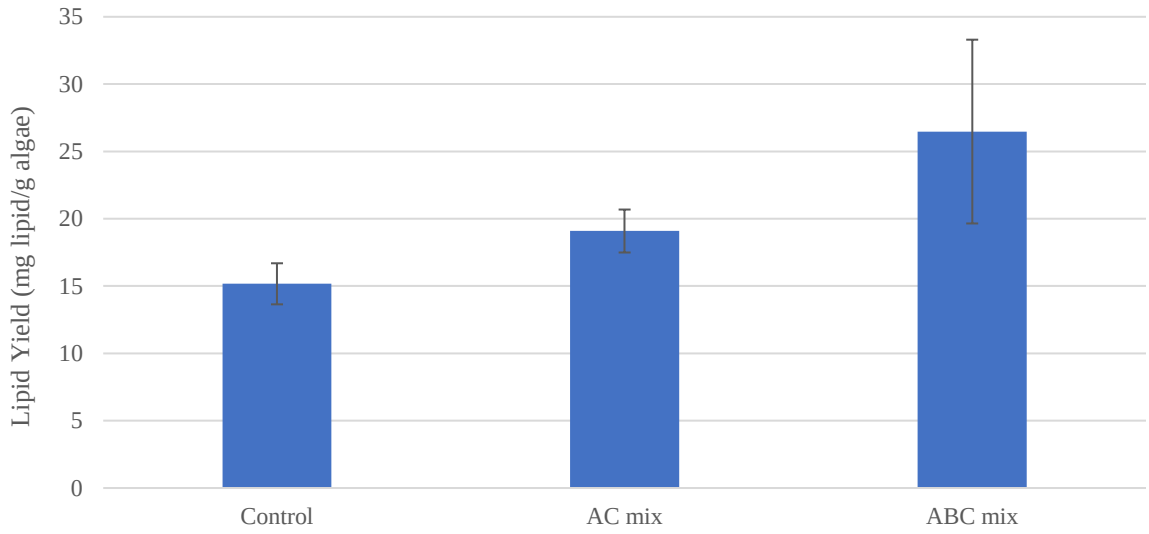


Figure 4-5: Yields of total lipids from Batch B microalgae after various enzyme treatments

Tables 4-5 and 4-6 describe the ANOVA and t-tests performed on the lipid yields from Batch B microalgae subjected to enzyme tests. Though there is a clear difference visually, Table 4-5 shows that since the F value is lower than the critical F value, there is no significant difference between using either enzyme mix. Similarly, the t-tests in Table 4-6 give similar results, and only by decreasing the confidence from 95% to 90% will the yields be significantly different ($p <$

0.1). These can both be explained by the large variance in the lipid yields from the three-enzyme treatment. Though the suspension was mixed evenly and spread into three boats equally, the lipid amounts in each boat were still 6.2, 16.6 and 9.1 mg. The wide spread in lipid amounts lead to a larger standard error and wider variance of lipid yields. However, it is also important to note that while lysozyme can increase the settling rate of microalgae, the enzyme also decreases the electron density of the outer cell wall and increases permeability of certain enzymes [50], hence a possible explanation for the three-enzyme mix giving the highest lipid yield is that lysozyme is allowing other enzymes to permeate and act on substrates found deeper within the cell wall.

Table 4-5: ANOVA test performed on the lipid yields of enzyme treatments for Batch B

Source of Variation	**SS**	**df**	**MS**	**F**	**P-value**	**F crit**
Between Groups	81.8277	1	81.8277	1.1105	0.3514	7.7086
Within Groups	294.7342	4	73.6836			

Table 4-6: t-tests comparing the lipid yields of enzyme treatments for Batch B

Comparison	**p-value**	**Significant (p<0.05)?**
Control with ABC	0.0906	No
Control with AC	0.0751	No

There are more noticeable differences between the lipid yields of steam exploded microalgae from Batch A and Batch B, demonstrated in Figure 4-7. These results contradict Aguirre's results [47] yet agree with Song [56]: yields decrease with an increase in temperature but increase with time spent in steam explosion. The discrepancy between the results of this study and Aguirre's

study can be explained largely by the method of calculation. The average solids amount for steam exploded microalgae at 400 °C with an additional 45 minutes is 129.97 mg, yet the average solids amount for 500 °C at the same additional time is 412.53 mg. The average amount of solids for the control was 186.67 mg, which means that additional mass is responsible for the low lipid yield from 500 °C steam explosion tests. Since the device was rinsed until there was barely any colour change in the water, the origin of this unknown mass in all six 500 °C trials is likely microalgae that could not be cleaned off. The colour of the additional mass matches a description of slurry observed by Aguirre [47]. She had noticed that at an internal temperature of 200 °C and a pressure of 147 psi, biomass liquefied into slurry. While the internal temperature of the 500 °C trials were around 120 °C, the pressure reached 175 – 260 psi, so it is possible that for the 500 °C trials, slurry from past trials was pulled into the suspension. However, trials at 400 °C did not have this issue, and the highest average lipid yield achieved was 124.39 ± 15.46 mg/g from the trials with an additional 45 minutes of steam explosion. Though, the highest average lipid amount was 23.75 mg from the 500 °C, 45-minute additional time experiments. Aguirre obtained a yield of 198.54 mg/g algae for a similar microalgae concentration, harvesting time and oven temperature. The higher yield may be attributed to the methods used in the study: lipids were extracted for 12 hours rather than a few minutes, and the microalgae were subjected to a sonicator for 15 minutes before steam explosion. The combination of two pretreatment methods may have further broken down the cell walls, allowing more lipids to be accessible.

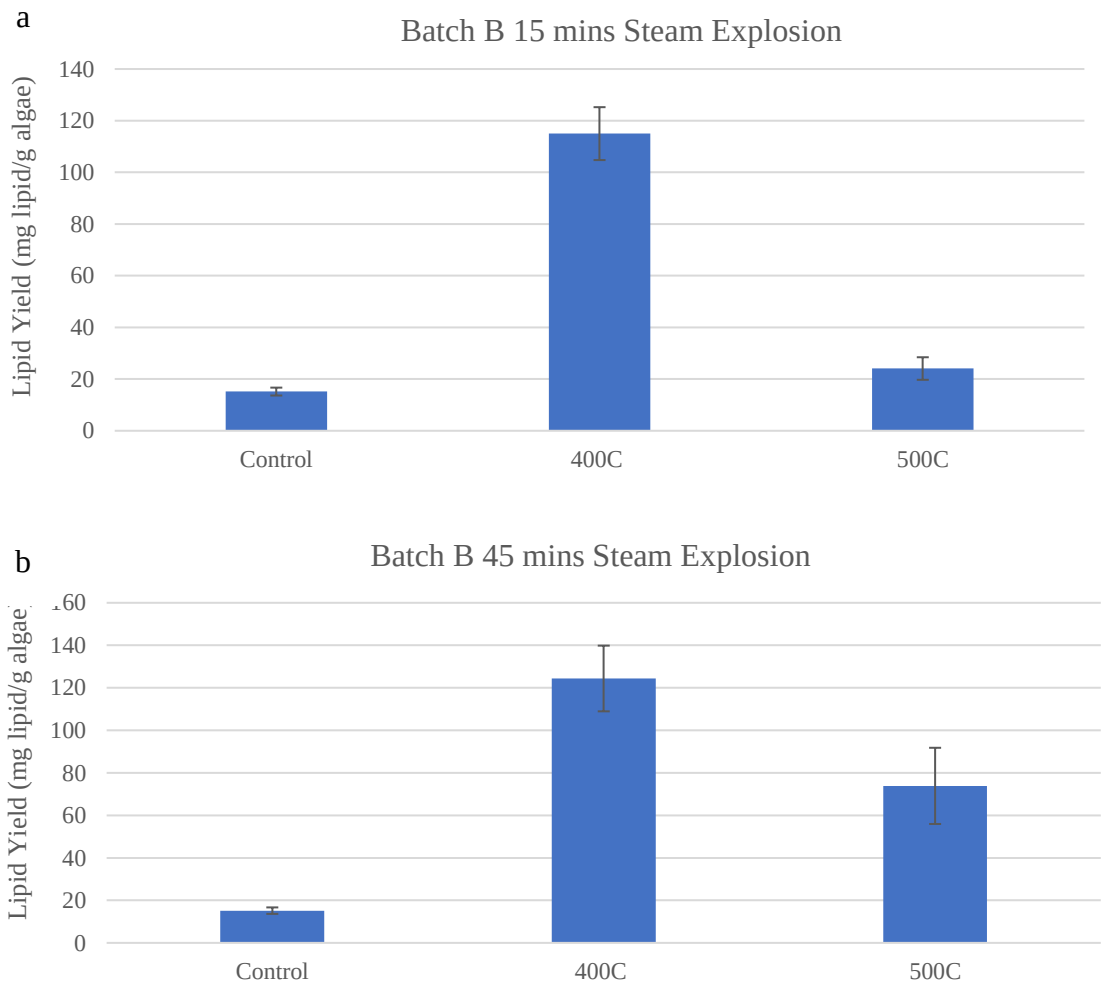


Figure 4-6a & b: Yields of total lipids from Batch B microalgae after steam explosion treatments of 15 (a) and 45 minutes (b) additional time

Tables 4-7 and 4-8 describe the statistical analyses performed on the lipid yields of steam exploded microalgae from Batch B. Interestingly, Table 4-7 shows that the significant variables have almost inverted from Batch A: now the interaction effect is not significant at 95% and is only significant at 80% confidence ($p < 0.2$). The effect of time is nearly significant ($p \approx 0.05$), and the effect of temperature on lipid yields is much more significant ($p < 0.05$). However, since interaction effects may arise from outliers [58] as observed in Batch A (600 °C, 15 mins additional time), this may be a sign that the data in Batch B is consistent and has no major outliers. According to Table 4-8, all lipid yields are significantly different from the control,

except for the yield of steam exploded microalgae at 500 °C with 15 minutes additional time, which only becomes significant at a confidence level of 90% ($p < 0.1$).

Table 4-7: ANOVA test on the lipid yields of steam exploded microalgae from Batch B

Source of Variation	**SS**	**df**	**MS**	**F**	**P-value**	**F crit**
Time	2626.999	1	2626.999	5.1193	0.0535	5.3177
Temperature	15001.49	1	15001.49	29.2339	0.0006	5.3177
Interaction	1222.686	1	1222.686	2.3827	0.1613	5.3177
Within	4105.228	8	513.1534			

Table 4-8: t-tests comparing the lipid yields of steam exploded microalgae from Batch B

Comparison	**p value**	**Significant (p<0.05)?**
Control with 400°C, 15 mins	0.0003	Yes
Control with 400°C, 45 mins	0.0011	Yes
Control with 500°C, 15 mins	0.0629	No
Control with 500°C, 45 mins	0.0155	Yes

The best steam explosion and enzyme treatments were combined to determine if more lipids could be extracted from the microalgae. Figure 4-7 shows the results of this, and it is immediately known that the lipid yield of microalgae which underwent steam explosion and enzymatic treatment is much lower than that of microalgae which was only subjected to steam explosion. Once again, this may be explained by the calculation used: an average of 13.6 mg lipids were extracted from microalgae under steam explosion and enzymatic treatment, whereas the average lipid amount for microalgae under steam explosion only is 16.23 mg. The average

solids amount for the former trial is 394.45 mg, which drastically decreases the yield to 34.27 ± 2.55 mg/g.

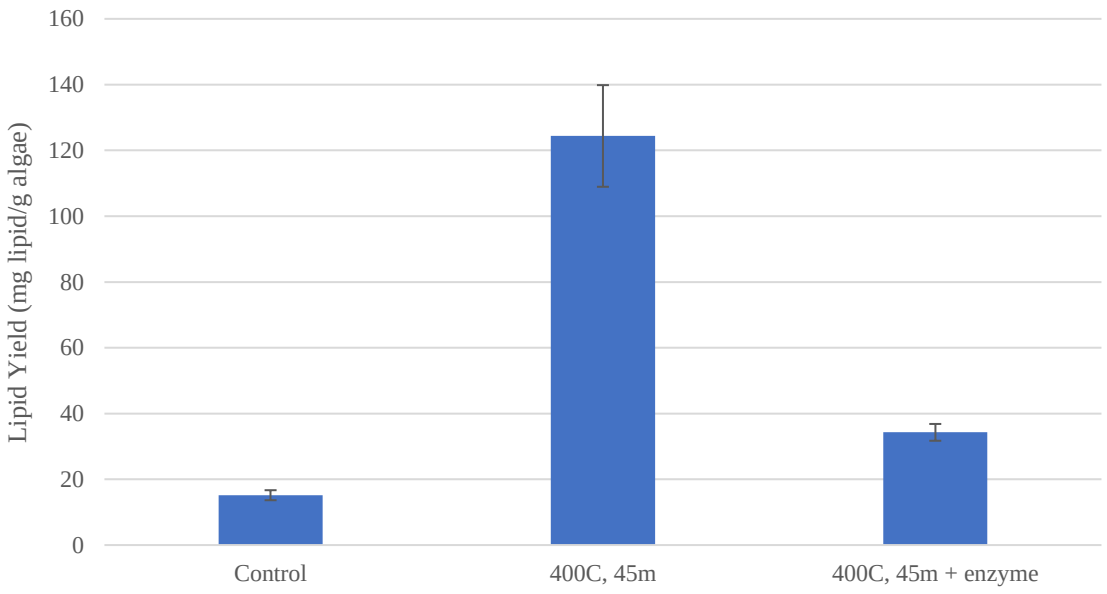


Figure 4-7: Yields of total lipids from combined steam explosion and enzymatic treatment (ABC)

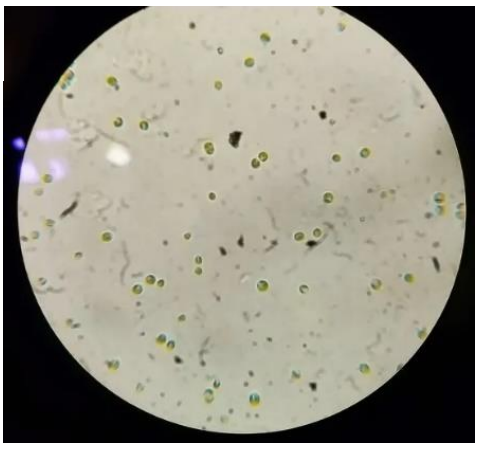
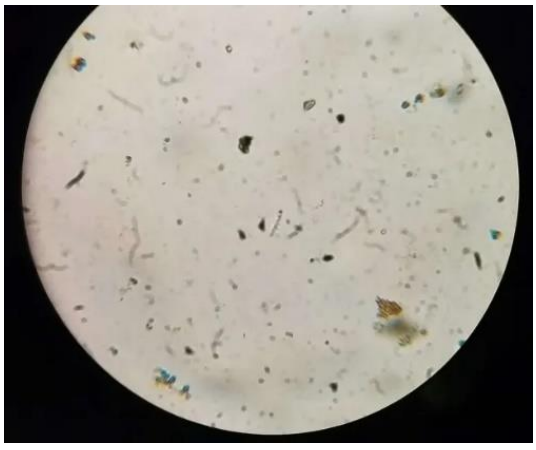
Table 4-9 shows the t-tests performed on lipid yields from both combined and steam explosion experiments. All differences are significant at 95% confidence ($p < 0.05$). Another possible explanation for the stark difference, caused by a lower lipid amount, is that lipid oxidation was accelerated due to exposure to cold fluorescent light, according to a study by Pignitter et. al. [59]. The study shows that oxidation can occur within as little as one week, which is when the combined test was performed.

Table 4-9: t-tests comparing the lipid yields of combined treatment on microalgae from Batch B

Comparison	**p value**	**Significant (p<0.05)?**
Control with 400°C, 45 mins	0.0003	Yes
Control with 400°C, 45 mins + enzyme	0.0015	Yes
400°C, 45 mins with 400°C, 45 mins + enzyme	0.0008	Yes

4.1.1 General Discussion of Lipid Tests

In general, since *C. vulgaris* can have a lipid content ranging from 5 to 58% [60], most of the results from both batches fit the expected lipid yields. The lower yields (less than 3%) may be explained by the relatively higher amounts of solids compared to the lipids. It may be uncertain if the cell walls of the microalgae were broken, but Figure 4-8 provides visual proof that the cells had changed structure. The control sample shows cells that are round and intact, whereas with steam explosion, there is a slight colour change to brown, and formation of clumps of cell debris. With enzymatic treatment, there is an almost total loss of chlorophyll.

a  b 

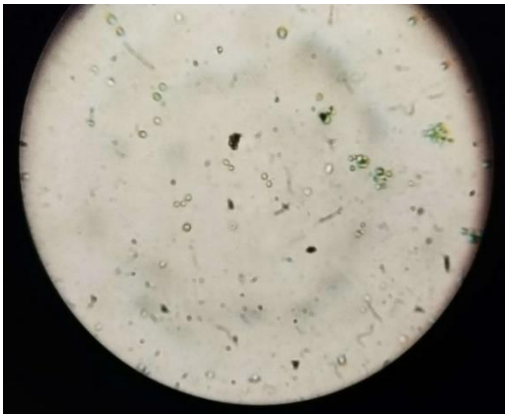
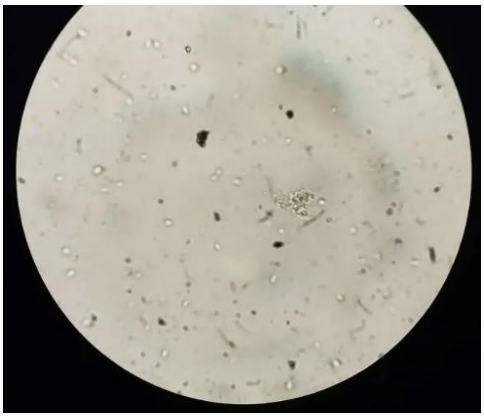
c  d 

Figure 4-8a, b, c & d: Microscope samples of control algae (a) and algae after steam explosion at 400 (b) and 500°C (c), and enzymatic treatment (d)

The differences in colours of suspension may also be observed outside of the microscope, in Figure 4-9. The change of the control specimen from a bright green to a duller olive-brown may be explained by the degradation of chlorophyll. It has been shown that high temperatures promote the formation of pheophytin in processed vegetables [61], and that temperatures as relatively low as 60 °C can induce this reaction [62]. However, this does not explain why at a lower temperature, enzymatic treatment completely changed the colour of the suspension. This can be explained by the damage of algal species (via enzymatic action) resulting in chlorophyll being released from the thylakoid membranes and degraded quickly to avoid further cellular damage [63]. In algae, these membranes are found in chloroplasts [64], which are in turn found in the cell wall.

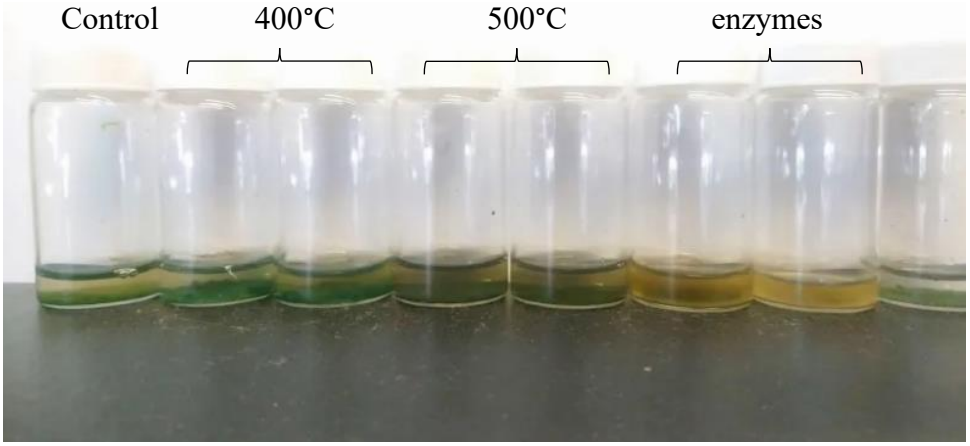


Figure 4-9: Colour changes of algal suspensions after various treatments: control, 400 °C & 500 °C steam explosion, enzymatic treatment (AC)

4.2 Biodiesel

To find the best combination of alcohols, catalysts and reaction time for the production of biodiesel from microalgal lipids, transesterification reactions were performed on canola and waste cooking oil. The results are shown in Table 4-10 below. None of the experiments reached the maximum FAME yield seen in literature, of 95% [40]. The highest total yield obtained was 62.07% on waste cooking oil, and 60.67% on canola oil. These discrepancies can be explained by the fact that less than 2% wt catalyst was used for each study, but the literature study used 15% wt sulphuric acid, which allowed more of the alcohol and fatty acids to react.

Table 4-10: Yields and conditions of biodiesel experiments performed throughout the study. WCO = waste cooking oil

Oil	Time (h)	Temp. (°C)	Alcohol (Ratio)	Catalyst/Glycerol	Yield (g biodiesel/g oil)
Canola	2	80	Ethanol (12:1)	0.8% KOH, 8% glycerol	0.488
Canola	2	80	Methanol/Ethanol (6:6:1)	1.3% NaOH, 8% glycerol	0.6067

Canola	2	80	Methanol/Ethanol /Isopropanol (3.5:3.5:3.5:1)	1% KOH, 5% glycerol	0.26
WCO	2	80	Methanol (12:1)	3.5% KOH, 3.5% glycerol	0.2907
WCO	2	70	2 stage - ethanol (9:1), ethanol (15:1)	0.5% H2SO4, 1 % NaOH, 12% glycerol in first stage	0.404 total
WCO	2, 1.5	80, 75	2 stage - ethanol (15:1), ethanol (9:1)	1.5% H2SO4, 1.2% NaOH, 1% H2O	0.4953 acid, 0.0893 base
WCO (22.3% FFA)	1.5, 3	70	2 stage - ethanol (10:1), ethanol (10:1)	0.75% H2SO4, 1% KOH, 2%/2% glycerol	0.3587 acid, 0.262 base
WCO (26% FFA)	1.5, 2.5	75	2 stage - ethanol (3:1), ethanol (9:1)	0.5% H2SO4, 1 % NaOH, 4.5% glycerol in second stage	0.1253 total

The best conditions for canola oil transesterification were selected (yield of 60.67% - second experiment) and these were used on extracted lipids from control and steam exploded microalgae. Figure 4-10 shows the biodiesel yields, and they are extremely low compared to literature. The lowest yield in the literature examined was 12.05% [42] yet the study gives a yield much less than this. This can be attributed to using an excess of methanol and ethanol, as the major fatty acid in oil from *C. vulgaris* is palmitic acid [65] and separating the palmitate ester from ethanol and glycerol is difficult at high ethanol concentrations [66].

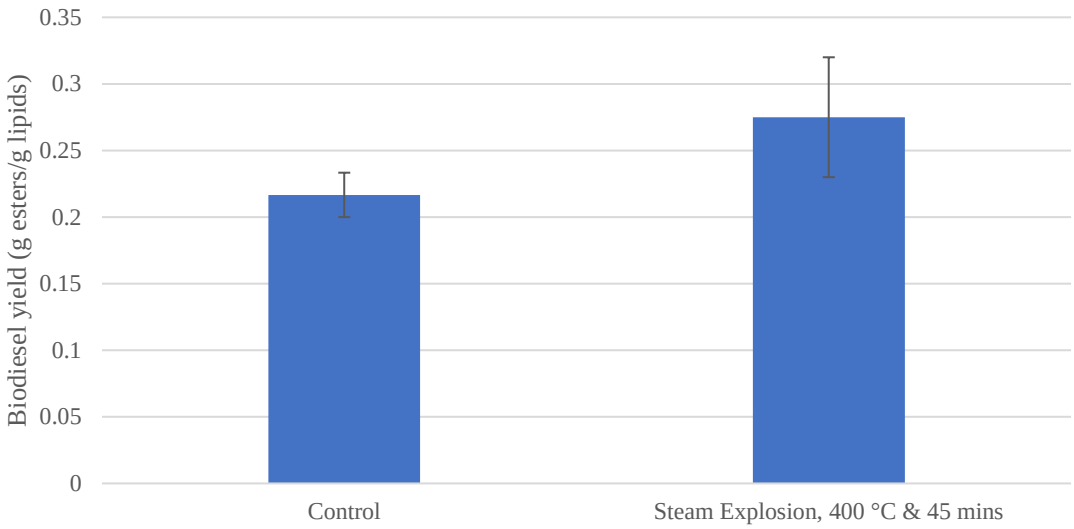


Figure 4-10: Biodiesel yields for lipids derived from control and steam exploded microalgae

The p-value for the t-test is 0.1741 for this trial. The difference is not significant ($p > 0.05$) at 95% confidence but is significant at 80% ($p < 0.2$). However, any difference is largely due to having a higher lipid amount at the start of the experiment. Lipid amounts were around 3 mg for the control, and 10 mg for the steam exploded microalgae in this trial. Nonetheless, this shows that yield can be increased using pre-treatment strategies. The highest average total yield percentage (g esters per 100 g dry microalgae) is for microalgae steam exploded at 400 °C for an 45 mins, and is 3.42 ± 1.00%.

4.3 Bioplastics

A wide variety of pectin-films containing glycerol and microalgae were produced during the course of the study, and the possible composites are shown in Figure 4-11. Table 4-11 shows the properties and description of these composites. Generally, using more pectin than glycerol resulted in tougher materials, whereas the converse is true for higher glycerol ratios. Using no pectin at all results in a liquid slurry that does not set (not shown), whereas using only pectin

gives an extremely tough, solid structure. Since tougher materials were more difficult to shape into dog-bones necessary for the ASTM D638 tests, films were made, which used higher microalgae ratios and kept an equal amount of pectin and glycerol.

a

b

Figure 4-11a & b: A range of possible biocomposites (a) and films (b) made by varying the ratio of pectin and glycerol

Figure 4-11b shows the results of forming films, using ratios of 95:2.5:2.5 algae/glycerol/pectin (AGP) on the left, decreasing the microalgae amount by 5, to films with an AGP ratio of 80:10:10. The same observations arose: using less microalgae and more pectin and glycerol results in tougher films. The easiest film to used an AGP ratio of 90:10:10.

Table 4-11: Description and densities of biocomposites made over the course of the study

AGP Ratio	**Description**	**Density (g/cm^3)**
50/20/30	Very tough disk made from old concentrated slurry	1.4286
70/15/15	Cubed 'candy', tough in texture but air pockets made it spongy	1.08

70/10/20	Tough texture, similar to golf ball and bouncy	0.8267
70/10/20	Able to sculpt into toy with tough texture	1.33337
70/10/20	Hard disk, with pectin settling on bottom, due to using ethanol	1.425
70/10/20	Tough disk made from old concentrated slurry	1.2
70/25/5	Rubbery film made from old concentrated slurry	0.84
70/30	No glycerol used, extremely tough like a poker chip	1.52
80/5/15	Flaky, non-uniform, tough and could be shaped into cup - broke off from top, floated in water	0.84447
80/5/15	Flaky, non-uniform, tough and could be shaped into cup - broke off from bottom, sunk in water	1.1
90/5/5	Flexible film, similar texture to flexi-ruler	1
90/5/5	Flexible film, felt like plastic sheet and slightly tough when pulled	1.4667
90/5/5	Hardened clump from leftover steamed algae, suspension settled on bottom: gave uncharacteristically tough, flaky, rubbery, dendritic mold	1.04
90/2.5/7.5	Tough, thin film, similar to potato chip	0.78
90/7.5/2.5	Thin film, not as tough as large disk	0.8
90/9/1	Very flimsy film, feels a little 'wet' due to glycerol leaking	0.8667
94/3/3	Formed into disc, very tough, thin film due to adding beeswax	0.86
95/1/4	Tough film, could be shaped into thin plastic bowl	0.85

Due to most of the composites having different materials added, or not being processed in a vacuum (unlike the films tested in the previous section), different densities and properties were shown. The average density for composites that sunk in water was 1.26 g/cm^3 and the average density for those which floated in water was 0.83 g/cm^3.

4.3.1 Tensile Tests

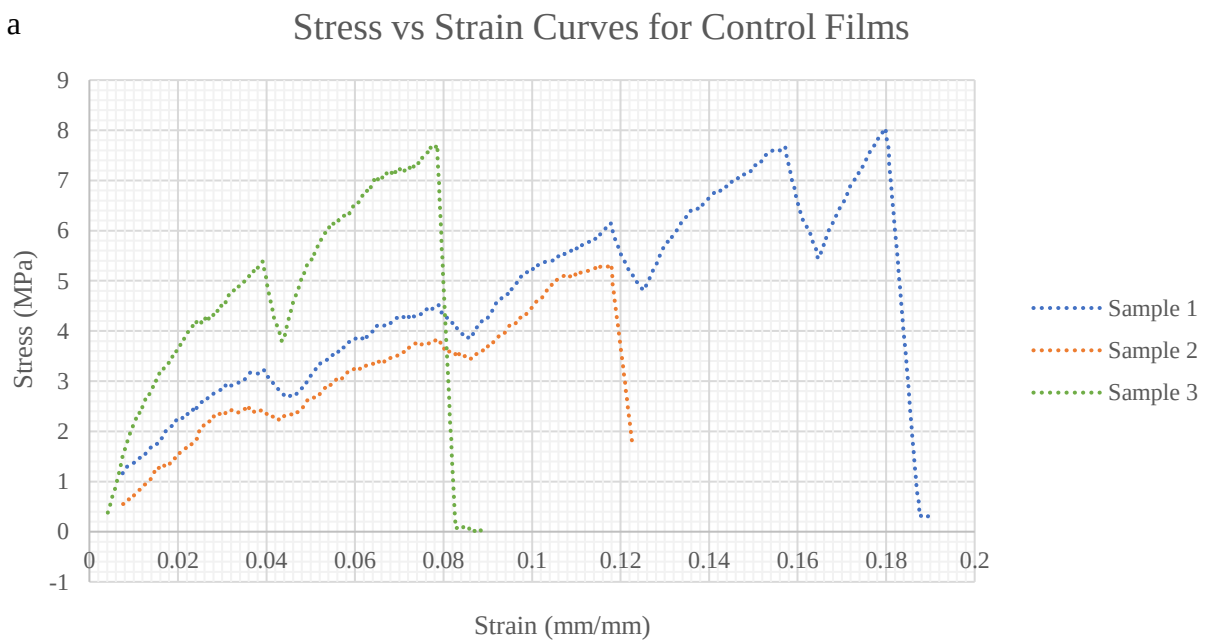
Table 4-12 displays the average thicknesses of each strip used in the tensile test. They generally showed a consistent thickness, except for sample 2 from the films with microalgae, and sample 1 from the films with steam-exploded microalgae. These anomalies may be explained by convection of air in the incubator causing minute temperature gradients across the surface of the film, hence resulting in different surface tensions along the suspension [67] and causing non-uniform thickness. Strips with algae, pectin and glycerol are thicker than those without algae (control) due to the increased volume of material added to the film. There is a contrasting effect when comparing the thickness of strips with treated algae: the thinness of these films are a result of the destruction of the cell wall potentially causing a decrease in the volume of algae added.

Table 4-12: Average thicknesses of various strips from different films used in tensile test

Film Type	Sample	Thickness (mm)
Control (only pectin and glycerol)	1	0.18
	2	0.17
	3	0.18
Algae (contains *C. vulgaris* that has not been treated)	1	0.22
	2	0.13
	3	0.21
400 °C, 15 min (contains steam-exploded *C. vulgaris*)	1	0.04
	2	0.11
	3	0.13
AC Enzymes (contains *C. vulgaris* after treatment with cellulase and macerozyme)	1	0.16
	2	0.16
	3	0.14

Figure 4-12 shows the results from the tensile tests performed on the films made from lipid-extracted microalgae. The highest maximum tensile strength achieved was 11.2 MPa, and the highest elongation before break is 0.18%. All curves have dips due to resetting the machine and releasing some of the tension before starting the test again. The curves look similar to those done in a study by Sharaby et. al. [68]: the control films with solely pectin and a plasticiser have higher elongations of break and smaller ultimate tensile strengths than the films with material added. In Sharaby's study, crystalline nanocellulose (CNC) were added to the control films. CNC increased tensile strength due to its homogenous distribution and interfacial interactions with the pectin matrix. However, higher concentrations decreased tensile strength, possibly from aggregation and self-networking. In this study, the microalgae, enzymes and cell debris found in steam exploded microalgae may have a similar effect to CNC in Sharaby's study.

a



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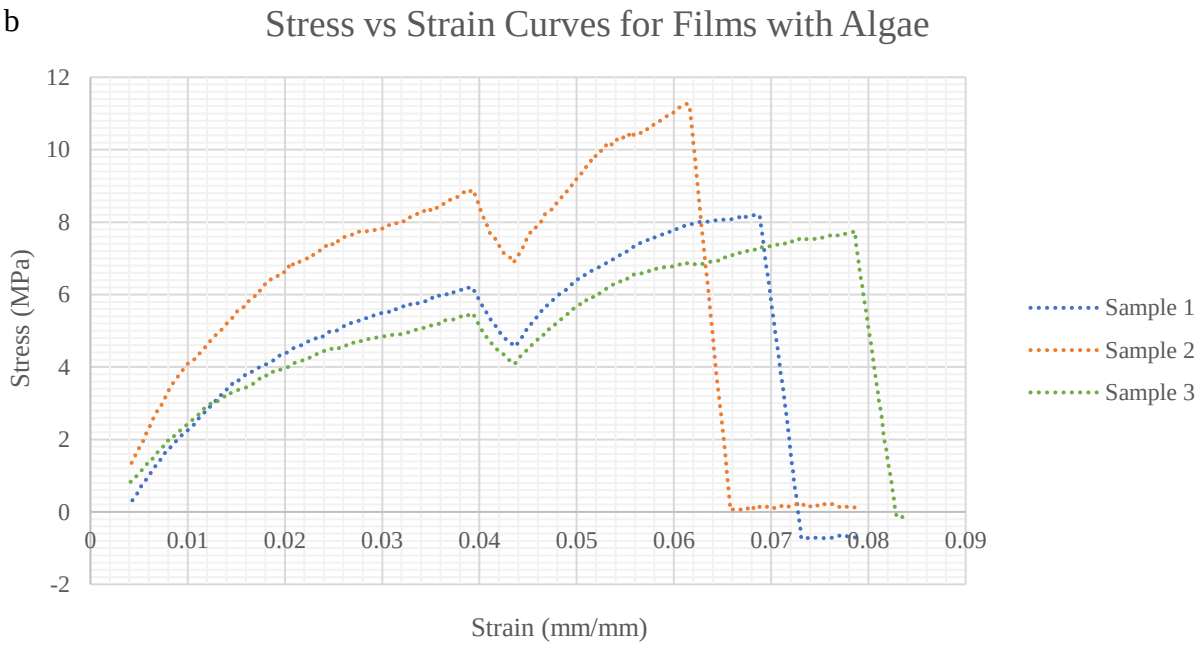
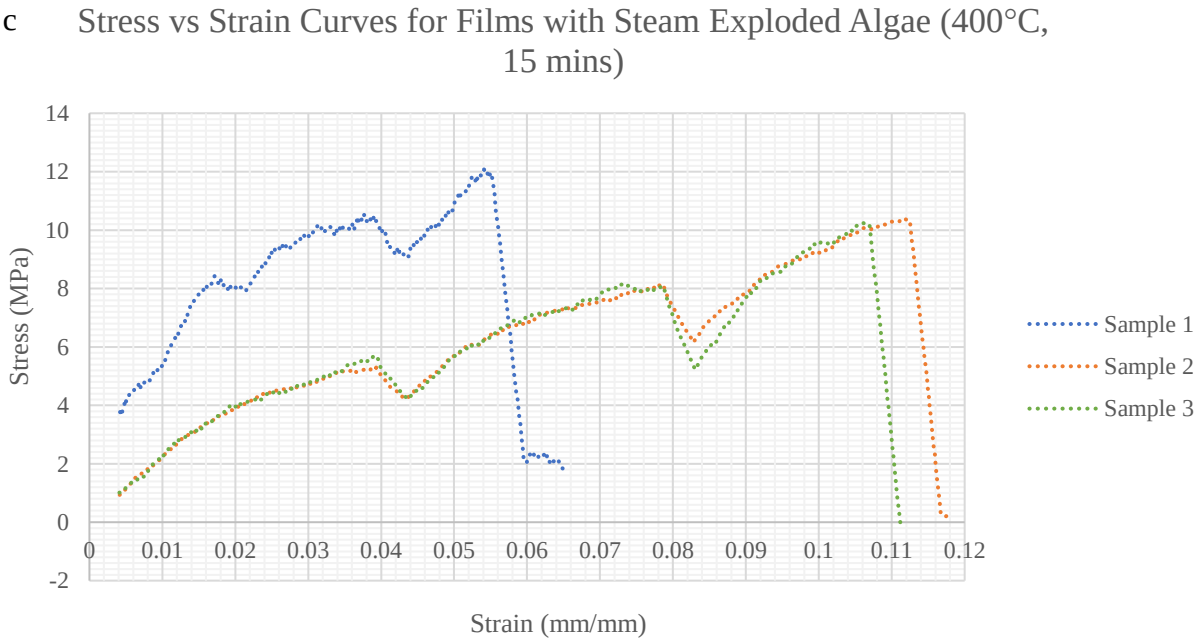

Stress vs Strain Curves for Films with Algae
Stress (MPa)
12
10
8
6
4
2
0
-2
0
0.01
0.02
0.03
0.04
0.05
0.06
0.07
0.08
0.09
Strain (mm/mm)
Sample 1
Sample 2
Sample 3

c


Stress vs Strain Curves for Films with Steam Exploded Algae (400°C, 15 mins)
Stress (MPa)
14
12
10
8
6
4
2
0
-2
0
0.01
0.02
0.03
0.04
0.05
0.06
0.07
0.08
0.09
0.1
0.11
0.12
Strain (mm/mm)
Sample 1
Sample 2
Sample 3

d

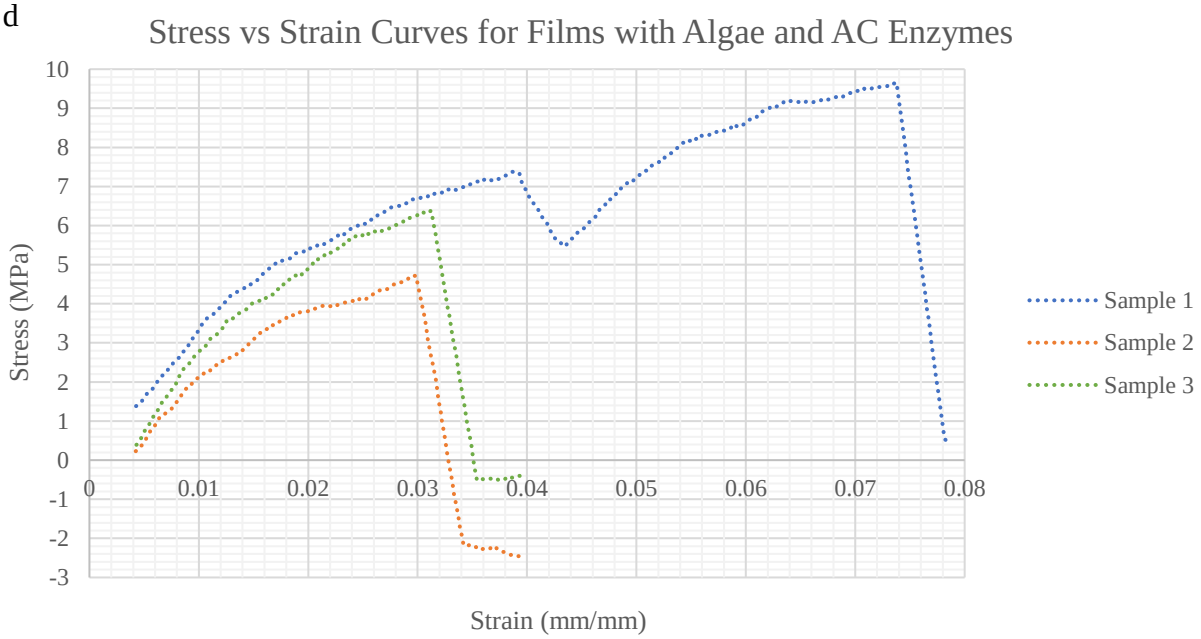


Figure 4-12a, b, c & d: Stress vs strain curves for various pectin films

Figure 4-13 shows the change in maximum tensile strength and Young's Modulus as material is added to control films. Both Young's Modulus and maximum tensile strength of the control in this study was higher than that of a study by Meerarsi and Sothornvit [69]. This may be due to the films in this study being about twice as thick, and hence sturdier, than those used in Meerarsi's study. The highest Young's Modulus achieved was 347.31 ± 72.60 MPa when microalgae was dissolved in pectin films, and the highest ultimate tensile strength was 10.87 ± 0.57 MPa from steam exploded microalgae dissolved in pectin films.

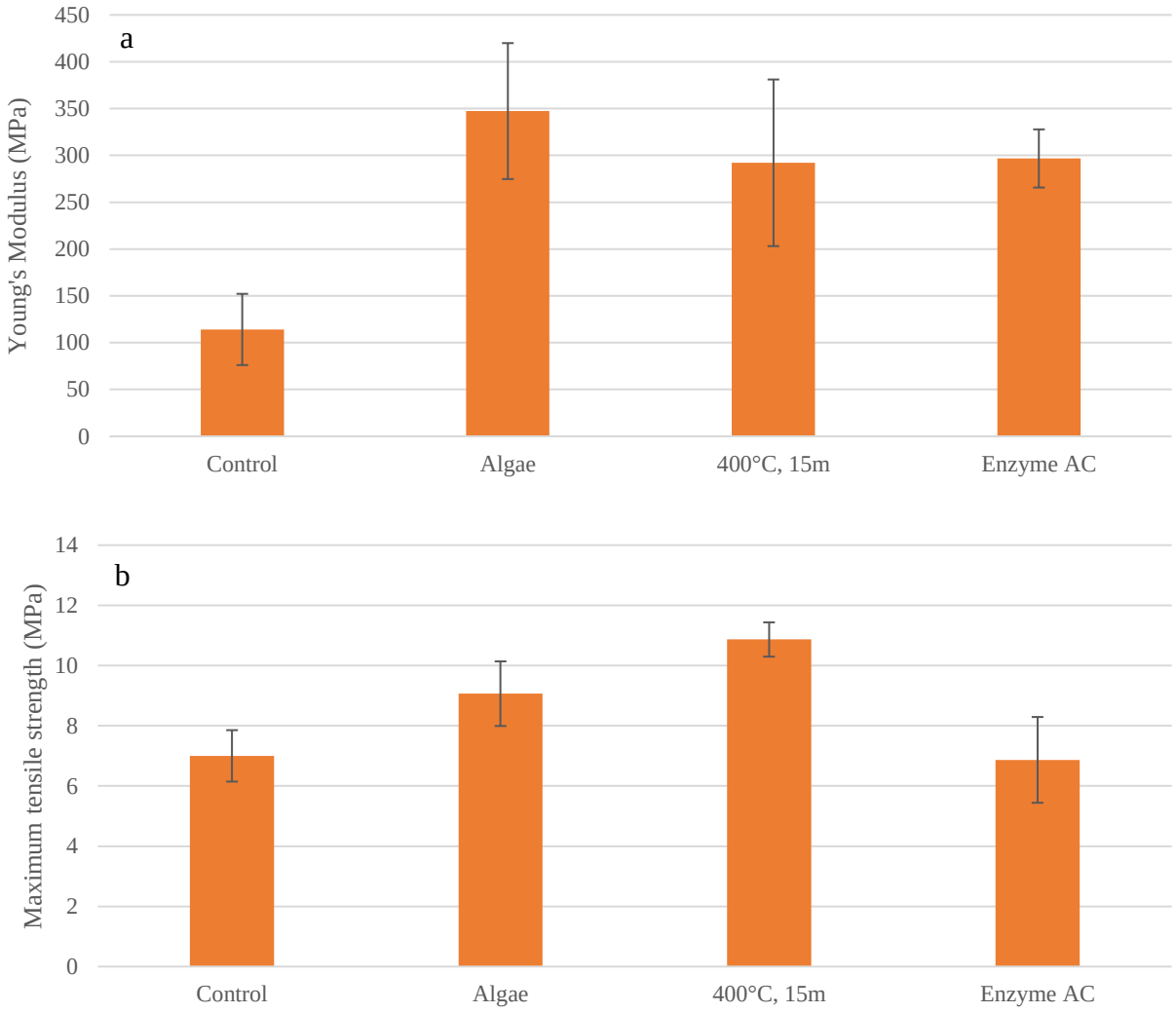


Figure 4-13a & b: Young's Moduli (a) and maximum tensile strengths (b) of different pectin films

Tables 4-13 and 4-14 give the results of the t-tests performed on the Young's Moduli and ultimate tensile strengths of all pectin films created in this study. Young's Modulus is significantly increased when microalgae is added to pectin films, and when enzyme-treated microalgae is added. There is a clear visual difference between the control and steam exploded algae, but the large variance reduces its significance. When comparing the microalgae to its treatments, there is no significant difference. A possible explanation for the significant difference of Young's Modulus' when comparing the control films with algae and enzyme films in Table 4-

13 is that the material was uniformly distributed with no clumps of solids in the film. This meant that functional groups in the pectin matrix could form interactions with the hydroxyl groups of the EPS's of algae or those found on the aromatic rings of cellulase (structure obtained from the National Library of Medicine, PubChem). A similar effect was observed in a study by Wei and A. Pascall [70], where pectin interacted with the phenolic hydroxyl groups of green tea extract. These interactions may provide resistance when the film is elongated. Table 4-14 shows no significant differences in tensile strengths, except when comparing control films to films made with steam-exploded microalgae. This difference could possibly be the result of the agglomeration of cell debris contributing to more interactions within the pectin matrix. With the cell wall damaged, more biomolecules would be available to bind with the functional groups of pectin. Sharaby [68] observed a similar effect with zinc oxide.

Table 4-13: t-tests comparing Young's Moduli of various pectin films

Comparison	**p value**	**Significant (p<0.05)?**
Control with Algae	0.0233	Yes
Control with 400°C, 15 mins	0.0699	No
Control with Enzyme AC	0.0103	Yes
Algae with 400°C, 15 mins	0.3278	No
Algae with Enzyme AC	0.2783	No

Table 4-14: t-tests comparing maximum tensile strengths of various pectin films

Comparison	**p value**	**Significant (p<0.05)?**
Control with Algae	0.1032	No
Control with 400°C, 15 mins	0.0098	Yes

Control with Enzyme AC	0.4700	No
Algae with 400°C, 15 mins	0.1063	No
Algae with Enzyme AC	0.1425	No

4.3.2 Water Solubility Tests

The solubilities of films are shown in Figure 4-14. There is hardly any difference between the solubilities of films, except for the film which used steam-exploded microalgae. This had the highest solubility percentage of 54.19 ± 1.82%. The results mostly showed a similar result to a concept shown in a study Layuk et al [71], which demonstrated that varying the concentration of chitosan while keeping the pectin levels constant results in similar solubilities. Layuk's study also showed solubilities of films that were >98%. The higher solubilities may be explained by the time the films were left in solution (not recorded in the article), and the fact that films are thinner and use less pectin and glycerol (1% each) than this study, resulting in more water permeation within the film structure. Pectin levels were also kept constant in this study, and hence the solubilities do not differ significantly from each other at 95% confidence.

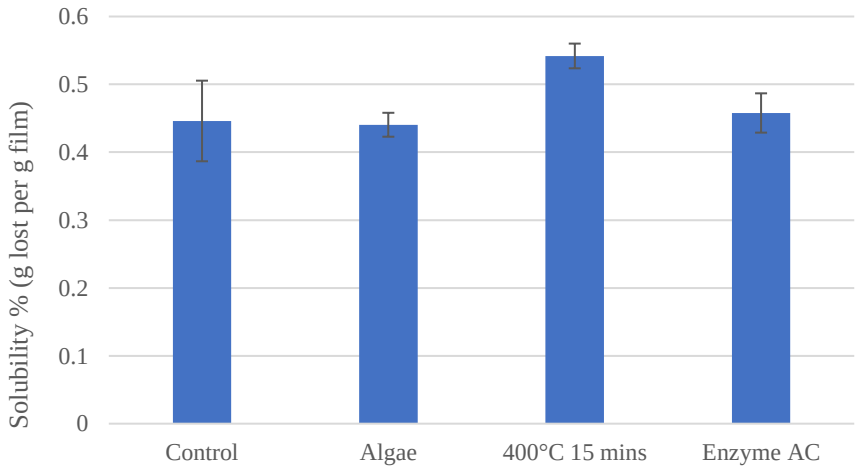


Figure 4-14: Solubilities of various pectin films

Table 4-15 shows that, according to the t-test, there is no significant difference between the solubilities of the films. However, the significant difference between the control film and film using steam exploded microalgae may be explained by a study by Pereira et. al. [72]. This study posits that the additional water-soluble salts in the films increased the water solubility compared to the control, hence water-soluble proteins from damaged cell walls in the microalgae may have contributed to an increase in solubility for this study. Since proteins and carbohydrates in microalgae degrade at 209 and 164 °C [54] and the maximum internal temperature reached during steam explosion at 400 °C is 80-145 °C (Figure 4-2), there is a chance that soluble biomolecules were not degraded and dissolved in the surrounding water, leading to the increased solubility.

Table 4-15: t-tests to compare the solubilities of various pectin films

Comparison	**p value**	**Significant (p<0.05)?**
Control with Algae	0.4662	No
Control with 400°C, 15 mins	0.0990	No
Control with Enzyme AC	0.4337	No
Algae with 400°C, 15 mins	0.0081	Yes
Algae with Enzyme AC	0.3177	No

A major limitation in this study was the inability of pectin to bind with microalgae which had been steam exploded at 500 °C, as the films formed were tough and flaky, and could not be used in tensile testing. This is seen in Figure 4-15 and may be explained by the reduction of water activity increasing the speed of gelation [73]. Since the suspension had turned yellow, this suggests the water's activity had been reduced. Another possible explanation is that magnesium

from the chlorophyll may have been displaced and formed a complex with pectin. Magnesium has a lower gelling ability [74], and therefore magnesium pectate may not have been able to form properly.

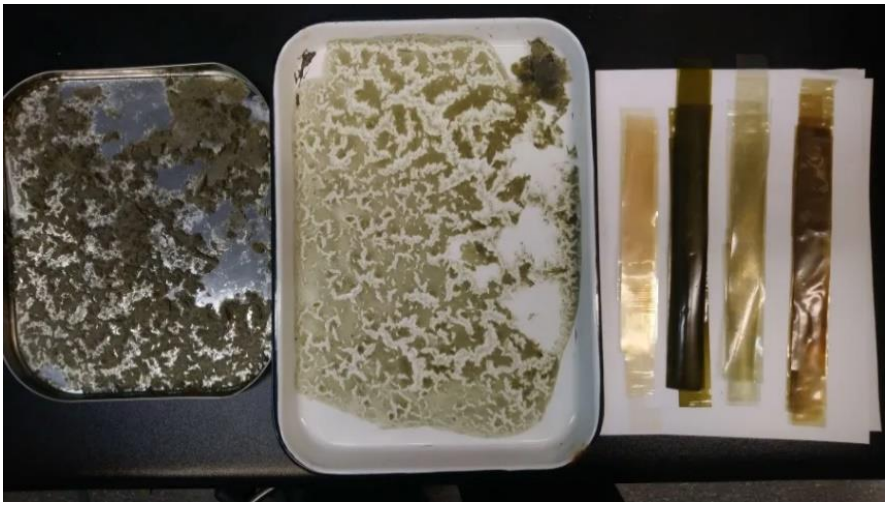


Figure 4-15: Photo of pectin films made with steam exploded microalgae at 500 °C (left) and all other films (control, algae, steam explosion, enzyme treatment)

CHAPTER 5: CONCLUSIONS AND RECOMMENDATIONS

In this study, the effects of temperature and duration of steam explosion were investigated. Several conclusions can be made with regards the values of these parameters, as well as the properties of bioproducts from the waste of the steam explosion and lipid extraction process:

- The use of a combination of cellulase, lysozyme and macerozyme can improve lipid yields. The highest yield with enzymatic treatment was almost a twofold increase compared to the control.
- Steam explosion significantly improved lipid yield. Though steam explosion at 500 °C for an additional 45 minutes produced the highest amount of lipids (23.75 mg) with algal concentrations of ~4 g/L, it also introduces the highest amount of solids and decreases the yield. The best steam explosion temperature and duration for the optimal lipid yield is 400 °C for an additional 45 minutes, which resulted in an increase of yield from 15.16 ± 1.52 mg lipids/g microalgae to 124.39 ± 15.46 mg/g. Steam explosion may also be used to improve biodiesel yield.
- Microalgae from lipid extraction (untreated or steam exploded at 400 °C) may be incorporated into pectin films to significantly improve their modulus of elasticity and tensile strength. Using untreated microalgae increased the Young's Modulus from 114.13 ± 38.04 to 347.31 ± 72.60 MPa, while 400 °C steam exploded microalgae increased the ultimate tensile strength from 7 ± 0.85 to 10.87 ± 0.57 MPa.
- The solubility of pectin films is mostly unaffected by the addition of microalgae, but pre-treating the culture using steam explosion may result in an increase of solubility.

Overall, this study has successfully demonstrated the ability of steam explosion to increase lipid yields in two experiments, as well as improve tensile properties of pectin films. However, there are certain limitations which may affect the validity of these conclusions:

- Gravimetric methods introduce error via the mass balance itself. In addition to instrumental error, there is an uncertainty associated with measurements. The minimum weight which allows a tolerance of 0.1% is 10 mg, and weights below this are subject to higher tolerances and hence greater error.
- It is not possible to control how much solid material and lipids are deposited in the steam explosion device during testing. Therefore, the amount of microalgae and lipids available after inverting the device may vary widely within the triplicate.
- The volume of algae suspension varies with each treatment, and its viscosity increases greatly with the addition of pectin. Similarly to the previous point, the volume of pectin suspension that remains in the flask cannot be controlled, hence the volume of suspension added to the tray is not consistent with each film.

There are many paths towards future research, and beyond the limitations, there are elements of the current research which may be improved. Some recommendations if this research is to be carried forward are to:

- Perform a life cycle analysis on the entire process to better understand the energy consumption and determine the environmental impacts of the procedure should it be scaled up.
- Experiment with a higher range of variables with smaller intervals, for example: taking a range of oven temperatures from 350 to 550 °C with intervals of 50 °C and using 15 to 60

minutes of additional steam explosion, with intervals of 15 minutes. Additionally, the effect of the ratio of pectin and glycerol on the tensile strength may also be studied.

- Investigate waterproof coatings (beeswax and ethanol) for the pectin films, and its effect on tensile strength and solubility.
- Research the thermal properties of pectin films (thermogravimetric analysis, differential scanning calorimetry) to decide if the polymer could be used in 3D printing. An example of the 'ink' is shown in Figure A-3.
- Explore the use of high lipid-yielding microalgal species, such as *Nannochloropsis* sp. and *Scenedesmus obliquus* [30].
- Start scaling up the process and incorporate wastewater, as *Chlorella vulgaris* has been shown to remove nutrients from wastewater [75]. A possible concept for a pilot plant is given in Figure A-4.
- Store lipids from microalgae in an organic solvent in the freezer at -20 °C, and not exposed to light. This ensures that oxidation of the lipids is slowed down for longer term storage [76]. Additionally, lipid extraction should be performed immediately after steam explosion tests, and the yield calculations must remain consistent.

REFERENCES

1. Walker TR, Fequet L. Current trends of unsustainable plastic production and micro(nano)plastic pollution. Trends Analyt Chem. 2023;160(116984):116984.
2. Knothe G, Razon LF. Biodiesel fuels. Prog Energy Combust Sci. 2017;58:36–59.
3. Luque R, Lovett JC, Datta B, Clancy J, Campelo JM, Romero AA. Biodiesel as feasible petrol fuel replacement: a multidisciplinary overview. Energy Environ Sci. 2010;3(11):1706.
4. Vasudevan PT, Briggs M. Biodiesel production--current state of the art and challenges. J Ind Microbiol Biotechnol. 2008;35(5):421.
5. Patel KR, Dhiman VD. Effect of hybrid nanoparticles MgO and Al2O3 added water-in-diesel emulsion fueled diesel engine using hybrid deep neural network-based spotted hyena optimization. Heat Transfer. 2022 Apr 16;51(6):5748–88.
6. Jackson RB, Le Quéré C, Andrew RM, Canadell JG, Peters GP, Roy J, et al. Warning signs for stabilizing global CO2 emissions. Environmental Research Letters. 2017 Nov 13;12(110202):1–4.
7. Jørgensen SV, Hauschild MZ, Nielsen PH. Assessment of urgent impacts of greenhouse gas emissions—the climate tipping potential (CTP). Int J Life Cycle Assess. 2014;19(4):919–30.
8. Yanamala N, Hatfield MK, Farcas MT, Schwegler-Berry D, Hummer JA, Shurin MR, et al. Biodiesel versus diesel exposure: Enhanced pulmonary inflammation, oxidative stress, and differential morphological changes in the mouse lung. Toxicol Appl Pharmacol. 2013;272(2):373–83.

9. Li L, Zuo J, Duan X, Wang S, Hu K, Chang R. Impacts and mitigation measures of plastic waste: A critical review. Environ Impact Assess Rev. 2021;90(106642):106642.
10. Sharma S, Sharma V, Chatterjee S. Contribution of plastic and microplastic to global climate change and their conjoining impacts on the environment - A review. Sci Total Environ. 2023;875(162627):162627.
11. Lloyd AC, Cackette TA. Diesel engines: environmental impact and control. J Air Waste Manag Assoc. 2001;51(6):809–47.
12. Spain O, Plöhn M, Funk C. The cell wall of green microalgae and its role in heavy metal removal. Physiol Plant. 2021;173(2):526–35.
13. Wahlen BD, Morgan MR, McCurdy AT, Willis RM, Morgan MD, Dye DJ, et al. Biodiesel from microalgae, yeast, and bacteria: Engine performance and exhaust emissions. Energy Fuels. 2013;27(1):220–8.
14. Weber S, Grande PM, Blank LM, Klose H. Insights into cell wall disintegration of *Chlorella vulgaris*. PLoS One. 2022;17(1):e0262500.
15. Colusse GA, Carneiro J, Duarte MER, Carvalho JC de, Noseda MD. Advances in microalgal cell wall polysaccharides: a review focused on structure, production, and biological application. Crit Rev Biotechnol. 2022;42(4):562–77.
16. Kim D-Y, Vijayan D, Praveenkumar R, Han J-I, Lee K, Park J-Y, et al. Cell-wall disruption and lipid/astaxanthin extraction from microalgae: *Chlorella* and *Haematococcus*. Bioresour Technol. 2016;199:300–10.
17. Xiao R, Zheng Y. Overview of microalgal extracellular polymeric substances (EPS) and their applications. Biotechnol Adv. 2016;34(7):1225–44.

18. Alhattab M, Kermanshahi-Pour A, Brooks MS-L. Microalgae disruption techniques for product recovery: influence of cell wall composition. J Appl Phycol. 2019;31(1):61–88.
19. Ziegler-Devin I, Chrusciel L, Brosse N. Steam Explosion pretreatment of lignocellulosic biomass: A mini-review of theorical and experimental approaches. Front Chem. 2021;9:705358.
20. Dekker RF, Wallis AF. Enzymic saccharification of sugarcane bagasse pretreated by autohydrolysis-steam explosion. Biotechnol Bioeng. 1983;25(12):3027–48.
21. Onumaegbu C, Mooney J, Alaswad A, Olabi AG. Pre-treatment methods for production of biofuel from microalgae biomass. Renew Sustain Energy Rev. 2018;93:16–26.
22. Kataria R, Mol A, Schulten E, Happel A, Mussatto SI. Bench scale steam explosion pretreatment of acid impregnated elephant grass biomass and its impacts on biomass composition, structure and hydrolysis. Ind Crops Prod. 2017;106:48–58.
23. Sakthivel R, Ramesh K, Purnachandran R, Mohamed Shameer P. A review on the properties, performance and emission aspects of the third generation biodiesels. Renew Sustain Energy Rev. 2018;82:2970–92.
24. Pascoe Ortiz S. Are bioplastics the solution to the plastic pollution problem? PLoS Biol. 2023;21(3):e3002045.
25. Nandakumar A, Chuah J-A, Sudesh K. Bioplastics: A boon or bane? Renew Sustain Energy Rev. 2021;147(111237):111237.
26. Gurram R, Souza Filho PF, Taherzadeh MJ, Zamani A. A solvent-free approach for production of films from pectin and fungal biomass. J Polym Environ. 2018;26(11):4282–92.

27. Ren W, Qiang T, Chen L. Recyclable and biodegradable pectin-based film with high mechanical strength. Food Hydrocoll. 2022;129(107643):107643.
28. Matheus JRV, Nogueira TB de B, Pereira APA, Correia TR, de Sousa AMF, Pastore GM, et al. Antibacterial films made with persimmon (*Diospyros kaki L.*), pectin, and glycerol: An experimental design approach. J Food Sci. 2021;86(10):4539–53.
29. Zhou J, Wang M, Saraiva JA, Martins AP, Pinto CA, Prieto MA, et al. Extraction of lipids from microalgae using classical and innovative approaches. Food Chem. 2022;384(132236):132236.
30. Mat Husin MA, Mohd Yasin NH, Takriff MS, Jamar NH. A review on pretreatment methods for lipid extraction from microalgae biomass. Prep Biochem Biotechnol. 2023;1–16.
31. Lee SY, Khoiroh I, Vo D-VN, Senthil Kumar P, Show PL. Techniques of lipid extraction from microalgae for biofuel production: a review. Environ Chem Lett. 2021;19(1):231–51.
32. Nagappan S, Devendran S, Tsai P-C, Dinakaran S, Dahms H-U, Ponnusamy VK. Passive cell disruption lipid extraction methods of microalgae for biofuel production – A review. Fuel (Lond). 2019;252:699–709.
33. Russell C, Rodriguez C. Lipid extraction from *Chlorella vulgaris* & *Haematococcus pluvialis* using the switchable solvent DMCHA for biofuel production. Energy (Oxf). 2023;278(127983):127983.
34. Rokicka M, Zieliński M, Dudek M, Dębowski M. Effects of ultrasonic and microwave pretreatment on lipid extraction of microalgae and methane production from the residual extracted biomass. Bioenergy Res. 2021;14(3):752–60.

35. Krishnamoorthy A, Rodriguez C, Durrant A. Optimisation of ultrasonication pretreatment on microalgae *Chlorella Vulgaris* & *Nannochloropsis Oculata* for lipid extraction in biodiesel production. Energy (Oxf). 2023;278(128026):128026.
36. Alavijeh RS, Karimi K, Wijffels RH, van den Berg C, Eppink M. Combined bead milling and enzymatic hydrolysis for efficient fractionation of lipids, proteins, and carbohydrates of *Chlorella vulgaris* microalgae. Bioresour Technol. 2020;309(123321):123321.
37. Nazloo EK, Moheimani NR, Ennaceri H. Biodiesel production from wet microalgae: Progress and challenges. Algal Res. 2022;68(102902):102902.
38. Ideris F, Zamri MFMA, Shamsuddin AH, Nomanbhay S, Kusumo F, Fattah IMR, et al. Progress on conventional and advanced techniques of in situ transesterification of microalgae lipids for biodiesel production. Energies. 2022;15(19):7190.
39. Dasan YK, Lam MK, Yusup S, Lim JW, Lee KT. Life cycle evaluation of microalgae biofuels production: Effect of cultivation system on energy, carbon emission and cost balance analysis. Sci Total Environ. 2019;688:112–28.
40. Moradi P, Saidi M. Biodiesel production from *Chlorella Vulgaris* microalgal-derived oil via electrochemical and thermal processes. Fuel Process Technol. 2022;228(107158):107158.
41. Felix C, Ubando A, Madrazo C, Gue IH, Sutanto S, Tran-Nguyen PL, et al. Non-catalytic in-situ (trans) esterification of lipids in wet microalgae *Chlorella vulgaris* under subcritical conditions for the synthesis of fatty acid methyl esters. Appl Energy. 2019;248:526–37.
42. Alam MA, Wu J, Xu J, Wang Z. Enhanced isolation of lipids from microalgal biomass with high water content for biodiesel production. Bioresour Technol.

43. Kalsum U, Mahmuddin, Mahfud M, Roesyadi A. Biodiesel Production from *Chlorella vulgar*is via Homogenous Acid Catalyzed In situ Transesterification with Microwave Irradiation. IOP Conf Ser Earth Environ Sci. 2018;175:012018.
44. Tien Thanh N, Mostapha M, Lam MK, Ishak S, Kanna Dasan Y, Lim JW, et al. Fundamental understanding of in-situ transesterification of microalgae biomass to biodiesel: A critical review. Energy Convers Manag. 2022;270(116212):116212.
45. Mathimani T, Baldinelli A, Rajendran K, Prabakar D, Matheswaran M, Pieter van Leeuwen R, et al. Review on cultivation and thermochemical conversion of microalgae to fuels and chemicals: Process evaluation and knowledge gaps. J Clean Prod. 2019;208:1053–64.
46. Kim S, Kim M, Chang YK, Kim D. Lipid production under a nutrient-sufficient condition outperforms starvation conditions due to a natural polarization of lipid content in algal biofilm. SSRN Electron J. 2022.
47. Aguirre A-M. Study of high pressure steaming on lipid recovery from microalgae. [Chemical and Biochemical Engineering]: The University of Western Ontario; 2014.
48. Axelsson M, Gentili F. A single-step method for rapid extraction of total lipids from green microalgae. PLoS One. 2014;9(2):e89643.
49. Kim H-Y. Statistical notes for clinical researchers: Understanding standard deviations and standard errors. Restor Dent Endod. 2013;38(4):263–5.
50. Gerken HG, Donohoe B, Knoshaug EP. Enzymatic cell wall degradation of *Chlorella vulgaris* and other microalgae for biofuels production. Planta. 2013;237(1):239–53.
51. Dhar R, Chakraborty S. Enzyme hydrolyzed bael fruit liquefaction and its kinetic study. Food Biosci. 2022;47(101779):101779.

52. Arora N, Philippidis GP. Insights into the physiology of *Chlorella vulgaris* cultivated in sweet sorghum bagasse hydrolysate for sustainable algal biomass and lipid production. Sci Rep. 2021;11(1):6779.

53. Russ A, Fišerová M, Letko M, Opálená E. Effect of Steam Explosion Temperature on Wheat Straw Hydrolysis. Wood Research. 2015 Nov;61(1):65–74.

54. Chen W-H, Chu Y-S, Liu J-L, Chang J-S. Thermal degradation of carbohydrates, proteins and lipids in microalgae analyzed by evolutionary computation. Energy Convers Manag. 2018;160:209–19.

55. Szabo Z, Marosvölgyi T, Szabo E, Koczka V, Verzar Z, Figler M, et al. Effects of repeated heating on fatty acid composition of plant-based cooking oils. Foods. 2022;11(2):192.

56. Song W, Huang R, Guo H, Yin C, Wang C, Cheng J, et al. Characterization of wet microalgal cells pretreated with steam for lipid extraction. Chin J Chem Eng. 2021;37:114–20.

57. Kong F, Zeng Q, Li Y, Di X, Ding Y, Guo X. Effect of steam explosion on nutritional components, physicochemical and rheological properties of brown rice powder. Front Nutr. 2022;9:954654.

58. Brown MB. Exploring interaction effects in the ANOVA. J R Stat Soc Ser C Appl Stat. 1975;24(3):288.

59. Pignitter M, Stolze K, Gartner S, Dumhart B, Stoll C, Steiger G, et al. Cold fluorescent light as major inducer of lipid oxidation in soybean oil stored at household conditions for eight weeks. J Agric Food Chem. 2014;62(10):2297–305.

60. Ru ITK, Sung YY, Jusoh M, Wahid MEA, Nagappan T. *Chlorella vulgaris*: a perspective on its potential for combining high biomass with high value bioproducts. Applied Phycology. 2020;1(1):2–11.
61. Yilmaz C, Gökmen V. Chlorophyll. In: Encyclopedia of Food and Health. Ankara, Turkey: Hacettepe University; 2015. p. 37–41.
62. Weemaes CA, Ooms V, Van Loey AM, Hendrickx ME. Kinetics of chlorophyll degradation and color loss in heated broccoli juice. J Agric Food Chem. 1999;47(6):2404–9.
63. Kariola T, Brader G, Li J, Palva ET. Chlorophyllase 1, a damage control enzyme, affects the balance between defense pathways in plants. Plant Cell. 2005;17(1):282–94.
64. Liberton M, Austin JR 2nd, Berg RH, Pakrasi HB. Insights into the complex 3-D architecture of thylakoid membranes in unicellular cyanobacterium *Cyanothece sp*. ATCC 51142. Plant Signal Behav. 2011;6(4):566–9.
65. Zhang K, Sun B, She X, Zhao F, Cao Y, Ren D, et al. Lipid production and composition of fatty acids in *Chlorella vulgaris* cultured using different methods: photoautotrophic, heterotrophic, and pure and mixed conditions. Ann Microbiol. 2014;64(3):1239–46.
66. Xia S, Luo H, Ma P. Ternary liquid-liquid equilibrium for systems of fatty acid methyl ester(methyl palmitate/methyl stearate)+ethanol+glycerol at atmospheric pressure. Trans Tianjin Univ. 2015;21(5):392–9.
67. Fedyushkin AI. The effect of convection on the position of the free liquid surface under zero and terrestrial gravity. J Phys Conf Ser. 2020;1675(1):012039.
68. Sharaby MR, Soliman EA, Abdel-Rahman AB, Osman A, Khalil R. Novel pectin-based nanocomposite film for active food packaging applications. Sci Rep. 2022;12(1):20673.

69. Meerasri J, Sothornvit R. Characterization of bioactive film from pectin incorporated with gamma-aminobutyric acid. Int J Biol Macromol. 2020;147:1285–93.

70. Wei H, Pascall MA. Evaluation of structural and functional properties of citrus pectin film enriched with green tea extract. Polymer Engineering. 2023 Jun 1;63(8):2522–33.

71. Layuk P, Winanda E, Lintang M, Lukas A, Ramlah S, La Ola LO. Characteristics of sago and pectin edible film with the addition of chitosan and its use in hard candy wrappers. IOP Conf Series: Earth and Environmental Science. 2023 Dec 12;1230(4):1–10.

72. Pereira DGM, Vieira JM, Vicente AA, Cruz RMS. Development and characterization of pectin films with *Salicornia ramosissima*: Biodegradation in soil and seawater. Polymers (Basel). 2021;13(16):2632.

73. Said NS, Olawuyi IF, Lee WY. Pectin hydrogels: Gel-forming behaviors, mechanisms, and food applications. Gels. 2023;9(9).

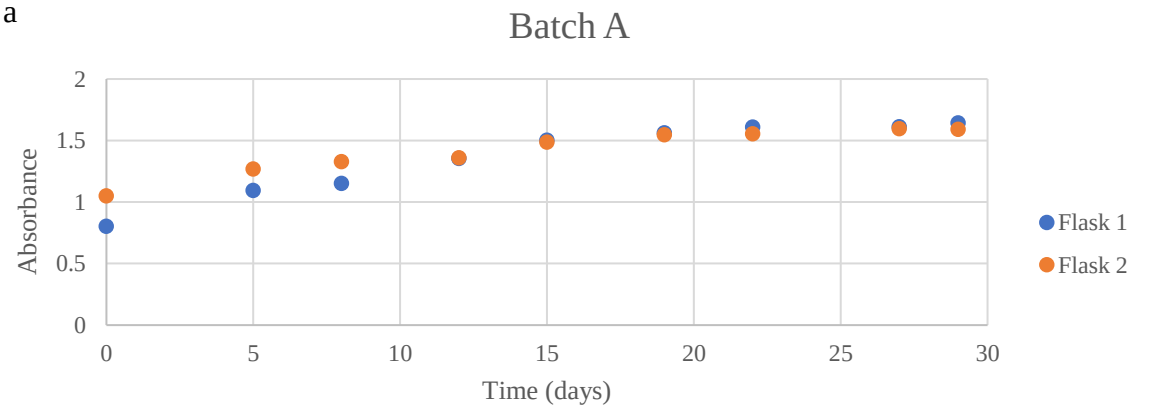
74. Moslemi M. Reviewing the recent advances in application of pectin for technical and health promotion purposes: From laboratory to market. Carbohydr Polym. 2021;254(117324):117324.

75. Znad H, Al Ketife AMD, Judd S, AlMomani F, Vuthaluru HB. Bioremediation and nutrient removal from wastewater by *Chlorella vulgaris*. Ecol Eng. 2018;110:1–7.

76. Ulmer CZ, Koelmel JP, Jones CM, Garrett TJ, Aristizabal-Henao JJ, Vesper HW, et al. A review of efforts to improve lipid stability during sample preparation and standardization efforts to ensure accuracy in the reporting of lipid measurements. Lipids. 2021;56(1):3–16.

APPENDICES

a



b

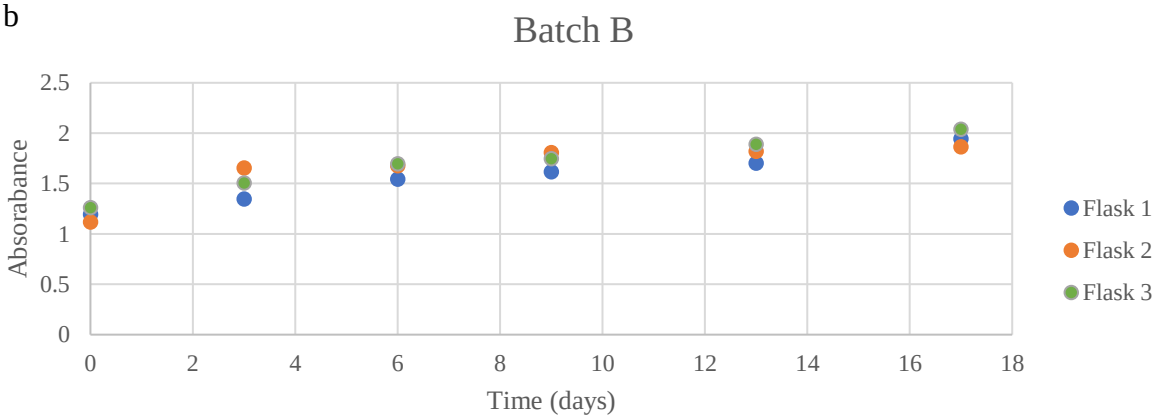


Figure A-1a & b: Growth of Batch A (a) and Batch B (b) of Chlorella vulgaris over time

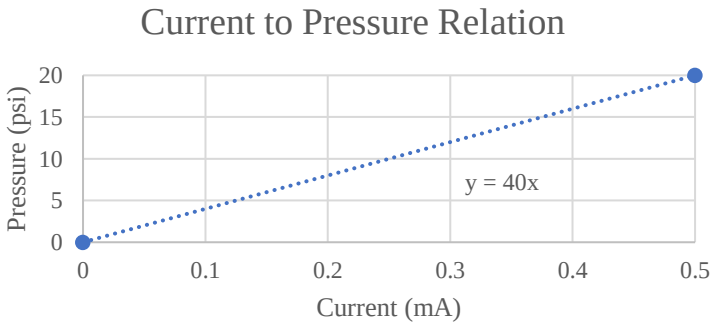


Figure A-2: Calibration of pressure transducer

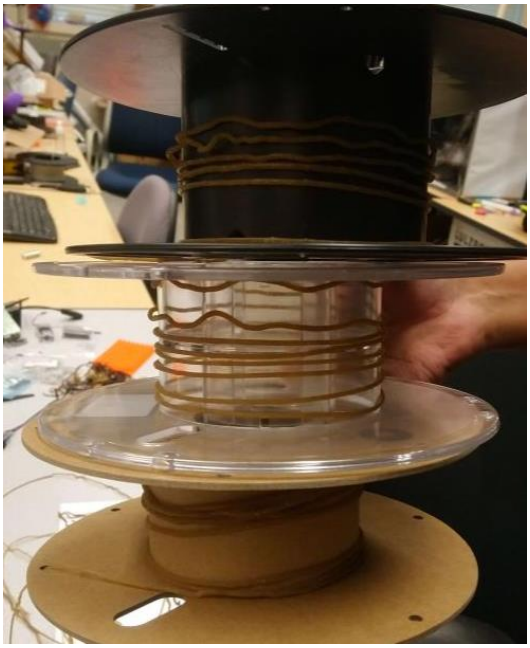


Figure A-3: Demonstration of threading of algae-pectin films for 3D printing

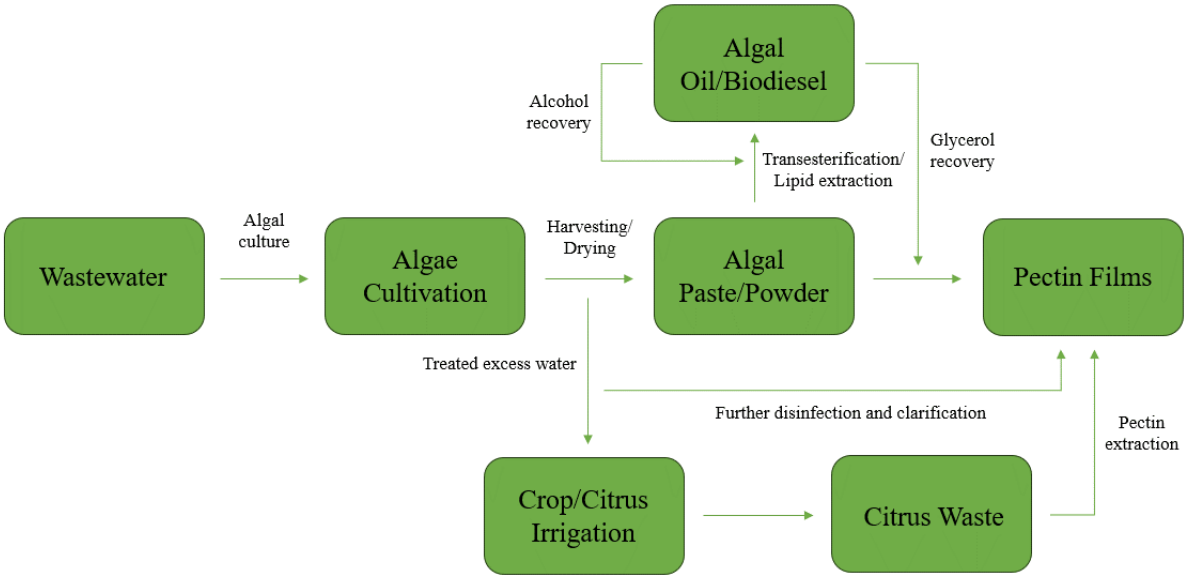


Figure A-4: Concept for a wastewater to bioproducts plant, based on this study

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