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Optimization of Growth and Lipid Production of the Chlorophyte Microalga *Chlorella vulgaris* as a Feedstock for Biodiesel Production

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Abstract: The present study aimed to throw light on the effect of nitrogen deficiency, salt stress and iron concentration on growth, lipid content and fatty acid profile of *Chlorella vulgaris*. The present results showed that lipid content of *C. vulgaris* is inversely related to the growth rate at different treatments. Nitrogen deficiency of *C. vulgaris* of 0.1 mM of $NaNO_3$ and 0.45 mM of NaCl resulted in the highest lipid content (54.88 % and 43.2 % of CDW, respectively) after 12 days of incubation, which represents 85 % and 45 % higher than their corresponding control, respectively. Treatment with ferrous sulphate showed the highest lipid content (34.7 of CDW, 17 % higher than the control) at 35 µM after 12 days of incubation. Total saturated fatty acids percentages represented 50-59 % of total fatty acids, while unsaturated fatty acids represented 41-50 % of total fatty acids. The predominant saturated and unsaturated fatty acids of *C. vulgaris* were C16:0 and C18: 2, respectively. Also, it was found that all of the treatments resulted in formation of lignoceric acid (C24:0) which wasn't recorded in the lipid profile of untreated alga.

Key words: Algal oil · Biodiesel · *Chlorella vulgaris* · Iron stress · Nitrogen deficiency · Salinity

INTRODUCTION

Nowadays, modern world is currently facing a dangerous energy problem due to the increase of world consumption of energy [1]. As a result, new alternatives and sustainable energy sources are vital [2, 3] and biodiesel exhibits a particular promise between these alternatives [4, 5]. Biodiesel is an alternative liquid fuel produced by chemical reaction between a plant oils or an animal fats and alcohol in the presence of acid or base as a catalyst to produce fatty acid methyl esters (Biodiesel), which is eco-friendly and renewable [6, 7]. Furthermore, the use of edible vegetable oils for fuel production competes significantly with food uses and this would results in undesirable increase in food and biodiesel costs [8]. Consequently, algal oils were found to be a good alternative for the production of biodiesel rather than vegetable oils [9, 10]. Microalgae, like seed crops, are sunlight-driven cell factories that convert carbon dioxide to potential biofuels [11, 12]. But, unlike traditional oil from seed crops, microalgae can grow in places away from the farmlands and forests such as ponds, fermentation units and even wastewater [13]. Therefore, using of algae as a feedstock for biodiesel minimizes the damages caused to the eco- and food-chain systems [14].

The biochemical composition of algae can be modulated with altering environmental conditions [15, 16]. Mine *et al.* [17] reported that a number of environmental factors including physical stimuli, such as light and gravity, invoke localized and generalized cellular reactions. Chemical stimuli could be nutrients limitation which increases lipid accumulation as well as lipid

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composition [18, 19]. Of these nutrients, nitrogen limitation [20], high salinity [21] and iron concentration [22] could modify and improve algal lipids. The present work was intended to throw some light on optimization of *Chlorella vulgaris* growth conditions for high lipid production to be used as a feedstock for biodiesel. Hence the present study focused on the effect of nitrogen deficiency, salt stress and iron stress on growth, lipid content and fatty acid profile of *C. vulgaris*.

MATERIALS AND METHODS

Alga Strain and Growth Conditions: The unicellular green microalga *C. vulgaris* was obtained from the Lab of Phycology, Faculty of Science, Zagazig University, Egypt. *C. vulgaris* was axenically grown on bold basal medium (BBM) according to Nichols [23]. 100 ml of BBM in 250 ml Erlenmeyer flasks were inoculated with 5 ml of precultured *C. vulgaris* under aseptic conditions, then incubated under photoperiod of light:dark regime of 16:8 h at temperature 25 ± 2 °C and light intensity of 5000 lux. Cultures were illuminated by tubular fluorescent lamps (PHILIPS Master TL-D 85 W / 840) for 12 days.

BBM medium, containing 3 mM sodium carbonate, 0.4 mM sodium chloride and 17 µM ferrous sulphate, was used as a control. The effect of different concentrations of sodium nitrate (0.10 mM, 1.00 mM, 2.00 mM and 5.00 mM), sodium chloride (0.30 mM, 0.42 mM and 0.45 mM) and ferrous sulphate (26 µM, 35 µM and 44 µM) on growth and lipid content were studied. A certain volume of exponentially growing *C. vulgaris* cells was inoculated in 500 ml of BBM medium in 1 L Erlenmeyer flasks at an initial OD_{450} of 0.1. Sterile filtered air enriched with 3 % (v/v) CO_2 was continuously applied to the cultures. Biomass and lipid content were measured every other day.

Biomass Assay: Algal growth was monitored using the optical density of the culture at 450 nm (OD_{450}) according to Hsieh and Wu [24] and by determination of algal cellular dry weight (CDW, g l^{-1}). The mean growth rate (R`) was calculated according to Robert [25].

Lipid Extraction and Determination: Oil content of *C. vulgaris* was determined according to Sadasivam and Manickam [26] by Soxhlet apparatus using n-hexane as the extraction solvent for 6 hours under reflux [27]. The lipid extracts were dried under a stream of argon. The pre-weighted glass vials containing the lipid extracts were dried at 80°C for 30 min, cooled in a desiccator and weighted, lipid content was determined as a percent of CDW.

Fatty Acid Analysis: The extracted lipids were saponified overnight with ethanolic KOH (20 %, w/v) at room temperature. Fatty acids were librated from their potassium salts by acidification with 5 N hydrochloric acid, followed by extraction with petroleum ether at 40-60°C. The ether extract containing fatty acid methyl esters was washed three times with distilled water and dried over anhydrous sodium sulfate [28]. To determine the fatty acid profiles, 1 µl of fatty acid methyl esters was injected into a 6 feet × 1/8 inch internal diameter column packed with 20 % diethylene glycol succinate (DEGS) on chromsorb 60-80 mesh by using Hewlett-Packard GC-MS.

Statistical Analysis: Results are presented as the mean ± standard deviation (SD) from three replicates. The statistical analyses were carried out using SAS (v 6. 12). Data obtained were analyzed statistically to determine the degree of significance using one way analysis of variance (ANOVA, p = 0. 05).

RESULTS AND DISCUSSION

A major bottleneck in microalgal biodiesel production is lipid content [29]. High lipid content is one main criterion for the selection of microalgae strains as a renewable source for the biodiesel production [10]. The present study was intended to study the optimization of *C. vulgaris* growth conditions for high lipid production to be used as a feedstock for biodiesel. As a general finding, the relation between lipid content and growth rate was found to be inversely under all the studied treatments. This finding in agreement with those obtained by Abomohra *et al.* [10] who reported inverse relation between the growth and lipid content of seven studied algal species. The effect of different concentrations of $NaNO_3$ on the growth of *C. vulgaris* was recorded as mean growth rate at 2 days interval for 12 days of incubation. The obtained results in Figure 1a revealed that decreasing of $NaNO_3$ concentration led to reduction of the growth rate while increase in $NaNO_3$ above control (3 mM) led to increasing of the growth rate. The maximum growth rate was recorded after 6 days of incubation at 5 mM of sodium nitrate (R` = 0.37 d^{-1}).

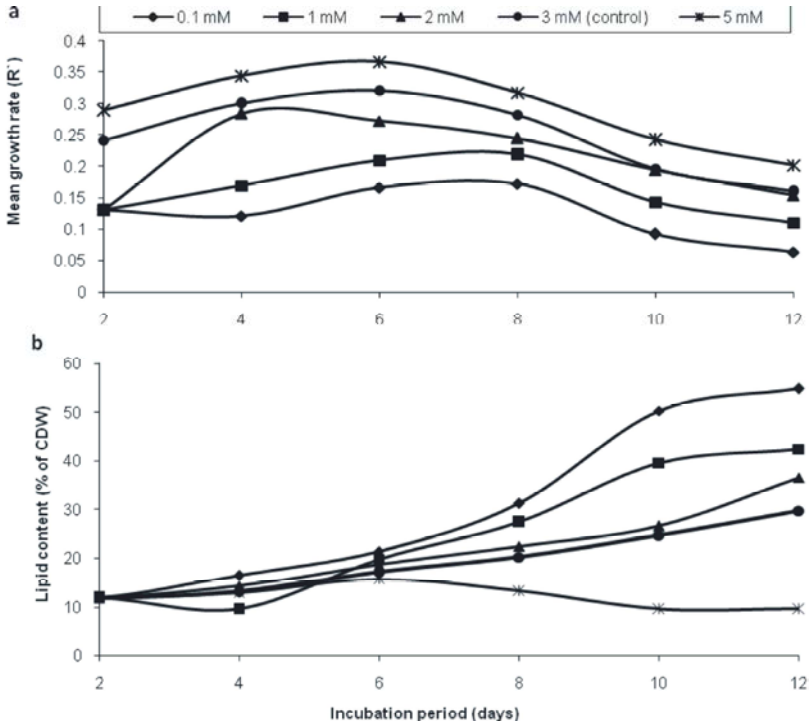

Fig. 1: Effect of different concentrations of $NaNO_3$ on growth (a) and lipid content (b) of *C. vulgaris* and cultivated for 12 days in bold basal medium.

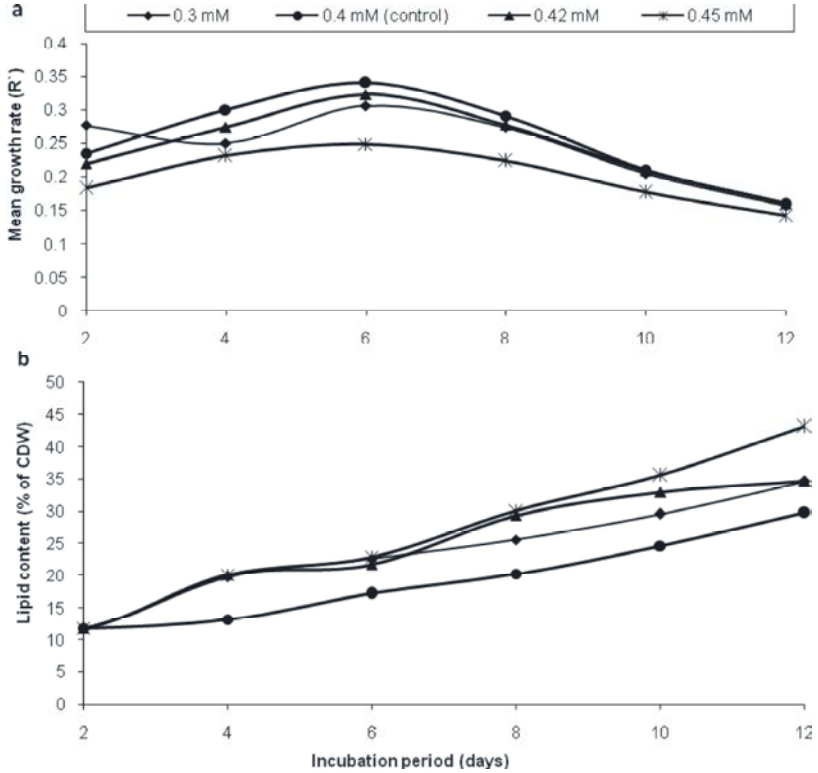

Fig. 2: Effect of different concentrations of NaCl on growth (a) and lipid content (b) of *C. vulgaris* cultivated for 12 days in bold basal medium.

On the other hand, the rate of growth was subsequently shifted to the lowest value as nitrate deprives to the level of 0.1 mM (R` = 0.17 d^{-1} after 6 days of incubation). The biosynthesis and accumulation of lipids of *C. vulgaris* under different concentrations of nitrate is shown in Figure 1b. It was revealed that the biosynthesis and accumulation of lipids of *C. vulgaris* was maximally recorded to 54.9 % of CDW as nitrate deprives to the level of 0.1 mM after 12 days of incubation. This value was about two folds more than the corresponding control. On the other hand, the lipid content of *C. vulgaris* was inhibited to 9.8 % of CDW after 12 days of incubation at high sodium nitrate concentration (5 mM). These results are in agreement with those obtained by Illman *et al*. [30], who recorded lipid content (40 % of CDW) of *C. vulgaris* under nitrogen starvation two times more than that in the control. The obvious increase in the lipid content under nitrogen limitation could activate diacylglycerol acyltransferase, which converts acyl-CoA to triglyceride or may be attributed to the inhibition of cell division under the environmental stresses [31]. Ahlgren and Hyenstrand [32] reported that under nitrogen-deficient conditions, algal cells often accumulate carbon metabolites as lipids. It was also reported that microalgae respond to the nitrogen starvation condition by degrading nitrogen containing macromolecules and accumulating carbon reserve compounds, as fats [33-35]. On the other hand, the growth of *C. vulgaris* at nitrogen deficiency did not parallel to the increase in lipid production. Such results were coincident with Rodolfi *et al*. [19] who found that N-deficiency could stimulate the lipid accumulation, but the biomass production was reduced. El-Sheekh *et al.* [35] reported that biomass reduction in nitrogen deficient cultures may be due to the reduction of pigment contents and photosynthetic activity caused by nitrogen limitation.

The mean growth rates of *C. vulgaris* grown at different salinities (0.3, 0.4, 0.42 and 0.45 mM NaCl) are shown in Figure 2a. Results indicated that the mean growth rate of *C. vulgaris* was subsequently decreased with increasing or decreasing the level of salinity about the control. The maximum percentage of reduction was recorded as 26.7 % below the control after 6 days of incubation in 0.45 mM NaCl. Meanwhile these parameters were maximally attained at 6 days old culture in untreated control (0.4 mM NaCl) where the mean growth rate was recorded 0.34 d^{-1}. Our results are in agreement with Sinha and Häder [36] who found that NaCl concentration inhibited the growth of *Anabaena* sp. We suggest that, inhibition of the growth of *C. vulgaris* in response to salinity could be a result of shifting the metabolites to the synthesis of osmoregulant compounds rather than synthesis of cellular constituents. The biosynthesis and accumulation of lipids of *C. vulgaris* under salinity stress was recorded in Figure 2b. The overproduction of lipids was significantly increased with increasing the level of salinity to 0.45 mM where the maximum lipid content was recorded (43.2 % of CDW) after 12 days of incubation. As indicated from the present results, salinity is a feasible tool for the overproduction of lipids which was coinciding with inhibition of the growth. Stimulation of lipid production under salt stress is common in plants as well as in algae [35, 37, 38, 39]. Walsby [21] suggested that the increases in lipid content under hypertonic conditions makes the plasma membrane more viscous and fluid to increase the turgor pressure of the cell and thus prevent the out flux of water from the cells as a mechanism of adaptation.

Results shown in Figure 3a revealed that there was a variation in the growth of *C. vulgaris* under the effect of different concentrations of ferrous sulphate. Using 26 µM and 35 µM of ferrous sulphate showed increases in the growth rate with 10.34 % and 6.89 %, respectively, over their corresponding control after 6 days of incubation. While high concentration of ferrous sulphate (44 µM) resulted in 51.7 % inhibition of the growth rate with respect to the control. The maximum growth rate (0.32 d^{-1}) was recorded using 35 µM of ferrous sulphate after 6 days of incubation. The biosynthesis and accumulation of lipids of *C. vulgaris* under the stress of ferrous sulphate is presented in Figure 3b, where the overproduction of lipids was slightly increased with increasing the level of ferrous sulphate up to 35 µM, but any further increase resulted in inhibition of the lipid accumulation as the lipid content decreased with 28.94 % below the corresponding control after 12 days of incubation of *C. vulgaris* at 44 µM of ferrous sulphate. The maximum lipid content was recorded (34.7 % of CDW) in 12 days old culture treated with 35 µM of ferrous sulphate which represents 17 % above the corresponding control. Regard Liu *et al*. [22] concluded that the relatively high ferrous sulphate concentration can induce the considerable lipid accumulation, but the exceeded iron in the media might restrict the metabolic pathways related to the lipid synthesis. The previous results could be explained on the basis that Iron is one of the most important elements required by most microalgae because ferric ions are involved in fundamental enzymatic reactions,

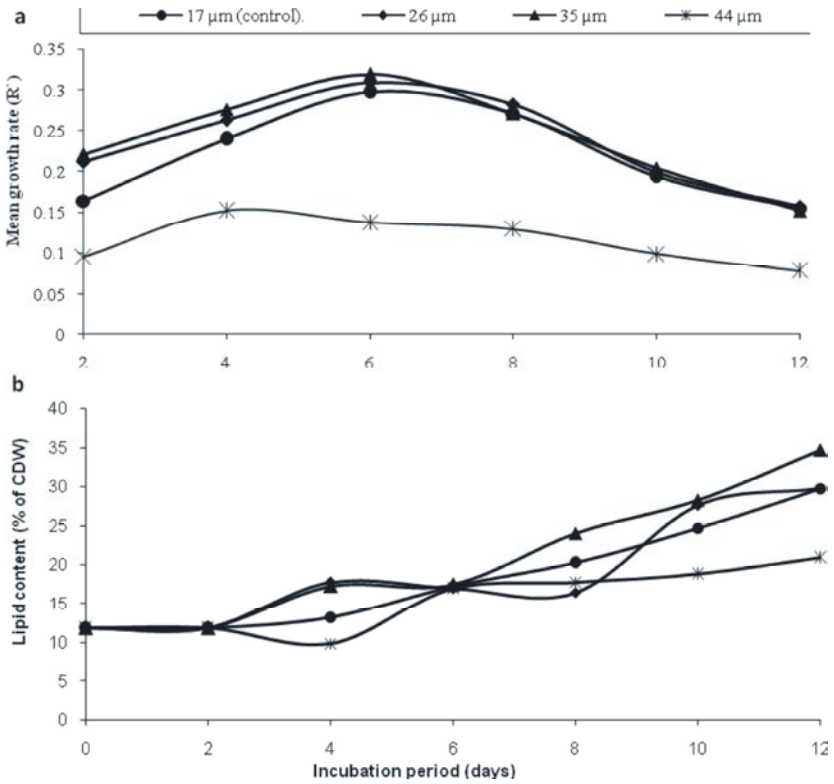

Fig. 3: Effect of different concentrations of $FeSO_4.7H_2O$ on growth (a) and lipid content (b) of *C. vulgaris* cultivated for 12 days in bold basal medium.

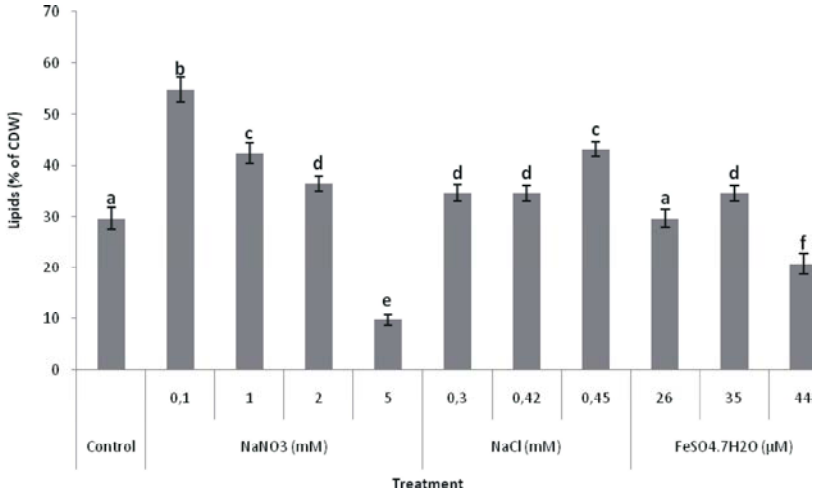

Fig. 4: Effect of nitrogen deficiency, salinity and iron concentration on lipid content of *C. vulgaris* (as % of CDW). Lipids were measured gravimetrically after 12 days of incubation. Error bars represent the SD from three replicates. Columns with the same letters showed insignificant difference at P = 0.05

photochemistry in photosystem II and nitrogen consumption and chlorophyll synthesis in the algal cells [40]. Deficient or excessive iron can generally reduce the photosynthetic efficiency of microalgae [41] which results in reduction of algal growth. In general, all treatments with $NaNO_3$, NaCl and $FeSO_4.7H_2O$ (except 26 µM $FeSO_4.7H_2O$) showed significant effect, with respect to the corresponding controls, (one way ANOVA, p = 0.05) on the lipid content of *C. vulgaris* after 12 days of incubation (Figure 4).

Table 1: Fatty acid profile of C. vulgaris treated with the best nutrient concentrations for lipid production and measured after 12 days of incubation. Values are given as % of total fatty acids.

Fatty acids	Control	0.1 mM $NaNO_3$	0.45 mM NaCl	35 µM $FeSO_4.7H_2O$
Capric (C10:0)	1.38 ± 0.08	*nd*	2.5 ± 0.08	2.36 ± 0.18
Lauric (C12:0)	4.67 ± 0.51	*nd*	2.16 ± 0.45	2.53 ± 0.19
Myristic (C14:0)	14.57 ± 0.71	1.45 ± 0.04	7.04 ± 0.07	6.46 ± 0.32
Palmitic (C16:0)	19.84 ± 0.95	11.17 ± 0.55	12.94 ± 0.65	14.24 ± 0.85
Palmitoleic (C16:1)	7.56 ± 0.59	*nd*	3.74 ± 0.15	4.47 ± 0.29
Margaric (C17:0)	2.79 ± 0.25	*nd*	0.46 ± 0.01	2.75 ± 0.13
Stearic (C18:0)	5.54 ± 0.12	44.12 ± 0.91	2.34 ± 0.24	5.52 ± 0.28
Oleic (C18:1)	10.69 ± 0.54	38.51 ± 1.08	11.49 ± 0.52	14.66 ± 0.77
Linoleic (C18:2)	17.65 ± 0.69	1.64 ± 0.12	16.49 ± 0.61	17.50 ± 0.79
á-Linoleic (C18: 3)	12.24 ± 0.64	0.77 ± 0.11	14.29 ± 0.55	13.22 ± 0.62
Arachidic (C20:0)	1.58 ± 0.12	*nd*	0.69 ± 0.06	2.27 ± 0.16
Behenic (C22:0)	1.49 ± 0.11	*nd*	1.58 ± 0.18	1.30 ± 0.03
Lignoceric (C24:0)	*nd*	2.34 ± 0.33	24.28 ± 0.85	12.72 ± 0.56
Saturated fatty acid (SFAs)	51.86 ± 2.85	59.08 ± 1.83*	53.99 ± 2.59ns	50.15 ± 2.7ns
Unsaturated fatty acid (UFAs)	48.14 ± 2.46	40.92 ± 1.31*	46.01 ± 1.83ns	49.85 ± 2.47ns

nd not detected

Each value is the mean of three replicates ± standard deviation.

* Significant difference with respect to the corresponding control. $p \leq 0.05$

ns Non significant difference with respect to the corresponding control. $p \leq 0.05$

For biodiesel production, lipid composition plays an important role from the economic point of view [35]. In the European Union, the standard EN 14214 is one of standards used for testing biodiesel intended for vehicle use [42]. One of those parameters used to test the quality of oil as a feedstock for biodiesel is the degree of unsaturation [43]. In the present study, the potential of *C. vulgaris* oil as raw material for diesel fuel was estimated with regard to the fatty acid composition. The relative percentages of fatty acids extracted from *C. vulgaris* are presented in Table 1. Thirteen fatty acids were identified from capric (C10:0) to lignoceric (C24:0) acids. Generally, total SFAs percentages ranged from 50-59 % of total fatty acids (TFAs) and the maximum significant percentage of saturated fatty acids (SFAs) was found in the lipid extract of cultures treated with 0.1 mM of $NaNO_3$. Treating of *C. vulgaris* with 0.1 mM of $NaNO_3$ and 0.45 mM of NaCl resulted in an obvious increase in SFAs with percent 14 % and 4 %, respectively, from their corresponding control. On the other hand, treating the alga with 35 µM ferrous sulphate resulted in decrease in SFAs with percentages of 3 % lower the corresponding control. Data also showed that the predominant saturated and unsaturated fatty acids (UFAs) of *C. vulgaris* were C16:0 and C18: 2, respectively. Furthermore, it was found that all of the treatments in the present work resulted in formation of lignoceric acid (C24:0) which wasn't recorded in the lipid profile of the control. Under nitrogen deficiency and salt stress, the portion of UFAs decreased in favor of SFAs. Lipids of *C. vulgaris* were composed of 59 and 54 % SFAs under nitrogen deficiency and salt stress, respectively, which high lighting the oxidative stability of *C. vulgaris* biodiesel.

In conclusion, the present study suggests that the most effective approach to enhance lipid production in *C. vulgaris* is to cultivate it in nitrogen deficient medium with 0.1 mM $NaNO_3$; this stimulates the lipid content by 85 % over the control, with 14 % increase in SFAs content over control. In addition, using 0.45 mM of NaCl stimulates the lipid content by 45 % over the control, with SFAs content 4 % over the control. These results make the fatty acid composition of *C. vulgaris* eligible for further research as a feedstock for biodiesel. Measurements of other parameters including density, viscosity, flash point, cold filter plugging point, ester content, cetane number and saponification value are important to detect the suitability of *C. vulgaris* oil for biodiesel.

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