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Research Article

Optimization cultivation of *Chlamydomonas reinhardtii* in a tubular photobioreactor (2000 Liter) for biomass and green bioenergy (biodiesel) production

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Abstract

The biodiesel can be produced from diverse microalgae lipids as alternative and renewable fuel. Thus, the aim of this study was to optimize the *Chlamydomonas reinhardtii* promising species as biodiesel feedstock for large-scale cultivation in Egypt. To understand some of the triggers required for the metabolic pathway switch to lipid accumulation, the effect of carbon sources and the three elements availability (N, P, S) in *C. reinhardtii* growth medium was determined. A local microalgae *C. reinhardtii* was cultured in modified Sueoka medium containing various concentrations of CO_2 and bicarbonate ($NaHCO_3$) (in 2-liter flasks) as a carbon source. The optimal source in term biomass, high lipid productivity (10.3 $mgL^{-1}d^{-1}$) and a higher lipid content (22.76%) were obtained in 6% CO_2 culture. Then, the availability of N, P, S (various concentrations of N, P and S) nutrients elements was added to 6% CO_2 culture, for produce a highest lipid content and lipid productivity. As expected, under low availability N-1.78 mM; P-0.14mM and S-0.10 mM mediums, *C. reinhardtii* showed a high accumulation lipid content. Therefore, to improve the economic feasibility of microalgae biofuels production, its concentrations were selected to combine (N+P+S) in order to cultivation of *C. reinhardtii* in a multi-tubular photobioreactor (400 liter) to produce high lipid contents. Under limited condition, the biomass dry weight, biomass productivity, lipid content and lipid productivity were found to be 3.11 (gL^{-1}), 0.15±0.012 ($g^{-1}L^{-1}d^{-1}$), 22.76% (w/w %) and 1.9± 0.35 ($mg^{-1}L^{-1}d^{-1}$), respectively. The extracted lipid was found to have physical and chemical properties similar that plant oils using for biodiesel production. The FAME profiling of prepared biodiesel shows the presence of considerable amount of 36.97% saturated fatty acids (palmitic acid and stearic acid, together) with 27.33% unsaturated (oleic acid and linoleic acid) fatty acids. The FAME had a low iodine value and high CN, which meet with the appropriate of biodiesel standards (EN 14214 and ASTM D6751). Thus, *C. reinhardtii* appears to be more feasible for high quality biodiesel production.

Keywords: biodiesel production; *C. reinhardtii*; lipids; photobioreactor

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Introduction

The extensive high usage of fossil fuels had a significant negatively impacted on global climate change. Therefore, to solve of these problems, could be by reduction of carbon emission, hydrocarbon (HC, CH_4), nitrogen oxides (NO_x), carbon monoxide (CO), particulate matter (PM) and selected renewable biomass as alternative energy source (Singh *et al.*, 2018; Goh *et al.*, 2019). The biofuel derived from biomass are developed as an environmentally (characterizes by sulphur-free, nontoxic and biodegradable) and socially acceptable substitute for fossil fuels (Lee and Kim, 2017; Cesar *et al.*, 2019). Recently, several strains of microorganisms rich in lipid contents (e.g., fungus, bacteria and microalgae) have been reported as ideal as an excellent feedstock for biofuel production (Abd El Baky and El Baroty, 2016; Patel *et al.*, 2017). However, many factors such as nutrients stress (nitrogen, phosphorus, iron and salt); temperature, pH, light intensity and photoperiod had a directly impact on biomass and lipid content of microalgae (Abd El Baky *et al.*, 2012; Singh *et al.*, 2015).

In general, a high quantity of lipid and high quality of the fatty acid compositions should be considered when seeking efficient biomasses for biofuel production. The fuel properties of biodiesel produced from feedstocks significantly depend on the fatty acids compositions. In this trend, the main compositions of the lipid of microalgae are containing unsaturated fatty acids, especially palmitolleic (16:1), oleic (18:1) and linoleic (18:2) and the saturated fatty acids of palmitic (16:0) (Sajjadi *et al.*, 2018). The ideal biodiesel 16:0 and C18:1 fatty acid methyl esters (FAMEs/) with high biodiesel conversion is produced via the trans-esterification reaction of lipids and short-chain alcohols with appropriate homogeneous catalysts (e.g. sodium hydroxide, sulphuric acid etc.) (Singh *et al.*, 2015; Patel *et al.*, 2017). Thus, the microalgae have been proved as promising candidates for large-scale biodiesel production. That have high photosynthesis efficiency, fast and short growth cycle (can be double their biomass weight within 24 h), high lipid content, and no culturing land and can be grown in wastewater or in seawater (Ma *et al.*, 2018; Sun *et al.*, 2018).

The biodiesel production is still challenging towards a large-scale application of microalgae lipid, and is still a technical challenge due to low yields and commoditization of biotechnological products. The lipid accumulation of lipids in microalgae is essential to improve the commercial feasibility of biodiesel production (Singh *et al.*, 2015). In order to increase the biomass productivity, the high-density culture of microalgae had been carried out in photobioreactors (airlift tubular and bubble column) with minimal nutrients (for large scale cultivation), high illumination surfaces and high biomass transfer rates (Fan *et al.*, 2008). The cultivation of microalgae in open or closed photobioreactor (PBR) systems, that can be controlled by operating parameters leading to higher productivities and improve its performance (Martins *et al.*, 2018). However, the biochemical characteristic parameter and harvesting yield of microalgae biomass culture in PBRs have not been extensively investigated (Lam and Lee, 2014). In previous work, we demonstrated that some microalgae species can grow much faster in the tubular photobioreactor with high biomass productivity and high lipid contents (Abd El Baky and El Baroty, 2016). Generally, the lipid productivity could be enhanced by nutrient stress to increases the lipid content of microalgae (Fan *et al.*, 2008; Abd El Baky and El Baroty, 2016). In response to various cultivation conditions, metabolic pathway of algae cells could be change toward lipid accumulation, thereby increase the enzyme activity of acetyl-CoA carboxylase a key enzyme in fatty acid biosynthesis (Karpagam *et al.*, 2015). A strategy to gained a lipid high-yielding from microalgae through nutrient limitation conditions (i.e., nitrogen deprivation, iron starvation and phosphorus depletion) and specific growth environment condition (stimuli high light or osmotic stress) were determined (Benvenuti *et al.*, 2015; Karpagam *et al.*, 2015; Zhang *et al.*, 2019). Under limited condition, lipid accumulation can be optimized by selecting a suitable microalgae species and manipulating the initial fatty acid profile by varying the growth conditions for large-scale production, which can be used as a source of biodiesel (Abd El Baky *et al.,* 2014). In the green algae *Chlamydomonas reinhardtii* accumulates increased quantity of lipids under conditions of macro- or micro nutrient depletion such as sulfur, nitrogen, phosphorus and iron or in physical stresses conditions, e.g. temperature, pH, salinity and high light (Kropat *et al.*, 2011; Benvenuti *et al.*, 2015; Wang *et al.*, 2018).

This study aimed to find suitable carbon source (gaseous CO_2 or bicarbonate) and nutrient availability (nitrogen N, phosphor P and sulfur S) levels for cultivating a *C. reinhardtii* strain (isolated from a freshwater reservoir in Egypt), to optimize lipid productivity. To enable economic production of algal biodiesel, the individual concentration P, S and N produces a high lipid yield, were selected for cultivation of *C. reinhardtii* in combined nutrients (P +S+N) at large scales in a bubble column photobioreactor that (2000 Liter) to maximizes the microalgae biomass and lipid productivities for biodiesel production. The FAME profiling and quality biodiesel of prepared were investigated.

Materials and Methods

Reagent and chemicals

All the chemicals and solvents were purchased from Sigma Aldrich and E. Merck CO. Other fine chemicals not mentioned here were of analytical grade and obtained from standard sources. The Millipore Milli Q plus system was used to prepare the high purely deionized water.

Microalgae strain

Microalga strain and pre-culture condition

Chlamydomonas reinhardtii strain was isolated from freshwater samples from the El Dokki region, Giza, Egypt), was preserved in a modified Sueoka (MS pH 7.0) medium under optimum conditions, as reported in a previous work (Kim *et al*., 2016). The pure strain had been deposited in Egyptian culture collection (accession number EMCCN 3043), National Research Centre (Giza, Egypt). For optimize the nutrient grown, employing different concentrations of (CO_2 and bicarbonate) as a carbon sources, bicarbonate at 0.2, 0.4 and 0.8 g L^1 and CO_2 enriched air supply 3, 6, 12 % in two-liter Erlenmeyer flasks each containing 1800 mL of MS medium, was achieved, that to ensure the better results for accumulation high lipid contents. The *C. reinhardtii* strain was grown photo-autotrophically under 10 white florescent light lamps (Philips 40 W) provided an illumination of 2500 lux. After the first steady-state condition was reached, CO_2 at 6% as a carbon source was selected due to given higher biomass dry weight and biomass productivity, further treatments, by study the effect of the three elements availability (N, P, S) in *C. reinhardtii* growth medium was determined to optimize and develop the economic viability of *Chlamydomonas reinhardtii* as feedstock biodiesel. Therefore, the *C. reinhardtii* was growing in different medium containing serial concentrations of nitrogen (9, 7.5, 0.93 mM), phosphorus (1.13, 0.70, 0.14 mM), sulfur (0.43, 0.25, 0.1 mM) to enhance lipid yield and high lipid productivity. In all cultivated flasks, conductivity, salinity, pH and temperature were measured every two with Hanna (HI 09812-5) conductivity meter. The purity of cultures was periodically checked by microscopic observation following taxonomy guidelines.

Cultivation of C. reinhardtii in 2000 L photobioreactor

A *C. reinhardtii* was cultivated in 2000 liter (large scale) tubular photobioreactor (Figure 1) modified Sueoka medium containing a combination of limited three element (0.1 Sulfur, 7.0 Nitrogen and 0.43 mM phosphorus, S+N+ P, is the most suitable nutrient levels for enhance lipid accumulation for biodiesel production (Sueoka *et al*., 1967). However, our designs, the photobioreactor (PBR) as showing in Fig. 1, which made of low-cost polypropylene-based (CPP) tubes, had high transparence and durability of the material. The working volume of these PBRs was 2000 L (8 tubes, with dimensions of 25 cm inner diameter, 6 mm wall thickness, and 2 m height to provide a total volume of 100 liter each). A 2 stone sparger was used to input gases into the PBRs, and 8 spargers for the serial-column connection. The base tube was mounted on a stainless-steel support stand. The PBR was continuously aerated by compressed-air from an air pump through the static sparger and air flow rate was controlled by a flow meter. The cultivation system was maintained at 24-hour

photo-period via ten cool-white fluorescent light (Philips, TL-D 36W/54-765) that was illuminated with an intensity of 60-70 µmol $m^{-2}s^{-1}$.

Harvesting

At the end of each batch run (the experiment was repeated 3 times and average), cultures were collected, filtered, washed with distilled water to remove any residual soluble salts, centrifuged at 3000 x g for 15 min at 4 °C and the pellets was frozen until using.

Growth measurement

The growth of *C. reinhardtii was* assessed every two days, during the 16 days of the cultivation period (log and stationary phase), using the dry cell weight method and optical density of the culture suspension. The optical density (OD) was read at 680 nm, and the biomass density of the culture suspension was calculated. A standard curve was established by correlation of the absorbance values (OD 680) and dry cell weight (g/L) as follows: Dry weight g/L = 1.074 X OD_{680} + 0.09855.

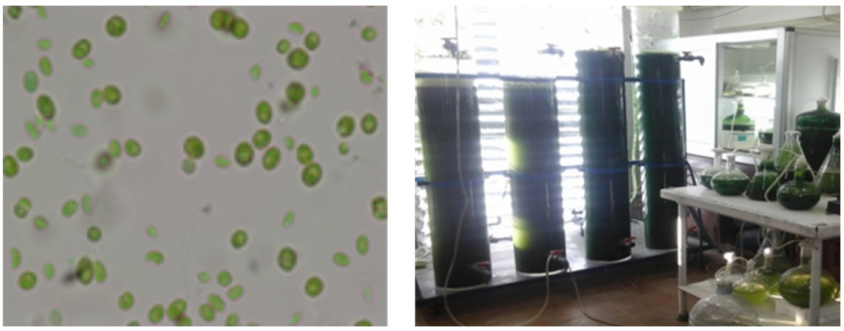

Figure 1. *Chlamydomonas reinhardtii* cells and large-scale cultivation in tubular photobioreactor

Determination of dry cell weight

To determine the dry cell weight, a known volume of (10 ml) culture suspension was centrifuged at 3000 x g for 15 min. The collected cells pellet was several times washed with distilled water to get rid of medium of any trace's nutrients. Then, the biomass pellet was dried in an oven at 60 °C to get the constant weight and kept for further analysis.

Microalgae growth parameters

During the cultivation period, growth parameters were determined as follows:

Biomass productivity (PB)

The dry biomass produced ($gL^{-1}day^{-1}$), during the stationary growth phase was determined. A known volume of the algal suspensions was centrifuged (2000 x g, 15 min) and the resulting pellets were washed with de ionized water, dried at 70 °C for 48 h and their dry weights were determined gravimetrically.

Specific growth rate (SGR, µg/ day)

The specific growth rate (SGR µg/ day) of *C. reinhardtii* culture was determined using the following equation: SGR (µg/ day) = ln $(X_1- X_2)/ t_2 – t_1$: Where, X_1 = Biomass concentration at the end of selected time interval, X_2 = biomass concentration at the beginning of selected time interval, and $t_2 –t_1$ = elapsed time between selected time in the day.

Determination of total chlorophyll a (Chl a)

A known volume (10 ml) from each culture was harvested by centrifuging, and then algal pellet was washed several times with distilled water and re-suspended in 10 ml of 95 % methanol. The tubes containing algal suspension were extracted aided by ultrasonic processor, centrifuged again to remove the cell debris. The supernatant containing the pigment was transferred to a volumetric flask (10 ml) and volume was made up to 10 ml by adding pure methanol. A blank with 100% methanol was run simultaneously. The Chl *a* content in the biomass was spectrophotometrically at 648.6 nm and 664.2 nm (Lichtenthaler 1983/7) using the follows Eq. Chl A= (13.36 A_{664} – 5.19 A_{648}) x 8.1/dry weight (mg/ g dw).

Determination of total lipids contents

To determine the total lipid content in dried algal cells *C. reinhardtii,* 2 g of biomass was extracted with 30 ml hexane: methanol (v/v, 1:1) and then 3 ml of distilled water was added. The contents were mixed for 10 min in a shaker, and then left at room temperature 25 °C for 10 min. The organic layer was separated by a 50 ml separating funnel, several times washed with 5% NaCl solution. Then, the organic extract was evaporated under vacuum to smallest volume, and evaporated under nitrogen stream. The lipid yield of *C. reinhardtii* was calculated in percentage gravimetrically (%).

Lipid productivity (LP)

Lipid productivity (LP, $mgl^{-1}day^{-1}$) of *C. reinhardtii* was calculated according to the Liu equation (Liu *et al*., 2011) LP = ¼ PB - LC

Determination of physical-chemical properties of C. reinhardtii lipid

Based on AOCS official method (1998), acid value (AV, Ca 5a-40), saponification value (cd 3-25), iodine value (IV, Cd 1-25) and peroxide value (PV Cd 8-53) of algal lipids were determined. The molecular weight of the oil (M) was calculated from saponification and acid value according to M= 168300/ SV–AV formula. The viscosity was measured with capillary viscometer in a constant temperature in bath at 40 °C.

Biodiesel preparation

Biodiesel from algal lipids was derived by acidic trans-esterification method. Algae lipid was mixed with methanol with 1:56 ratio (weight ratio) and the reaction was carried out at 45 °C for 4 h in the presence of sulfuric acid (H_2SO_4) as catalyst with 1:1 weight ratio of catalyst to lipids. The upper biodiesel layer was separated by a separating funnel, washed several times with 5% NaCl solution to remove any traces of methanol and glycerol. Then, the biodiesel was dried over anhydrous sodium sulfate and collected and evaporated at 45 °C to constant weight for analysis. The biodiesel yield from microalgae biomass was determined gravimetrically by the following equation: Biodiesel yields (%) = [biodiesel mass (g) / algae mass (g) X lipid content %] x 100%

Algal biodiesel fatty acid methyl ester profile by GC/MS

The fatty acid methyl ester (FAME) composition of algal biodiesel was analyzed by using GC-MS. The GC-MS detection was performed with an HP-6890 gas chromatograph connected to an HP 5973 mass selective detector at 70eV (*m/z* 50-550 amu; source at 230 °C and quadruple at 150 °C) in the electron impact mode with a TR-FAME-ms (75% cyanophenyl-silxane (Thermo (USA) capillary column (30m × 0.25mm i.d., 0.25µm film thickness). The oven temperature was programmed for 2 min at 80 °C and raised to 280 °C at 4 °C/min and maintained for 5 min at 280 °C. Helium was used as carrier gas (flow rate of 1.2 ml/min). The inlet temperature was maintained at 300 °C. Structural assignments were based on interpretation of mass spectrometry fragmentation (NIST 11 MS spectra) and confirmed by comparison of retention times as well as fragmentation patterns of authentic standard FAMEs Mix (Supelco 37 Component FAME Mix, purity >98.0% by GC, Sigma-Aldrich).

Characterization of properties of C. reinhardtii biodiesel

The physical and chemical properties of *C. reinhardtii* biodiesel was evaluated based on ASTM biodiesel standard or AOCS methods. Physical and chemical characteristics include: density at 15 °C (ASTM D4052), kinematic viscosity at 40 °C (ASTM D445), iodine value (AOCS 1998), acid value (ASTM D664), peroxide value (AOAC, 1999), saponification value. The other important biodiesel quality properties were determined mathematical following the empirical equations published by Ramos *et al.* (2009) and Wu and Miao (2014): cetane number (CN, 46.3+5458/SV- 0.225 IV), degree of un saturation /saturation (DU), long-chain saturated factor (LCSF), and cold filter plugging point (CFPP). All determinations for biodiesel properties were conducted at least three times for each sample, and the results were averaged.

Results and Discussion

A *Chlamydomonas reinhardtii* green microalgae is grown in the laboratory for research in biofuels study's due to its fast growth rates and accumulate lipids. This study aimed to optimize the productivity of lipid content for biodiesel production. Thus, the carbon (sources and concentrations) and three elements availability (N, P, S) in *C. reinhardtii* growth medium required for the metabolic pathway switch to lipid accumulation was determined. To achieve this *C. ireinhardtii* are cultured in different nutrient conditions continuously under laboratory scales (2-liter flasks) (Table 1 and Figure 2).

Table 1. Impact of carbon scours and concentrations on *Chlamydomonas reinhardtii* biomass

Carbon scours and culture age (days)					
Concentrations	Culture Age (days)				
CO_2	0	3	6	9	12
3% CO_2	0.651	0.916	2.11	2.78	3.22
6% CO_2	0.584	0.995	2.56	3.99	5.11
9% CO_2	0.679	0.864	1.13	1.89	2.34
$NaHCO_3$	0	3	6	9	12
2.5 mmol L^{-1}	0.611	0.854	1.99	2.68	3.75
5.00 mmol L^{-1}	0.634	0.915	2.89	3.86	4.32
7.50 mmol L^{-1}	0.656	0.851	1.89	2.96	3.43

Firstly, *C. ireinhardtii* cultured in Sueoka medium containing optimal concentration of N, S and P and different concentrations of carbon sources (carbonate and CO_2, as a rich R, optimal O and limited L concentrations). The data of revealed that biomass yields (BM) and total lipid contents (TL) were significantly different (P>0.5%) among all tested cultures (CO_2 3, 6, 12%) and sodium bicarbonate salt 2.5, 5.0 and 0.5mmol L^{-1}). The biomass yields (as dw) were 2.9 g L^{-1} in 6% CO_2 (2.9 g L^{-1}) and 3.22 g L^{-1} in 3% CO_2 culture. Whereas, these values were 0.122, 0.191 and 0.201 mg/L, respectively in cultures supplemented with 2.5, 5.0 and 7.50mmol L^{-1} $NaHCO_2$. The lower yield of biomass in carbonate cultures suggests the occurrence of stress conditions induced by high bicarbonate levels in the medium due to excess osmotic pressure (Table 2 and Figure 2). However, after 12 days of cultivation, the highest oil content (%) and oil yield were found to be (2.9 and 2.6 $gd^{-1}L^{-1}$), oil contents (20% and 27%, of dw.) and lipid productivity (32 and 36 mg L^{-1} d^{-1}) were observed, respectively in 6% and 9% cultures. It is interesting to note that oil yield in 6% CO_2 culture was higher that obtained in 9% CO_2 culture. Moreover, higher lipid productivity of *C. ireinhardtii* cultures supplied of gaseous CO_2 could be more economically sustainable than that supplied by an exogenous organic carbon source (Davey *et al.*, 2014; Juergens, *et al.*, 2016). Due to the high lipid productivity and lipid contents are considered as the most important desirable characteristic of chosen the microalgae strains to use for biodiesel production (Griffiths and Harrison, 2009), thus, 6.0 % was used for as carbon source for further cultivation *C. ireinhardtii* to provide the best result in terms of higher oil yield and biomass. Quit similar concentration level were

reported for cultured the *M. pusillum O. multisporus, Dunaliella, chlorella* and *Spirullina* cultured grown at 5% CO_2 (Tang *et al*., 2011; Abd El Baky *et al*., 2012, 2014).

Table 2. Impact of carbon scours and concentrations on *Chlamydomonas reinhardtii* oil content (%) and oil yield

Carbon scours and concentrations		Culture Age (days)	Culture Age (days)			Oil yield mg/L
CO_2	0	3	6	9	12	
3% CO_2	1.44	1.78	2.23	2.93	3.41	0.109
6% CO_2	1.44	2.15	2.97	3.87	4.67	0.238
9% CO_2	1.44	2.98	3.56	4.32	5.78	0.135
$NaHCO_3$	0	3	6	9	12	
2.5 mmol L^{-1}	1.44	1.71	2.23	2.56	3.26	0.122
5.00 mmol L^{-1}	1.44	1.86	2.66	3.22	4.43	0.191
7.50 mmol L^{-1}	1.44	1.95	2.98	3.56	5.88	0.201

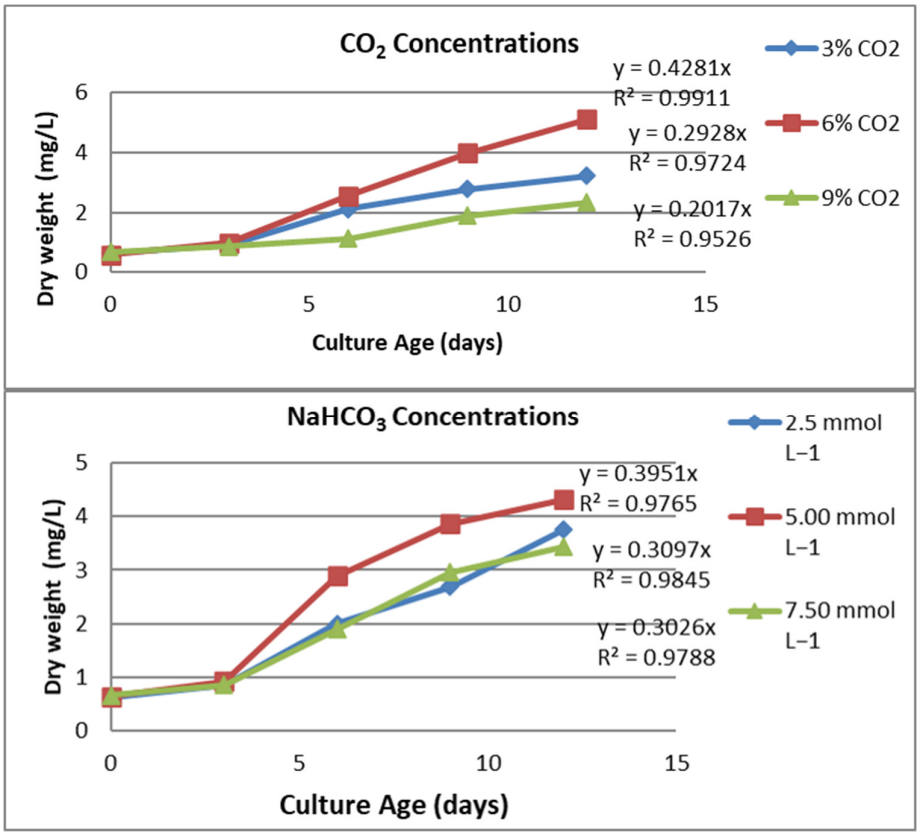

Figure 2. Impact of carbon scours and concentrations on *Chlamydomonas reinhardtii* biomass

Impact of the element's availability (N, P, S) on *C. reinhardtii* growth and lipid contents

Sulfur, N and P elements are the most important nutrients for autotrophic growth of microalgae and their supply is central tools to microalgae biotechnology. These nutrients are playing important roles as enzyme cofactors and in photosynthetic pathways. It has been notes that many microalgae, when stressed to the sub point of growth inhibition, metabolic pathway switch to lipid accumulation (Karpagam *et al*., 2015), a phenomenon is interesting to researchers for production of biodiesels (Abd EL Baky and EL Baroty, 2016).

Therefore, the availability of N, P and S (replete and deplete conditions) was assessed to accumulate high lipid contents in *C. reinhardtii* microalgae.

Phosphorous (P)

The effect of availability of P (concentrations: 1.13 mM P-rich, PR, 0.28 mM P-optimal, PO and 0.14 mM P-limited, PL) in Sueoka medium on *C. reinhardtii* growth (in term of dry weight, dw.) and lipid contents are shown in Table 3 and 4 and Figures 3 and 4. The final dry weight and lipid contents in the different P cultures were significantly deference's. The dry weight of P-rich culture was higher than in the P-optimal or P-limited ($p<0.01$). P-limited culture is yielding lowest DW than that in P-optimal or P-rich cultures. Where, high lipid content was obtained in P-Limit culture (0.246 mg/L) was than of P-optimal (0.183 mg/L) or P-Rich (0.163 mg/L). Although, the P-limited culture had high lipid and less DW compared to P-optimal or P-rich cultures. Phosphorus starvation has lesser effects on microalgae photosynthesis in comparison to nitrogen starvation. However, phosphorus starvation is found to induce a six-fold increase in lipid production in some algae species (Goh *et al.*, 2019).

Table 3. Impact of elements concentrations on *Chlamydomonas reinhardtii* biomass

Elements and concentrations	Culture Age (days)				
	0	3	6	9	12
Nitrogen					
7.5 mM N	0.595	0.976	2.43	3.57	4.54
3.75 mM N	0.562	0.795	1.89	2.56	3.67
1.87 mM N	0.511	0.764	1.59	2.12	3.51
0.93 mM N	0.567	0.756	1.25	1.79	2.63
Phosphorus	0	3	6	9	12
1.13 mM P	0.588	0.763	2.65	3.43	4.45
0.28 mMP	0.654	0.712	1.76	1.96	3.32
0.14 mM P	0.674	0.724	1.54	2.41	3.83
Sulfur	0	3	6	9	12
0.43 mM S	0.677	0.789	2.11	3.47	4.33
0.21 mM S	0.681	0.793	1.45	2.48	3.45
0.10 mM S	0.598	0.674	1.23	1.56	3.11

Table 4. Impact of nitrogen concentrations on *Chlamydomonas reinhardtii oil* content (%) and oil yield

Elements and concentrations		Culture age (days)				Oil yield mg/L
Nitrogen	0	3	6	9	12	
7.5 mM N	1.23	1.51	2.35	3.11	3.87	0.17
3.75 mM N	1.23	1.85	3.45	4.76	5.66	0.207
1.87 mM N	1.23	2.11	4.53	6.55	7.54	0.264
0.93 mM N	1.23	2.34	5.67	7.32	8.56	0.225
Phosphorus	0	3	6	9	12	
1.13 mM P	1.23	1.57	1.99	2.65	3.67	0.163
0.28 mMP	1.23	1.85	2.14	3.87	5.54	0.183
0.14 mM P	1.23	2.11	3.76	4.85	6.43	0.246
Sulfur	0	3	6	9	12	
0.43 mM S	1.23	1.75	2.47	2.81	3.66	0.158
0.21 mM S	1.23	1.99	2.95	3.89	4.75	0.163
0.10 mM S	1.23	2.22	3.73	4.84	5.49	0.171

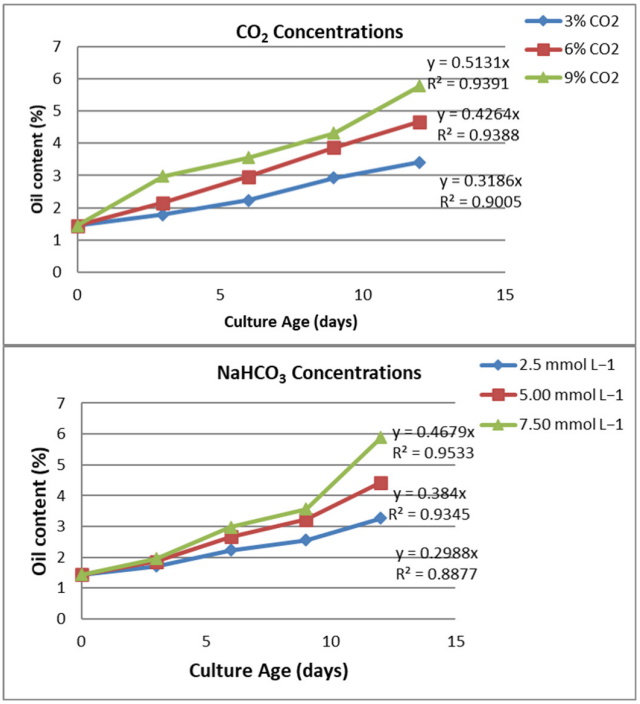

Figure 3. Impact of carbon scours and concentrations on Chlamydomonas reinhardtii oil content (%)

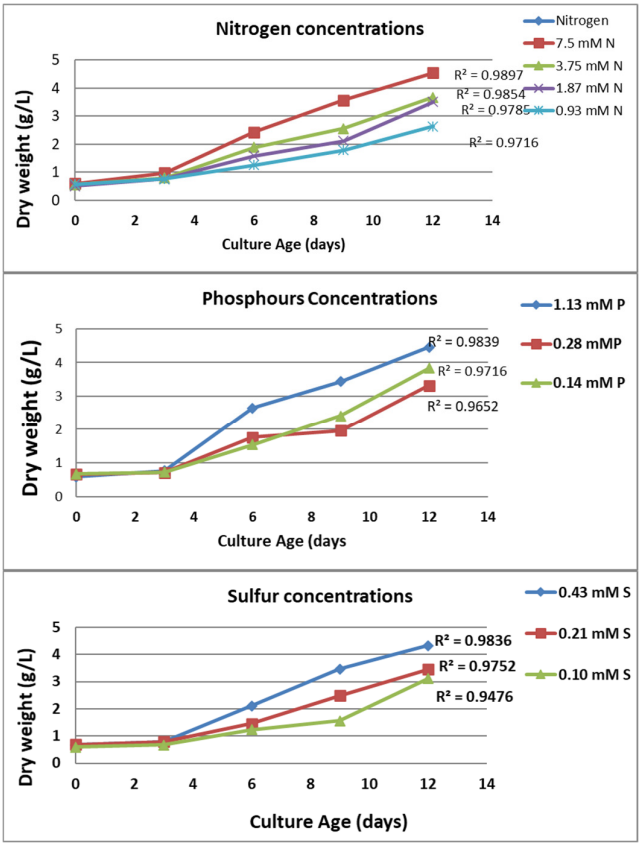

Figure 4. Impact of elements concentrations on *Chlamydomonas reinhardtii* biomass

Effect of N-concentrations on C. reinhardtii growth and oil yield

Microalgae, as biological system have received considerable attention due to their high accumulation of high lipid yield when grown under N-limited condition, which could be used for bio-fuel production (Table 4 and Figure 5). The impact of supplement of Sueoka medium by sodium nitrate as a N-source (0.50 mM N-rich, NR, 3.75 mM N-optimal, NO, 1.78 mM N-limited, NL and 0.93 mM N-Sub-limited, NSL) on *C. reinhardtii* growth (in term of dry weight, dw.) are shown in Table 2 and Figure 2. The results demonstrated that the cell biomass (dw.) was increased gradually as function of incubation times. After 15 days of incubation, the biomass (mg/L) were found to be 4.54, 3.67, 3.51 and 2.62 for NR, NO, PL and SNL cultures, respectively. In regarded with total lipid contents, the negatively relation was found between N- concentration and lipid accumulation. The values of total lipid content (5 of dw.) and oil yield (in parenthesis, mg/L) of NR, NO, PL and NSL *C. reinhardtii* cultured for 12 days were 3.78 (0. 17), 5.66 (0.207), 7.55 (0.264) and 8.56 (0.225), respectively. Under N-limited condition, algae move to toward increasing of lipid biosynthesis (Abd El Baky and El Baroty, 2012). Therefore, biomass was direct proportional to N- concentration but TL was indirect to N-concentration. In the most our researches we observed that phenomenon under N-limitation is a proportional reduction of dry weight due to reduce the total proteins and simultaneously inducing lipids biosynthesis due to the reduced nutrient availability (Abd El Baky *et al.*, 2015; Abd El Baky and El Baroty 2016). Abd El Baky *et al.* (2012) reported that microalgae grown under Fe or N-limitation had high carbon rich compounds due to accumulation of carbohydrates or lipids. Under nutrients stress, many microalgae have the ability to produce substantial high amounts (20-50% dw) of triacylglycerols as a storage lipid (James *et al.*, 2011; Karpagam *et al.*, 2015).

Effect of sulfur (S) concentration on C. reinhardtii growth and oil yield

The effect of supplement of Sueoka medium by sodium sulfate as a sulfate source at different S-concentrations (0.43 mM S-rich, SR; 0.21 mM S-optimal, SO; and 0.10 mM S-limited, SL) on *C. reinhardtii* growth (in term of dry weight, dw) are presented in Tables 2 and 3 and Figures 3 and 4. The results revealed that the *C. reinhardtii* biomass (dw) was increased gradually as function of incubation period. After 12 days, the biomass (mg/L) were found to be 4.33, 3.45 and 3.11 for SR, SO and SL cultures, respectively. Also, the levels of total lipid content and oil yield (in parenthesis) were 3.66% (1.58 mg/L), 4.75% (0.163 mg/L), 5.49% and (0.1781 mg/L), respectively. Thus, afterwards, the oil content significantly increased with the decrease in S levels. Previous research by Hu *et al.* (2008) Singh *et al.* (2015) using many microalgae species reported an increase in lipid productivity and lipid content when cultured under S-limited condition.

The N, P and S elements are the key factors required for microalgae growth and lipid accumulation (Abd El Baky and El Baroty, 2016). The change in N, P, and S levels led to significantly alteration of biomass (dw) and lipid contents. In rich C, N, S and P individual growth cultures, biomass may reach about two-fold, of that did in limited medium. In contrast, the values of the total lipid TL content and oil yield OY were significantly increased in all element limited of *C. reinhardtii* cultures compared with that obtained in rich or optimal cultures.

Growth and lipid accumulation properties in C. reinhardtii cultured at large scale in 2000-liter photobioreactor for production biodiesel

It is essential to optimize the nutrient growth conditions to improving the lipid accumulation without significantly biomass loss in the nutrient limited cells, which becomes a potential cost-effective strategy for keeping the production cost of biofuels at a minimum. Thus, the best possible conditions for ***C. reinhardtii*** growth were chosen for cultured at large-scale to improve the economic feasibility of lipid content for biodiesel production. According to above data, the explore the conditions required for accumulation high lipid content in *C. reinhardtii* grown under limitation availability of (P+N+S) in large scale 2000-liter serial-column photo-bioreactor PBR. The nutrient volume, inoculums density, temperature, CO_2 flow rate and levels of combined

N, S N element levels (N-1.78 mM, P- 0.14 mM and S-limited 0.10 mM) concentration was achieved to make sure the good cells grow and produce a suitable cell density and high lipid contents.

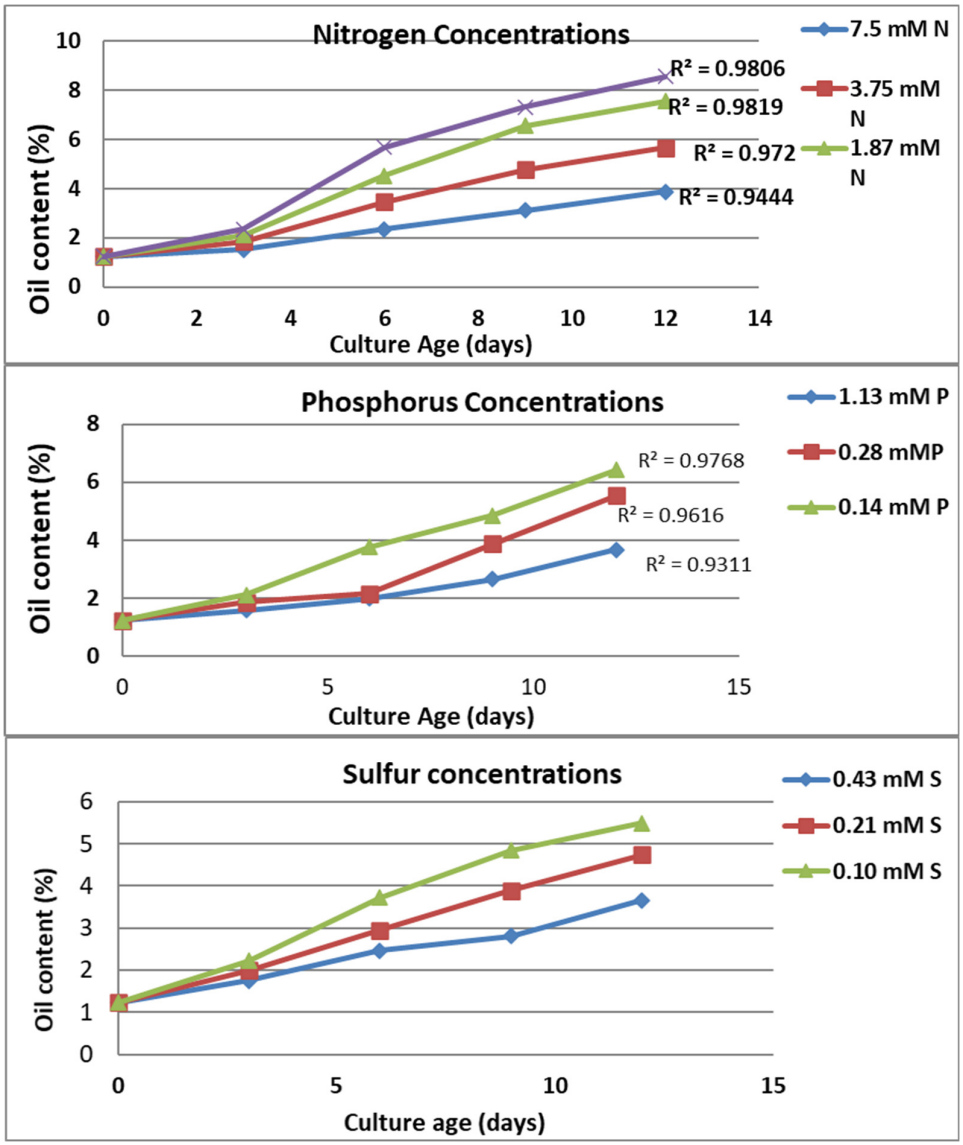

Figure 5. Impact of elements concentrations on *Chlamydomonas reinhardtii oil* content (%)

After 15 days, the cultures reached the higher biomass productivity, and harvested for lipid analysis. As shows in Table 5 and Figure 6, after 15 days of cultivation, the *C. reinhardtii* culture, had an early stationary phase with higher growth rate, and this trend was continuously reached to 15 days of cultivation. The yield of biomass of cultures was increased gradually with varying degree during the cultivation periods. After 15 day of cultivation, the levels, of dry weight (dw.), biomass productivity, chlorophyll a (Chl a), lipid contents and oil yield (d^{-1}) was found to be 6.14 (g/L), 108.61 (mg/L), 22.76 (%) and 1.22 (g/L), respectively. These values were increased over all that in those induce in each basic individual concentration medium by several times, which the lipid percentage (%) can reach to 1.7 - fold. These results showed that the induce increase lipid accumulation in *C. reinhardtii* microalgae cells were associated with values of specific growth rates of cells (0.114 g/day), Chla content (31 mg/day) and OY (0.127 g/day) recorded under this condition. The statistical

analysis also revealed that good correlation was found between the dw as factor of microalgae growth and the incubation time with (R^2 of 0.9778, y=0.2681x+0.9751). These results are confirmed by these previous reports that the *C. reinhardtii* microalga, showed that the metabolic effect of limitation P, N and S, related to the increasing lipid accumulation and also may causes cell growth inhibition and decreases the total biomass. (Hu *et al*., 2008; Griffiths and Harrison, 2009). As already previously described by Abd El Baky and El Baroty (2014, 2016, 2017), that the nutrient limitation decreases partially the biomass yield and enhance accumulation of lipid content in microalgae cells due to the metabolism shift from synthesis of protein and other N biomolecular to synthesis of neutral lipid molecules (e.g., triacylglycerols and carotenoids). However, the increased lipid productivity is reflecting the independent impact of N or P levels on both growth and lipid contents (Sajjadi *et al*., 2018). Abd El Baky and El Baroty (2016) reported that the cultivation conditions of microalgae including nitrogen, sulfur, phosphate and CO_2 concentrations and light intensity had a high influenced impact on cell growth and lipid accumulation.

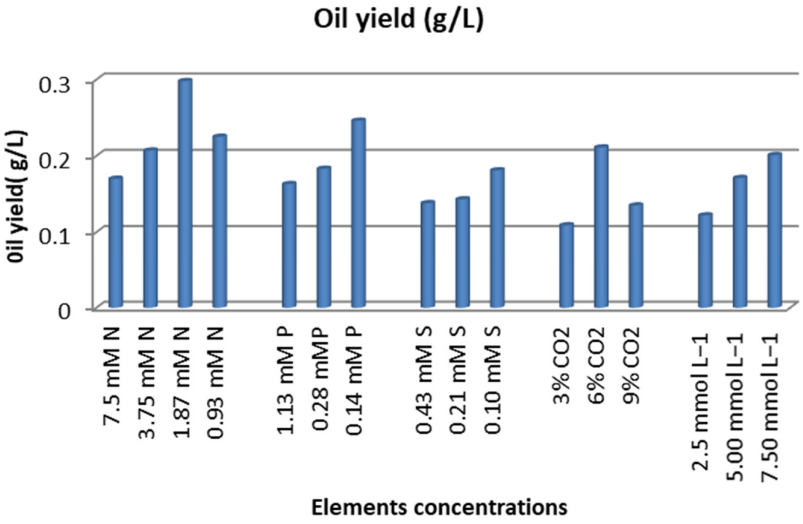

Figure 6. Oil yield of *Chlamydomonas reinhardtii* grown under different element concentrations

Table 5. Specific growth rate (SGR g/day) of cell, chlorophyll a (chll-a) and lipid of *Chlamydomonas reinhardtii* grown in nitrogen (1.87 mM L^{-1}N), Phosphorus (0.14 mM L^{-1} P) and sulfur (0.10 mM L^{-1}S) limitation and CO_2 (6%)

Parameters	*Chlamydomonas reinhardtii*
SGR of cell (g/day)	0.114
SGR of Chll-a (g/day)	0.278
SGR of lipid (g/day)	0.0127

Physical-chemical properties (PCP) of oils

The lipid of *C. reinhardtii* grows in limited of (N+P + S) medium in PBR allowed to producing high lipid accumulation. Table 6, showed some physicochemical properties of *C. reinhardtii* lipid includes: refractive index (1.55± 0.11, at 40 °C), viscosity (mm^2/S), density (g/cm^3 24 °C), saponification value (SV185.54±2.48 mg of KOH/g of oil), acid value (AV, 0.43 ± 0.037 mg of KOH/one gram of oil), iodine value (IV 75.9± 0.55 mg of I_2/100 g of oil), peroxide value (PV 0.376± 0.034 meq O_2 kg^{-1}). These values showed that *C. reinhardtii* lipid is suitable for produce biodiesel production, that are similar to those reported for plant oils like soybean, palm and rapeseed crops that are suitable for biodiesel production the most restrictive biodiesel standards, i.e. the EN 14214 and STAM D 6571 (Giakoumis, 2013).

Table 6. Physicochemical properties of *Chlamydomonas reinhardtii* oils

Physiochemical properties	*Chlamydomonas reinhardtii* oil properties
Molecular weight (Da)	929
Refractive index (40 °C)	1.55 ± 0.11
Viscosity (mm^2/S)	6.87 ± 0.45
Density (g/cm^3) 24 °C	1.54 ± 0.14
Saponification value (mg KOH g^{-1} oil)	185.54 ± 2.48
Acidity (mg KOH / g oil)	4.43 ± 0.37
Iodine value (mg of I/100 g of oil)	75.9 ± 0.55
Peroxide value meq O_2 /kg oil	0.01 ± 0.001
Conjugated diene ($g^{1\%}1cm$ (ƛ 232)	0.01
Conjugated triene (e1%1cm (ƛ270)	0.01

The molecular weight of the oil was calculated from saponification and acid value according to: {M= 168300/ SV−AV}, where M is the molecular weight of the oil, SV the saponification value, and AV is the acid value.

Biodiesel production from *C. reinhardtii* lipids

According to our previous work (Abd EL Baky and El Baroty, 2016), the direct transesterification TE reaction condition was optimizated as a way to improve the biodiesel yield. In this study, microalgae biomass of *C. reinhardtii* cultured at large scales in limited combinations of (N+ S+ P) medium was subjected to a directly TE in one step. The biodiesel yield (BY %) and biodiesel productivity ($mg^{-1}L^{-1}d^{-1}$) were found to be was about 87.96% and 1.67±0.23, respectively (Table 7).

Table 7. *Chlamydomonas reinhardtii* cultivation under high lipids production conditions at large scale tubular photobioreactor

Parameters	*Chlamydomonas reinhardtii*
Biomass productivity (g^{-1} L $^{-1}$ d^{-1})	0.15±0.012
Specific growth rate μ (d^{-1})	0.298
R^2	R^2 = 0.9759
Max. lipids contents (w/w %)	22.76
Max. lipids productivity (mg^{-1} L $^{-1}$ d^{-1})	1.9± 0.35
R^2	R^2 = 0.9338
Max. biodiesel contents (w/w %)	87.96
Max. biodiesel productivity (mg^{-1} L $^{-1}$ d^{-1})	1.67±0.23

The FAME profile of *C. reinhardtii* biodiesel

The FAME profile (FAME, fatty acids methyl esters) of *C. reinhardtii* biodiesel was analyzed by GC/MS methods. The data shows that the main fatty acids were long-chain fatty acids with 16 and 18 carbon atoms, including palamitic acid (C16:0), oleic acid (C18:1) and linoleic acid (C18:2). Other FA, including myristic acid (C14:0), stearic acid (C18:0), linolenic acid (γ-C18:3), pentadecanoic (C15:0) were percent in significant quantity. The FA composition of *C. reinhardtii* was similar to the crop seed oils compositions, considered as a potential source for biodiesel production, which have noticeably high C16 saturated (SFA 36.97% TFAs) and C18:1 (27.33%, of TFAs) of fatty acids (Table 8). This observation in *C. reinhardtii* biodiesel was quite similar to that found in previous works, where the palamitic acid and oleic were identified as a major FAs in most species of microalgae (El Kassas, 2013; Abd EL Baky *et al*., 2014, 2015). James *et al.* (2011) demonstrated that *C. reinhardtii* starch mutants had high levels of 16:0, 18:1, 18:2 and low levels of long chain fatty acids (20:0, 20:1, 20:2, 22:0, and 24:0), when nitrogen starved, which may be suitable for conversion to liquid fuels. Abd El Baky *et al.* (2012, 2014) reported that microalgae grown in N and P-limited condition, had a higher level of unsaturated fatty acids than that in cells grow on nutrient sufficient medium. However, N-stress has been found to decrease the saturated fatty acid of C16:0, which could improve the

Cetane number and oxidative stability of the biodiesel produced (Sajjadi *et al.*, 2018). However, total polyunsaturated fatty acids (TPUFA) in microalgae culture were percent in lower quantity. The values of TFAs and TSFAs were a direct proportion with ratios of saturated FA/ unSFA/ (unsaturation degree DU), oxidation degree (OR) (Table 9). The *C. reinhardtii* had a moderate Du (1.21), TU/TS (1.69) and OR (8.33) degrees as that recorded in some microalgae biodiesel. However, the lipid composition has considerable impact on the technology of biodiesel production and product quality (Demirbas, 2009). In general *C. reinhardtii* had a high SFA and MUSFA, in a good balance, which lead to a good oxidative stability and low-temperature properties and promotes the quality of biodiesel.

Table 8. Fatty acid methyl esters profile of *Chlamydomonas reinhardtii* biodiesel

Molecular formula	*Chlamydomonas reinhardtii biodiesel*
	Relative fatty acids content %
$C_{14:0}$	.....
$C_{15:0}$	.....
$C_{16:0}$	33.76
$C_{16:1\ 9c}$	4.68
$C_{16:2\ 7,10c}$	1.99
$C_{16:3\ 7,10c}$	2.43
$C_{16:4\ 7,10c}$	2.36
$C_{17:0}$	.....
$C_{18:0}$	3.21
$C_{18:1\ (9c)}$	20.32
$C_{18:2\ (9,12\ c)}$	14.94
$C_{18:3\ (6,9,12\ c)}$	9.54
$C_{18:4\ (5,\ 9,12,15\ c)}$	3.72
$C_{20:1}$	1.11
$C_{22:1}$	1.22

[a]: Fatty acid was identified based on the retention time of standard fatty acids and MS spectra

[b]: The percentage of the fatty acid was evaluated through the peak area.

Table 9. Evaluation criteria of *Chlamydomonas reinhardtii* biodiesel

Lipid criteria (%)	*Chlamydomonas reinhardtii biodiesel*
Total saturated fatty acids	36.97
Total monounsaturated fatty acids	27.33
Total polyunsaturated fatty acids	34.98
Total unsaturated fatty acids	62.31
TU/TS	1.69
DU	1.21
RO	8.33

TU/TS: Total unsaturated / Total saturated

DU: Degree of Unsaturated: TMSFA/100 + 2 [Tdi= FA/100] +3 [T Tri= FA /100] + 4 [Tetra (x) FA/100]

RO: Rate of oxidation = [%UFA 1 = x1 /100] + T di (x) FA/100] + T trii (x) FA/100] + [%UFA 4 (x)50 /100]

(x) = Number of double pounds

The biodiesel properties of *C. reinhardtii*

Biodiesel (FAME) properties derived *C. reinhardtii* lipid cultured at large scales PBR on combined limited (N+P+S) nutrient levels (Table 10) including the density (0.871± o.12 $Kg^{-1}L$), viscosity (4.14±0.23 mm^2S^{-1}, at 40 °C), acid value (AV 0.45±0.01 mg KOH/one gram oil), peroxide value (PV less than 0.1 meqO_2 kg-1 of oil), saponification value (SV, 189.33 ± 1.79 mg of KOH/100 g), iodine value (IV, g I_2/100 g) were determined. Also, the cetane number (CN 64.8), oxidation ability (OX 0.344) and degree of unsaturation (DU 97.29) were calculated based on empirical equations extracted from the GC/MS fatty acid profile (Table 6). All chosen parameter was found to be within that limit values reported in the international standards, EN 14214 and ASTM D6751 for biodiesel and agree with that findings in the literatures for biomass biodiesel (Kaisan *et al.,* 2015). Moreover, the biodiesel had low values of conjugated diene ($g^{1\%}$1cm absorbance A =0.01 at ƛ 232 nm was 0.1) and conjugated triene ($g^{1\%}$1cm A = 0.01 at ƛ 270 nm was 0.1) which indicating good quality of biodiesel due to the high oxidative stability (against auto oxidation reaction) and high shelf life time. Further, the ratio of SFA and unsaturated fatty acids are tented to produce optimal heating value (HV) and high CN (min) that is related to the shorter ignition delay time (Moser 2014). On the other hand, other quality checking parameters (based on empirical equations) of biodiesel like average of molecular weight (mw, Da 999), heating value (HV, 41.36 MJ/kg), flash point (FP, 44.0 °C), LCSF 33.33 and CFPP 88.23 were found to be appropriate as per the given in international standard (Nascimento *et al.,* 2013. Thus, the results the *C. reinhardtii* biodiesel property was found in accordance with the biodiesel specifications are given by the most international standards (ASTM D6751 US and EN 14214 Europe (Hoekman *et al.,* 2012). Also, all those properties of *C. reinhardtii* biodiesels are similar to that reported in literatures for plant oils and some microalgae and macroalgae species used for production of high biodiesel quality (Moser, 2009; Ramos *et al.*, 2009; Demirbas, 2011; Abd El Baky and El Baroty, 2016, 2015). Thus, this result indicates that lipids of microalgae grow under limited (S+P+N) condition have a high potential as economical feedstock for biodiesel production, due to the fact that limited nutrient can reduce the essential nutrients consumption and it could make the production of *C. reinhardtii* lipid is feasible and as a promising strategy.

Table 10. Comparison of physiochemical properties of *Chlamydomonas reinhardtii* biodiesel, diesel fuel and biodiesel standard

Property	Units	Biodiesel of *Chlamydomonas reinhardtii*	Diesel[a] fuel	ASTM[a] biodiesel standard
Density	($Kg^{-1}L$)	0.871± 0.12	0.838	0.86 - 0.9
Viscosity	$mm^2 S^{-1}$, at 40 °C	4.14± 0.23	1.9 - 4.1	3.5 - 5.0
Acid value (AV)	mg KOH $^{-1}$ g	0.45± 0.01	Max 0.5	Max 0.5
Peroxide number	meq O_2 /kg oil	ND	ND	
Saponification value (SV)	mg KOH g^{-1} oil	189.33 ± 1.79	.....	.....
Iodine value	g I_2/100 g	45.78±2.96	120	Max 120
Cetane number (CN min)		64.8	45.9	≥47
Oxidizability (OX)		0.344	.....	>0.7
DU		97.29	.....	.....
Heating value	(MJ/kg) HV	41.36		
Flash point (FP)	(°C)	44.00		93
LCSF		33.33		.....
CFPP		88.23		.....

a: The data about diesel and ASTM biodiesel standard were taken from published literature; DU = MUFA + (2X PUSFA); CN= (46.3+ [5458/SV]) -(0.225 X IV); Oxidizability (OX)= [0.02(%Oleic) + %linoleic + 2 (%linolenic)] / 100; HHV = 0.4625 ν +39.450, Higher Heating Value of biodiesel from their viscosity (ν); FP = (HHV -32.12) /0.21; LCSF =(0.1 X C_{16})+ (0.5 XC_{18}) +(1 XC_{20})+ (1.5 X C_{22})+ (2 X C_{24}); CFPP= (3.1417 X LCSF) - 16.477

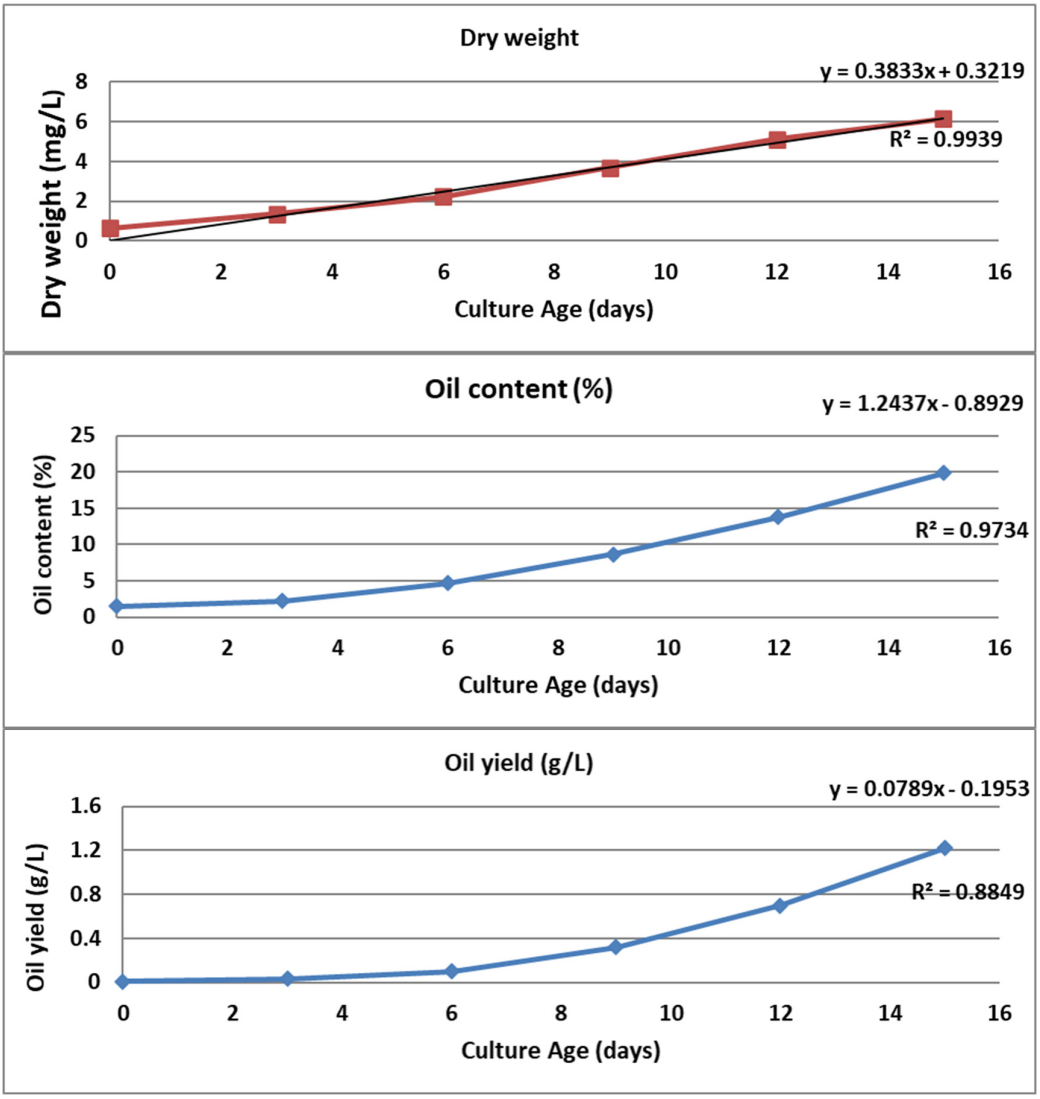

Figure 7. Dry weight, oil content and oil yield of *Chlamydomonas reinhardtii* grown in photobioreactor under nitrogen, phosphorus and sulfur limitation

Conclusions

The study demonstrated feasibility of lipid production from *C. reinhardtii* cultivation modes, among which limited viability of combined elements S+P+N condition under large scale PBR (2000 Liters) cultured, as the effect strategy for the lipid accumulation. Thus, it is insight to *C. reinhardtii* strain thought for cost-effective biofuels production. The *C. reinhardtii* lipid was subjected to acidic TE process for producing the biodiesel. The GC/MS analysis of fatty acids presented in the biodiesel showed a predominance of mono unsaturated and saturated fatty acids, considered ideal for the production of high-quality biodiesel. The properties of biodiesel were in accordance with international specification established by EN 14214 and ASTM 6571. However, this study may assess the importance and helps to cover the gap of the microalgae cultivated in Egypt for biodiesel production and other application.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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