INVESTIGATION OF THE EFFECT OF PREPROCESSING PARAMETERS ON THE EFFICACY OF CHLOROPHYLL REMOVAL FROM *MYCHONASTES HOMOSPAERA* FOR BIOFUEL PRODUCTION

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Degree of Master of Science

Department of Chemical and Process Engineering

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Thesis submitted in partial fulfillment of the requirements for the degree Master of Science in Chemical and Process Engineering

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DECLARATION

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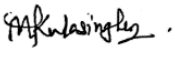

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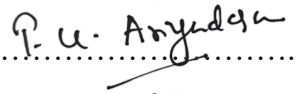

04/05/2021

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Abstract

Microalgal lipids have become a potential candidate for biofuel production in recent years. High lipid accumulation and shorter doubling time enabling higher growth rate are foremost factors in microalgae to compete with first and second-generation biodiesel feedstocks. However, high levels of chlorophyll in feedstock limit its large-scale application. Chlorophyll makes oil more susceptible to photo-oxidation, decreases the storage stability, causes low-quality oil with a dull and dark color, and decreases the transesterification efficiency and combustion efficiency of biodiesel. This study aimed to develop a novel preprocessing method to identify the best solvent ratios, temperature, and reaction time for chlorophyll removal from the selected microalgae to synthesize high-quality biodiesel. *Mychonastes homosphaera* isolated from Beire Lake, Colombo, Sri Lanka with a doubling time, and the lipid accumulation of 2.89 d and 58 % (w/w) was selected for the study. The results indicated that the best solvent ratio (NaOH: ethanol), temperature, and reaction time were 7:3, 60 ^{0}C, and 90 min, respectively.

Keywords - biodiesel, biomass, chlorophyll, fatty acids, lipids, microalgae

DEDICATION

Dedicated to my mother, father and husband for their unconditional love, endless support, and encouragement

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I owe my deepest gratitude to the people mentioned below without whom the successful completion of this research would not have been possible.

Foremost, I would like to express my heartfelt gratitude to my supervisor Dr. H.L.T.U.Ariyadasa for the immense support and the technical guidance provided throughout this research. Her immense knowledge and vast experience in the field of microbiology and third generation biofuel production helped me tremendously whenever I had come across with problems. Also, I am very much grateful for the inspiration and motivation given by her to proceed the research.

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LIST OF ABBREVIATIONS

Abbreviation	Description
BBM	Bold basal medium
CN	Cetane number
CTAB	Cetyltrimethylammonium bromide
ER	Endoplasmic reticulum
FAME	Fatty acid methyl esters
FAS	Fatty acid synthesis
FTL	Fischer-Tropsch synthesis
GHG	Green House Gas
GL	Glycerolipids
IV	Iodine value
LCSF	Long chain saturation factor
MUFA	Monounsaturated faty acids
PL	Polar lipids
PUFA	Polyunsaturated fatty acids
SFA	Saturated fatty acids
SMC	Sodium magnesium chlorophyllin
SV	Saponification value
TAGs	Triacylglycerides
TL	Total lipids
USFA	Unsaturated fatty acids

1. INTRODUCTION

The world's total energy utilization segregates into 85% from non-renewable and 15% from renewable energy sources [1]. The energy consumption has increased over time, creating an imbalance between demand and supply [2]. Excessive energy usage leads to environmental issues such as greenhouse gas (GHG) emission, acid rains, global warming, ozone layer depletion, air pollution, and climate change [3]. Drilling to acquire oil affects the natural landscape, biodiversity, and wildlife. Oil combustion and coal mining contribute to high levels of pollution towards the biosphere [4], [5].

The depletion of fossil fuels encourages scientists to discover sustainable energy sources that are renewable, ecofriendly, and carbon-neutral to replace non-renewable fuels [6]. Renewable energy sources include wind, sunlight, hydropower, geothermal energy, and biomass [7]. Biofuels are derived from biomass and can be mainly categorized into three generations based on the primary source [8]. Animal fats, subsistence crops, and food grains are the major sources of first-generation biofuels [9]. Plant materials such as lignocellulosic biomass is utilized as the feedstock in second-generation biofuels [8]. Aquatic biomass, including macroalgae and microalgae, are the promising feedstock for third generation biofuel production [8]. The third-generation feedstock is more suitable than the first and second-generation biomass for biofuel production due to high lipid yield and less land requirement [8].

Microalgae are considered as an optimal feedstock for biodiesel production [10]. Chlorophyll in algae limits its application in diesel engines due to the low combustion efficiency [11]. The existence of chlorophyll in biodiesel reduces the storage stability of oil and photo-oxidize by oxygen and light [12]. This can impact biodiesel quality by lowering the long-chain saturation factor (LCSF), saponification value (SV), and cetane number (CN) [11].

Chlorophyll is a nonpolar compound due to alcoholic phytol and soluble in nonpolar organic solvents [13]. These organic solvents include ether, ethanol, chloroform, methanol, and acetone [14]. Nevertheless, chlorophyll removal from algal biomass should be economically feasible and reduce total lipid loss [15]. Conventional chlorophyll removal methods are physical adsorption, phosphoric acid degumming,

and oxidative treatment followed by solvent extraction [16]. The biodiesel quality was significantly improved by surface adsorption after using a catalyst in the transesterification reaction, according to the study conducted by Baroi et al. (2013) [17]. Przybylski et al. used activated charcoal for bleaching, and the amount of chlorophyll in the feedstock was reduced by 60% [18]. According to Bahmaei et al., chlorophyll removal efficiency in canola oil was reported as 97.6% by reacting with sulfuric acid and phosphoric acid (8:7, v/v) at 50 ^{0}C for 5 min [19].

Further, the chlorophyll removal in *Scenedesmus* sp was carried out by Chen et al. (2012) using bleaching agents [18], [20]. According to the results, the chlorophyll in the algae biomass was reduced by 98.06% [14]. These methods caused a high net lipid loss during chlorophyll removal. Therefore, these methods were not suitable for large-scale industrial applications in biodiesel production. T. Li et al. (2016) investigated pigment removal from biomass using different organic solvents [21]. Results showed that more than 90 % of total lipids could be lost when reacting with absolute organic solvents [11]. Incorporating NaOH with an organic solvent in the reaction mixture was beneficial as the decrease in chlorophyll (more than 90%) was more significant than the lipid loss [13].

Solvent extraction is based on the permeability of cell membrane and chloroplast membranes to the solvent for dissolving lipids and lipoproteins [22]. Many studies have conducted different cell wall disruption mechanisms to extract chlorophylls from biological feedstocks [23]. However, these techniques required a series of post-extraction steps and have many disadvantages [24], [25].

Even though there are many research conducted on biofuel production from microalgae to date, information regarding chlorophyll removal from microalgae oil is scarce [26]. The optimization of temperature, reaction time, and solvent ratios for chlorophyll removal has not been conducted. This study investigates the effect of preprocessing parameters such as temperature, reaction time, and solvent ratio for chlorophyll removal from the freshwater green algae *M. homosphaera* for biofuel production.

The current study was conducted under four objectives. The first objective was to isolate and identify indigenous microalgae species from a natural water body in Sri Lanka. Secondly, the determination of the rate of growth and lipid accumulation in the isolated microalgae was performed. Further, the efficiency of chlorophyll removal from the selected microalgae with the highest growth rate was analyzed under different preprocessing conditions. Finally, the total lipid content and fatty acid profiles of chlorophyll removed biomass for biodiesel production was determined.

2. LITERATURE REVIEW

2.1 Biofuels as a renewable energy source

The depletion of existing petroleum-derived fuels and environmental concerns have caused scientists to discover alternative renewable energy sources [27]. This attempt has impacted and changed the existing trend in the world's fossil fuel consumption. Global energy expenditure depends on the availability and accessibility to resources and socio-economic development [28]. Fossil fuel combustion can create environmental issues such as climate change, global warming, depletion of the ozone layer, and acid rains [29]. Contrastingly, biofuels are carbon neutral and reduce greenhouse gas (GHG) emissions. Biofuels provide 4% of world transport fuel and have reached 4.3% in 2020 [30], [30]. Table 2-1 shows the expected global renewable energy demand by 2040 [30]. European Renewable Energy Council stated that around half of the world's energy supply would be achieved from renewable energy resources in 2040 [11]. During the last few decades, there was an increase in biofuel production by microalgae and will escalate in the following decades. Algae biomass can produce a variety of fuels [1].

Table 2-1. The expected global renewable energy demand by 2040 [30]

Total consumption	**2001**	**2010**	**2020**	**2030**	**2040**
(million-ton oil equivalent)	10 038	10 549	11 425	12 352	13 310
Biomass	1 080	1 313	1 791	2 483	3 271
Large hydro	22.7	266	309	341	358
Geothermal	43.2	86	186	333	493
Small hydro	9.5	19	49	106	189
Wind	4.7	44	266	542	688
Solar thermal	4.1	15	66	244	480
Photovoltaic	0.2	2	24	221	784
Solar thermal electricity	0.1	0.4	3	16	68
Tidal	0.05	0.1	0.4	3	20
Total renewable energy source contribution	1 365.5	1 745.5	2694.4	4 289	6351
Total renewable energy source contribution %	13.6	16.6	23.6	34.7	47.7

2.1.1 Evolution of biofuels

Biofuels are generated from biological feedstocks and municipal solid wastes [31]. These could be potentially converted into fuels, as shown in Figure 2-1. Two predominant processes can be used to produce biofuels. They are (1) direct processing of raw plant materials, animal fats, and sewage into one type of fuel, and (2) the extraction of products after natural processes such as anaerobic degradation of compounds into bioethanol by bacteria [32].

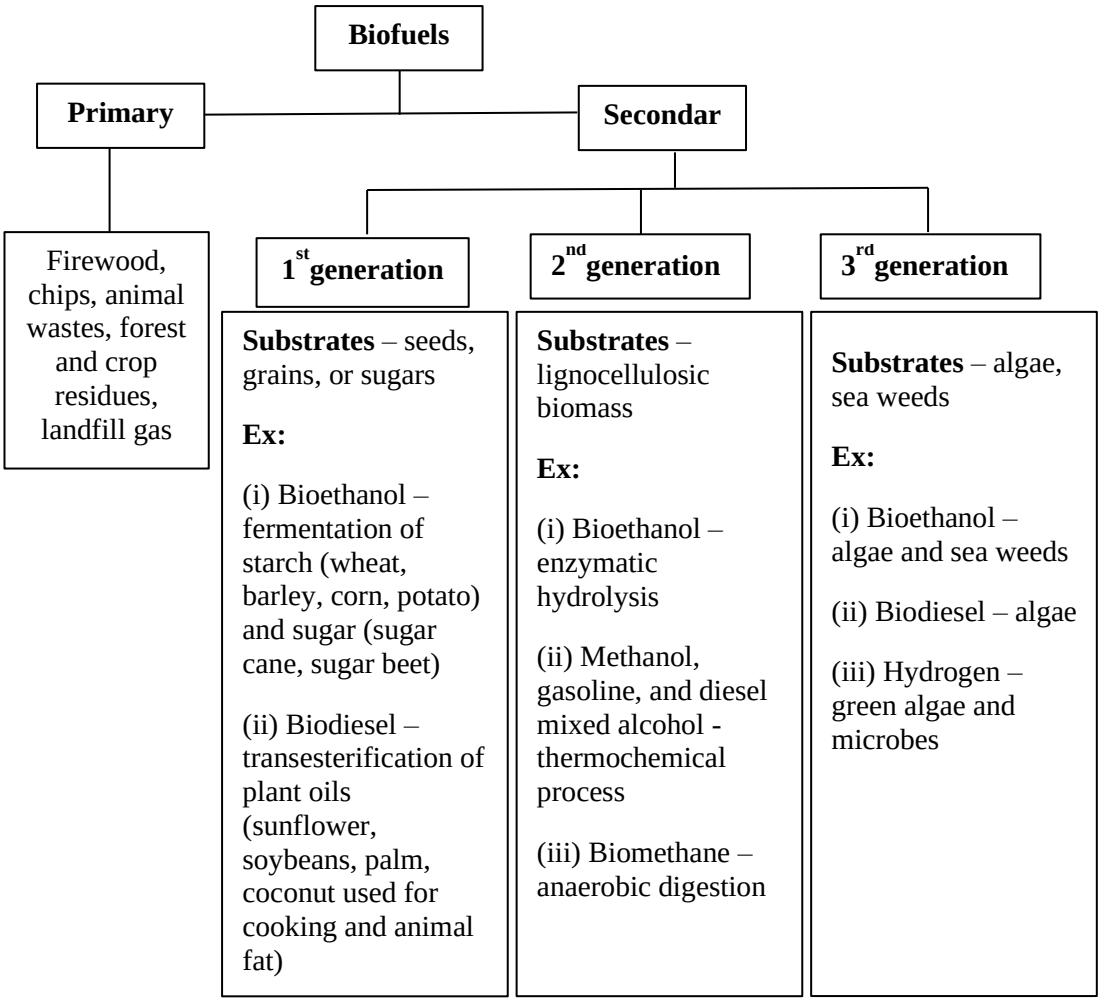

Figure 2-1. Production of different types of fuels from biomass

2.1.2 First-generation biofuels

Biodiesel, bioethanol, and biogas are the three main types of commercially available first-generation biofuels. Bioethanol is made by fermentation of sugar (sugar cane) or starch (corn) crops [4]. Initially, the sugar (glucose) is extracted from the raw materials and converted to ethanol by yeast [3]. The desired concentration of ethanol can be obtained via distillation and dehydration. Further, the ethanol can be directly used as fuels or blended with petroleum fuels [33]. Biogas is generated by anaerobic digestion of biodegradable materials such as biomass, municipal waste, sewage, and energy crops by anaerobic microorganisms. Biogas is mainly composed of carbon dioxide (CO_2), methane (CH_4), and hydrogen sulphide (H_2S), and water vapor (H_2O) [4]. Biodiesel is considered the most common type of biofuel made by transesterification of oils or fat [34]. Non-edible oils extracted from plants such as *Jatropha curcas*,

rapeseed, soybean are the main source for biodiesel [7]. Biodiesel has no sulphur, no aromatics, and more oxygen (10-12 % by weight), which helps complete combustion [30]. Therefore, biodiesel is contemplated as a clean fuel. This substantially reduces carbon monoxide emissions, partially burnt hydrocarbons, and exhaust particulate matters in conventional diesel engines. Because of its higher cetane number, ignition quality is increased even when blended with the petroleum diesel [35].

First-generation biomass comprises edible plant materials and crops. Therefore, they were untenable for trading due to the food-versus-fuel disputation [36]. Thus, first-generation biofuels have many drawbacks such as (1) social repudiation and ethical concerns due to permutation of food crops as feedstocks for biofuel production, (2) lack of variety in the components in the feedstock, and (3) conflicts of utilizing of cultivable lands for cultivation of fuel crops rather than subsistence crops [37].

2.1.3 Second-generation biofuels

The second-generation feedstock is based on lignocellulosic materials [28]. They include a high percentage of cellulose (30–60%), hemicellulose (20–40%), and lignin (15–25%) [38]. These constituents have the potential for being converted into energy-condensed hydrocarbons. The conversion routes engage biochemical and thermochemical pathways and technologies. Biomass can be converted into liquid fuels by pyrolysis, liquefaction, and fermentation [4]. Conversely, gaseous fuels can be synthesized via gasification and methanation, and gas to liquid conversion is done by Fischer-Tropsch synthesis (FTL) and syngas fermentation [4].

Second-generation biofuels such as ethanol and butanol are produced through biochemical processes, whereas the rest is produced thermochemically [39]. The necessary steps for producing these include pre-treatment, simultaneous saccharification and fermentation, and purification by boiling and condensation [40]. Extra cellular enzymes secreted by microorganisms are used to hydrolyze complex polysaccharides such as cellulose & hemicellulose in biomass to monosaccharides prior to fermentation to produce ethanol [24]. Methanol, refined FTL, and dimethyl ether (DME) are second generation thermochemical fuels [20] produced by thermochemical gasification or pyrolysis. Further, hydrothermal gasification technologies convert solid biomass into gaseous fuel called synthesis gas ($CO+H_2$).

Synthetic diesel or renewable aviation turbine fuel are produced from lignocellulosic syn gas [41].

The second-generation biofuels eliminate the barriers to compete with food crops to generate fuels and decrease the demand for first-generation biofuels. Moreover, second-generation biofuels emit significantly lower GHGs compared to first-generation biofuels during their life cycle [42]. Perennial grasses are considered as ideal energy crops in second-generation biofuels as they offer some advantages over food crops because of the high growth speed, year-round availability, high biomass production, low nutrient demand, cost-effectiveness, less agricultural practices and labor cost, and ability to recycle soil nutrients and resistance to diseases and climate conditions [43].

2.1.4 Third-generation biofuels

Microalgae and macroalgae are used as third-generation feedstock for biofuel production [44]. They are naturally found in freshwater, brackish water, and saltwater [45]. Freshwater microalgae such as *Chlorella* sp, *Desmodesmus* sp, and *Scenedesmus* sp are commonly used for biofuel production due to their minimum doubling time and high lipid yield [24]. Marine biomass such as microalgae and macroalgae has the ability to produce biofuels, especially biodiesel [46]. Aquatic biomass is an advanced feedstock for biodiesel production due to its perennial growth, high biomass accumulation, and less competition with energy crops for light, nutrients, and space [8],12]. Algae can be processed to produce biodiesel, biohydrogen, bioethanol, and syngas [2].

Based on the current scientific advancements and technology projections, microalgal biodiesel is free from obstacles related to first and second-generation biofuels [35]. Figure 2-2 illustrates the generation of biofuel continued from the first generation to the third generation. Integrated biofuels production from microalgae (Figure 2-3) includes the cultivation of algae, followed by harvesting, lipid extraction, and transesterification of lipids into FAME [23].

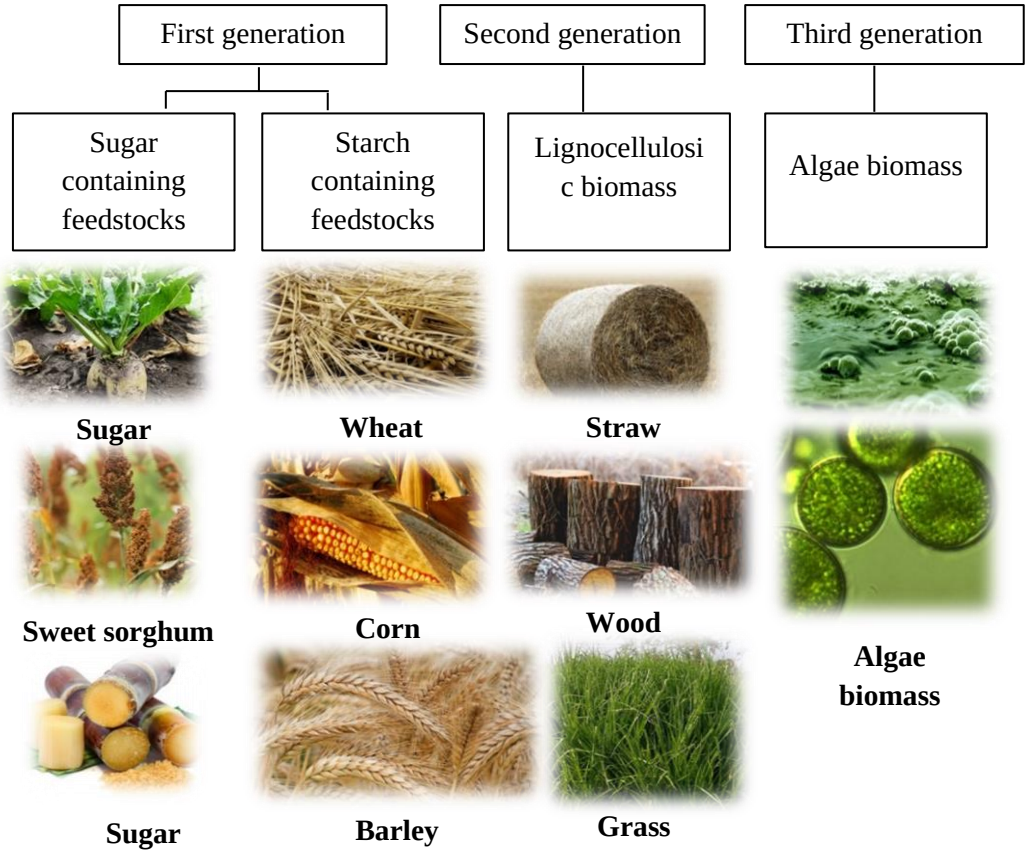

Figure 2-2. Representative diagram of the evolution of biofuel feedstock from the first generation to third generation

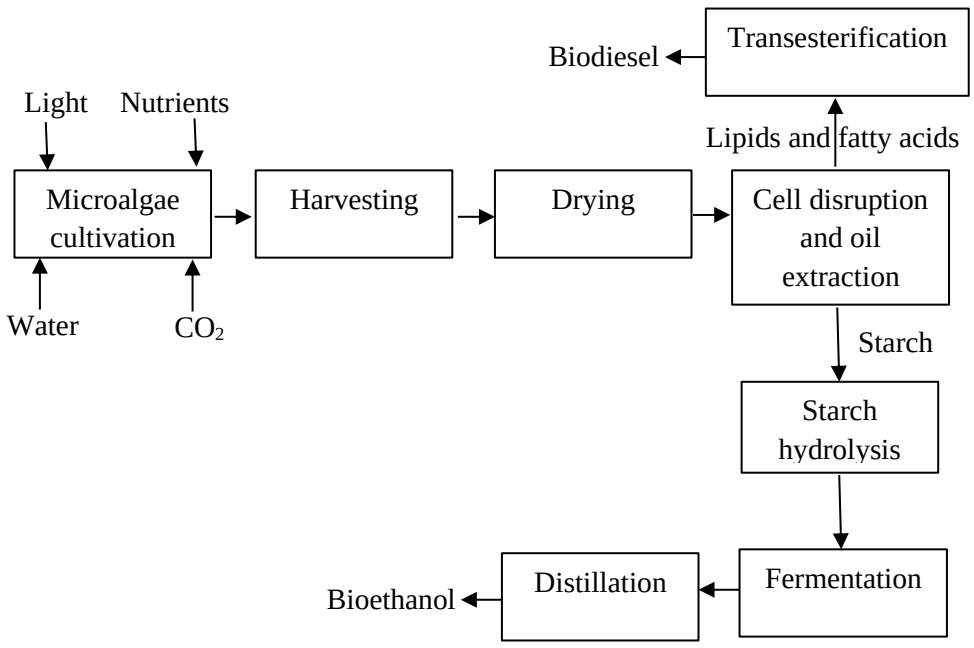

Figure 2-3. Integrated production of biofuels from microalgae

2.2 Biology of microalgae and fatty acid synthesis

Around the 1970s, considerable attention was given to microalgae lipids as a feedstock for biofuel production [1]. The major reason for this was the oil crisis during 1970 where the USA [47] and Japan developed research areas on algal-based biofuel production [48]. The aquatic species program to produce biodiesel from microalgae was initiated by the department of energy in United State to investigate microalgae as a feedstock of energy [30]. The project continued another two decades, and they have screened more than 3000 algal strains on developing biodiesel. According to the results obtained, green algae (chlorophyta) and diatoms (golden brown algae) were found as best candidates towards biofuel production [49]

The use of microalgae biomass to produce liquid fuels (biodiesel and bioethanol) is not a novel concept. Microalgae biomass can be converted into energy either by biochemical conversion or thermochemical conversion [50]. Microalgae can produce 15-300 folds more oil per area than conventional crops such as sugar canes and starch-containing grains [40]. Conventional energy crops can be harvested up to twice a year [4], while microalgae exhibit a short life cycle and can be harvested within 10-25 d of cultivation period [3]. Additionally, they eliminate the use of pesticides and herbicides, which reduce cost and pollution. Furthermore, the CO_2 in flue gases emitted by fossil fuel combustion is sequestered in microalgae photosynthesis, decreasing the emissions of major GHGs [2]. Industrial and municipal wastewaters and agricultural run-off (rich in NH_4^+, PO_4^{3-} and NO_3^-) can be treated using microalgae. Microalgae can be grown under different habitats such as in saline water, seawater, brackish water, and non-arable lands [7].

Advancements in biotechnology have shown rapid improvements of algal strains without affecting the lipid yield [9]. Furthermore, nutritional and environmental stresses are known to induce different metabolic pathways thereby stimulating high lipid accumulation in microalgae [51]. Moreover, depending on the species used, valuable by-products that have commercial applications in different industrial sectors can be extracted.

2.2.1 Biology of microalgae

The microalgae taxonomy has undergone significant changes in recent decades. The classification is mostly based on pigment types, cell wall constituents, chemical nature of storage products, and additional morphological and cytological characteristics such as occurrence and structure of flagella [11]. In the last decades, an increasing number of studies applying molecular phylogeny for classification have become popular [8]. Microalgae are either autotrophic (utilize inorganic C) or heterotrophic (use organic C). Further, they can be photoautotrophs (utilize solar energy) or chemotrophs (utilize chemical energy), while a combination of heterotrophy and autotrophy results in mixotrophy [52]. Large scale production of microalgae and commercialization of biofuel will reduce the cost for nutrients compared to the cultivation of energy crops [9].

2.2.2 Fatty acid biosynthesis in microalgae

Microalgae fatty acid biosynthesis pathway mainly consists of three stages; *de-novo* fatty acid synthesis in plastids, lipid accumulation, assembling, and lipids modification in the endoplasmic reticulum (ER), and a final assortment of oil bodies as presented in Figure 2-4. The chloroplasts contain fatty acid synthase (FAS); a multi-enzyme protein complex utilized for synthesizing aliphatic fatty acid [12]. This is taken place in the chloroplast, and newly synthesized fatty acids are broken down by a thioesterase and developed by desaturases and converted to complex prokaryotic lipid using plastid acyltransferases [50]. These esters are transported into ER for further modification, elongation, and packaging into lipids or storage triacylglycerides (TAG). Lipid bodies are small (0.2-2.5 μm), spherical shaped sac-like structures wrapped by a membrane and found in the cytosol of microalgae [7].

2.3 The production of biodiesel from microalgae fatty acids

Transesterification is the process that converts fatty acids into biodiesel [12]. The drying and harvesting mechanisms in lipid conversion are highly energy consuming and expensive [53]. Figure 2-5 shows the acid-catalyzed direct transesterification reaction, where the glycerol backbone and the fatty acids are separated from the lipid compound [13]. Direct transesterification of lipids from wet biomass using switchable polarity solvents is a method that eliminates the drying step [1].

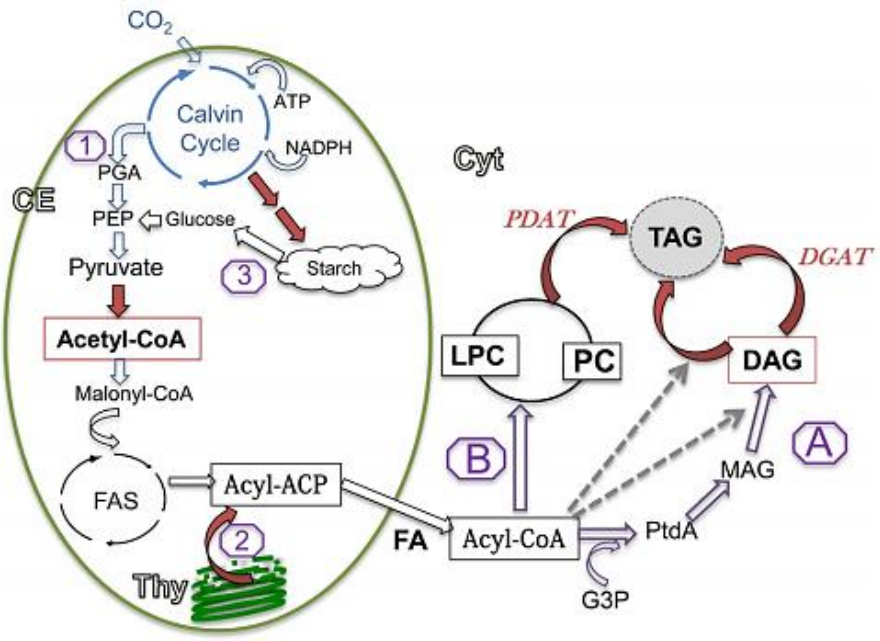

Figure 2-4. Illustration of the microalgae fatty acid synthesis pathway

Microalgae fatty acid synthesis pathway (Metabolic pathways for TAG biosynthesis in green algae. FA synthesis pathways: (1) integration of newly assimilated carbon; (2) degradation of pre-formed polar lipids. (3) degradation of starch. Enzymes and substrates that are regulated under N deprivation are marked in italics. TAG synthesis pathways: (A) the Kennedy pathway, (B) PC acyl editing. CE, chloroplast envelope; Cyt, cytoplasm; FAS, fatty acid synthase pathway; ACP, acyl-carrier protein; PEP, phosphoenol pyruvate; PGA, 3-phosphoglyceric acid; G3P, glycerol-3-phosphate; PtdA, phosphatidic acid; MAG, monoacylglycerol; DAG, diacylglycerol; LPC, lyso-phosphatidylcholine; PDAT, phospholipid diacylglycerol acyltransferase; DGAT, diacylglycerol acyltransferase) [17].

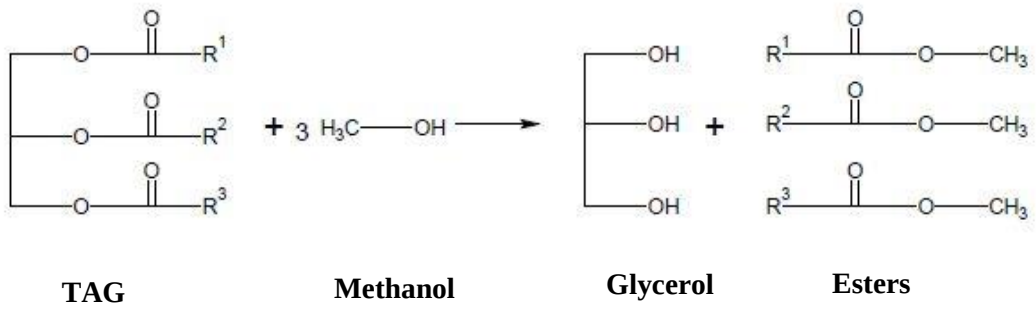

Figure 2-5. Simplified flow diagram of direct transesterification using methanol.

2.4 Chlorophylls in microalgae

Chlorophylls are green pigments present in photosystems of plants and green algae. Two major forms of chlorophylls commonly found in plants and photosynthetic organisms are chlorophylls a and b [6]. Chlorophyll a is dominant over chlorophyll b (3:1) [13].

The chlorophyll has a closed ring structure due to four peripheral pyrrole groups (tetrapyrroles) coupled with a central magnesium atom, as shown in Figure 2-6 [9]. Pyrrole ring is consisted of one central nitrogen atom and four carbon atoms [54]. Each nitrogen atom arranges as a circle creating a central hole where magnesium ions combine [55].

The major structural difference of chlorophyll b from chlorophyll a is the replacement of the methyl group in seventh carbon atom by a formyl group [5, 7]. Due to this structural difference chlorophyll b absorbs 640-652 nm wavelengths and transmit yellow green color and chlorophyll a molecules absorb 660-665 nm wavelengths and transmit blue/green color [56].

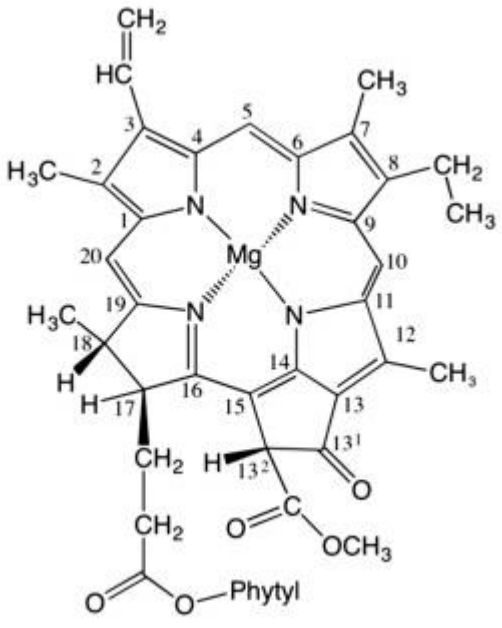

Figure 2-6. The basic structure of chlorophyll pigments [2]

2.4.1 Effects of chlorophyll for biofuel production in microalgae

Chlorophyll makes oil more susceptible to photo-oxidation [57]. Oxygen is an electrophile that readily reacts with fatty acids and derivatives via cycloaddition reactions [58].

There are two oxidation mechanisms called autoxidation and photo-oxidation due to the presence of two forms of oxygen (triplet and singlet) [63]. The singlet form of oxygen is more reactive than the triplet form [64]. Photo-oxidation of oil mainly occurs due to the action of the singlet form and exposure to ultraviolet radiation in the atmosphere and chlorophyll pigments [65]. The photo-oxidation of unsaturated oils occurs through the absorption of ultraviolet radiation by photosensitive molecules such as chlorophyll and the transfer of the absorbed energy to triplet oxygen, generating singlet oxygen [65].

The singlet oxygen readily reacts with the double bonds of unsaturated fatty acids by means of addition reactions to form hydroperoxides [59]. The hydroperoxides are unstable and can decompose to form various secondary oxidation products, including aldehydes, alcohols, shorter-chain carboxylic acids [35]. This process of oxidation leads to changes in the biodiesel properties, mainly viscosity, acid number, and oxidation stability. In autoxidation reactions, oxygen molecules directly attack the double bonds present in the alkyl chains of biodiesel [66]. The extent of the autoxidation reactions depends on the oil composition, number, position, and geometry conjugation of the double bonds, as well as the presence of antioxidants and storage conditions [35].

Oxidation of biodiesel causes an increase in acidity due to the formation of free acids, and the viscosity increases because of the formation of undesirable insoluble compounds and polymeric compounds [59]. Viscosity is an important parameter of biodiesels at low temperatures [60]. High viscosity affects the fluidity of the biofuel, combustion process and the power generation of the fuel engine [61]. Further, poor fuel atomization, partial combustion, and subsequent increase of particulate matters in exhaust smoke can occur. This makes the biofuel unfit for use in vehicles [62].

According to the literature, presence of chlorophyll causes rapid deterioration of oil changes the color of biodiesel, and develops an undesirable odor [23], thereby decreasing the economic value of the biodiesel [24]. Presence of chlorophyll in the feedstock reduces the transesterification efficiency and storage stability [18], and decreases the combustion efficiency of microalgal biodiesel [5]. Therefore, high levels of chlorophyll in feedstock limits the large-scale application of biodiesel [67].

In addition, the distinct green color that occurred due to the presence of pigments reduces biodiesel quality. Long-chain saturation factor (LCSF), cetane number (CN), saponification value (SV), and iodine value number (IV) and are quality parameters of biodiesel [2]. Generally, CN should be above 50 for diesel engines to perform better on biodiesel [7]. Higher CN reduces the ignition delay. Studies reported that CN is increased (48 -52.35) in fuels produced by chlorophyll removed biomass compared to the fuels produced by untreated biomass [12]. This indicates that chlorophyll removal has increased the combustion efficacy of biofuel [68]. Low SV value is treated biomass increases the combustibility of biodiesel [8]. This reduces the emission of unburnt carbon during combustion. Further, chlorophyll removal impacts increasing the degree of saturation, which prevents the oxidation of oil in the presence of oxygen and increases the storage time of the oil [17].

2.4.2 Chlorophyll removal from microalgal lipids

Few effective studies were conducted for microalgal chlorophyll removal towards biodiesel production. T. Li et al. successfully conducted chlorophyll removal with different organic solvents such as 100 % ethanol, 100 % acetone, 100 % methanol + 1 % NaOH, 100 % ethanol + 1 % NaOH, 100 % acetone + 1 % NaOH, 100 % methanol, and 1 % sodium hydroxide (NaOH) [7].

Results showed that absolute organic solvents cause high total lipid loss, and the incorporation of NaOH to the mixture is beneficial for the saponification of chlorophyll [6]. Based on the results observed from the previous studies, the present study developed a novel preprocessing method to find the best solvent ratio along with the best temperature and reaction time that can be used to remove chlorophyll from microalgae.

2.4.3 Saponification reaction in chlorophyll removal

NaOH saponifies chlorophyll pigments into chlorophyllin and phytol [17].

$C_{55}H_{72}O_5N_4Mg + 2NaOH \rightarrow C_{34}H_{30}O_5N_4MgNa_2 + 2CH_3OH + C_{20}H_{39}OH$ [19].

Saponification reaction is used to separate chlorophyll and carotenoids from other liposoluble substances present in microalgae [20]. NaOH can react with both chlorophyll and TAGs [8]. Therefore, it is beneficial to use a mixture of NaOH and ethanol in chlorophyll removal, as discussed in the present study. In consideration of the cellular structure of microalgae (e.g. cell wall consisted of cellulose, membrane-bound oil bodies), it is assumed that the storage lipids are not saponified by saponification reaction [8]. Also, the Gibbs free energy for saponification of chlorophyll is lesser than storage lipids. Therefore, chlorophyll saponifies more easily than lipids [12].

2.4.4 Quantification of chlorophyll

The absorbance value of the supernatant of the chlorophyll removal mixture is measured at 665.2 nm, 652.4 nm, and 470 nm wavelengths, respectively. The following equations can be used to determine the total chlorophyll content in microalgae biomass [69].

$$C_a = 16.72 \times A_{665.2} - 9.16 \times A_{652.4} \longrightarrow (1)$$

$$C_b = 34.09 \times A_{652.4} - 15.28 \times A_{665.2} \longrightarrow (2)$$

$$\text{Total carotenoids} = (1000 \times A_{470} - 1.63 \times C_a - 104.96 \times C_b)/221 \longrightarrow (3)$$

(C_a and C_b are the concentrations of chlorophyll a and chlorophyll b (mg L^{-1}), $A_{665.2}$, $A_{652.4}$, and A_{470} are the absorbance of the solution at 665.2 nm, 652.4 nm, and 470 nm, respectively).

In transportation sector microalgal biofuels can overcome most of the environmental constraints that occur from fossil fuel combustion. However, the operating costs of biomass processing, and biodiesel production should be minimized to compete with the existing petroleum-based fuels. Use of genetic modification and recombinant DNA technology to obtain high yielding microalgae strains, developing efficient biorefinery

pathways and optimization of biomass preprocessing parameters will make biodiesel more competitive in the fuel market [7]. The present study investigates the growth rate and the effect of different parameters for chlorophyll removal and compares the lipid yields and fatty acid profiles in chlorophyll removed biomass [69]. Microalgae *M. homosphaera* with highest growth rate was used to conduct the chlorophyll removal. Further, the efficiency of chlorophyll removal was assessed by varying solvent ratio, temperature, and reaction time. Thus, a novel preprocessing method was developed to remove chlorophyll whilst significantly reducing total lipid loss.

3. MATERIALS AND METHODS

3.1 Isolation of microalgae

3.1.1 Microalgae strain

The microalgae species *Mychonastes homosphaera* isolated from Beire Lake, Colombo, Sri Lanka, was employed in the current study.

3.1.2 Preparation of stock solutions

Culture medium was prepared by adding macronutrients and trace elements including vitamins in the correct proportions. Bold Basal medium (BBM) was used for microalgae culturing in the present study. BBM is contained with inorganic salts and extensively used to culture freshwater microalgae. Stock solutions are prepared using analytical grade chemicals purchased from Sigma Aldrich, United States (US). The media composition of BBM is shown in Table 3-1. BBM is not prepared as a single stock solution including all the constituents as some chemicals are partially soluble and can form a precipitate. Therefore, the stock solutions were prepared separately for each constituent.

The following volumes of appropriate stock solutions are required to prepare 1 L of BBM media. Each stock solution should be added to a conical flask containing 900 mL distilled water in numerical order as given in Table 3-1. Distilled water is added to volume up to 1 L after adding required nutrients. The medium was stirred (HYCC SH-2 Laboratory) for 30 min placed on a mechanical stirrer for 30 min to assure proper mixing. After stirring, the pH was adjusted to 7.

3.1.3 pH measurement

The pH measurement during the culturing was conducted using a laboratory pH meter (OHAUS Starter 2100). The two-point calibration was used to calibrate the pH meter with the aid of buffer solutions (pH 4 and pH 7) [70]. The pH of the media was adjusted to 7 using 1 M HCl and 1 M NaOH.

Table 3-1. Media composition of BBM [71]

Stock solution no.	**Chemical name**	**Formula**	**Weight (g)**	**Distilled water (mL)**
1	Di-potassium hydrogen orthophosphate	K_2HPO_4	1.875	250
2	Di-potassium hydrogen orthophosphate	KH_2PO_4	4.375	250
3	Magnesium sulphate	$MgSO_4.7H_2O$	1.875	250
4	Sodium nitrate	$NaNO_3$	6.250	250
5	Calcium chloride	$CaCl_2.2H_2O$	0.625	250
6	Sodium chloride	NaCl	0.625	250
7	EDTA tetrasodium salt Potassium hydroxide	EDTA - Na_4 KOH	5.000 3.100	100
8	Ferrous sulphate Sulphuric acid conc. (1.84 g mL^{-1})	$FeSO_47H_2O$ H_2SO_4	0.498 0.1mL	100
9	Boric acid	H_3BO_3	1.142	100
10	Zinc sulphate	$ZnSO_4.7H_2O$	0.353	25
11	Manganese chloride	$MnCl_2.4H_2O$	0.058	25
12	Cupric sulphate	$CuSO_4.5H_2O$	0.063	25
13	Cobaltous nitrate	$Co(NO_3)_2.6H_2O$	0.020	25
14	Sodium molybdate	$Na_2MoO_4.2H_2O$	0.048	25
Stock solution no. 1-6 7-9 10-14				 10 1 0.1

3.1.4 Sample collection and microalgae isolation

Water samples with visible microalgal growth were collected from two different locations of the Beire Lake, Colombo, Sri Lanka (N: 6.9294, E: 79.8542, N: 6.9169, E: 79.8545). Samples were transported in an ice box in sterilized sampling bottles to the microbiology lab. Particles and debris were filtered and separated using filter papers. Further, 150 µL of filtrate was spread on BBM solid medium (100 × 15 mm petri plates) at pH 7.

Next, agar plates were incubated for 7 days for colony formation. After obtaining single colonies, microalgae were streaked on BBM agar plates. Microalgae were maintained at 25 ^{o}C with a light intensity of 27 µE/m/s (12:12 light: dark photoperiod cycle) [34].

3.2 Identification of microalgae

3.2.1 Morphological observation and identification of algae by biological microscope

Morphological identification of microalgae samples was achieved by light microscopy (Axio Lab A1, Biological microscope). Water samples were centrifuged at 3500 rpm for 10 min at room temperature to obtain algae for morphological identification. The filtrate was discarded, and the pellet was dissolved in distilled water. Then, 10 µL of the algal sample was observed under Axio Lab A1 biological microscope. Digital images of isolated strains taken from the light microscopy with digital imaging system were compared with the photomicrographs of known species in algae strain catalogue for the morphological identification [9]. Figure 3-1 illustrates the simplified flow chart for axenic algal colony isolation.

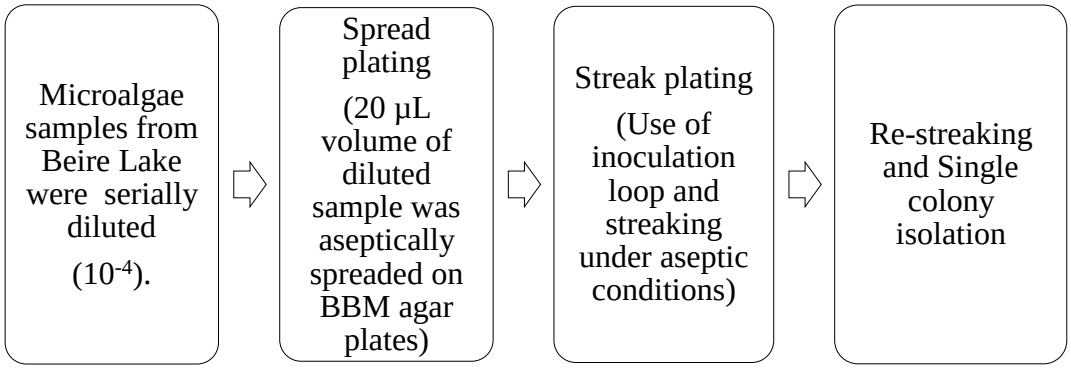

Figure 3-1. The simplified steps for the spread plate and streak plate procedure to achieve single colonies under aseptic conditions.

3.2.2 Molecular characterization of microalgae

3.2.2.1 Isolation of DNA

Microalgae cells isolated from the exponential phase were centrifuged (EPPENDORF 5804R) for 3500 g at 10 min. Cell pellet was separated and freeze-dried using LGC Alpha 1-4 Loplus lyophilizer. The method involves CTAB (cetyltrimethylammonium bromide) extraction was conducted for DNA extraction of isolated strain as shown in Figure 3-2 [13].

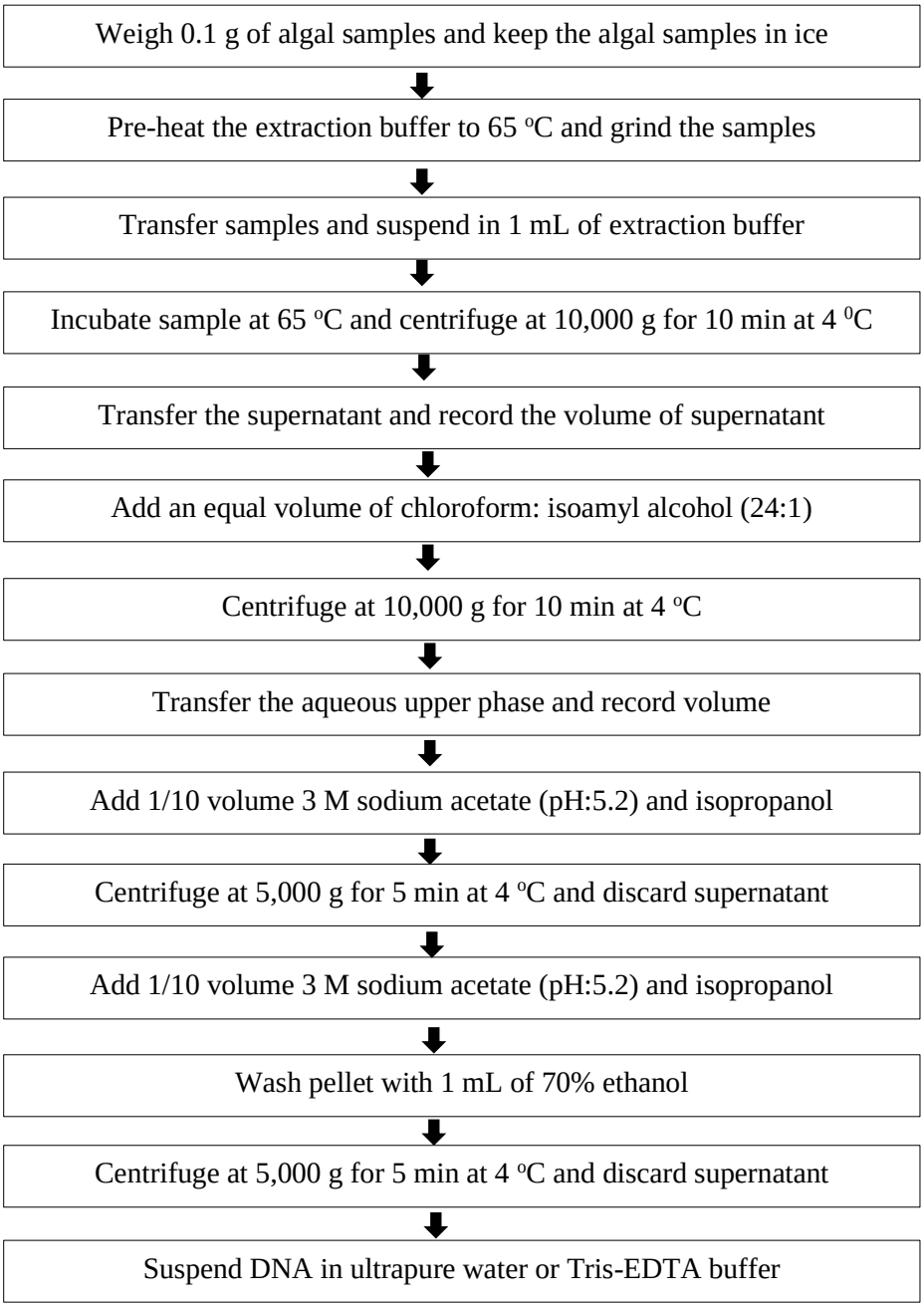

Figure 3-2. The total genomic DNA isolation procedure according to the CTAB method [13].

3.2.2.2 Quantitation and assessment of DNA

The NanoDrop 1000 Thermo scientific spectrophotometer (220-750 nm) was used to measure the concentration of extracted DNA. The ratio of OD 260/OD 280 (optical density) was used as a secondary measure of nucleic acid purity [72]. The ITS2 rDNA region was amplified via Polymerase Chain Reaction (PCR) containing stock 10X PCR buffer, 2 mM dNTP mix, 5 U Taq polymerase, 25 mM $MgCl_2$, and 10 µM concentration of reverse primer SQITS2 and forward primer (BIO-RAD T100) [16].

The DNA sequence with respect to the internal transcribed sequence 1 and 2 (ITS 1 and 2) in 18S ribosomal RNA (rRNA) was amplified using the following forward and reverse primers [45]:

p23SrV_f1 (5' GGA CAG AAA GAC CCT ATG AA 3') and p23SrV_r1 (5' TCA GCC TGT TAT CCC TAG AG 3').

3.2.2.3 PCR amplification and cloning of ITS2 region

PCR conditions (BIO-RAD T100) were maintained at 95 ^{o}C for 5 min for pre-heating and further 30 s at 95 ^{o}C, 30 s at 55 ^{o}C and 1 min 72 ^{o}C for 29 cycles, and 5 min at 72 ^{o}C [9]. Amplified DNAs were separated using agarose gel electrophoresis including tris base, acetic acid and EDTA buffer, and visualization of bands stained with ethidium bromide was done using ultraviolet illumination. Nucleospin gel extraction kit (Macherey-Nagel™ 740609.50) was used for the purification of PCR products [9].

3.2.2.4 Cloning of PCR products

According to manufacturers, PCR products were cloned by TA cloning and applied colony PCR using 5U Taq polymerase, 10 mM dNTP mix, reverse primer (M13), forward primer (M13), and 10X PCR buffer. PCR optimum condition for initial heating was adjusted at 95 ^{o}C for 15 min. Further heating was carried out at 95 ^{o}C for 30 s, at 53 ^{o}C for 30 s, and at 72 ^{o}C for 1 min for 25 cycles, and at 72 ^{o}C for 5 min [11]. The purified PCR products of the isolates were sequenced, and the phylogenetic relationship was determined by comparing the sequencing data with sequences available through the GenBank database of the National Centre for Biotechnology Information (NCBI) [8] Samples were outsourced to Genetech, Sri Lanka for analysis.

3.3 Microalgae cultivation

Pure cultures obtained during the isolation process were grown in separate 2 L bioreactors containing liquid BBM for 20 d to determine the growth in terms of cells mL^{-1} [9]. Growth conditions were adjusted to 30 ºC of temperature, 12 h:12 h light: dark photoperiodic cycle and shaking at 300 rpm. Aerated bioreactor and shaking incubator (Thermo scientific MAXQ8000) were used to grow cultures.

3.4 Microalgae harvesting

Centrifugation was performed to harvest microalgae at 7000 rpm for 5 min. Next, the solution was filtered through filter papers, and the supernatant was discarded. Pellet was oven dried (Memmert UN110) at 80 °C for 24 h and further kept in a desiccator for measuring dry weight [11].

3.5 Determination of microalgae growth

The growth rate of the isolated microalgal species was determined by:

(i) Direct cell counting method using a hemocytometer (Assistant Thoma Neu, Germany)

(ii) Measuring turbidity of the solution at 750 nm using a UV visible spectrophotometer (SHIMADZU UV- 1800) [10].

According to the Beer-Lambert law, absorbance is directly proportional to the analyte concentration that was used to draw the growth curve for the isolated species [10].

$$A = \lambda b c \longrightarrow (4)$$

Where,

A is the measured absorbance, λ is the absorptivity coefficient (a measure of how well a chemical species absorbs given wavelength / $mol^{-1} L cm^{-1}$), b is the path length of the cuvette (constant, 1 cm), and c is the concentration of the analyte (mol L^{-1}) [73].

The total number of cells per milliliter was plotted against time to calculate the growth rate of the microalgae. The doubling time of the selected species was calculated by the following equations. It is assumed that the doubling time remains constant during the exponential growth phase [5].

$$\mu = (Log_{10}N_1 - Log_{10}N_0) = (t_1 - t_0) \longrightarrow (5)$$

$$\mu = (Log_{10}N_1 - Log_{10}N_0) = (t_1 - t_0) \longrightarrow (6)$$

$$G = (0{:}301/\mu) \longrightarrow (7)$$

Where,

μ is the specific growth constant, N_0 is the number of cells at t_0, N_1 is the number of cells at t_1, G is the doubling time, t_0 is the initial time, and t_1 is the final time [4].

3.6 Microalgae cell disruption

Manual grinding was carried out in the presence of liquid nitrogen for 10 min using motor and pestle.

3.7 Lipid extraction and fatty acid analysis

Bligh and Dyer method was involved in determining the total lipid content in *M. homosphaera* as described previously (Bligh, 1959) using chloroform: methanol: water 1:1:0.9 (v/v) ratio in the final solvent [13].

Dried biomass was treated with chloroform and methanol (1:2 v/v) and vortexed (VELP SCIENTIFICA ZX3) for 5 min. Further, water and chloroform were added to the mixture and shaken for 15 min in a separatory funnel for proper mixing. Then water was added to induce the transition from the monophasic system to the biphasic system and left for 12 h. The methanol, chloroform, and water volumes were maintained at a ratio of 1:1:0.9 respectively. The chloroform layer extracts the microalgae lipids as shown in Figure 3-3 [13].

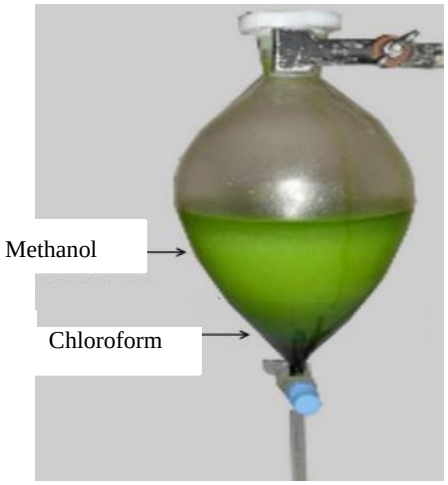

Figure 3-3. Lipid extraction by Bligh and Dyer method

3.7.1 Transesterification and analysis of fatty acid methyl ester (FAME) content

Transesterification of extracted lipids was performed using 4:1 v/v 5 % acid and 100 % methanol, as shown in Figure 3-4 [14].

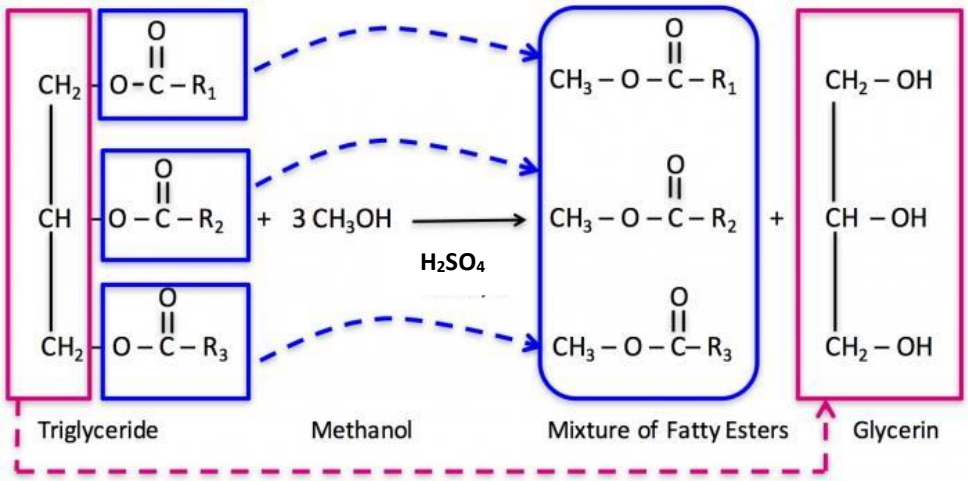

Figure 3-4. The chemical reaction for transesterification of extracted lipids

Gas Chromatography with flame ionization detector (GC-FID) was used to determine the FAME composition of transesterified lipids. A fused silica capillary column (SP-2560 – 60 m × 0.25 mm ID, 0.2 μm) was used as the GC column. The temperature of the GC oven was initially maintained at 100 ^{0}C for 5 min and increased to 240 ^{0}C at a rate of 4 ^{0}C/ min for 15 min. H_2 gas was used as a carrier gas at a constant flow of 1.2 mL min^{-1}. The temperature of FID and inlet was adjusted to and 280 ^{0}C and 250 ^{0}C, respectively [5].

Palmitic acid, oleic acid, and linoleic acids were used in the standard solution to compare the retention time and peak size of microalgae FAME.

3.8 Chlorophyll removal

The treatment solutions were consisted of different ratios of 100 % ethanol and 1 % NaOH; S1 (1:9), S2 (1:4), S3 (3:7), S4 (2:3). Dried biomass (70 mg) was dissolved in 7 mL of treatment mixture and incubated at 70 ^{0}C for 1 h in a water bath. Pellet from the sample was separated by centrifugation at 3500 rpm for 5 min and oven-dried at

80 ^{0}C. Further, the oven-dried samples (70 mg) were treated with 7 mL of 100 % methanol to conduct chlorophyll analysis. Then the residue was removed via centrifugation at 3500 rpm for 5 min and the absorbance of the supernatant was measured at 665.2 nm, 652.4 nm, and 470 nm wavelengths [12]. The experiment was repeated with different solvent ratios of ethanol: NaOH to determine the best solvent ratio for highest chlorophyll removal efficiency (Figure 3-5). Removal of chlorophyll from *M. homosphaera* was determined by varying temperature and reaction time. Temperature was varied from 30 – 70 ^{0}C while reaction time was varied from 30- 120 min for the selected solvent ratio.

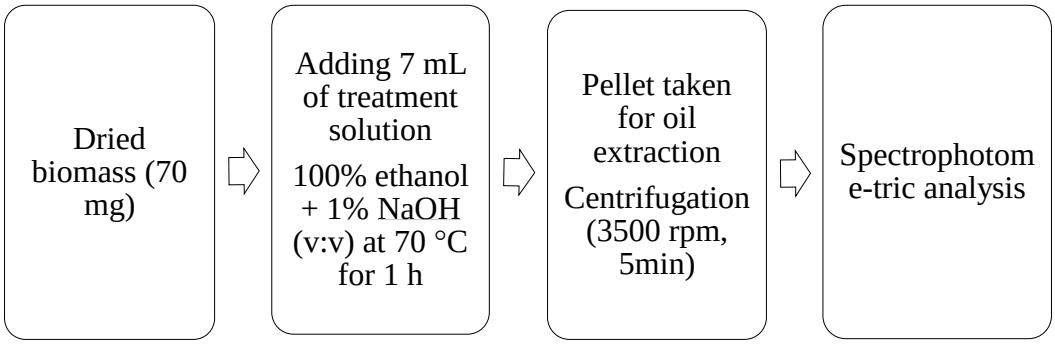

Figure 3-5. Simplified flowchart for the process of chlorophyll removal from dried biomass

4. RESULTS

4.1 Microalgae isolation

Microalgae were isolated by spread plating and streak plating, as shown in Figures 4-1 and 4-2. They were grown in BBM agar plates, and colonies appeared in dark green. The algae were unicellular and filamentous in structure, and ten to fifteen colonies were observed after 7 days of growth in each agar plate. Microalgae were observed under light microscopy, and morphological identification was carried out based on the cellular morphologies such as the shape and size of the cell, shape, and location of chloroplasts, presence of pyrenoids and flagella [35]. The photomicrographs of isolated species are shown in Figure 4-3.

All ten viable species (*Pseudochlorella, Scenedesmus, Nitzchia, Desmodesmus,* and few unknown species) were sub-cultured into BBM for biomass production. The minimum time for colony formation was observed in unknown species 1 (Un 1) and reported as 4 days, as shown in Table 4-1. Further, the isolated colonies were cultured in BBM (liquid medium) for 20 days. After 20 days of the culturing period, Un 1 showed significantly high biomass production, while the rest showed lower biomass accumulation. The highest growth rate was achieved at pH 7 [36].

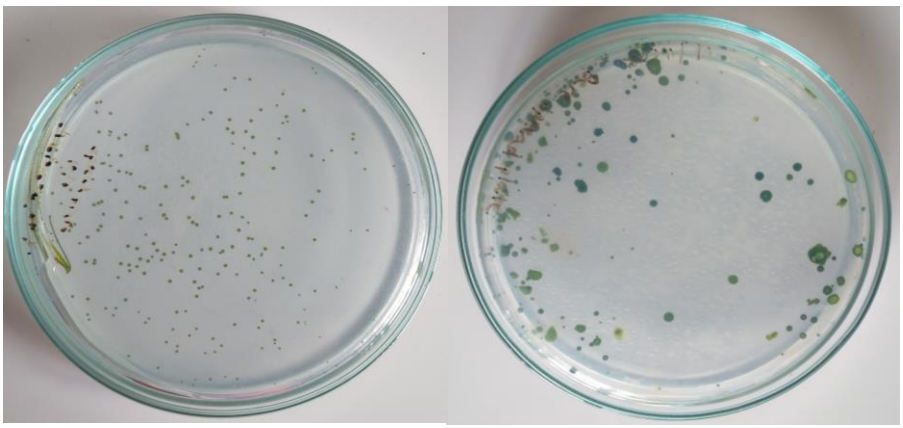

Figure 4-1. Spread plates (Samples were taken from Beire Lake, Colombo, Sri Lanka)

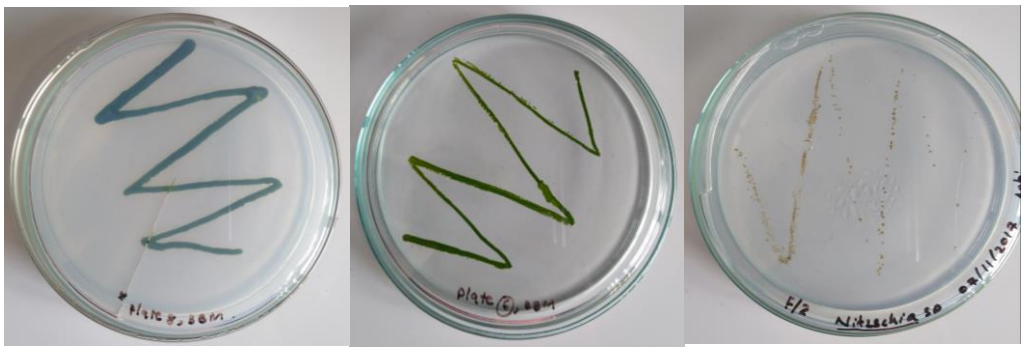

Figure 4-2. Streak plates of isolated strains

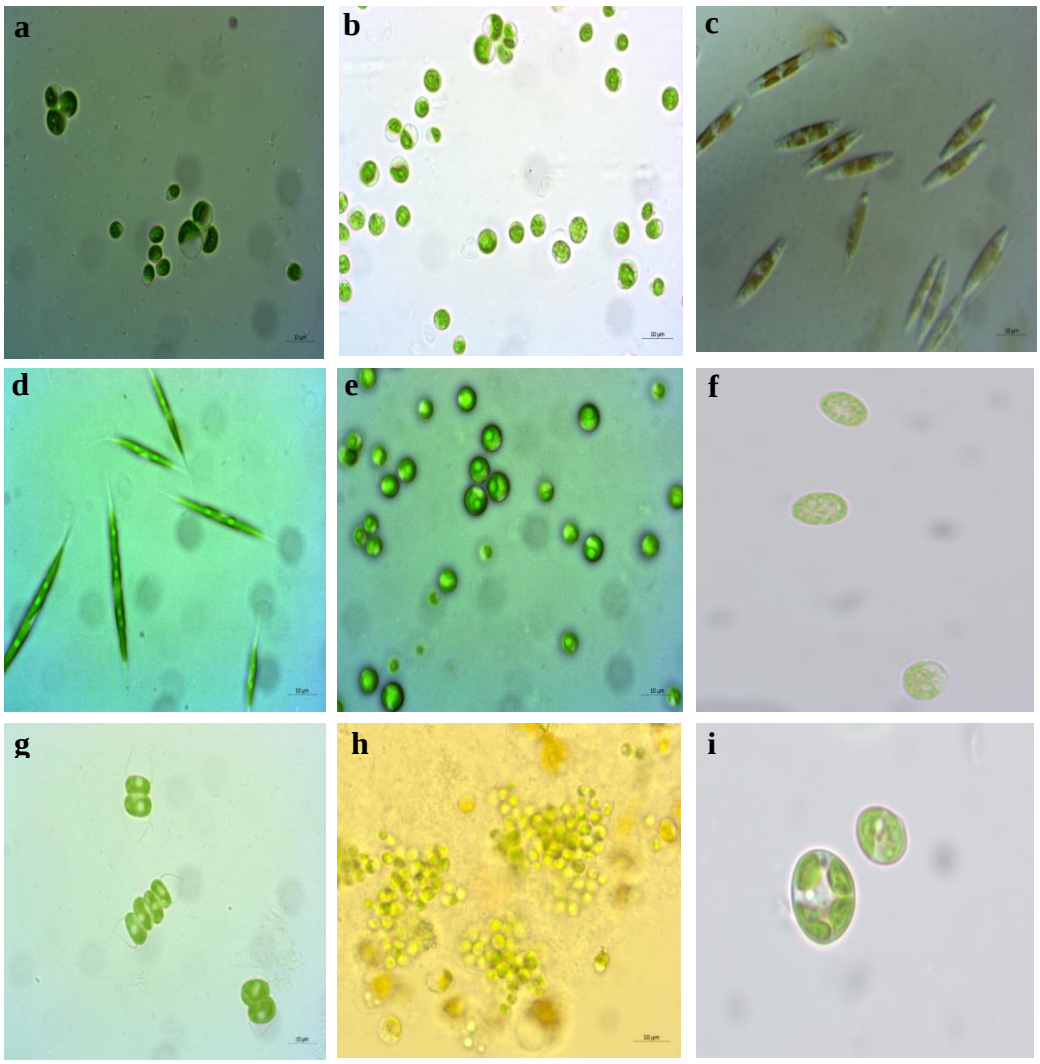

Figure 4-3. Photomicrographs of different morphologies of the microalgae obtained at the end of the isolation process (a) *Pseudochlorella* b) Un 1 c) *Nitzchia* d) *Chlosteriopsis* e) *Nanochloropsis* f) Un 2 g) *Chaetocerous* and h) *Desmodesmus*)

Table 4-1. Minimum days required for colony formation in isolated strains

Name of the species	**Minimum time required for colony formation (days)**
Scenedesmus sp	10
Nitzschia sp	5
Unknown species 1 (Un 1)	4
Pseudochlorella sp	5
Closteriopsis	7
Nannochloropsis sp	6
Chaetocerus sp	14
Unknown species 2 (Un 2)	8
Unknown species 3 (Un 3)	8
Unknown species 4 (Un 4)	6

4.2 Microalgae identification

The microalgae strain (Un 1), which showed the highest growth rate was identified as *M. homosphaera* as shown in Figure 4-4. The total sequence obtained from the 18s rRNA sequencing was aligned to the NCBI BLAST software to compare the obtained result with the Genebank database for the identification purpose [53]. Blast results are showed in Figure 4-5 in the form of a phylogenetic tree.

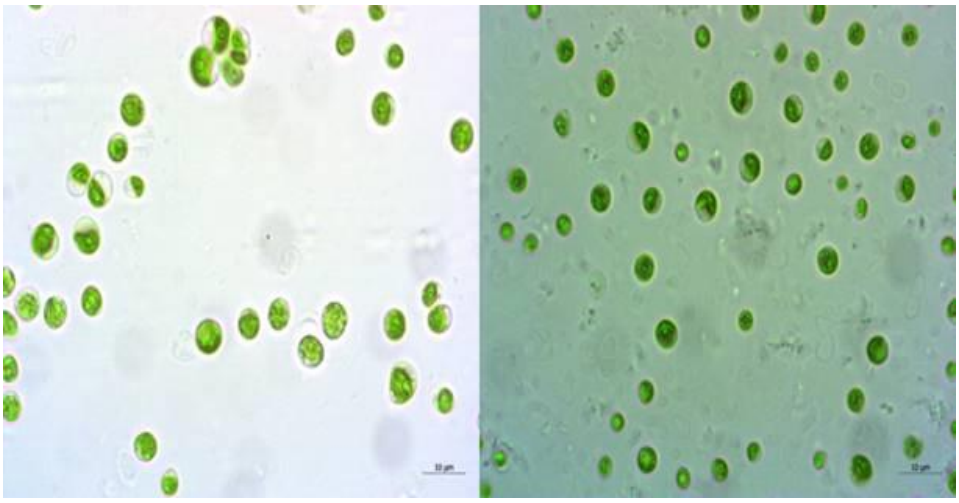

Figure 4-4. Micrograph of the *M. homosphaera* by light microscope

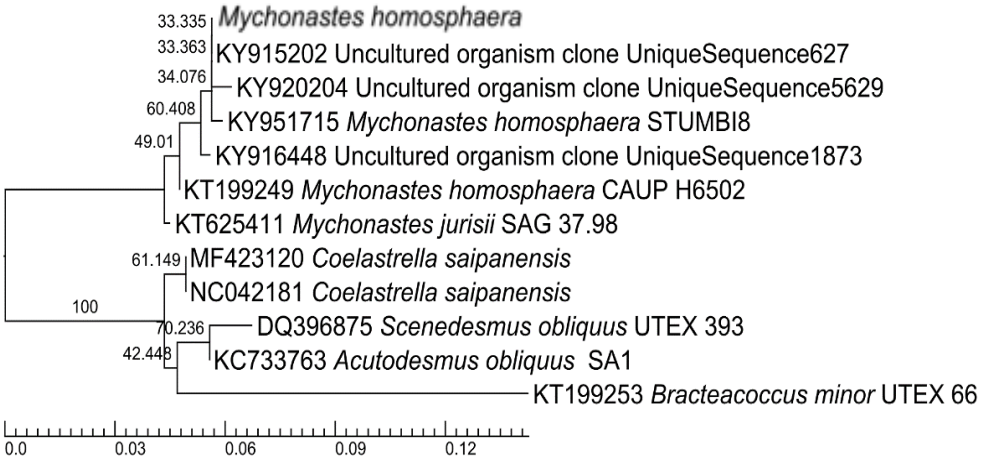

Figure 4-5. Phylogenetic tree of isolated microalgae; *M. homosphaera* from NCBI genes bank database

4.3 Determination of growth rate

The growth rate of isolated strains was determined by measuring the absorbance of cell suspension at 750 nm by spectrophotometer (Figure 4-6). The wavelength of 750 nm was used in the present study to avoid light absorption by chlorophyll pigments and carotenoids [8]. The *M. homosphaera* obtained the highest growth rate, and it was used for lipid characterization. The total number of cells in a unit volume was counted using the hemocytometer (Figure 4-7). The log number of cells was plotted against time (Figure 4-8) to calculate the specific growth rate constant (μ) and doubling time (G). G is a measure to determine how fast the population doubles. Shorter G indicates a higher growth rate. The algae growth curve consists of four major phases: lag phase, log or exponential phase, stationary phase, and death phase [3]. The growth slowly increases during the lag phase as algae start to adjust to the new environment. The exponential phase is the linear portion of the growth curve, where the population doubles rapidly. The linear part of the curve was used to calculate the doubling time (G) of *M. homosphaera*, which was 2.89 days. During the stationary phase, the population's number remains constant as the cell division rate equals the death rate [67]. The growth curve's final stage includes the death phase, where the death rate is higher than the cell division rate [26].

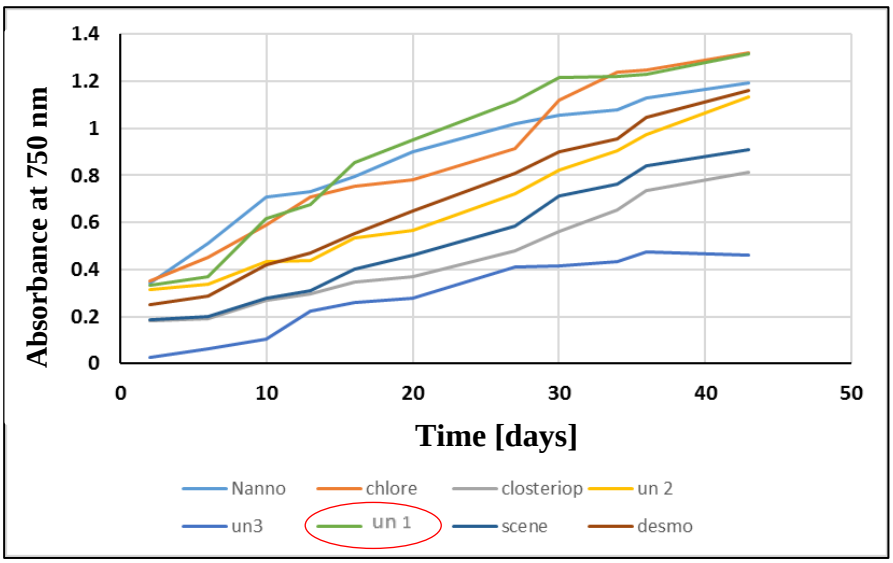

Figure 4-6. The rate of growth of microalgal strains

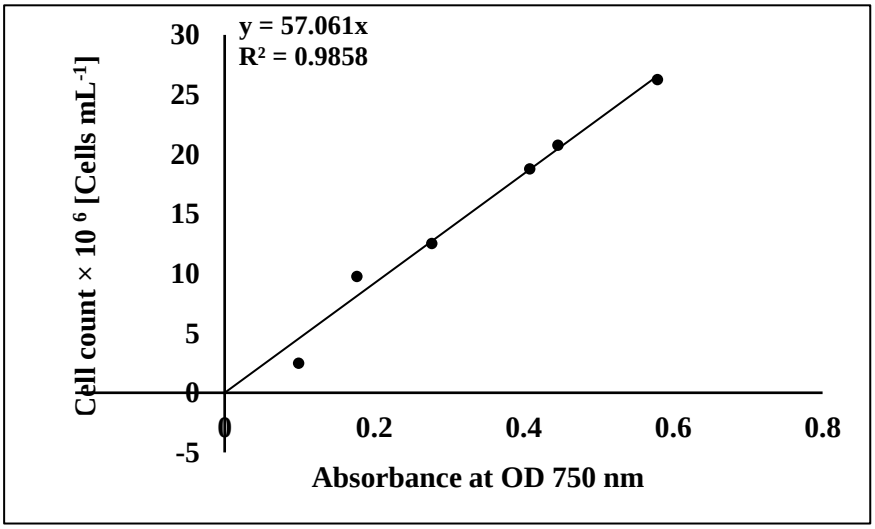

Figure 4-7. The plot of number of cells vs absorbance at 750 nm wavelength for *M. homosphaera*

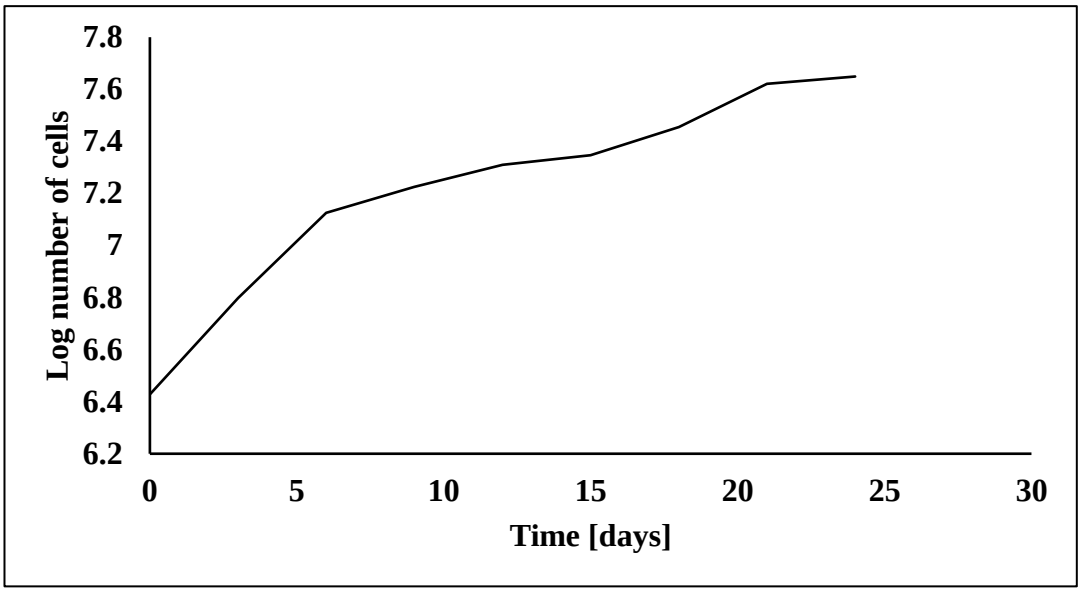

Figure 4-8. The plot of log number of cells vs time

4.4 Effect of solvent ratios for chlorophyll removal, total lipid content, and fatty acid profile

Chlorophyll removal was performed by varying reaction time, temperature, and solvent ratios. NaOH: ethanol mixture was used to remove chlorophyll from algal biomass in this study because lipids can be easily saponified when they are exposed to absolute NaOH [30]. Further, employing 100% organic solvents for chlorophyll removal causes high total lipid loss and dissolve polar lipids along with chlorophylls [7]. Therefore, the present solvent ratio does not affect the fatty acid composition and cellular structures [26]. A high percentage of NaOH in the pre-treatment ratio facilitates chlorophyll's saponification and a low percentage of ethanol aids to solubilize chlorophyll [13]. It is speculated that chlorophyll might be saponified more easily than total lipids with NaOH due to the different Gibbs free energy for the saponification reactions of chlorophyll and triacylglycerides [7]. The chlorophyll removal efficiency with ethanol's various molar ratios: NaOH in the treatment mixture was analyzed and compared in Figure 4-9 (a).

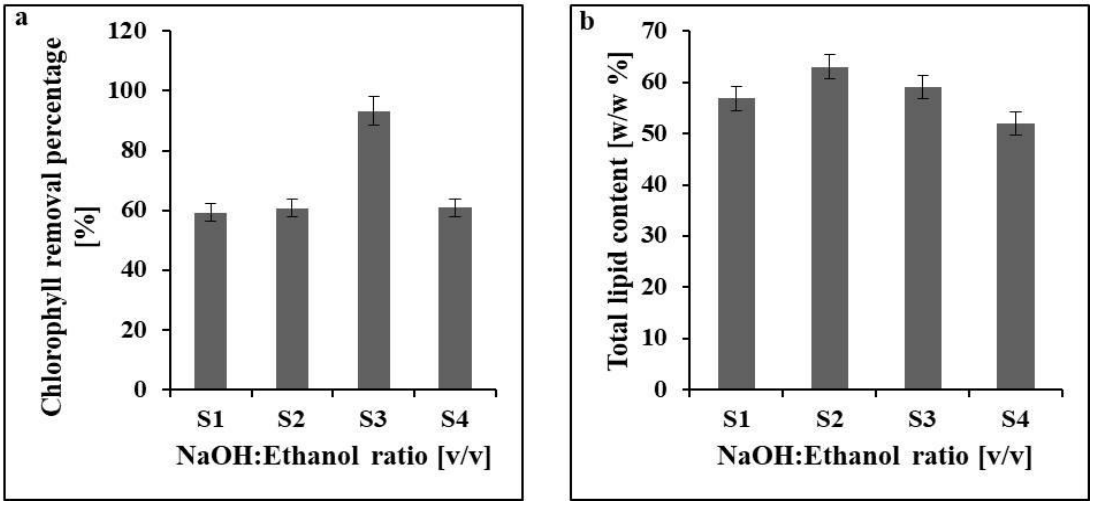

Figure 4-9. Comparison of the (a) Chlorophyll removal percentage and (b) Total lipid content in different solvent ratios of NaOH: ethanol (S1-9:1, S2-8:2, S3-7:3, S4-6:4)

According to the results obtained in the current study, the S3 mixture (NaOH: ethanol, 7:3) received the highest chlorophyll removal percentage (93.25 %) over S1 (59.24 %), S2 (60.79 %), and S4 (60.91 %) mixtures. The total lipid content was obtained as 58 % (w/w) in the S3 mixture, as shown in Figure 4-9 (b). Therefore, the S3 mixture was selected as the best solvent ratio for chlorophyll removal in *M. homosphaera*. NaOH diffuses into cells and reacts with chlorophyll to generate water-soluble sodium magnesium chlorophyllin (SMC) and transports outside of the cells [4]. Ethanol in the treatment solution contributes to the solubilization of chlorophyll [26].

Microalgal biofuels are mainly derived from lipids. Based on the chemical structure, lipids can be subdivided into polar lipids (PL), neutral lipids (NL), and glycolipids (GL) [8]. NL is a storage lipid and less reactive than PL [26]. PL can be saponified easily and can cause a net lipid loss [8]. Research conducted by Wenzhou Xiang found that the proportion of NLs and GLs of de-chlorophyll oil increased by 3.8% and 1.9%, and the proportion of PLs decreased by 0.8% compared to the crude oil [9]. NL and GL remain in the cells and are transesterified into FAME [12]. Therefore, NL is more suitable as a feedstock for biofuel production [9].

The fatty acid profile of biodiesel can be varied in the different feedstock. Oleic acid (C18:1), linoleic acid (C18:2), and palmitic acid (C16:0) were the three main compounds in algal biodiesel [15]. Fatty acids can be saturated fatty acids (SFA) and

unsaturated fatty acids [74] (monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA)). The higher SFA content would cause higher oxidative and thermal stability. This leads to a slower lipid deterioration rate and lowers NO_2 emissions compared to diesel [10]. The presence of unsaturated fatty acids can cause major drawbacks such as photo-oxidation and rancidification [17]. Unsaturated fatty acids can only be utilized for biodiesel production in a cold climate as they could increase cold flow properties [16]. The fatty acid composition of the samples pre-treated with S1 to S4 solutions was analyzed using GC-FID as shown in Figure 4-10.

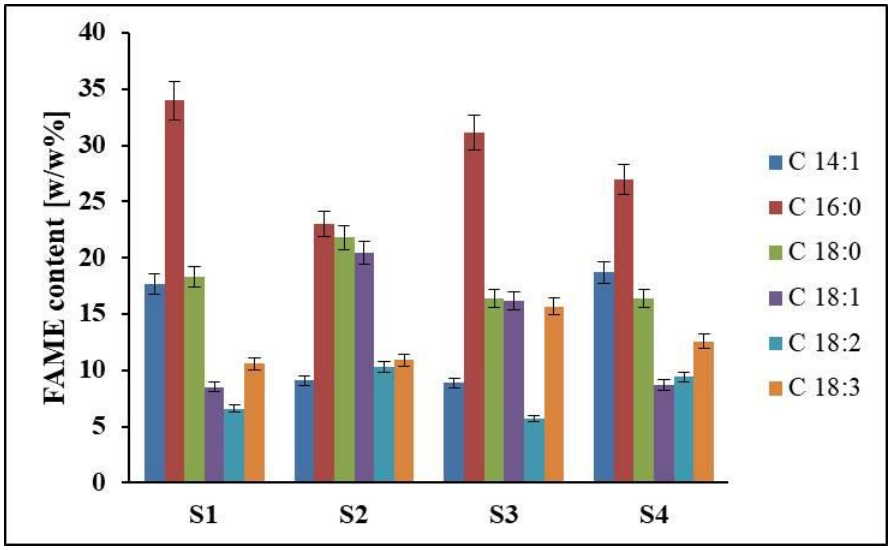

Figure 4-10. Comparison of the fatty acid profile in different solvent ratios (S1-S4)

The total SFA, MUFA, and PUFA were recorded as 50.4 %, 26.1 %, and 10.5 %, respectively, in the S3 mixture. There is a decrease of PUFA in treated biomass (8.3 %) than the control (22 %). Also, an increase of SFA (8.9 %) and a decrease of MUFA (9 %) of treated biomass compared to the chlorophyll removed biomass. This observation proved that there is an effect of chlorophyll removal for FAME composition [9].

The lipid yields vary with the type of organic solvents used, and the ratio of polar solvents to nonpolar solvents [26]. PL can be easily degraded in the presence of polar organic solvents. An ideal solvent for the lipid extraction should be free of toxicity, easy to remove, and more selective towards target products [15]. These characteristics

have been found in NaOH: ethanol mixture. Low NaOH: ethanol (v/v) ratio results in less chlorophyll removal efficiency as less NaOH is available to saponify chlorophyll [26]. Therefore, a high amount of PL can be present in the lipid extract, including more PUFA. Neutral lipids mainly contain saturated fatty acids (SFAs) and monounsaturated fatty acids (MUFAs), such as C16:0, C16:1, and C18:1. The accumulation of SFAs or MUFAs coupled with the decrease in the level of PUFAs was also observed under a moderately high NaOH: ethanol (7:3) ratio.

4.5 Effect of temperature for chlorophyll removal, total lipid content, and fatty acid profile

According to Figure 4-11 (a), the chlorophyll removal percentage is increased from 30 ^{0}C to 60 ^{0}C, with the highest chlorophyll removal percentage is obtained at 60 ^{0}C. The molecule fraction that exceeds the activation energy of the saponification reaction increases with increasing temperature [46]. Therefore, the rate of chlorophyll removal and total lipid content gradually increases from 30-60 ^{0}C. Further increase of temperature from 60-70 ^{0}C resulted a decrease in chlorophyll removal percentage (from 81.1-63 %) and lipid content (from 72-58 %) as illustrated in Figure 4-11 (b). In high temperatures, protein denaturation takes place while altering the membrane properties [18]. Therefore, the lipid degradation rate is accelerated at high temperatures [9]. As shown in Figure 4-12, the total SFA, MUFA, and PUFA contents are gradually increased up to 60 ^{0}C and the highest SFA 43.5 % including 35.1 % of C16:0, 8.4 % of C18:0, and the lowest of PUFA 9.2 % are obtained at 60 ^{0}C.

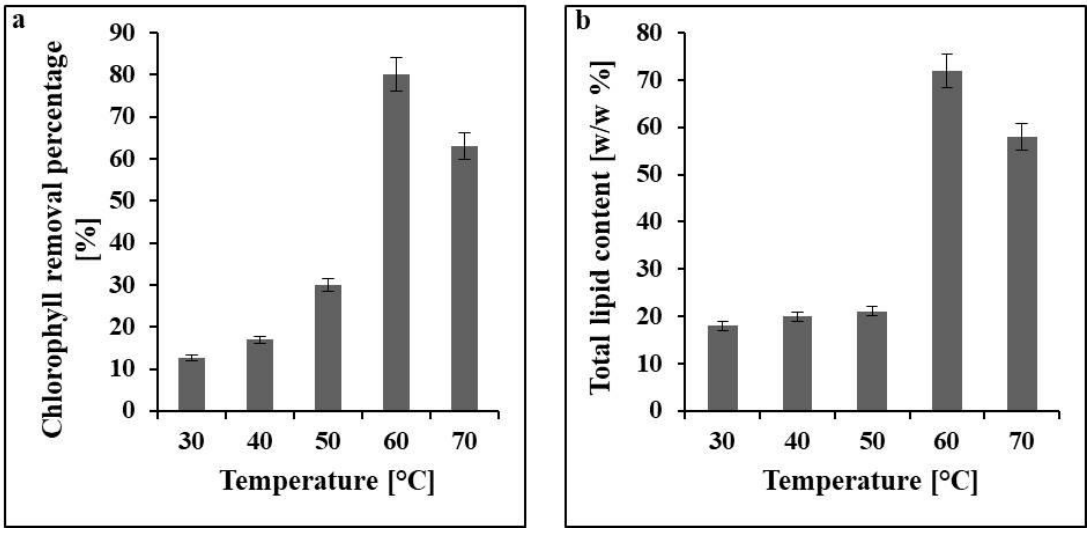

Figure 4-11. The comparison of (a) chlorophyll removal percentage and (b) Total lipid content in different temperatures (30, 40, 50, 60, 70 ^{0}C)

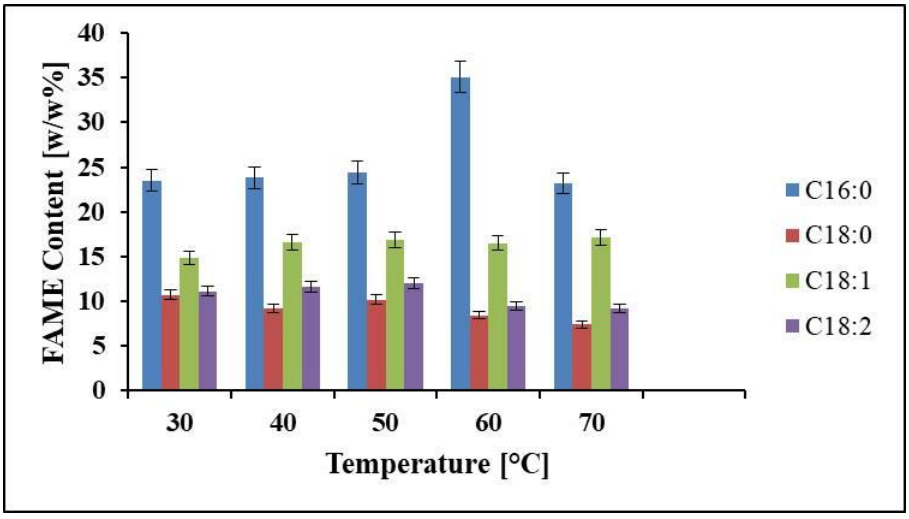

Figure 4-12. The comparison of fatty acid profiles at different temperatures

Transesterification was carried out to convert fatty acids into FAME so that they can be analyzed using GC-FID.

4.6 Effect of reaction time for chlorophyll removal, total lipid content, and fatty acid profile

The chlorophyll removal percentage remained less than 5 % during the first 30 min. The highest chlorophyll removal was achieved at 90 min. As observed in Figure 4-13

(a), a gradual increase in the chlorophyll removal percentage can be observed from 30-90 min. The highest percentage of chlorophyll removal was obtained at 90 min, while a further increase showed a slight decrease in chlorophyll removal. Prolonged exposure of chlorophyll molecules with the treatment solution provides adequate time to react with NaOH and solubilize in ethanol [26].

The total lipid content is increased from 21-61 % from 30-45 min and fluctuated around 50 % from 60-120 min, as shown in Figure 4-13 (b). Figure 4-14 shows that the higher SFA (33.7 %), MUFA (17.6 %), and lowest PUFA (9.1 %) contents were reported at 90 min compared to other samples. Therefore, 90 min was taken as the best reaction time for chlorophyll removal.

Therefore, the best preprocessing parameters for microalgal chlorophyll removal were NaOH: ethanol 7:3, 60 ^{0}C, and 90 min.

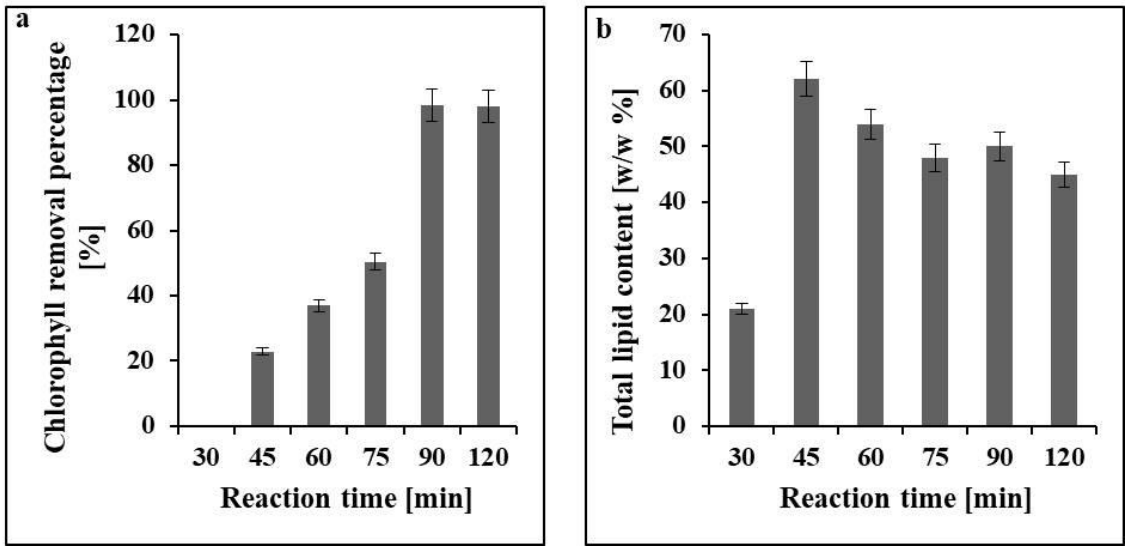

Figure 4-13. The comparison of (a) chlorophyll removal percentage and (b) Total lipid content at different reaction times (30, 45, 60, 75, 90 and 120 min)

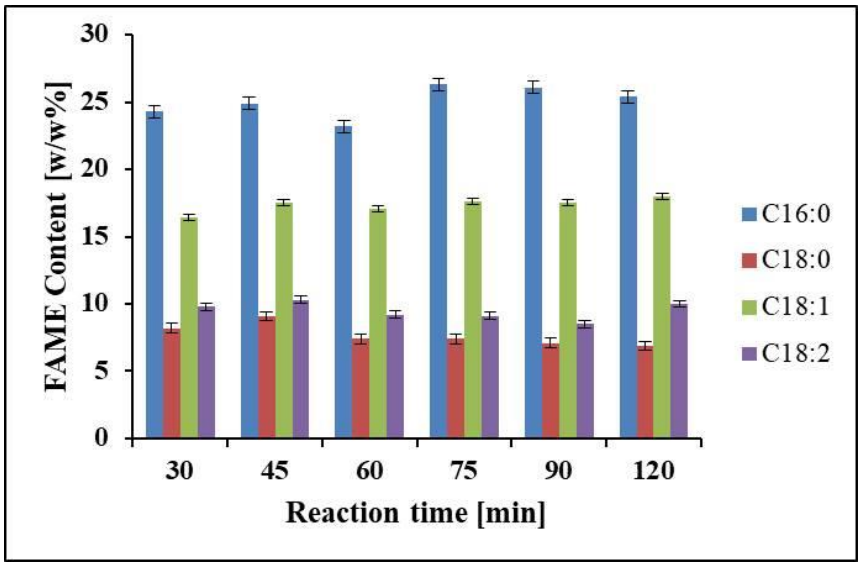

Figure 4-14. The comparison of fatty acid profiles at different reaction times

5. CONCLUSION

The effect of solvent ratio, reaction time and temperature for the removal of chlorophyll pigments from microalgae was investigated by varying a single parameter at a time. Lipid profile and FAME content of chlorophyll removed biomass were compared [69]. Prolonged extraction time and elevated temperatures could impact on plasma membrane and deteriorate lipids during saponification. Conversely, chlorophyll removal efficiency was high when the biomass is treated under moderate temperature and high reaction time. Therefore, prolonged exposure of the biomass in the treatment mixture (varying solvent ratios) allows chlorophyll molecules to migrate to the solution. After determining the best solvent ratio, the study was continued with the selected ratio to investigate other preprocessing parameters on the efficacy of chlorophyll removal from the isolated strain. The best experimental conditions for the removal of chlorophyll from algae feedstock with minimum total lipid loss and maximum SFA and MUFA yield are the solvent ratios of 7:3 (NaOH: ethanol), 60 ^{0}C, and 90 min of reaction time.

The study aimed to investigate the effect of preprocessing parameters (solvent ratio, temperature, and reaction time) for chlorophyll removal in *M. homosphaera*. This method could be adopted for other algal strains to remove chlorophyll for biodiesel production. Further, future research could focus on investigating the effect of surfactants for chlorophyll removal, as surfactants play a pivotal role in the extraction performance and increase the extraction yield of highly hydrophobic compounds from biomass, such as chlorophylls.

REFERENCES

[1] R. R. Judkins, W. Fulkerson, and M. K. Sanghvi, “the Dilemma of Fossil Fuel Use and Global Climate Change.,” no. Masters 1987, 1990.

[2] M. A. Rajaeifar, B. Ghobadian, M. D. Heidari, and E. Fayyazi, “Energy consumption and greenhouse gas emissions of biodiesel production from rapeseed in Iran,” *J. Renew. Sustain. Energy*, vol. 5, no. 6, pp. 1–14, 2013.

[3] A. K. Azad, M. G. Rasul, M. M. K. Khan, and S. C. Sharma, “Review of biodiesel production from microalgae: a novel source of green energy,” *Int. Green Energy Conf.*, no. May, pp. 1–9, 2014.

[4] M. F. Demirbas, “Biofuels from algae for sustainable development,” *Appl. Energy*, vol. 88, no. 10, pp. 3473–3480, 2011.

[5] K. Singh, D. Kaloni, S. Gaur, S. Kushwaha, and G. Mathur, “Current research and perspectives on microalgae-derived biodiesel,” *Biofuels*, vol. 7269, no. January, pp. 1–18, 2017.

[6] I. Dincer, “Environmental Issues : II-Potential Solutions,” vol. 8312, no. January 2000, pp. 83–92, 2001.

[7] P. T. Vasudevan and M. Briggs, “Biodiesel production-current state of the art and challenges,” *J. Ind. Microbiol. Biotechnol.*, vol. 35, no. 5, pp. 421–430, 2008.

[8] D. Sarkar and K. Shimizu, “An overview on biofuel and biochemical production by photosynthetic microorganisms with understanding of the metabolism and by metabolic engineering together with efficient cultivation and downstream processing,” *Bioresour. Bioprocess.*, vol. 2, no. 1, p. 17, 2015.

[9] G. Philippidis, “Commentary: Powering America with sustainable energy in the 21st century,” *J. Renew. Sustain. Energy*, vol. 4, no. 6, pp. 1–10, 2012.

[10] T. Ren, “Primary Factors Affecting Growth of Microalgae Optimal Light

Exposure Duration and Frequency," 2014.

[11] C. D. Calixto *et al.*, "Productivity and fuel quality parameters of lipids obtained from 12 species of microalgae from the northeastern region of Brazil," *Renew. Energy*, vol. 115, no. January 2019, pp. 1144–1152, 2018.

[12] Q. Hu *et al.*, "Microalgal triacylglycerols as feedstocks for biofuel production: Perspectives and advances," *Plant J.*, vol. 54, no. 4, pp. 621–639, 2008.

[13] J. M. Lv, L. H. Cheng, X. H. Xu, L. Zhang, and H. L. Chen, "Enhanced lipid production of Chlorella vulgaris by adjustment of cultivation conditions," *Bioresour. Technol.*, vol. 101, no. 17, pp. 6797–6804, 2010.

[14] R. Halim, A. Hosikian, S. Lim, and M. K. Danquah, "Chlorophyll extraction from microalgae: A review on the process engineering aspects," *Int. J. Chem. Eng.*, vol. 2010, 2010.

[15] D. K. Y. Lim and P. M. Schenk, "Microalgae selection and improvement as oil crops: GM vs non-GM strain engineering," *AIMS Bioeng.*, vol. 4, no. 1, pp. 151–161, 2017.

[16] C. Prommuak, P. Pavasant, A. T. Quitain, M. Goto, and A. Shotipruk, "Microalgal lipid extraction and evaluation of single-step biodiesel production," *Eng. J.*, vol. 16, no. 5, pp. 157–166, 2012.

[17] Y. Li, M. Horsman, B. Wang, N. Wu, and C. Q. Lan, "Effects of nitrogen sources on cell growth and lipid accumulation of green alga Neochloris oleoabundans," *Appl. Microbiol. Biotechnol.*, vol. 81, no. 4, pp. 629–636, 2008.

[18] J. W. Richardson, M. D. Johnson, X. Zhang, P. Zemke, W. Chen, and Q. Hu, "A fi nancial assessment of two alternative cultivation systems and their contributions to algae biofuel economic viability ☆," *ALGAL*, vol. 4, pp. 96–104, 2014.

[19] Y. C. Sharma and B. Singh, "Development of biodiesel: Current scenario,"

Renew. Sustain. Energy Rev., vol. 13, no. 6–7, pp. 1646–1651, 2009.

[20] G. Kumar, S. Shobana, W. Chen, and Q. Bach, "A review of thermochemical conversion of microalgal biomass for biofuels : chemistry and," 2016.

[21] D. P. Sartory and J. U. Grobbelaar, "Extraction of chlorophyll a from freshwater phytoplankton for spectrophotometric analysis," *Hydrobiologia*, vol. 114, no. 3, pp. 177–187, 1984.

[22] R. J. A. Saracanlao, J. O. Nacorda, and H. P. Hernandez, "Influence of Different Extraction Methods on Fatty Acid Composition of Lipid Extracts of Chlorella vulgaris Beijerinck from Laguna De Bay , Philippines," vol. 148, no. March, pp. 95–104, 2019.

[23] B. Liu *et al.*, "Rapid characterization of fatty acids in oleaginous microalgae by near-infrared spectroscopy," *Int. J. Mol. Sci.*, vol. 16, no. 4, pp. 7045–7056, 2015.

[24] P. Kumar, P. K. Sharma, P. K. Sharma, and D. Sharma, "Micro-algal lipids: A potential source of biodiesel," *J. Innov. Pharm. Biol. Sicences*, vol. 2, no. 2, pp. 135–143, 2015.

[25] F. Nazari, "Genetic Engineering of Microalgae for Enhanced Biodiesel Production Suitable Fuel Replacement of Fossil Fuel as a Novel Energy Source," *Am. J. Life Sci.*, vol. 3, no. 1, p. 32, 2015.

[26] T. Li *et al.*, "A Saponification Method for Chlorophyll Removal from Microalgae Biomass as Oil Feedstock," no. 1960, pp. 1–19, 2016.

[27] M. R. Brown and S. I. Blackburn, "Biofuels from microalgae," *Sustain. Energy Solut. Agric.*, pp. 277–321, 2014.

[28] J. C. Liao, L. Mi, S. Pontrelli, and S. Luo, "Fuelling the future: Microbial engineering for the production of sustainable biofuels," *Nature Reviews Microbiology*, vol. 14, no. 5. 2016.

[29] I. Dincer, "Renewable energy and sustainable development: a crucial review,"

Renew. Sustain. Energy Rev., vol. 4, no. 2, pp. 157–175, 2000.

[30] L. Gouveia and A. C. Oliveira, "Microalgae as a raw material for biofuels production," *J. Ind. Microbiol. Biotechnol.*, vol. 36, no. 2, pp. 269–274, 2009.

[31] P. J. McGinn, K. E. Dickinson, S. Bhatti, J. C. Frigon, S. R. Guiot, and S. J. B. O'Leary, "Integration of microalgae cultivation with industrial waste remediation for biofuel and bioenergy production: Opportunities and limitations," *Photosynth. Res.*, vol. 109, no. 1–3, pp. 231–247, 2011.

[32] J. A. McGarvey, W. G. Miller, R. Zhang, Y. Ma, and F. Mitloehner, "Bacterial population dynamics in dairy waste during aerobic and anaerobic treatment and subsequent storage," *Appl. Environ. Microbiol.*, vol. 73, no. 1, pp. 193–202, 2007.

[33] V. Makareviciene, M. Gumbyte, V. Skorupskaite, and E. Sendzikiene, "Biodiesel fuel production by enzymatic microalgae oil transesterification with ethanol," *J. Renew. Sustain. Energy*, vol. 9, no. 2, 2017.

[34] T. M. Mata, A. A. Martins, and N. S. Caetano, "Microalgae for biodiesel production and other applications: A review," *Renew. Sustain. Energy Rev.*, vol. 14, no. 1, pp. 217–232, 2010.

[35] M. A. Islam, M. Magnusson, R. J. Brown, G. A. Ayoko, M. N. Nabi, and K. Heimann, "Microalgal species selection for biodiesel production based on fuel properties derived from fatty acid profiles," *Energies*, vol. 6, no. 11, pp. 5676–5702, 2013.

[36] G. B. Leite, A. E. M. Abdelaziz, and P. C. Hallenbeck, "Algal biofuels: Challenges and opportunities," *Bioresour. Technol.*, vol. 145, pp. 134–141, 2013.

[37] K. Hegde, N. Chandra, S. J. Sarma, S. K. Brar, and V. D. Veeranki, "Genetic Engineering Strategies for Enhanced Biodiesel Production," *Mol. Biotechnol.*, vol. 57, no. 7, pp. 606–624, 2015.

[38] R. Kumar, M. Tabatabaei, K. Karimi, and I. S. Horváth, “Recent updates on lignocellulosic biomass derived ethanol - A review,” *Biofuel Res. J.*, vol. 9, pp. 347–356, 2016.

[39] U. Jena and K. C. Das, “Comparative evaluation of thermochemical liquefaction and pyrolysis for bio-oil production from microalgae,” *Energy and Fuels*, vol. 25, no. 11, pp. 5472–5482, 2011.

[40] T. Mutanda, D. Ramesh, S. Karthikeyan, S. Kumari, A. Anandraj, and F. Bux, “Bioprospecting for hyper-lipid producing microalgal strains for sustainable biofuel production,” *Bioresour. Technol.*, vol. 102, no. 1, pp. 57–70, 2011.

[41] a Dauta, J. Devaux, F. Piquemal, and L. Boumnich, “Growth rate of four freshwater algae in relation to light and temperature,” *Hydrobiologia*, vol. 207, pp. 221–226, 1990.

[42] I. Ahmad, A. K. Sharma, H. Daniell, and S. Kumar, “Altered lipid composition and enhanced lipid production in green microalga by introduction of brassica diacylglycerol acyltransferase 2,” *Plant Biotechnol. J.*, vol. 13, no. 4, pp. 540–550, 2015.

[43] C. Y. Chen, K. L. Yeh, R. Aisyah, D. J. Lee, and J. S. Chang, “Cultivation, photobioreactor design and harvesting of microalgae for biodiesel production: A critical review,” *Bioresour. Technol.*, vol. 102, no. 1, pp. 71–81, 2011.

[44] D. B. Stengel and S. Connan, “Natural Products From Marine Algae,” *Fat. acids, Total lipid, protein ash contents Process. edible Seaweeds*, vol. 1308, no. July, pp. 103–108, 2015.

[45] E. A., “Algal Biomass and Biodiesel Production,” *Biodiesel - Feed. Process. Technol.*, no. May, 2012.

[46] L. Xin, H. Hong-ying, and Z. Yu-ping, “Growth and lipid accumulation properties of a freshwater microalga Scenedesmus sp. under different cultivation temperature,” *Bioresour. Technol.*, vol. 102, no. 3, pp. 3098–3102, 2011.

[47] M. Paul Abishek, J. Patel, and A. Prem Rajan, “Algae Oil: A Sustainable Renewable Fuel of Future,” *Biotechnol. Res. Int.*, vol. 2014, pp. 1–8, 2014.

[48] K. Yoo *et al.*, “Comparison of fatty acid analysis methods for assessing biorefinery applicability of wastewater cultivated microalgae,” *Talanta*, vol. 97, pp. 103–110, 2012.

[49] D. B. De Alencar *et al.*, “Fatty acid composition from the marine red algae pterocladiella capillacea (S. G. gmelin) santelices & hommersand 1997 and osmundaria obtusiloba (C. agardh) R. E. norris 1991 and its antioxidant activity,” *An. Acad. Bras. Cienc.*, vol. 90, no. 1, pp. 449–459, 2018.

[50] J. Fan, C. Andre, and C. Xu, “A chloroplast pathway for the de novo biosynthesis of triacylglycerol in Chlamydomonas reinhardtii,” *FEBS Lett.*, vol. 585, no. 12, pp. 1985–1991, 2011.

[51] G. Markou, I. Angelidaki, and D. Georgakakis, “Microalgal carbohydrates: An overview of the factors influencing carbohydrates production, and of main bioconversion technologies for production of biofuels,” *Appl. Microbiol. Biotechnol.*, vol. 96, no. 3, pp. 631–645, 2012.

[52] P. G. Roessler *et al.*, “Genetic Engineering Approaches for Enhanced Production of Biodiesel Fuel from Microalgae,” *Enzymatic Conversion of Biomass for Fuels Production*. pp. 255–270, 1994.

[53] W. A. C. Evers *et al.*, “Analysis of Fatty Acid Content and Composition in Microalgae,” *J. Vis. Exp.*, no. 80, pp. 1–8, 2013.

[54] P. Schlagermann, G. Gerold, R. Dillschneider, R. Rosello-sastre, and C. Posten, “Composition of Algal Oil and Its Potential as Biofuel,” vol. 2012, 2012.

[55] H. Tang, S. O. Salley, and K. Y. S. Ng, “Recent developments in microalgae for biodiesel production,” *Biofuels*, vol. 1, no. 4, pp. 631–643, 2010.

[56] P. Hidalgo, C. Toro, G. Ciudad, and R. Navia, “Advances in direct

transesterification of microalgal biomass for biodiesel production," 2013.

[57] S. Markossian, P. Becker, H. Märkl, and G. Antranikian, "Isolation and characterization of lipid-degrading Bacillus thermoleovorans IHI-91 from an icelandic hot spring," *Extremophiles*, vol. 4, no. 6, pp. 365–371, 2000.

[58] P. Asadi, H. A. Rad, and F. Qaderi, "Lipid and biodiesel production by cultivation isolated strain Chlorella sorokiniana pa . 91 and Chlorella vulgaris in dairy wastewater treatment plant effluents," 2020.

[59] M. Meira, P. M. B. Santana, A. S. Araújo, C. L. Silva, J. R. L. L. Filho, and H. T. Ferreira, "Oxidative degradation and corrosiveness of biodiesel," vol. 32, no. Figure 1, pp. 143–161, 2014.

[60] R. Ganesan *et al.*, "A review on prospective production of biofuel from microalgae," *Biotechnol. Reports*, vol. 27, p. e00509, 2020.

[61] A. B. Culaba, A. T. Ubando, P. M. L. Ching, and W. Chen, "Biofuel from Microalgae : Sustainable Pathways," 2020.

[62] S. Nagappan, P. Tsai, S. Devendran, V. Alagarsamy, and V. K. Ponnusamy, "Enhancement of biofuel production by microalgae using cement flue gas as substrate," 2019.

[63] Y. J. Choong, H. Y. Y. M. M. K. Lam, Y. Uemura, Y. K. Dasan, and W. N. A. Kadir, "The potential of using microalgae for simultaneous oil removal in wastewater and lipid production," *Int. J. Environ. Sci. Technol.*, no. 0123456789, 2020.

[64] A. Sathish, R. Sims, and B. Wood, "DigitalCommons @ USU Biodiesel Production from Mixed Culture Algae Via a Wet Lipid Extraction Procedure," 2012.

[65] U. T. C. All, "Photosynthesis , Photooxidation of Chlorophyll and Fluorescence of Normal and Manganese-deficient Chlorella with and without Hydrogenase Author (s): Erich Kessler Published by : Springer Stable URL :

http://www.jstor.org/stable/23368743 Photosynthesis , Photooxidation of Chlorophyll and Fluorescence," no. 1970, pp. 222–234, 2016.

[66] L. Yao, J. A. Gerde, S. L. Lee, T. Wang, and K. A. Harrata, "Microalgae lipid characterization," *J. Agric. Food Chem.*, vol. 63, no. 6, pp. 1773–1787, 2015.

[67] R. S. Menezes *et al.*, "Evaluation of fatty acid composition of the microalgae Choricystis minor var. minor according to two different nutrient feeding strategies," *J. Renew. Sustain. Energy*, vol. 7, no. 4, 2015.

[68] G. Vidal, A. Carvalho, R. M??ndez, and J. M. Lema, "Influence of the content in fats and proteins on the anaerobic biodegradability of dairy wastewaters," *Bioresour. Technol.*, vol. 74, no. 3, pp. 231–239, 2000.

[69] Y. M. Kulasinghe, "Development of a Novel Preprocessing Method for Removal of Chlorophyll from Microalgae," pp. 505–509, 2020.

[70] Y. Matsumiya, D. Wakita, A. Kimura, S. Sanpa, and M. Kubo, "Isolation and Characterization of a Lipid-Degrading Bacterium and Its Application to Lipid-Containing Wastewater Treatment," vol. 103, no. 4, pp. 325–330, 2007.

[71] S. Mandal and N. Mallick, "Microalga Scenedesmus obliquus as a potential source for biodiesel production," *Appl. Microbiol. Biotechnol.*, vol. 84, no. 2, pp. 281–291, 2009.

[72] R. S. Dungan and A. B. Leytem, "The characterization of microorganisms in dairy wastewater storage ponds.," *J. Environ. Qual.*, vol. 42, no. 5, pp. 1583–8, 2013.

[73] L. L. Beer, E. S. Boyd, J. W. Peters, and M. C. Posewitz, "Engineering algae for biohydrogen and biofuel production," *Curr. Opin. Biotechnol.*, vol. 20, no. 3, pp. 264–271, 2009.

[74] A. M. Brooksbank, J. W. Latchford, and S. M. Mudge, "Degradation and modification of fats, oils and grease by commercial microbial supplements," *World J. Microbiol. Biotechnol.*, vol. 23, no. 7, pp. 977–985, 2007.

6. PUBLICATIONS

1. The best research paper under the sustainable energy category at Moratuwa Engineering Research Conference (MERCon), 2020.

Y. M. Kulasinghe and T. U. Ariyadasa, "Development of a Novel Preprocessing Method for Removal of Chlorophyll from Microalgae," 2020 Moratuwa Engineering Research Conference (MERCon), Moratuwa, Sri Lanka, 2020, pp. 505-509, doi:10.1109/MERCon50084.2020.9185262.

2. Research publication at 4th Interdisciplinary Conference of Management Researchers.

Development of a novel method for microalgae chlorophyll removal towards biofuel production, 2019 (Abstract).