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Nitrogen Source, an Important Determinant of Fatty Acid Accumulation and Profile in *Scenedesmus obliquus*

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The potential of algae-based fuel technologies for manufacturing renewable biofuels has been attracting interest from the scientific community. Biomass productivity and cellular lipid content are important parameters affecting the feasibility of using algae oil for biodiesel production. This study compares utilization of $NaNO_3$, NH_4Cl and urea as different nitrogen sources in terms of their effects on biomass productivity, fatty acid profile and accumulation in *Scenedesmus obliquus*. Cellular lipid accumulation was analyzed by gravimetric, fluorometric, and flow-cytometric methods, besides collecting spectrophotometric data for biomass productivity analysis. In addition, fatty acid profiles were compared by using gas chromatography–mass spectrometry. The alga can utilize all tested nitrogen sources successfully however growth rates demonstrate differences. Gravimetric lipid content analysis showed approximately a ≈1.5-fold increase in total lipid accumulation under NH_4Cl regime when compared to that of $NaNO_3$ and a ≈2-fold increase when compared to that of urea at the end of ten days cultivation course. Fatty acid profiles under different nitrogen regimes present variations especially under NH_4Cl regime. Moreover, all lipid extracts mostly consist of saturated, straight- and branched-chain hydrocarbons of different chain lengths ranging from C16–C20 which grant a suitable profile for biodiesel production. *Scenedesmus obliquus* is a suitable species for biodiesel production. The results obtained from this study provide a better understanding of cultivation characteristics of this important species and support potential, future biodiesel production.

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1. Introduction

The global state of increasing energy consumption and demand pressurizes the scientific community to find new sustainable energy resources. Among different prospective options, biodiesel has recently garnered interest because of its environmental benefits and renewable characteristics.

The use of alkyl esters of long-chain fatty acids in diesel engines without expensive modifications makes biodiesel an attractive candidate when compared to other options such as hydrogen or solar power. Microalgae, due to their fast growth and small production area compared to other terrestrial plants, have been proposed as a very good potential feedstock of sustainable biodiesel production. Furthermore, using oilseed crops instead of algae for biofuel production creates a negative tension with food security because of the projected increased need for agricultural food production on the horizon [1].

Another desirable characteristic of algae is that environmental changes or stress conditions result in alterations in the lipid biosynthetic pathways and the formation and accumulation of increasing amounts of lipids [20–50% dry cell weight (DCW)], generally in the form of triacylglycerol (TAG). Unlike other lipids, TAGs do not perform a structural role but mainly serve as a storage form of carbon and energy. After synthesis, TAGs are deposited in densely packed lipid bodies in the cytoplasm of algal cells. Therefore, after following the isolation of algal lipids, biodiesel can be produced simply by transesterification of the TAGs with methanol and a catalyzer, generally NaOH [2].

Many algal species are already known for their lipid contents and hence their potentials for use in the production of biofuel [3]. Examples of such algae are *Botryococcus braunii* [4], *Nannochloropsis oculata* [5], *Dunaliella tertiolecta* [6], *Haematococcus pluvialis* [7], and *Chlorella vulgaris* [8] in terms of the effects of nutrient deficiencies and different cultivation conditions on their lipid accumulation and biomass production rates. It is also known that different nitrogen sources can be successfully utilized by most of microalgae species [9]. Therefore, some nitrogen sources may promote higher lipid accumulation and different fatty acid profiles. However, limited information has been demonstrated to date.

According to a study the optimal biomass and lipid productivity, of *S. obliquus* were determined as 292.50 mg L^{-1} day^{-1}, 78.73 mg L^{-1} day^{-1} (38.9% lipid content per dry weight of biomass), respectively [10]. In addition, under the nutrient-deficient condition, the

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micro-alga lipid was found mainly composed of C16–C18 fatty acids (accounting for 89% of total fatty acids), a composition thought suitable for biodiesel production [10]. Another study analyzed the potential of *S. obliquus* for biodiesel production: according to the study, the lipid contents of experimental groups under nitrogen depleted conditions increased up to 40-fold higher when compared to that of control groups [11].

On the basis of previous findings, it is clearly stated that *S. obliquus* suggests itself as one of the most prominent candidate algal species for biodiesel production. Its high biomass and lipid productive, well-adaptive characteristics prompted us to choose *S. obliquus* in this study. Additional benefit of previous studies has led us to focus on our comparison of the effects of $NaNO_3$, NH_4Cl and urea as different nitrogen sources on growth, lipid profile and accumulation so as to attain a better understanding of cultivation characteristics of this important species and support potential, future biodiesel production.

2. Materials and methods

2.1. Organisms and culture conditions

S. obliquus UTEX 393 was obtained from the Culture Collection of Algae at the University of Texas at Austin, and maintained in modified bold 3N growth medium prepared according to the instructions of the UTEX Culture Collection of Algae. Cultivations were done at 24 °C, 150 rpm, with continuous illumination of 150 μmol/(m^2 s) in batch cultures. The medium contains 5 mM nitrogen by adding one of the different sources ($NaNO_3$/urea/NH_4Cl) along with $CaCl_2{\cdot}2H_2O$ 0.17 mM, $MgSO_4{\cdot}7H_2O$ 0.3 mM, K_2HPO_4 0.43 mM, KH_2PO_4 1.29 mM, NaCl 0.43 mM, $Na_2EDTA{\cdot}2H_2O$ 2 mM, $FeCl_3{\cdot}6H_2O$ 0.36 mM, $MnCl_2{\cdot}4H_2O$ 0.21 mM, $ZnCl_2$ 0.037 mM, $CoCl_2{\cdot}6H_2O$ 0.0084 mM, $Na_2MoO_4{\cdot}2H_2O$ 0.017 mM, vitamin B_{12} 0.1 mM, biotin 0.1 mM, thiamine 6.5 mM and 5 mM $NaCO_3$ was supplied as a carbon source.

2.2. Effect of different nitrogen sources on growth and lipid accumulation

To study the effect of different nitrogen sources on growth and lipid accumulation, algae cells were grown in media with different nitrogen sources. Growth was achieved by equimolar concentrations (5 mM) of nitrogen by adding the appropriate amounts of urea, $NaNO_3$ or NH_4Cl in a cultivation medium. The experiments were done in triplicate, in 1 L Erlenmeyer glass flasks in 500 mL volume.

2.3. Biomass measurement

Biomasses were determined each day by measuring the optical density (OD) of samples at 600 nm. Samples were diluted by a ratio of 1:2 to ensure that the measured OD_{600} values ranged in 0.2–0.6. The correspondence between optical density and DCW was determined by plotting OD_{600} values against DCW of appropriate serial dilutions of samples with different biomass concentrations. On the basis of this noun, biomass concentration was calculated by multiplying OD_{600} values with 0.373 ($r^2 = 0.993$). The conversion factor DCW of the samples was determined gravimetrically after centrifugation drying (6,000 rpm, 10 min) of the algal cells collected from samples and washing twice with dH_2O.

2.4. Growth analysis

Specific growth rate was calculated according to the equation

$$K' = \ln(N_2/N_1)/(t_2 - t_1),$$

where N_1 and N_2 is biomass at t_1 and t_2, respectively ($t_2 > t_1$). Divisions per day and the generation or doubling time were calculated according to the equations below, once the specific growth rate was known divisions per day:

$$Div.day^{-1} = K'/\ln 2,$$

generation time:

$$Gen.t = Div.day^{-1}.$$

2.5. Extraction and measurements of lipid contents

The lipid was extracted according to Bligh and Dyer wet extraction method. Briefly, to a 15 mL glass vial containing 100 mg dried algal biomass, 2 mL methanol and 1 mL chloroform were added and kept for 24 h at 25 °C. The mixture was then vortexed for 5 min. 1 mL of chloroform was again added, and the mixture shaken vigorously for 1 min. Subsequently, 1.8 mL of distilled water was added and the mixture vortexed again for 2 min. The aqueous and organic phases were separated by centrifugation for 10 min at 2,000 rpm. The lower (organic) phase was transferred into a previously weighed clean vial (V1). Evaporation occurred in a thermo-block at 95 °C, and the residue was further dried at 104 °C for 30 min. The weight of the vial was again recorded (V2). Lipid content was calculated by subtracting V1 from V2, and expressed as % dcw.

2.6. Micro plate nile red fluorescence assay

A stock solution of nile red (NR) (Sigma, 72485) was prepared by adding 3 mg of NR to 100 mL of acetone. The solution was kept in an opaque bottle and stored in the dark at –20 °C. Algal cells cultivated with different nitrogen sources were transferred to 1.5 mL eppendorf tubes for 10 min centrifugation at 6,000 rpm, washed twice with dH_2O, and measured in a spectrophotometer at 600 nm. Each sample was adjusted to an OD_{600} of 0.3 in a 1 mL final volume by dilution with H_2O. 5 μL of nile red solution was added to each tube and well mixed, followed by 20 min incubation in dark. Finally, cellular neutral lipids were quantified using a 96-well microplate reader (SpectraMAX GEMINI XS) with an excitation wavelength of 485 nm and an emission wavelength of 595 nm.

2.7. Flow cytometric analysis

5 μL of a working solution of NR and acetone (0.03 mg/mL) was added to 1 mL of a cell suspension at an OD_{600} of 0.3. This mixture was gently vortexed and incubated for 30 min at 37 °C in darkness. NR uptake was determined with a BD-FACS Canto flow cytometer (Becton Dickinson Instruments), equipped with a 488 nm argon laser. Upon excitation by a 488 nm argon laser, NR exhibits intense yellow-gold fluorescence when dissolved in neutral lipids. The optical system used in the FACS Canto collects yellow and orange light (560–640 nm, corresponding to neutral lipids). The fluorescence of NR-emission for neutral lipids was thus determined. Approximately 10,000 cells were analyzed using a log amplification of the fluorescent signal. Non-stained cells were used as an auto fluorescence control. Data were expressed as mean fluorescence intensity.

2.8. Protein, chlorophyll and carotenoid analyses

Protein content was determined following the Bradford protein assay [12]. Cellular chlorophyll and carotenoid isolations were achieved with the methanol extraction method; chlorophyll and carotenoid contents were calculated according to the formula for methanol extraction described by Lichtenthaler [13].

2.9. GC-MS fatty acid profile determination

1% (V/V) dilutions of each oil sample dissolved in chloroform were subjected to GC/MS analysis by injecting 2 µL sample into a Rtx®-5MS fumed silica column (30 m, 0.25 mm ID, 0.10 µm.df). The injection temperature was set to 200 °C and column temperature program began at 50 °C for 5 min. Column temperature was further increased by 15 °C/min to 300 °C where it was kept for 5 min to complete the run. The GC was coupled to a quadrupole mass spectrometer, the interface temperature and ion source temperature was adjusted to 280 °C. MS analysis was started at 2.5 min which was set to solvent cut time.

3. Results and discussion

3.1. Growth analysis of S. obliquus cultivated under different nitrogen sources

Utilization of different nitrogen sources and their effects on cell growth, lipid accumulation and fatty acid profile of green alga *S. obliquus* were analyzed. Previous studies have demonstrated the influence of altered nutrient conditions on biomass and lipid productivity in various algae species [14–16]. According to the previous findings, the strategy of altering nutrient conditions especially limitations of nitrogen and phosphorus may result in increased lipid accumulation; however fatty acid profiles generally were not determined under experimented conditions in most studies. Green alga *S. obliquus* was selected for this study because several studies have demonstrated the alga as a promising species in biodiesel production [10, 11]. This study showed that different nitrogen source utilization results in altered lipid accumulation besides significantly changed fatty acid profile in *S. obliquus* suggesting that altered nutrient conditions might be also one of the important factors for deciding biodiesel quality.

All tested nitrogen sources were found to be successfully promoting growth of *S. obliquus* as shown in Fig. 1. According to the results, specific growth rates were found to be 0.34, 0.32, and 0.37 for $NaNO_3$, NH_4Cl and urea experimental groups. The best biomass concentration was found under urea regime which was 659 mg/L, compared with 272 mg/L and 524 mg/L for NH_4Cl and $NaNO_3$ regimes, respectively. Doubling times were found as 2.06, 2.14, and 1.90. Divisions per day of different groups were found as 0.49, 0.47, and 0.53 in the order of $NaNO_3$, NH_4Cl and urea regimes.

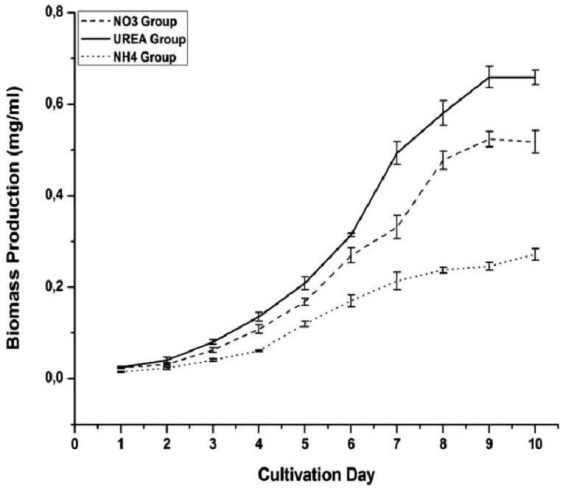

Fig. 1. Growth analysis of *S. obliquus* cultivated under different nitrogen regimes. Data are presented as the mean value of triplicates with ± error bars.

3.2. Gravimetric, fluorometric and flow-cytometric lipid content analysis of S. obliquus cultivated under different nitrogen sources

Nitrogen depletion is well-known factor which increases lipid production in many algal species shown in various studies [10, 14–16], however relatively low information has been presented about different nitrogen source utilization and the effects on cellular metabolic response. Therefore, we focused specifically on the influence of different nitrogen source utilization.

Microalgae biomass was harvested after ten days of cultivation and subjected to lipid extraction for gravimetric lipid content analysis. The biomass obtained from NH_4Cl group has the highest lipid content, 25.6%, followed by 19.7%, and 11.04% for $NaNO_3$ and urea medium, respectively, seen in Fig. 2. The nile-red fluorometric assay was previously shown to be a powerful method for comparing lipid contents of many tissues and various cells of different organisms, especially algal species [17–20]. After ten days of cultivation, cells were harvested, stained with nile-red fluorometric dye, and subjected to 96-well

microplate fluorometric assay. In accordance with the gravimetric lipid content measurements, results obtained from fluorometric assays showed similar results seen also in Fig. 2. According to the results, gravimetric and fluorometric methods are significantly correlated and support each other with significant R^2 values.

According to Li et al., *N. oleoabundans* has the best lipid accumulation under $NaNO_3$ regime [9]. Lin et al. [14] demonstrated that *S. rubescens* like microalgae accumulated more fatty acid methyl esters (FAME) under $(NH_4)_2CO_3$-N regime. Moreover, $NaNO_3$ was not the most favorable nitrogen source both in biomass and FAME productivity [14]. Under the light of previous studies, it can be discussed that each algal species may utilize different nitrogen source and demonstrate different lipid and biomass productivities besides different fatty acid profiles under different nitrogen regimes. Here, it is reported that a slight decrease in biomass productivity of the NH_4Cl group was found and lipid productivity of *S. obliquus* was demonstrated to be higher than that of other tested nitrogen sources.

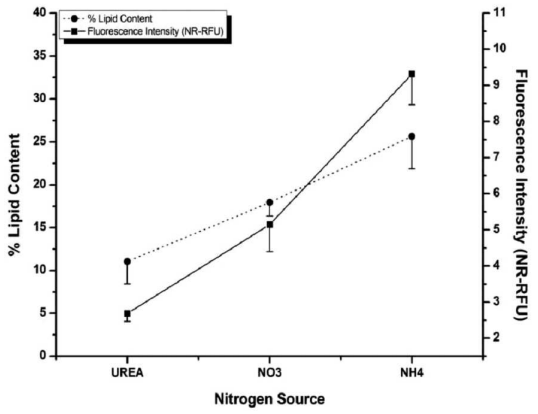

Fig. 3. Lipid content analysis of *S. obliquus* cultivated under different nitrogen regimes. Data represent the % lipid contents of *S. obliquus* cultivated with different nitrogen sources and 96-well microplate fluorometric nile-red staining analysis represented as relative fluorescent units. Data are presented as the mean value of triplicates with ± error bars.

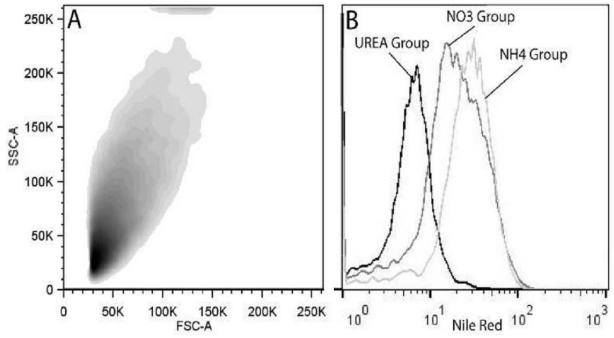

Fig. 2. Representative data of flow-cytometric lipid content analysis of *S. obliquus* cultivated under different nitrogen regimes. (A) Forward scatter (FSC) histogram of analyzed *S. obliquus* cells 11% of total particles were determined as viable cells based on their cellular size and granulation and further analyzed in terms of their lipid contents. (B) Histogram of flow-cytometric analysis of lipid contents of *S. obliquus* cells cultivated with 5 mM urea (black line), $NaNO_3$ (gray line), and NH_4Cl (light gray line). Experiments were done in triplicates for each experimental group.

The use of flow-cytometric analysis for determination of lipid accumulations in algae cells were successfully shown in previous studies [21]. Because gravimetric and microplate fluorometric analyses could not give information about the single cell level, flow-cytometric analysis was also conducted for determination of the response of single cells. Results shown in Fig. 3 NH_4Cl regime resulted in increased lipid accumulation compared to other tested nitrogen sources, $NaNO_3$ and urea.

For a successful lipid comparison in algae cells, different methodologies should be used in combinatory manner and support to each other. Each of the three different methods used in this study has their own advantages and disadvantages. For the gravimetric method, quantifying cellular lipid content is mostly time consuming process. It requires the extraction of lipid from a high amount of cellular biomass using dangerous organic solvents such as hexane or chloroform, evaporation of the solvents, and determination of the amount of lipid by weighing the dried extract, which might not be precise due to technical bottlenecks.

Fluorometric measurements may be more robust for determination of cellular lipid content if a suitable lipid standard is able to be used. Nevertheless, optimization of lipid standard is difficult, and even if standard curves are of good quality, the fluorometric technique would not give the precise *in vivo* lipid content of cells. If lipids were extracted from cells and allowed to form micelles in aqueous solutions, lipid standards would give more accurate results with fluorometric methods. Some studies have proposed techniques using lipid standards for fluorometric algal lipid content analyses [20].

What is more, regarding the 96-well microplate fluorometric method, it is very important to avoid cell clustering due to stress conditions, as the difference between emissions from single and clustered cells would lead to misleading measurements. Also there is no way to separate living and dead cells during measurements. For this reason, flow-cytometric analysis has advantages over other techniques such as the possibility of characterizing and separating subpopulations, distinguishing between living and dead cells during measurements, and the ability to measure at the single cell level and avoid misreading of clustered cells. However, flow-cytometry might not be suitable for all algal species, especially not for filamentous species which do not have round and well-shaped cellular morphology; moreover, the doubling effect must be avoided during measurements for correct readings.

In order to prevent possible misleading, results from the weaknesses were mentioned for each method. In this study, gravimetric, fluorometric and flow-cytometric techniques were all used for better and precise comparison of cellular responses of green alga *S. obliquus* in terms of lipid accumulation. In addition, *S. obliquus* cells were successfully subjected to flow-cytometric nile-red analysis because of its suitable, unicellular morphological characteristics. Such features easing high-throughput analysis methods are crucial for fast and reliable selection of algal species/strains for biodiesel production.

3.3. Pigment and protein analysis of S. obliquus *cultivated under different nitrogen sources*

It is known that nitrogen conditions affect all metabolic processes including lipid, protein, and pigment synthesis in algae species [22]. From this rationale, chlorophyll, carotenoid, and protein analyses were also conducted to better understand the effects of different nitrogen sources on important metabolic processes shown in Fig. 4. According to the results, chlorophyll a, chlorophyll b, and total carotenoid contents for the $NaNO_3$ group were found to be 0.30–0.19–0.04 µg/gfw, respectively, the same pigment contents for the NH_4Cl group was found to be 0.23–0.13–0.05 µg/gfw, and finally for the urea group chlorophyll a-b and carotenoid contents were determined as 0.41–0.24–0.04 µg/gfw in the same manner. Total protein contents were found to be 0.28 µg/µL, 0.21 µg/µL and 0.33 µg/µL corresponding to 53%, 36% and 60% in terms of cellular protein contents for $NaNO_3$, NH_4Cl and urea, respectively.

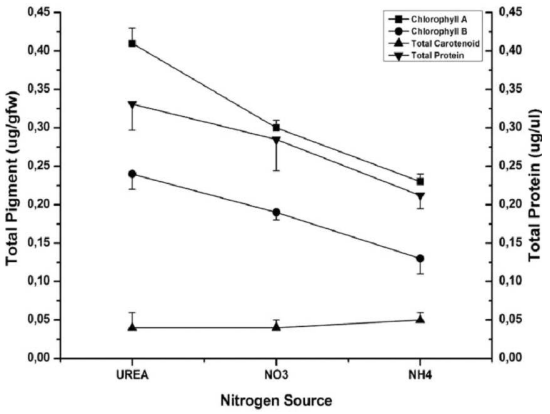

Fig. 4. Protein and pigment content analysis of *S. obliquus* cultivated under different nitrogen regimes. Data present the mean value of each triplicate represented as µg/gfw (microgram per gram fresh weight) and µg/µl with ± error bars for pigments and total protein contents, respectively.

Algae species can utilize different inorganic and organic substances as nitrogen sources [23, 24]. Therefore, green alga *S. obliquus* also could successfully use $NaNO_3$, NH_4Cl and urea. Chlorophyll a-b and protein contents shown in Fig. 4 demonstrate the successful utilization of all tested nitrogen sources. Total carotenoid synthesis was found slightly increased upon increased level of lipid accumulation in NH_4Cl group compared to other tested nitrogen sources which may indicate a crosstalk between carotenoid and lipid synthesis.

3.4. GC-MS analysis of S. obliquus *cultivated under different nitrogen sources*

Polyunsaturated fatty acids containing four or more double bonds are common in microalgae lipids. These bonds can be oxidized easily during storage, reducing the acceptability of microalgae oil for production of biodiesel. In contrast, Mandal et al. demonstrated that the biodiesel from *S. obliquus* contains mainly saturated and mono-unsaturated fatty acids (≈75% of the total fatty acyl methyl esters), which grants that oil obtained from *S. obliquus* has higher oxidative stability [11]. Thus, *S. obliquus* should be considered as a potential organism for biodiesel production. Another study concluded that if the purpose is to produce biodiesel from one algal species, *S. obliquus* presents the most adequate fatty acid profile [25]. On the basis of the previous studies, it was characterized that the fatty acid profiles of *S. obliquus* cultivated with different nitrogen sources by using GC-MS. For each oil sample, individual fatty acid percentages

TABLE I

Fatty acid profiles of *S. obliquus* cultivated with $NaNO_3$, NH_4Cl and urea as nitrogen sources. Data are presented as the mean value of triplicates with ± errors.

Type	fraction [%]	Total [%]
$NaNO_3$		
C16+0	5.54±0.2	
C16+1	1.70±0.05	
C17+0	5.73±0.09	saturated
C18+0	34.32±1	73.75±2.06
C19+0	0.95±0.07	
C19+1	9.03±0.6	unsaturated
C19+2	10.60±0.5	26.25±1.17
C20+0	27.19±0.7	
C22+1	4.90±0.02	
NH_4Cl		
C17+0	9.50±0.5	
C17+3	0.27±0.03	saturated
C18+0	39.18±0.2	76.98±1.4
C18+2	1.50±0.09	
C19+2	13.28±2	unsaturated
C19+3	13.32±0.1	23.02±2.22
C20+0	30.22±0.7	
Urea		
C17+0	7.72±0.3	
C17+3	0.47±0.01	saturated
C18+0	36.28±1.2	72.53±3.1
C18+2	2.66±0.09	
C19+2	12.49±1	unsaturated
C19+3	11.82±0.9	27.47±2
C20+0	28.52±1.6	

were estimated from total integrated peak areas. Irrelevant peaks were not included to the calculations. As shown in Table I, total saturated fatty acid percentages are much higher than those of unsaturated fatty acids in each experimental group which is supported by previous studies. This result indicates that oil extracted from *S. obliquus* has higher oxidative stability. Data also indicates that fatty acid profiles are significantly divergent under different nitrogen regimes, especially NH_4Cl group demonstrates slightly different profile compared to $NaNO_3$ and urea groups. Last of all C18:0 and C20:0 were found as the most abundant oil types in all samples which demonstrate an appropriate oil characteristic for biodiesel production under all tested nitrogen regimes.

4. Conclusions

S. obliquus is a suitable species for manufacturing biodiesel, and therefore optimizing the cultivation process of this species is a worthwhile activity. The results obtained from this study show that $NaNO_3$, NH_4Cl and urea can be successfully utilized for growth of the green alga *S. obliquus*. NH_4Cl was found to result in increased lipid accumulation compared to alternative nitrogen sources $NaNO_3$ and urea under investigated conditions. Furthermore, fatty acid profiles show differences under different nitrogen regimes especially in NH_4Cl regime. In addition, flow-cytometric and microplate nilered fluorescent assays for the determination of lipid contents of algae were verified to be high-throughput, fast and reliable methods. For industrial applications, such studies could be scaled-up and repeated in photo bioreactors and open-cultivation conditions. Other nitrogen sources might be also examined so as to see the differences in growth and lipid accumulation responses.

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