JMBFS

Journal of Microbiology, Biotechnology and Food Sciences

International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

NITROGEN STARVATION INDUCED LIPID ACCUMULATION BY *Chlorococcum infusionum* (EAU-10) AS POTENTIAL RENEWABLE SOURCE OF LIPID FOR BIODIESEL PRODUCTION

*Eldrin DLR. Arguelles**

Address(es):
Philippine National Collection of Microorganisms, National Institute of Molecular Biology and Biotechnology (BIOTECH), University of the Philippine Los Baños, College, Laguna, Philippines, 4031.

*Corresponding author: edarguelles@up.edu.ph

https://doi.org/10.55251/jmbfs.1931

ARTICLE INFO

Received 12. 7. 2019
Revised 14. 10. 2021
Accepted 19. 10. 2021
Published 1. 4. 2022

Regular article

OPEN ACCESS

ABSTRACT

Microalgae are organisms effective of accumulating high quantity of industrially important lipids with promising characteristics as an excellent feedstock for biodiesel production. This study assess the possibility of using a green microalga, *Chlorococcum infusionum* (EAU-10) for biodiesel production by evaluating the growth characteristic, lipid yield and fatty acid profile of the microalga cultivated at varying concentrations of nitrogen source (0.375 - 1.50 g L^{-1} $NaNO_3$). High lipid accumulation was observed in nitrogen-starved cultivation condition (0.375 g L^{-1} $NaNO_3$) after 23 days of growth. Maximum biomass concentration of *Chlorococcum infusionum* (EAU-10) under nitrogen starved condition is 0.577 ± 0.003 g L^{-1} with 21.26% oil content per dry weight of algal biomass and lipid productivity of 22.08 mg L^{-1} day^{-1}. Nitrogen starvation caused an increase in the total oil content and a decrease in biomass production of the microalga. Profiling of fatty acids of the obtained algal biodiesel shows methyl palmitate (C16:0) and methyl nonadecanoate (C19:0) contribute to almost 70% of *Chlorococcum infusionum* (EAU-10) fatty acid methyl esters (FAME) profile. Overall, a total of 74.58% of saturated fatty acid (SAFA) methyl ester content is present in the algal lipid, which is exceedingly high in contrast to other similar studies. Analysis of *C. infusionum* FAME profile in relation to some important fuel properties showed that the algal oil has the potential to produce biodiesel with excellent fuel qualities.

Keywords: *Biodiesel, Chlorococcum infusionum, fatty acid methyl esters, lipids, microalgae, nitrogen starvation*

INTRODUCTION

Several species of microalgae are known as excellent feedstock to produce biodiesel since these organisms can accumulate high amount of industrially important lipids (reaching up to 50-60% of the total dried biomass in some species) (**Sheehan *et al.*, 1998; Chisti, 2007; Arguelles *et al.*, 2019; Arguelles and Sapin, 2021**). The utilization of algae as a third-generation biodiesel feedstock presents several distinct advantages such as high photosynthetic efficiencies, lack of use for arable or agricultural lands in mass production as well as excellent biomass productivities (algal biomass can doubled in less than one day) (**Hariskos and Posten, 2014; Tredici, 2010; Pugliese *et al.*, 2020**). In addition, neutral lipids (NLs) and triacylglycerols are considered as the main storage lipids in these organisms which can be esterified to FAMEs having fatty acid profile dominated with saturated fatty acids (C16 and C18) suitable for biodiesel production (**Ge *et al.*, 2017; Chen *et al.*, 2018; Arguelles and Sapin, 2020**). Microalgae also exhibit good adaptation strategies to different environmental conditions, making them easy to cultivate. For example, they can grow on both closed and open pond systems using wastewater as well as in marginal lands, CO_2-enriched gases, and organic wastes (such as crude glycerol) to provide nutrients and carbon as well as temperature maintenance (in a cold climate), attaining environmentally and economically sustainable waste bioremediation and biofuel production (**Ge and Champagne, 2016; Chen *et al.*, 2018**). Also, several routes of microalgal metabolisms can be used for high lipid production and enhanced growth. Usually, microalgae are cultivated autotrophically with carbon dioxide as the only source of carbon and light providing all the energy needed. However, some species of microalgae can be heterotrophically cultivated using only organic compounds, while other strains can grow mixotrophically using both CO_2 and organic compounds to support growth (**Sforza *et al.*, 2012; Chen *et al.*, 2018 Arguelles, 2020**).

Manipulation of algal growth is possible to produce particular groups of free fatty acids and lipids via alterations of some of the chemical and physical composition of its culture medium. Such manipulation can cause microalgae to produce atypical and useful fatty acids and lipids for several industrial applications (**Behrens and Kyle, 1996; Shokravi *et al.*, 2020**). Studies showed that oil-rich microalgae have the ability of producing triacylglycerols (20- 50% up to 80% of dried algal biomass) when subjected to simple alteration in culture growth conditions (e.g. nitrogen and phosphorus limitation, high temperature, high salt concentration, and high light intensity). Nitrogen starvation is a popular method in increasing triacylglycerol (TAG) accumulation in algal biomass, (**Breuer *et al.*, 2012; Yang *et al.*, 2018**). During nitrogen-limited growth condition, there is a rechanneling and remodeling on the use of reduced carbon inducing the synthesis and accumulation of lipids such as triglycerides (**Breuer *et al.*, 2012; Negi *et al.*, 2016; Shokravi *et al.*, 2020**). Also, changes such as increase in the carbohydrate and carotenoid content as well as cell volume are associated with nitrogen starvation in green microalgae. However, nitrogen limitation in culture media of green algae causes cell division to slow down or sometimes ceases which leads to reduced chlorophyll, protein, and total algal biomass production (**Cakmak *et al.*, 2012; Negi *et al.*, 2016**).

The kind of fatty acid methyl ester (FAME) profile dictates the physico-chemical properties of biodiesel. Generally, lipids with FAME profile that show high amounts of saturated and monounsaturated fatty acids as well as low concentration of linoleic acid are suitable for biodiesel production (**Pugliese *et al.*, 2020; Negi *et al.*, 2016**). Saturated fatty acids (SAFA) produce high oxidation stability and good cetane number but inferior low-temperature biodiesel properties while unsaturated FAMEs produce biodiesel that are suitable in low-temperature performance **(Knothe 2009; Arguelles *et al.*, 2019**). A study made by **Arguelles and Martinez-Goss (2021)** showed FAME profile of biodiesel obtained from *Chlorella* sp. and *Chlorolobion* sp. possess fuel properties that conforms the American (ASTM D6751) and European (EN 14214) biodiesel standards. *Chlorella* sp. biodiesel has low kinematic viscosity (2.78 mm^2 s^{-1}), good cetane number (68.79), oxidation stability (10.44 h), and low density (0.88 g cm^{-3}). On the other hand, *Chlorolobion* sp. has biodiesel properties with low kinematic viscosity (2.79 mm^2 s^{-1}), good cetane number (65.17), oxidation stability (8.93 h) and low density (0.89 g cm^{-3}). These algal strains are considered candidate microalgae that can be use as raw material for production of biodiesel with superior fuel quality.

Chlorococcum infusionum is a fast growing and easily cultured microalga commonly isolated in aquatic and terrestrial habitat with potential use as an alternative biomass feedstock for biodiesel production (**Arguelles and Monsalud,**

2017). The current investigation was conducted to evaluate the growth characteristic and lipid accumulation of *Chlorococcum infusionum* (EAU-10) via nitrogen starvation (modifying the concentrations of nitrate ($NaNO_3$) in Blue Green (BG11) medium). Lipid content and fatty acid composition were also assessed to know the impact of limiting nitrogen source in this microalga. In addition, characterization of the transesterified FAMEs were used to predict the fuel characteristics and check the acceptability of the microalgal oil as alternative biomass feedstock for biodiesel application.

MATERIAL AND METHODS

Sampling and microalgae pre-cultures

The Philippine National Collection of Microorganisms, National Institute of Molecular Biology and Biotechnology (BIOTECH), University of the Philippines Los Baños (UPLB), Philippines, provided the microalga, *Chlorococcum infusionum* (EAU-10) used in the present study. Stock culture of the microalga was initially kept at 25 ± 2 °C in test tubes exposed in a fluorescent light with an 18:6 h light–dark cycle (**Arguelles, 2019; Arguelles, 2021b**). Blue green (BG11) culture media (**Stanier *et al.*, 1971**) was used in maintaining the microalgal inocula and in the conduct of the experiments.

The microalgal seeds were cultured in 50 mL sterile BG11 culture medium in 100 mL Erlenmeyer flasks. The flasks were then incubated at 25 ± 2 °C under light intensity of 120 ± 2 µmol photons m^{-2} s^{-1} using artificial lights at 18:6 h light/dark cycles for 2-3 weeks. Regular agitations of each culture flasks were done for three times each day (**Arguelles, 2021a).**

Biomass production and nitrogen starvation

Chlorococcum infusionum (EAU-10) was grown in batch culture using BG11 medium with different cultivation condition including nitrogen-starved (1.5, 0.75, 0.50, and 0.375 g L^{-1} of $NaNO_3$) and control conditions, placed in three distinct 1000 mL Erlenmeyer flask containing 500 mL of fresh culture medium for 23 days (**Arguelles and Martinez-Goss, 2021**). In nitrogen starved culture condition, *C. infusionum* cells from the inoculum development were concentrated using a centrifuge (set at 5,000 rpm, 20°C for 10 minutes). The centrifuged algal cells were thoroughly rinsed using a normal saline solution thrice to eliminate contaminating cellular debris as well as excess sodium nitrate. The harvested algal biomass was then placed in 500 mL of nitrate-limited medium. The experiment was conducted in three replicates and the computed average values for the noted growth characteristics were accounted. Three fluorescent lamps placed at 7" away from the algal culture flasks were used as a source of illumination in the experimental set up. The fluorescent lights emitted a 120 µmol photons m^{-2} s^{-1} for its total light intensity. The aeration used in the experimental set ups were provided by one of the two air outlets of an aquarium air pump that was set at low aeration with computed air velocity of 300 mL min^{-1} (**Arguelles *et al.*, 2018**).

Microalgal growth parameter was analyzed by taking the optical density (OD) readings of algal sample collected every two days. The absorbance readings were assessed at 425 nm using a spectrophotometer (UV Vis Double Beam spectrophotometer, Shimadzu, UV-1800, Japan). The growth rate (µ), doublings per day (k), doubling time (T2) and biomass productivity of the microalga was computed using the formula given below:

Growth Rate (µ): $$\mu = \frac{\mathbf{Ln X_2 - Ln X_1}}{\mathbf{T}}$$

where:

X_2 = optical density at end of a selected interval
X_1 = optical density at start of a selected interval
T = number of days in the interval

Doublings per day (k): $$k = \frac{\mu}{\mathbf{Ln\ 2}}$$

Doubling Time (T2): $$T2 = \frac{\mathbf{Ln\ 2}}{\mu}$$

Biomass productivity (g L^{-1} day^{-1}) = biomass density (g L^{-1}) x µ (day^{-1})

Determination of microalgal oil yield

The biomass of *C. infusionum* was concentrated using a centrifuge set at 5,000 rpm for 10 minutes after 23 days of cultivation. The algal pellets were dried using a drying oven (set at 70-80 ^{0}C) for 48 hours. The dry weight of the centrifuged cells was calculated gravimetrically while microalgal growth was noted in terms of dry weight grams per litre. Dried biomass for each microalga was finely ground and placed to a screw capped tube containing a specific amount of chloroform-methanol (2:1) solvent for lipid extraction. After 24 hours of soaking, the algal biomass was set apart from the extracted lipid by filtration. The solvent part of the extract was vaporized by a water bath (set at 80°C) and the recovered algal crude oil was noted gravimetrically and kept for FAME profile analysis (**Arguelles *et al.*, 2018; Arguelles and Sapin, 2021**). The percent algal oil yield and lipid productivity was determined using the this formula:

$$\textbf{Percent lipid yield} = \left(\frac{\textbf{weight of oil}}{\textbf{weight of oven dried biomass}}\right) \times \mathbf{100}$$

$$\textbf{Lipid productivity}\ (\textbf{mg}\ \textbf{L}^{-1}\textbf{day}^{-1}) = \textbf{Biomass Productivity} \times \textbf{Lipid Yield}$$

where: biomass productivity (g L^{-1} day^{-1}), lipid yield = lipid yield as percent dry weight.

Transesterification and Fatty acid profile analysis

Fatty acid profiling and transesterification (using methanolic sodium hydroxide) of the extracted microalgal lipid was analyzed following the procedures done by **Arguelles *et al.* (2018).** Initially, 0.25 g of algal oil was mixed in 6 mL stock solution of 0.5 N methanolic NaOH in a 50-mL flask. The reaction mixture was heated at 90 °C for 5–10 min until all globules of lipids were totally cleared. Aliquot (7 mL) of boron trifluoride was then added in the reaction mixture and was further subjected to heating for 2 min. Heptane (5 mL) was then added in the mixture and refluxing of the sample was done for 1 min. After refluxing, a large volume of saturated sodium chloride was mixed in the treated algal oil sample in order to achieve phase separation. The top heptane layer (1 mL) of the reaction mixture was obtained and kept in a sterile vial with a small volume of anhydrous Na_2SO_4 to remove excess water (**Arguelles and Martinez-Goss, 2021).** Fatty acid profile analysis was done using a Shimadzu GC-2010 chromatograph provided with Supelco SP-2560 column (100 m × 0.25 mm × 0.2 µm film thickness) and flame ionization detector (FID). The following GC condition was employed in the analysis of the sample: Injection temperature was fixed at 260°C and the injector was set at a temperature 250°C. The temperature of the column oven was set from 140°C (5 min) to 240°C at 4°C min^{-1}. The carrier gas of the chromatograph is helium with a set flow rate of 1.12 mL min^{-1}. The identification of the fatty acid methyl esters were known by comparing the retention time of each FAMEs to that of Supelco 37 Component FAME Mix, CRM47885 (**Arguelles *et al.*, 2018**).

Statistical analysis

The analysis of variance was utilized to assess the interaction to each experimental runs and treatments from the data obtained in the study. Means of the experimental treatments were collated employing the Least Significant Difference (LSD) test at $p \leq 0.05$ using STAR [Statistical Tool for Agricultural Research (STAR) version 1.0 2013].

RESULTS AND DISCUSSION

Microalgal Biomass and Lipid Production by Nitrogen Limitation

Growth curve of the algal species, *Chlorococcum infusionum* (EAU-10) grown in batch culture using Blue Green (BG 11) culture medium with altered amounts of $NaNO_3$ is shown in Figure 1. The experimental treatments showing different concentrations of sodium nitrate (0.375 gL^{-1} - 1.5 g L^{-1} $NaNO_3$) were used to do comparative evaluation and describe its effect on growth characteristics and total lipid content of *Chlorococcum infusionum* (EAU-10). A sharp increase in the growth of the green microalga was observed following a brief lag phase of two days, which is succeeded by a log phase and reached stationary stage after 19 days of cultivation. As shown from the growth response curve (Figure 1), the higher the amount of $NaNO_3$ concentration, the growth of the microalga also increased.

Growth Kinetics, Biomass and Oil Productivity

Microalgal growth rate and amount of lipid concentration are two of the well-investigated criterion in search of a suitable microalgae for mass cultivation and biofuel production (**Nascimento *et al.*, 2013; Shokravi *et al.*, 2020; Pugliese *et al.*, 2020**). Average specific growth rate of *Chlorococcum infusionum* (EAU-10) is 0.18 with computed doublings per day (k) and doubling time (T2) of 0.26 and 3.85 respectively. Highest algal biomass concentration of 0.792 g L^{-1} for *Chlorococcum infusionum* (EAU-10) was obtained at day 19 in the control set up (BG 11 with 1.500 g L^{-1} $NaNO_3$) while a maximum algal biomass concentration of 0.577±0.003 g L^{-1} was observed in nitrogen-limited growth medium (0.375 g L^{-1} $NaNO_3$) (Table 1). Dry algal biomass concentration of 0.685, 0.616, and 0.577 g L^{-1} were observed in experimental algal culture set-up with 0.750 g L^{-1} $NaNO_3$ (1/2 of the control), 0.500 g L^{-1} $NaNO_3$ (1/3 of control), and 0.375 g L^{-1} $NaNO_3$ (1/4 of the control), respectively (Table 1). A statistically significant ($p \leq 0.05$) difference in the total amount of algal biomass concentration was observed for all the experimental treatments. Evident decrease in the amount of algal biomass recovered as the concentration of $NaNO_3$ decrease was observed. This observation is in accordance with the investigation made by **Kamalanathan *et al.*, (2016)** which reported a decreased growth pattern in the green microalga *Chlamydomonas*

reinhardtii, when subjected at nitrogen-limited and combined nitrogen and phosphorus (nutrient) deprived growth conditions. Also, physiological changes like increase in the amount of neutral lipid and a decline in the amount of protein concentration were also observed in *C. reinhardtii* biomass when grown under nutrient-limited growth condition as compared to the experimental controls. Another study made by **Saxena *et al.*, (2016)** showed a loss of algal biomass of the green alga, *Chlorella minutissima* when subjected to nitrogen starved culture conditions. In combination with a decrease in algal biomass, nitrogen starvation causes a significant increase in the average thickness of cell wall and sizes of some microalgal cells such as *Nannochloropsis* sp., *Chlorococcum* sp. and *Chlorella* sp. (**Yap *et al.*, 2016; Chen *et al.*, 2018; Shokravi *et al.*, 2020**). These differences in microstructural properties didn't appear to have an effect on the overall vulnerability of algal cells to mechanical fracture by homogenization under high-pressure. These observations are significant as it show that these algae when subjected at nitrogen-limited condition accumulate lipids and pose no unfavorable effects on the retrieval of intracellular lipids (**Yap *et al.*, 2016; Chen *et al.*, 2018; Shokravi *et al.*, 2020; Pugliese *et al.*, 2020**).

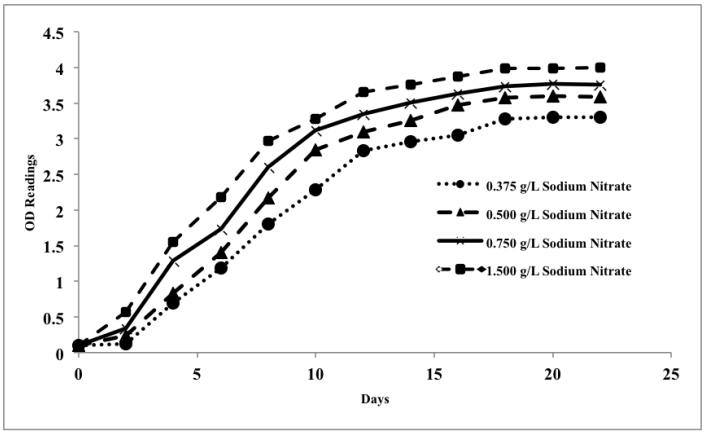

Figure 1 Growth curve of *Chlorococcum infusionum* (EAU-10) for 23 days under different concentration levels of sodium nitrate.

Microalgal oil yield of *Chlorococcum infusionum* (EAU-10) grown in varying concentration of sodium nitrate was assessed by extracting the microalgal oil using chloroform:methanol extraction solvent. Table 1 shows the lipid content of *Chlorococcum infusionum* (EAU-10) in various concentrations of nitrate after cultivating for a period of 23 days. A significant difference in the mean lipid content is observed for all of the experimental treatments ($p \leq 0.05$) and an increasing trend in the total microalgal oil concentration were observed under low concentration of nitrate. When the amount of nitrate was limited (0.375 g L^{-1} $NaNO_3$), the mean oil concentration in *C. infusionum* (EAU-10) was 21.26% as opposed to the control treatment (7.55%). In addition, high lipid productivity was also observed for nitrogen-limited *C. infusionum* of 22.08 mg L^{-1} day^{-1}. The observed high lipid yield and productivity were around three times greater than that acquired from the nitrogen-rich condition. In this investigation, as the nitrogen source was decreased from 1.5 g L^{-1} $NaNO_3$ to 0.375 g L^{-1} $NaNO_3$, lipid content after cultivation of the alga drastically increased. Based from this observation, the optimum initial nitrate concentration for the growth and biodiesel production of the microalga seems to be 0.375 g L^{-1} $NaNO_3$. At this concentration, a sharp enhancement in *Chlorococcum infusionum* (EAU-10) lipid accumulation was recorded which is about 14% higher as compared to that of the control. High oil yield was observed in algal biomass grown in nitrogen stressed media. This observation is due to the reported decrease in the rate of metabolism and induced lipid production in most microalga under nitrogen-starved conditions. Under such conditions, the amount of nitrogen is limited for the production of protein needed for cellular growth and multiplication, surplus of carbon derived from photosynthesis is being converted into cellular compounds like starch and triglycerides (**Scott *et al.*, 2010; Zhang *et al.*, 2013; Shokravi *et al.*, 2020; Arguelles and Martinez-Goss, 2021**). Several reports showed that lipid content in some green microalgae such as *Chlorella sorokiniana*, *Chlorella vulgaris* and *Nannochloropsis oculata* could be doubled or even tripled at nitrogen exhausted growth conditions (**Converti *et al.*, 2009; Zhang *et al.*, 2013**). In addition, growth of algal cells from nutrient rich to nitrogen starved culture media will cause an alteration in the lipid constituents from fatty acid-rich lipid to nearly all triglyceride-rich lipid (**Takagi *et al.*, 2000; Zhang *et al.*, 2013; Shokravi *et al.*, 2020; Pugliese *et al.*, 2020**).

Table 1 Correlation of biomass and oil yield of *Chlorococcum infusionum* (EAU-10) cultivated on BG 11 medium with altered initial $NaNO_3$ concentration.

$NaNO_3$ Concentration (g L^{-1}) in BG 11 medium	Average Algal Biomass Concentration (g L^{-1})	Average Oil Yield (%)
0.375	0.577 ± 0.003^{d}	21.263 ± 0.208^{a}
0.500	0.616 ± 0.013^{c}	16.040 ± 0.168^{b}
0.750	0.685 ± 0.010^{b}	11.904 ± 0.149^{c}
1.500	0.792 ± 0.009^{a}	7.553 ± 0.294^{d}

Means were compared using the LSD test at 0.05 probability level. The differences were not significant for groups with the same letter. The values reported are means of three replicates and standard deviation; *n*=3.

Approximately, 80% increase in microalgal oil content was reported in *Chlorella vulgaris* when cultivated in nitrogen-starved condition (**Agirman and Cetin, 2015**). This result is also similar with that of **Xia *et al.*, (2015)** where a strain of *Desmodesmus sp*. accumulated more than 40% of its dried biomass as lipids when subjected to nitrogen-limited growth condition. In this study, sudden and progressive nitrogen limitation conditions induced lipid formation and accumulation as well as protein and chlorophyll degradation (**Xia *et al.*, 2015; Pugliese *et al.*, 2020; Shokravi *et al.*, 2020**). The content of triacylglycerol in nitrogen stressed *Chlorella zofingiensis* cells was approximately 27.3% (dried biomass weight), which is about three times greater than the amount obtained under the control set ups (**Zhu *et al.*, 2015**). A direct linear correlation between the concentration of nitrogen source and oil content was also reported in *Chlorella*, which exhibited doubling in the amount of oil content under nitrogen-stressed growth conditions (**Widjaja *et al.*, 2009; Arguelles and Martinez-Goss, 2021**).

Analysis of the Fatty Acid Methyl Ester Composition

The acceptable attributes required in microalgal species for favorable biodiesel production are high biomass production (characterized by high growth rate) and high oil content. Also, the microalga must possess the desirable kind of FAMEs needed for the synthesis of high quality biodiesel. The crude oil extracts obtained from *Chlorococcum infusionum* (EAU-10) was examined to know the percent fatty acid composition. Fatty acids in the microalgal strain were analyzed in the form of FAMEs by doing tranesterification process. Chromatographic peaks of FAMEs identified and their corresponding percent composition are presented in Figure 2 and Table 3.

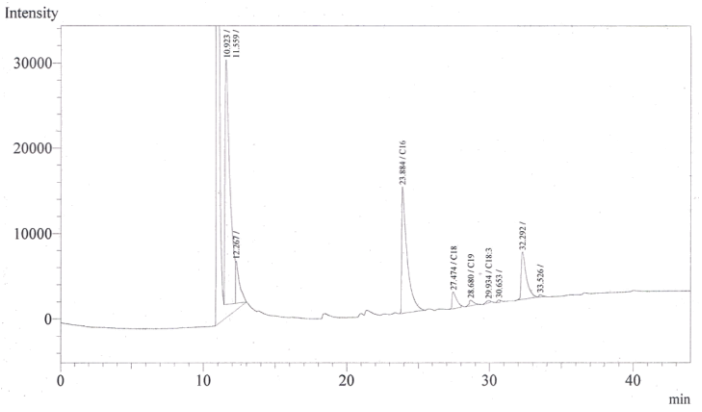

Figure 2 GC-FID chromatogram of *Chlorococcum infusionum* (EAU-10).

The FAME profile of microalgal lipids greatly influences the grade of the biodiesel being produced. The conventional guidelines being imposed for the development of high grade biodiesel recommends crude algal lipid extracts that are abounding in monounsaturated and saturated fatty acids since these lipids are critical in having superior oxidative stability and increasing energy yield of biodiesel (**Mussharraf *et al.*, 2012; Arguelles *et al.*, 2018; Shokravi *et al.*, 2020**). Also, the length of the carbon chain of both saturated and unsaturated fatty acids in crude lipid extracts in general affects some of the principal attributes of biodiesel like cold-flow properties and cetane number (**Mussharraf *et al.*, 2012; Pugliese *et al.*, 2020**). In this investigation, lipid profile of *Chlorococcum infusionum* (EAU-10) is mainly composed of saturated fatty acid methyl esters (74.58%), which is appropriate for biodiesel production. Methyl palmitate (C16:0) and methyl nonadecanoate (C19:0) contribute to almost 70% of *Chlorococcum infusionum* (EAU-10) FAME profile.

The level of FAMEs saturation dictates the stability and storability of biodiesel, therefore the existence of high percentage of saturated fatty acids in *Chlorococcum infusionum* (EAU-10) suggest an interesting avenue as good alternative feedstock for the synthesis of biodiesel. The concentration of linolenic acid (C18:3) should be less than 12% of the total FAME composition in order to meet the EN 14214 biodiesel standard. This requirement was met by *C. infusionum* since low concentration of C18:3 (Table 3) was noted when the microalga was cultivated under nitrogen-starved condition.

Table 3 Fatty acid methyl ester (FAME) composition of oil from *Chlorococcum infusionum* (EAU-10).

Fatty Acid methyl ester	Relative Content (%)
Methyl hexanoate (C6:0)	ND
Methyl octanoate (C8:0)	ND
Methyl decanoate (C10:0)	ND
Methyl laurate (C12:0)	ND
Methyl myristate (C14:0)	ND
Methyl pentadecenoate (C15:1)	ND
Methyl palmitate (C16:0)	48.40
Methyl heptadecanoate (C17:0)	ND
Methyl stearate (C18:0)	4.98
Methyl oleate (C18:1)	ND
Methyl linoleate (C18:2)	ND
Methyl linolenate (C18:3)	0.871
Methyl nonadecanoate (C19:0)	21.20
Methyl eicosapentaenoate (C20:5)	ND
Methyl behenate (C22:0)	ND
Methyl tricosanoate (C23:0)	ND
methyl lignocerate (C24:0)	ND

*ND = None Detected

Characterization of fatty acid methyl esters group

The diversity of FAMEs and its relative abundance on crude algal oil extract dictate biodiesel's physical and chemical properties. Thus, fatty acid profile of *C. infusionum* was determined (Table 3) and characterized. Several important fuel properties that influence the acceptability of an alternative feedstock as diesel fuel includes cetane number (CN), oxidation stability, cold filter plugging point (CFPP), density, kinematic viscosity, and heat of combustion. These fuel key attributes are highly determined by FAME profile of the biodiesel fuel. The description of the distinctive features of typical triglyceride FAMEs used in biodiesel production is given in Table 4. Fatty acids making up biodiesel are primarily made up of linolenic, palmitic, stearic, and oleic acids (**Knothe, 2008; Pugliese *et al.*, 2020**). In the current investigation, the length of the fatty acid chain of biodiesel produced by *C. infusionum* ranged from C16 to C19 with percentage composition presented below (Table 4).

In this study, description of some of the fuel properties of the FAME components of *C. infusionum* biodiesel was done.

***Cold flow*:** High concentration of saturated fatty acids (SAFAs) in biodiesel tends to exhibit higher cold flow attribute. In contrast, high concentration of polyunsaturated fatty acid (PUFA) methyl esters decreases the cold flow, the minimum temperature (usually reported in degrees Celsius) wherein the diesel begins to gel or crystallize and plug the engine's fuel filters (**Knothe, 2005; Mohammady, 2011; Pugliese *et al.*, 2020; Arguelles and Martinez-Goss, 2021**). The total amount of saturated FAMEs of *C. infusionum* is about 74.58%. This result corresponds to a cloud point that is below 0°C (**Knothe, 2008**). Therefore, cold flow of biodiesel produced from *C. infusionum* is regarded to be of superior quality.

***Cetane number*:** CN value or cetane number is a diesel attribute that characterizes the ignition property of diesel fuel. This property is a measure of ignition speed of a diesel. Diesel with cetane number that is high is considered to have a shorter ignition delay time than those fuels with lower cetane number (**Knothe, 2005; Pugliese *et al.*, 2020; Arguelles and Martinez-Goss, 2021**). The allowable cetane values for fuel of the American (ASTM D6751) and European (EN 14214) standards are ≥ 47 and ≥ 51 respectively. CN value (Table 4) of dominant fatty acid methyl esters of *C. infusionum* (E-AU10) is in accordance to the set cetane values of the described biodiesel standards. Higher cetane number values causes good combustion and improved proficiency of engine motors (**Ramos *et al.* 2009; Arias-Peñaranda *et al.*, 2013; Arguelles *et al.*, 2019**).

***Viscosity*:** Kinematic viscosity is the amount of flow movement resistance to a liquid caused by inner abrasion of fuel diesel in motion across another. It is a significant diesel attribute since it influences the atomization of diesel upon direct introduction of fuel into the combustion units of a combustion engine (**Saxena *et al.*, 2013; Pugliese *et al.*, 2020**). In general, viscosity of biodiesel rises when there is high degree of saturation and fatty acid carbon chain length. Nonetheless, only FAMEs with *cis* type configuration double bonds bring about an observable decrease of viscosity while FAMEs with *trans* double bonds shows diesel viscosity that resembles that of the saturated fatty acids (**Knothe and Steidley 2005; Knothe 2009; Arguelles and Martinez-Goss, 2021**). In biodiesel production, kinematic viscosity of FAME is lower in contrast to that of the fatty acids that are ethyl or branched. Fatty acid esterification by methanol has a major advantage as compared to other alcohols because of lower cost in production (**Knothe and Steidley 2007; Mohammady 2011**). Kinematic viscosity range are fixed to 3.5–5.0 $mm^2 s^{-1}$ and 1.9–6.0 $mm^2 s^{-1}$ as per EN 14214 and ASTM D6751 respectively (**Islam *et al.*, 2013**). In the current investigation, viscosity of the observed FAMEs of *Chlorococcum infusionum* is within the range of the kinematic viscosity limits of the standards used for biodiesel. This value meet the requirement for kinematic viscosity needed for biodiesel production (**Islam *et al.*, 2013; Arguelles *et al.*, 2019**).

Table 4 Characterization of typical triglyceride FAME in biodiesel obtained from *Chlorococcum infusionum* (EAU-10).

Fatty Acid Methyl Ester (FAME)	Molecular Formula	Molecular Weight (g mol^{-1})	Content (%)	Boiling point (°C)[a]	Cetane number[b]	Heat of Combustion[c] (kcal mol^{-1})	Kinematic viscosity[d] ($mm^2 s^{-1}$)	Density[e] (g cm^3)
Methyl palmitate (C16:0)	$C_{16}H_{32}O_2$	256.43	48.40	350	74.5	2550	4.32	0.865
Methyl stearate (C18:0)	$C_{18}H_{36}O_2$	284.48	4.98	360	86.9	2696.12	5.85	0.864
Methyl linolenate (C18:3)	$C_{18}H_{30}O_2$	278.44	0.871	230	28	nr[f]	3.14	0.901
Methyl nonadecanoate (C19:0)	$C_{20}H_{40}O_2$	312.53	21.20	nr	nr	nr	nr	nr

[a] Boiling point data from **Weast (1986); Gunstone *et al.*, (1994); Schenk *et al.*, (2008)**.
[b] Cetane number data were obtained from **Schenk *et al.*, (2008).**
[c] Heat of combustion data were obtained from **Freedman and Bagby (1989); Weast (1986).**
[d] Kinematic viscosity data were obtained from **Knothe (2005); Knothe and Steidley (2005); Gouw *et al.*, (1966).**
[e] Density data were obtained from **Ramírez-Verduzco *et al.*, (2012); Al-lwayzy *et al.*, (2014)**
[f] Not reported.

***Density*:** Fuel density is a critical property of biofuel that have a significant influence in engine performance (**Arguelles and Martinez-Goss, 2021**). The lowest density was noted for methyl stearate (C18:0) with 0.864 g cm^{-3}, while the highest density value is 0.901 g cm^{-3} for methyl linolenate (C18:3). These values

are similar to those obtained by **Al-lwayzy *et al.*, (2014)** and **Ramírez-Verduzco *et al.*, (2012)** who observed that for fatty acids with similar number of carbon chain length, the greater the number of double bonds the higher the density value. The ideal standard value set by the EN 14214 is 0.86–0.90 g cm^{-3} for fuel density. Fatty acids from FAME profile-derived density values of *Chlorococcum infusionum* (EAU-10) conforms the set range of the standard showing its potential for use as biodiesel

***Heat of combustion*:** Heat of combustion is a significant diesel attribute that shows acceptability of FAMEs for biodiesel production. The acceptable value for heat of combustion (HG) of triacylglycerols and FAMEs are within the limits of 1300 kcal mol^{-1} to about 3500 kcal mol^{-1} for FAMEs of C8–C22 (**Bridgwater and Maniatis 2004; Knothe, 2005; Mohammady 2011; Arguelles and Martinez-Goss, 2021**). The findings of this investigation shows that larger part of the identified FAMEs for *C. infusionum* fall within the carbon chain length of C16 to C19, and heat of combustions are in the limit of 2550 – 2696.12 kcal mol^{-1}, which is within the range of what is required of the biodiesel standard.

The significant findings obtained in the study showed the abundance of saturated FAMEs as principal fatty acids for *Chlorococcum infusionum* (EAU-10) crude oil extract. Polyunsaturated methyl esters were recovered in the sample but in low amounts. Thus, the limit of instability is very minimal. The physico-chemical attributes of diesel are highly affected by the diversity, length of the carbon chain, structural features and branching of the obtained FAMEs. Earlier studies showed that heat of combustion (HG), kinematic viscosity, and cetane number (CN) of FAMEs decrease with higher concentration of unsaturated fatty acids and increases with higher carbon chain length of FAME (**Knothe, 2005; Arguelles and Martinez-Goss, 2021**). Characterization of some of the important fuel properties using fatty acid profile reveals that the biodiesel obtained from *C. infusionum* falls within the set range of biofuel standard. Hence, the possibility of producing biodiesel from *Chlorococcum infusionum* (EAU-10) is commended for use as a diesel fuel. The present investigation showed that the algal strain (*Chlorococcum infusionum* (EAU-10) is a suitable microorganism for the production of high quality biodiesel.

CONCLUSION

The current study was done to assess the use of a green microalga, *Chlorococcum infusionum* (EAU-10) as possible alternative biomass feedstock for biodiesel production. Results showed that under nitrogen starved cultivation condition, *Chlorococcum infusionum* (EAU-10) exhibited high oil content (21.26%) which is mainly composed of saturated fatty acids (74.58%). However, high production of lipid caused by nitrogen limitation is correlated with slow growth and lower microalgal biomass concentration. Hence, *Chlorococcum infusionum* (EAU-10) is a suitable candidate for the synthesis of biodiesel with good quality. The findings of this study are preliminary results on the initial assessment of *C. infusionum* for biodiesel production. Optimization of culture growth condition of the microalga using minimal medium and other cheap organic nitrogen source is important for large-scale production and will be the subject of later papers of this research.

Acknowledgments: The author wishes to thank the financial and technical support of the Philippine National Collection of Microorganisms, National Institute of Molecular Biology and Biotechnology, University of the Philippines Los Baños (BIOTECH-UPLB) during the conduct of the study. The assistance of Ms. Allysa Mae P. Gargarino for the statistical evaluation of the experimental data in the study is acknowledged.

REFERENCES

Agirman, N., Setin A. K. (2015). Effects of nitrogen starvations on cell growth, protein and lipid amount of *Chlorella vulgaris*. *Fresenius Environmental Bulletin*, 24(11): 3643-3648.

AL-Lwayzy, S.H., Yusaf, T., Al-Juboori, R.A. (2015). Biofuels from the fresh water microalgae *Chlorella vulgaris* (FWM-CV) for diesel engines. *Energies*, 7(3):1829-1851. https://doi.org/10.3390/en7031829

Arguelles, E.D.L.R. (2021a). Biochemical composition and bioactive properties of *Chlorella minutissima* (Chm1) as a potential source of chemical compounds for nutritional feed supplement and disease control in aquaculture. *Current Applied Science and Technology*, 21(1): 65-77.

Arguelles, E.D.L.R. (2021b). Epixylic algae in periphyton mats from surfaces of submerged wood and bamboo poles of fish pen found in Laguna De Bay (Philippines). *Science and Technology Asia*, 26(2): 184-196.

Arguelles, E.D.L.R. (2020). Species composition of algal epiphyton of Pink Lotus (*Nymphaea pubescens* Willd) found in Laguna de Bay (Philippines). *Walailak Journal of Science and Technology*, *17*(3), 237-256. https://doi.org/10.48048/wjst.2020.5015

Arguelles, E.D.L.R., Sapin, A.B. (2021). Chemical composition and bioactive properties of *Sargassum aquifolium* (Turner) C. Agardh and its potential for pharmaceutical application. *Philippine Journal of Science*, 151 (S1): 9-24.

Arguelles, E.D.L.R., Sapin, A.B. (2020). Bioactive properties of *Sargassum siliquosum* J. Agardh (Fucales, Phaeophyta) and its potential as source of skin-lightening active ingredient for cosmetic application. *Journal of Applied Pharmaceutical Science*, 10 (7): 51-58. https://dx.doi.org/10.7324/JAPS.2020.10707

Arguelles, E.D.L.R., Martinez-Goss, M.R. (2021). Lipid accumulation and profiling in microalgae *Chlorolobion* sp. (BIOTECH 4031) and *Chlorella* sp. (BIOTECH 4026) during nitrogen starvation for biodiesel production. *Journal of Applied Phycology*. 33: 1-11. https://doi.org/10.1007/s10811-020-02126-z

Arguelles, E.D.L.R., Laurena, A.C., Monsalud, R.G., Martinez-Goss, M.R. (2019). High lipid and protein-producing epilithic microalga, *Desmodesmus* sp. (U-AU2): a promising alternative feedstock for biodiesel and animal feed production. *Philippine Journal of Crop Science*, 44:13–23

Arguelles, EDLR. (2019). Morphotaxonomic Study of Algal Epiphytes from *Ipomoea aquatica* Forssk. (Convolvulaceae) found in Laguna de Bay (Philippines). *Pertanika Journal of Tropical Agricultural Science*, 42(2): 817-832.

Arguelles, E.D.L.R., Laurena, A.C., Monsalud, R.G., Martinez-Goss, M.R. (2018). Fatty acid profile and fuel-derived physico- chemical properties of biodiesel obtained from an indigenous green microalga, *Desmodesmus* sp. (I-AU1), as potential source of renewable lipid and high quality biodiesel. *Journal of Applied Phycology*. 30: 411-419. https://doi.org/10.1007/s10811-017-1264-6

Arguelles, E.D.L.R., Monsalud, R.G. (2017). Morphotaxonomy and diversity of terrestrial microalgae and cyanobacteria in biological crusts of soil from paddy fields of Los Baños, Laguna (Philippines). *Philippine Journal of Systematic Biology*, 11(2): 25-36. https://doi.org/10.26757/pjsb.2017b11016

Arias-Peñaranda, M.T., Cristiani-Urbina, E., Montes-Horcasitas, C., Esparza-Garcia, F., Torzillo, G., Cañizares-Villanueva, R.O. (2013). *Scenedesmus incrassatulus* CLHE-Si01: A potential source of renewable lipid for high quality biodiesel production. *Bioresource Technology*, 140: 158–164. https://doi.org/10.1016/j.biortech.2013.04.080

Araujo, G.S., Matos, L.J.B.L., GOncalves, L.R.B., Fernandes, F.A.N., Farias, V.R.L. (2011). Bioprospecting for oil producing microalgal strains: Evaluation of oil and biomass production for ten microalgal strains. *Bioresource Technology*, 102 (8): 5248–5250. https://doi.org/10.1016/j.biortech.2011.01.089

ASTM. (2015). ASTM D6751-15ce1, standard specification for biodiesel fuel blend stock (B100) for middle distillate fuels. ASTM International, West Conshohocken www.astm.org

Barabás, I., Todorut, I-A. (2011). Biodiesel Quality, Standards and Properties, Biodiesel-Quality, Emissions and By-Products, Dr. Gisela Montero (Ed.), ISBN: 978-953-307-784-0, InTech, Available from: http://www.intechopen.com/books/biodiesel-quality-emissions-and-by-products/biodiesel-quality- standards-and-properties.

Behrens, P.W., Kyle, D.J. (1996). Microalgae as a source of fatty acids. *Journal of Food Lipids*, 3: 259–272. https://doi.org/10.1111/j.1745-4522.1996.tb00073.x

Breuer, G., Lamers, P.P., Martens, D.E., Draaisma, R.B., Wijffels, R.H. (2012). The impact of nitrogen starvation on the dynamics of triacylglycerol accumulation in nine microalgae strains. *Bioresource Technology*, 124:217–226. https://doi.org/10.1016/j.biortech.2012.08.003

Bridgwater, A., Maniatis, K. (2004). The production of biofuels by thermal chemical processing of biomass. In Archer M. and Barber J. (eds.). *Molecular Global Photosynthesis*. London: Imperial College Press.

Cakmak, T., Angun, P., Demiray, Y.E., Ozkan, A.D., Elibol, Z., Tekinay, T. (2012). Differential effects of nitrogen and sulfur deprivation on growth and biodiesel feedstock production of *Chlamydomonas reinhardtii*. *Biotechnology and Bioengineering*, 109:1947–1957. https://doi.org/10.1002/bit.24474

Chisti, Y. (2007). Biodiesel from microalgae. *Biotechnology Advances*, 25: 294–306. https://www.massey.ac.nz/~ychisti/Biodiesel.pdf

Chen, Z., Wang, L., Qiu, S., Ge, S. (2018). Determination of microalgal lipid content and fatty acid for biofuel production. *BioMed Research International*, Article ID 1503126, 17 pages. https://doi.org/10.1155/2018/1503126

Converti, A., Casazza, A.A., Ortiz, E.Y., Perego, P., Del Borghi, M. (2009). Effect of temperature and nitrogen concentration on the growth and lipid content of *Nannochloropsis oculata* and *Chlorella vulgaris* for biodiesel production. *Chemical Engineering and Processing: Process Intensification*, 48(6): 1146–1151. https://doi.org/10.1016/j.cep.2009.03.006

Freedman, B., Bagby, M. (1989). Heats of combustions of fatty esters and triglycerides. *Journal of the American Oil Chemist's Society,* 66 (11): 1601 – 1604.

Ge, S., Champagne, P., Plaxton, W.C., Leite, G.B., Marazzi, F. (2017). Microalgal cultivation with waste streams and metabolic constraints to triacylglycerides accumulation for biofuel production. *Biofuels, Bioproducts and Biorefining*, 11(2): 325–343. https://doi.org/10.1002/bbb.1726

Ge, S., Champagne, P. (2016). Nutrient removal, microalgal biomass growth, harvesting and lipid yield in response to centrate wastewater loadings. *Water Research*, 88: 604– 612. https://doi.org/10.1016/j.watres.2015.10.054
Gunstone, F., Harwood, J., Padley, F. (1994). *The Lipid Handbook*. London: Chapman and Hall. 551pp.
Gouw, T., Vlugter, J., Roelands, C. (1966). Physical properties of fatty acid methyl esters: VI. Viscosity. *Journal of the American Oil Chemist's Society*, 43(7): 433 – 434. https://doi.org/10.1007/BF02682408
Hariskos, I., Posten, C. (2014). Biorefinery of microalgae - opportunities and constraints for different production scenarios. *Biotechnology Journal*, 9(6): 739–752. https://doi.org/10.1002/biot.201300142
Hsieh, C.H., Wu, W.T. (2009). Cultivation of microalgae for oil production with a cultivation strategy of urea limitation. *Bioresource Technology*, 100 (17): 3921–3926. https://doi.org/10.1016/j.biortech.2009.03.019
Islam, M.A., Magnusson, M., Brown, R.J., Ayoko, G.A., Nabi, M.N., Heimann, K. (2013). Microalgal species selection for biodiesel production based on fuel properties derived from fatty acid profiles. *Energies*, 6(11): 5676-5702. https://doi.org/10.3390/en6115676
Kamalanathan, M., Pierangelini, M., Shearman, L.A., Gleadow, R., Beardall, J. (2016). Impacts of nitrogen and phosphorus starvation on the physiology of *Chlamydomonas reinhardtii*. *Journal of Applied Phycology*, 28(3): 1509-1520. https://doi.org/10.1007/s10811-015-0726-y
Knothe, G., Steidley, K.R. (2005). Kinematic viscosity of biodiesel fuel components. influence of compound structure and comparison to petrodiesel fuel components. *Fuel*, 84(9):1059–1065. https://doi.org/10.1016/j.fuel.2005.01.016
Knothe, G.H., Steidley, K.R. (2007). Kinematic viscosity of biodiesel components (fatty acid alkyl esters) and related compounds at low temperatures. *Fuel*, 86(16): 2560 – 2567. https://doi.org/10.1016/j.fuel.2007.02.006
Knothe, G.H. (2008). ''Designer" biodiesel: optimizing fatty ester composition to improve fuel properties. *Energy Fuel*, 22(2): 1358 – 1364. https://doi.org/10.1021/ef700639e
Knothe, G. (2009). Improving biodiesel fuel properties by modifying fatty ester composition. *Energy and Environmental Science*, 2: 759–766. https://doi.org/10.1039/B903941D
Mohammady, N.G. (2011). Characterization of the fatty acid composition of *Nannochloropsis salina* as a determinant of biodiesel properties. *Zeitschrift für Naturforshung C*, 66 (7-8): 328-332. https://doi.org/10.1515/znc-2011-7-802
Musharaff, S.G., Ahmed, M.A., Zehra, N., Kabir, N., Choudhary, M.I., Rahman, A. (2012). Biodiesel production from microalgal isolates of southern Pakistan and quantification of FAMEs by GC-MS/MS analysis. *Chemistry Central Journal*, 6:149. https://doi.org/10.1186/1752-153X-6-149
Nascimento, L.A., Marques, S.S.I., Cabanelas, L.T.D., Pereira, S.A., Druzian, J.I., De Souza, C.O., Vich, D.Y., De Carvalho, G.C., Nascimento, M.A. (2013). Screening microalgae strains for biodiesel production: Lipid productivity and estimation of fuel quality based on fatty acids profiles as selective criteria. *Bioenergy Research*, 6(1): 1–13. https://doi.org/10.1007/s12155-012-9222-2
Negi, S., Barry, A.N., Friedland, N., Sudasinghe, N., Subramanian, S., Pieris, S., Holguin, F.O., Dungan, B., Schaub, T., Sayre, R. (2016). Impact of nitrogen limitation on biomass, photosynthesis, and lipid accumulation in *Chlorella sorokiniana*. *Journal of Applied Phycology*, 28(2):803–812. https://doi.org/10.1007/s10811-015-0652-z
Ramírez-Verduzco, L.F., Rodríguez-Rodríguez, J.E., Jaramillo-Jacob, A.D.R. (2012). Predicting cetane number, kinematic viscosity, density and higher heating value of biodiesel from its fatty acid methyl ester composition. *Fuel*, 91(1): 102–111. https://doi.org/10.1016/j.fuel.2011.06.070
Pugliese, A., Biondi, L., Bartoci, P., Fantozzi, F. (2020). *Selenastrum capricornutum* a new strain of algae for biodiesel production. *Fermentation* 6:46. https://doi.org/10.3390/fermentation6020046
Ramos, M.J., Fernández, C.M., Casas, A., Rodríguez, L., Pérez, A. (2009). Influence of fatty acid composition of raw materials on biodiesel properties. *Bioresource Technology*, 100(1):261–268. https://doi.org/10.1016/j.biortech.2008.06.039
Rodolfi, L., Zittelli, G.C., Bassi, N., Padovani, G., Biondi, N., Bonini, G., Tredici, M.R. (2009). Microalgae for oil: strain selection, induction of lipid synthesis and outdoor mass cultivation in a low-cost photobioreactor. *Biotechnology and Bioengineering*, 102: 100–112. https://doi.org/10.1002/bit.22033
Saxena, P., Jawale, S., Joshipura, M.H. (2013). A review on prediction of properties of biodiesel and blends of biodiesel. *Procedia Engineering*, (51): 395 – 402. https://doi.org/10.1016/j.proeng.2013.01.055
Saxena, G., Kumar, L., Hariri, S.M., Roy, A., Kundu, K., Bharadvaja, N. (2016). Identification of potential culture conditions for enhancing the biomass production of microalga *Chlorella minutissima*. *Expert Opinion on Environmental Biology*, S1. http://dx.doi.org/10.4172/2325-9655.S1-005
Sheehan, J., Dunahay, T., Benemann, J., Roessler, P. (1998). A look back at the US department of energy's aquatic species program: biodiesel from algae. NREL/TP-580–24190, In: National Renewable Energy Laboratory. US Department of Energy, Washington, 1–100 pp.
Schenk, P., Thomas-Hall, S., Stephens, E., Marx, U., Mussgnug, J., Posten, C., Kruse, O., Hankamer, B. (2008). Second generation biofuels: High-efficiency microalgae for biodiesel production. *BioEnergy Research*, 1(1): 20 – 43. https://doi.org/10.1007/s12155-008-9008-8
Scott, S.A., Davey, M.P., Dennis, J.S., Horst, I., Howe, C.J., Et, A.L. (2010). Biodiesel from algae: challenges and prospects. *Current Opinion in Biotechnology*, 21(3): 277–286. https://doi.org/10.1016/j.copbio.2010.03.005
Sforza, E., Cipriani, R., Morosinotto, T., Bertucco, A., Giacometti, G.M. (2012). Excess CO_2 supply inhibits mixotrophic growth of *Chlorella protothecoides* and *Nannochloropsis nsalina*. *Bioresource Technology*, 104: 523–529. https://doi.org/10.1016/j.biortech.2011.10.025
Shokravi, Z., Shokravi, H., Chyuan, O.H., Lau, W.J., Koloor, S.S.R., Petru, M., Ismail, A.F. (2020). Improving 'lipid productivity' in microalgae by bilateral enhancement of biomass and lipid contents: A review. *Sustainability*. 12: 9083. https://doi.org/10.3390/su12219083
Stanier, R.Y., Kunizawa, R., Mandel, M., Cohen-Bazire, G. (1971). Purification and properties of unicellular blue green algae (order Chroococcales). *Bacteriological Reviews*, 35(2): 171-205.
Takagi, M., Watanabe, K., Yamaberi, K., Yoshida, T. (2000). Limited feeding of potassium nitrate for intracellular lipid and triglyceride accumulation of *Nannochloris* sp. UTEX LB1999. *Applied Microbiology and Biotechnology*, 54(1): 112–117. https://doi.org/10.1007/s002530000333
Tredici, M.R. (2010). Photobiology of microalgae mass cultures: Understanding the tools for the next green revolution. *Biofuels*, 1(1): 143–162. https://doi.org/10.4155/bfs.09.10
Weast, R. 1986. *Handbook of Chemistry and Physics*, 66th ed. Florida: CRC Press, D272.
Widjaja, A., Chien, C.C., Ju, Y.H. (2009). Study of increasing lipid production from freshwater microalgae *Chlorella vulgaris*. *Journal of the Taiwan Institute of Chemical Engineers*, 40(1): 13–20. https://doi.org/10.1016/j.jtice.2008.07.007
Xia, L., Yang, H., He, Q., Hu, C. (2015). Physiological responses of freshwater oleaginous microalgae *Desmodesmus* sp. NMX451 under nitrogen deficiency and alkaline pH-induced lipid accumulation. *Journal of Applied Phycology*, 27(2): 649-659. https://doi.org/10.1007/s10811-014-0371-x
Yang, L., Chen, J., Qin, S., Zeng, M., Jiang, Y., Hu, L., Xiao, P., Hao, W., Hu, Z., Lei, A., Wang, J. (2018). Growth and lipid accumulation by different nutrients in the microalga *Chlamydomonas reinhardtii*. *Biotechnology for Biofuels*, 11:40 https://doi.org/10.1186/s13068-018-1041-z
Yap, B.H.J, Crawford, S.A., Dagastine, R.R., Scales, P.J., Martin, G.J.O. (2016). Nitrogen deprivation of microalgae: effect on cell size, cell wall thickness, cell strength, and resistance to mechanical disruption. *Journal of Industrial Microbiology and Biotechnology*, 43(12): 1671-1680. https://doi.org/10.1007/s10295-016-1848-1
Zhang, Y.M., Chen, H., He, C.L., Wang, Q. (2013). Nitrogen starvation induced oxidative stress in an oil-producing green alga *Chlorella sorokiniana* C3. *PloS one*, 8(7): e69225. https://doi.org/10.1371/journal.pone.0069225
Zhu, S., Wang, Y., Shang, C., Wang, Z., Xu, J., Yuan, Z. (2015). Characterization of lipid and fatty acids composition of *Chlorella zofingiensis* in response to nitrogen starvation. *Journal of Bioscience and Engineering*, 120(2): 205-209. https://doi.org/10.1016/j.jbiosc.2014.12.018