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Research Article

Lipid Enhancement in Oleaginous *Nannochloropsis* sp. under Nitrate Limitation for Future Bioenergy Production

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Microalgae possess high oil content, exhibit rapid growth rates and biomass productivity, and leave a minimal environmental footprint, making them highly attractive for biofuel applications. Consequently, this present study is aimed at evaluating the impact of nitrogen starvation on the marine microalgae *Nannochloropsis* sp. to enhance lipid accumulation. The microalga culture was grown under an irradiance of $120\,\mu$mol photon m^{-2} s^{-1}, temperature $25 \pm 1^{\circ}$C, continuous air-CO$_2$, and with different concentrations of sodium nitrites over a 14-day cultivation period. From the results, it was found that the growth of the strain decreased under limited nitrogen conditions. After harvesting, the cells in the supernatant were regenerated by cultivating in double concentration of media. The results showed that on the 12th day, the highest lipid content was achieved, reaching 68.0 ± 2.3 wt.% of the dry weight under nitrogen limitation. The composition of the obtained lipid consisted of 18.0 wt.% saturated fatty acids, 72.38 wt.% monounsaturated fatty acids, and 8.95 wt.% polyunsaturated fatty acids. The lipid composition was dominated by monounsaturated oleic acid (70.97 ± 4.6 wt.%), saturated palmitic acid (13.68 ± 1.0 wt.%), and polyunsaturated linoleic acid (8.41 ± 0.4 wt.%). The findings of this study demonstrated that limiting nitrogen availability enhanced lipid accumulation in *Nannochloropsis* sp. Despite experiencing inhibited growth, this microalga species shows great potential for the large-scale cultivation of lipids and essential fatty acids. These results contribute to understanding the relationship between nitrogen availability and lipid metabolism in microalgae, providing valuable insights for optimizing lipid production in the context of biofuel and biotechnology applications.

1. Introduction

Microalgae offer a significant advantage over edible crops by mitigating the adverse effects of human food consumption and providing a sustainable and carbon-neutral alternative to petroleum fuels. While biodiesel derived from edible crops falls short in meeting the current demand for transportation fuels due to direct competition with food resources, microalgae present a promising solution as they can be grown on nonarable land [1, 2]. Microalgal biochemical compositions and growth are greatly influenced by the availability of nutrients such as nitrogen and phosphorus,

by which the limitation and depletion of nitrogen in the cultivation media can limit its growth with concurrent increases in lipid accumulation [3]. *Nannochloropsis* sp. is capable of accumulating triacylglycerols when exposed to nutrient limitation and responses to carbon dioxide fluctuation [4] particularly nitrate (N) and is therefore considered a promising organism for essential oil [5]. Moreover, *Nannochloropsis* sp. exhibits high biomass and lipid production potential under various stress conditions [6] and has been shown to be capable of growth in palm oil mill effluent [7–9].

Various species of *Nannochloropsis* have been extensively studied for lipid production due to their high lipid content of 37-64% [8, 10–14]. The interconnectedness between the environment and the activity of microalgae and cyanobacteria is crucial for environmental sustainability. Over the years, algae and their immediate descendants have played a significant role in sequestering vast amounts of atmospheric carbon in fossil deposits [1, 15]. Microalgae removed huge tonnages of carbon from the atmosphere to sequester it in mineral deposits and fossilized carbon [16]. These renewable biomass resources serve as valuable inputs to produce various goods and services such as biofuel production, aquafeed, and high-value products [17, 18]. A continuous flow process is developed for the recovery of the biomass of the marine microalga *Nannochloropsis salina* using flocculation methods and recovering more than 85% of the biomass [19]. *Nannochloropsis* species have drawn attention in lipid and biofuel research, due to their rapid growth in open ponds or photobioreactors and their ability to grow in seawater with high lipid yields (up to 60% of dry weight) [20]. Raceway ponds can also produce large-scale production of algal biomass [21]. Several microalga strains, including *Chlorella vulgaris*, *Spirulina* sp., *Chlamydomonas reinhardtii*, *Dunaliella* sp., *Scenedesmus obliquus*, and *Coelastrella* sp., could accumulate significant amounts of protein, lipids, and carbohydrates, making them ideal feedstocks for the production of biofuel [22].

In a continuous culture system operating at a steady state with a consistent biomass concentration, it is crucial for the dilution rate to match the specific growth rate [2]. *Nannochloropsis* species can accumulate more than 48% of their biomass as lipids in nitrogen-limiting conditions [13, 23]. This enhancement and lipid accumulation is consistent with predictions of the metabolic model of the microalgae [23–26]. Therefore, the focus of this study is to investigate the ability of *Nannochloropsis* sp. which can accumulate lipid components and their survivability culture under different concentrations of nitrogen environment. Additionally, an unconventional approach was carried out where the supernatants were subjected to repleted nitrogen media and then provided 24 hours of continuous light and aeration to initiate new growth. Besides that, the composition of lipids was analyzed and quantified for biodiesel production through gas chromatography-mass spectroscopy.

2. Materials and Methods

2.1. Strain and Culture Medium. *Nannochloropsis* sp. was obtained from the Algae Culture Collection Center and Laboratory, Faculty of Industrial Sciences and Technology, Universiti Malaysia Pahang Al-Sultan Abdullah, Pahang, Malaysia. The stock culture was maintained under sterile condition in 1 L Erlenmeyer flask with 500 mL standard f/2 medium with composition of $NaNO_3$ (8.82×10^{-4} M), $NaH_2PO_4H_2O$ (3.62×10^{-5} M), Na_2CO_3 (1.06×10^{-4} M), $FeCl_3 \cdot 6(H_2O)$ (1.17×10^{-5} M), $Na_2(EDTA)$ $2(H_2O)$ (1.17×10^{-5} M), $CuSO_4 \cdot 5(H_2O)$ (3.93×10^{-8} M), Na_2MoO_4 (2.60×10^{-8} M), $ZnSO_4 \cdot 7(H_2O)$ (7.65×10^{-8} M), $CoCl_2 \cdot 6(H_2O)$ (4.20×10^{-8} M), $MnCl_2 \cdot 4(H_2O)$ (9.10×10^{-7} M), thiamine HCI (2.96×10^{-7} M), biotin (2.05×10^{-9} M), and cyanocobalamin (3.69×10^{-10} M). The culture was grown under a light intensity of $120 \, \mu mol \, m^{-2} \, s^{-1}$ and with a photoperiod of 16 : 8 h light-to-dark cycle, optimum temperature ($25 \pm 1°C$), and provided agitation with filtered air through an air pump to prevent sediment. For nitrate starvation experiments, various concentrations of $NaNO_3$ were used, in addition to other standard compounds (0 M, 4.41×10^{-4}, 8.82×10^{-4}, and 1.76×10^{-3} M for zero nitrate, depleted nitrate, standard nitrate, and repleted nitrate, respectively).

2.2. Experimental Conditions. To determine the effect of nitrogen concentration on the growth, biomass, and lipid productivity of *Nannochloropsis* sp., cultures were grown for 14 days in batch mode. The f/2 medium was prepared with different concentrations of nitrate, for repleted experiments 1.76×10^{-3} M (double the amount as standard), for standard experiments 8.82×10^{-4} M, for limited experiments 4.41×10^{-4} M (half the amount as standard), and for N-free experiments zero nitrate, while other nutrients have remained in the concentration as stated above. The stock *Nannochloropsis* sp. culture was diluted with distilled water before being inoculated with each specific formulated f/2 medium at a 1 : 3 ratio ($v \, v^{-1}$) for the experiment. The initial cell concentration of each treatment was approximately 2.5×10^5 microalgal cells per milliliter. The environmental conditions such as light, agitation, and temperature were provided for the culture same as mentioned above.

2.3. Growth Measurement. Cell counts were determined using a hemocytometer, while optical density values at a wavelength of 680 nm were measured every two days using a Genesis UV-VIS spectrophotometer. After a 14-day period, the biomass was harvested by centrifugation at $2500 \times g$ for 10 minutes. The wet biomass was then rinsed with distilled water and subjected to another round of centrifugation. Before and after drying the biomass at 70°C for 24 hours, its weight was measured to ensure a constant weight. After the harvesting process, an unconventional approach was carried out, in which the supernatants were subjected to repleted nitrogen media and then provided 24 hours of continuous light and aeration to initiate the growth cycle of *Nannochloropsis* sp. The next growth of the cycle was monitored and studied to regenerate cells' growth rate for continuous biomass production. In this approach, continuous cycles of cultivation can be carried out, with less biomass wastage.

2.4. Lipid Extraction.

Lipids were extracted using methods described in Bligh and Dyer [27] and Smedes and Thomasen assisted with an ultrasound technique [28–30]. Dry biomass was weighed and dissolved in 50 mL of hexane (1 : 8, wv^{-1}) solution in a Falcon tube. The mixture was vortexed for 30 sec. The tube was placed in a water bath at $70 \pm 2°C$ for 60 min to disrupt the cells. The mixture was then centrifuged at $3293 \times g$, $10°C$ for 5 min, and the supernatant was collected. This step was repeated by adding 50 mL of hexane solvent to the biomass until the solution became colorless [29]. The supernatant containing the extracted lipid was collected after filtering through $0.45 \mu m$ PTFE disk filters. The hexane solvent was separated from the mixture, and the extract was evaporated at $50°C$, $269 \times g$ for 15 min by using a rotary evaporator. The separated lipid was weighed and collected in 2 mL of the centrifuged tube. The extracted lipid was transesterified by using hexane/methanolic-KOH (2 : 1, 1%) [31]. The mixture was heated at $70°C$ for 10 min and vortex for 30 sec. Two phases were formed in the mixture and the top phase by pipetting in a vial [32].

2.5. Fatty Acid Methyl Ester Analysis.

Gas chromatography-mass spectrometry was used to detect fatty acid methyl esters content. Fatty acid methyl esters (FAMEs) were measured and analyzed in an Agilent 7890A gas chromatography (GC) system equipped with a capillary mega wax MS column (30 m length $\times$ 0.32 mm diameter $\times$ 0.50 μm film thickness). The mass spectrometer detector used helium as the carrier gas at $1.0 \, mL \, min^{-1}$. The oven is programmed with the following time–temperature program: $190°C$ (2 min), 190–$230°C$ ($5°C \, min^{-1}$), and $230°C$ (2 min). The mass spectra were recorded at 70 eV. Mass range was 40-250 mz^{-1}. About 40 mg of fatty acid methyl ester sample was weighed in the vial; $1000 \mu L$ of internal standard dilute on octane and 15 mg mL^{-1} concentration were added. In homogenous mixture form, sample (1 μL) was injected into GC. The inlet temperature was maintained at $230°C$ with a split ratio of 50 : 1 selected. The individual peaks of FAMEs were identified through the chromatographic peaks by comparing retention times with those of standards [33].

2.6. Statistical Analysis.

All the experiments were carried out in triplicate ($n = 3$), and the observed standard deviations were reported accordingly.

3. Results and Discussions

3.1. Effect of Nitrate Concentration.

The effect of varying nitrate concentration on the growth of Nannochloropsis sp. (Figure 1) monitored through cell counts and OD at 680 nm is illustrated in Figure 2. The growth of cells over the day period was higher in the standard concentration of f/2 media when compared to the other nitrate treatments. The growth curve exhibited a lag phase on the first day, followed by an exponential phase starting from the second day, during which cell multiplication was rapid until day 12. Subsequently, the growth entered a stationary phase and showed a rapid decline on the 13th and 14th days in both the standard and repleted medium cultures. Both cell counts and optical density (OD) measurements confirmed that the standard nitrate concentration resulted in the highest growth, followed by repleted nitrate, limited nitrate, and finally nitrate-free media.

On the other hand, for a continuous cycle of cultivation, the supernatant was aerated and kept at the same temperature and light environment. The result found that the growth of the cell was still present, and it was boosted by adding double the concentration of media. The minimum amount of nutrients present in the supernatant initiates the growth cell. However, the addition of media increases biomass productivity at optimum conditions. This helps to minimize the usage of water reduce the loss of nutrients and reduce the cost of cultivation for biomass production.

Based on Figure 3, the highest dry biomass yield was obtained from standard media (1234 ± 35.6 mg L^{-1}) followed by repleted-N (1011 ± 32.1 mg L^{-1}), limited-N (850 ± 25.4 mg L^{-1}), and free-N (765 ± 35.6 mg L^{-1}) media after 14-day cultivation period. On the other hand, the lipid productivity trend differed in the order of limited-N (68.0 ± 2.3 wt.%), then free-N (62.3 ± 2.9 wt.%), standard-N ($51.2 + 2.5$ wt.%), and finally repleted-N (48.1 ± 2.8 wt.%). The optimum productivity of biomass and lipids is shown in Table 1. The results obtained in this study can be compared with previous studies mentioned in Table 2. Nannochloropsis sp. showed the least growth and biomass production in limited-N media, likely due to a reduction in their light harvesting efficiency and energy transduction, leading to decreased photosynthetic efficacy. Nitrogen deficiency in microalgae causes the loss of their photosynthetic efficacy of antenna, chlorophyll a, and photosystem II completely [34]. In response to nitrogen deficiency, microalgae can boost light intake and divert carbon metabolism toward lipid synthesis and carbohydrate storage. Intracellular nitrogen is reassigned through reducing biosynthetic pathways under sustained low nitrogen stress. Under nitrogen deficiency, protein synthesis and cell development both slowed [35, 36]. A similar result mentioned that the cellular density reduced when the NaNO$_3$ was reduced, where the photosynthetic efficiency was inhibited due to the response of microalgae to nitrogen limitation. The reason behind this condition is the relationship between pigment synthesis and nitrogen metabolism. However, increasing the NaNO$_3$ enhances the biomass dry weight cell of Chlorella sp. [37]. Nitrogen in the forms of nitrate, nitrite, urea, and ammonium can be assimilated by microalgae. Green microalgae favor ammonium because they can easily use it from a metabolic standpoint. Indeed, a series of enzymatic pathways is required to transform other forms [38]. A study [39] investigated the nitrogen limitation on Nannochloropsis oceanica, Isochrysis aff. galbana clone T-Iso, Rhodomonas baltica, and Phaeodactylum tricornutum. As a result of response to severe nitrogen limitation, all four species accumulated lipids, primarily in the form of TAG. In moderate nitrogen limitation, N. oceanica accumulated 51% of the dry weight as a lipid, up to 87% of the fatty acids in TAG. The only species where the fraction of polyunsaturated fatty acids, particularly the fraction of docosahexaenoic acid, was found was Isochrysis aff. galbana clone T-Iso. Another study by [40] found that under
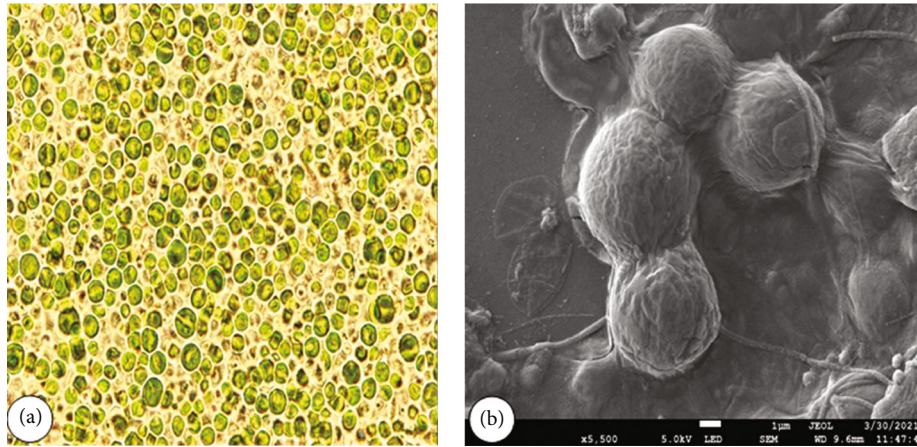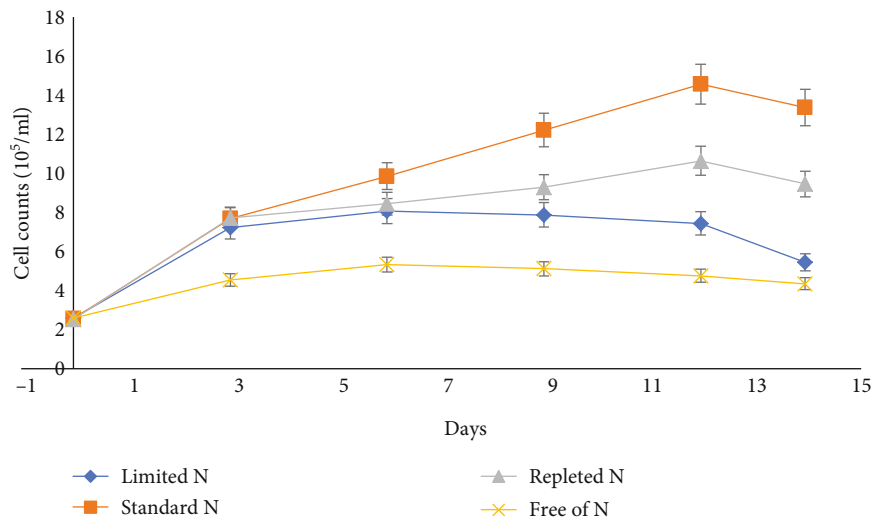
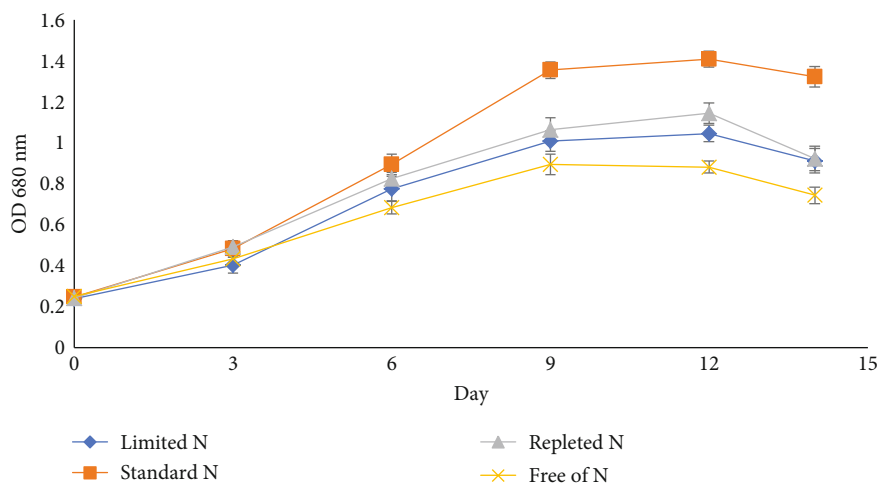FIGURE 1: *Nannochloropsis* sp. photomicrographs under a fluorescence microscope (×1000) (a) and image under a field emission scanning electron microscope (×5500) (b).

(a)

(b)

FIGURE 2: Growth curve (a) by cell counts and optical density (at 680 nm) (b) of *Nannochloropsis* sp. cultivated under various concentrations of nitrate.
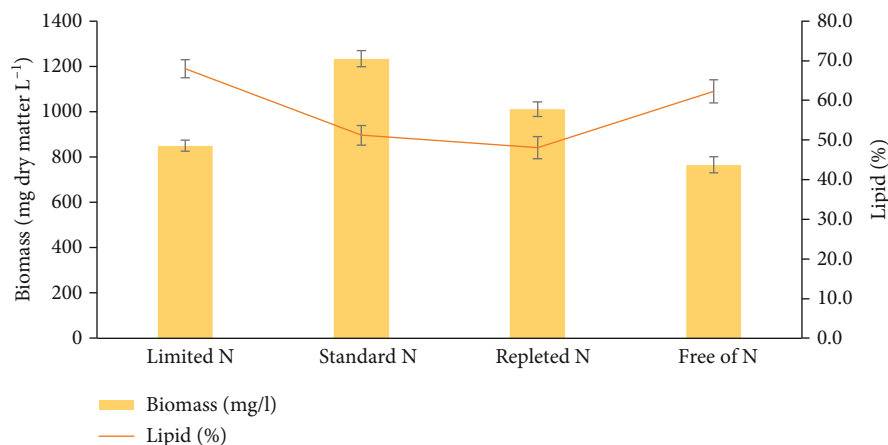
FIGURE 3: The final dry biomass (mg L^{-1}) and lipid productivity (%) of *Nannochloropsis* sp.

TABLE 1: Biomass and lipid outputs of *Nannochloropsis* sp. under N-starvation (4.41×10^{-4} M) condition. Conditions: light intensity 120 μmol photon m^{-2} s^{-1}, temperature $25 \pm 2°$C, and standard f/2 media with shaking 80 rpm.

Parameter	Value
Biomass concentration (mg L^{-1})	850.3 ± 25.4
Biomass productivity (mg L^{-1} d^{-1})	60.73 ± 0.9
Lipid in biomass (%, $w\,w^{-1}$)	68.0 ± 2.3

lower light and limited nitrogen conditions, the starch content of *Pseudochlorococcum* increased in the first 4 days and then began to decrease slightly on day 10. However, neutral lipid content begins to accumulate at day 10. Under high light with limited nitrogen, *Pseudochlorococcum* showed rapid growth and high nitrate reductase activity. Therefore, starch acts as the main carbon storage which can indicate that *Pseudochlorococcum* applies starch as the primary carbon storage product when it has grown under sufficient nitrogen conditions [40]. Therefore, the rate at which nitrogen (supplied as NaNO$_3$) is consumed during the synthesis of starch and lipids was monitored by measuring the concentration of nitrogen in the growth medium and the activity of nitrate reductase because the ability to take up and assimilate nitrogen frequently determines algal growth and basal metabolism.

3.2. Transesterification and Methyl Ester Analysis. Figure 4 depicts the GC chromatogram of transesterified lipid that is extracted from *Nannochloropsis* sp. The extraction and transesterification processes showed high efficiency and yielded the successful recovery of fatty acids. Microalgae have emerged as a possible source of highly unsaturated fatty acids, and *Nannochloropsis* sp. is observed as one of the most promising microalgae due to its fatty acid profile. Under nitrate limitation, the highest fatty acid content (oleic acid) from the lower biomass specific was obtained. The current work demonstrates that the fatty acyl compositions of the various major lipid classes are also distinctive. Fatty acid

methyl esters are the main component of biodiesel, a sustainable kind of transportation fuel. The size distribution and degree of unsaturation in FAME profiles, in particular, may have a major impact on the physical and chemical characteristics of biodiesel [45].

Table 3 compares the result of detected fatty acids of C16:0, C18:0, C20:0, C16:1, C18:1, C20:1, C18:2, and C18:3 under various concentrations of nitrite media. Oleic acid (C18:1, $70.97 \pm 4.6\%$), palmitic fatty acid (C16:0, $13.68 \pm 1.0\%$), and followed by linoleic acid (C18:2, $8.41 \pm 0.4\%$) are the most dominant fatty acids in each subgroup of fatty acids. Wang et al. [39] found that high percentage of oleic acid (C18:1) and other dominating fatty acids such as 14:0, 16:0, 16:1, and 20:5 in the fatty acid profile of *Nannochloropsis oceanica*. Hindarso et al. [49] reported that methyl palmitic 35.21% and methyl γ 15.98% weight as the highest composition of methyl ester from the *Nannochloropsis*. Since oleic acid (39.51%) and palmitoleic acid (31.24%) are the dominant components in the extracted oil, these unsaturated fatty acids will improve the cold performance of biodiesel. In turn, the produced biodiesel will be comparatively more suitable for export to cold climate regions. According to earlier research, the best biodiesel would have relatively low quantities of saturated fatty acids and polyunsaturated fatty acids to reduce oxidative stability and cold flow issues [45].

Biodiesel quantification was done according to EN14103 in which methyl heptadecanoate (C$_{17}$) was used as an internal standard. Peaks of methyl esters were identified by comparing them to their respective standards.

$$\text{Methyl ester content (wt.\%)} = \frac{(\Sigma Ai) - A_{\text{IS}}}{A_{\text{IS}}} \times \frac{C_{\text{IS}} - V_{\text{IS}}}{m} \times 100,$$
(1)

where ΣAi represents the sum of methyl ester peak area, A_{IS} is the area of internal standard, C_{IS} is the concentration of the internal standard in heptane (mg/mL), V_{IS} is the volume of internal standard (mL), and M is the weight of the sample. Accordingly, GC results confirmed that the transesterification reaction has yielded methyl ester conversion of $98.7 \pm$

TABLE 2: The biomass and lipid productivities of various strains of *Nannochloropsis* sp. from previous work.

Species	Maximum biomass (mg L^{-1})	Lipid productivity (mg L^{-1} d^{-1})	References
Nannochloropsis sp.	360	48	This study
Nannochloropsis gaditana	185	185	[41]
Nannochloropsis oculata	497	151	[42]
Nannochloropsis salina	610	228	[43]
Nannochloropsis sp.	440	108	[44]
Nannochloropsis oceanica IMET1	301 ± 0.02	158 ± 0.013	[43]
Nannochloropsis granulate CCMP525	216 ± 0.01	130 ± 0.037	[45]
Nannochloropsis oceanica NIOF15/001	75	28	[46]
Nannochloropsis limnetica	640		
	490 ± 0.01	173	[47]
Nannochloropsis gaditana 1049	540 ± 0.013	289 ± 0.02	[48]
N. oculata NCTU-3	480 ± 0.029	142 ± 0.049	[42]
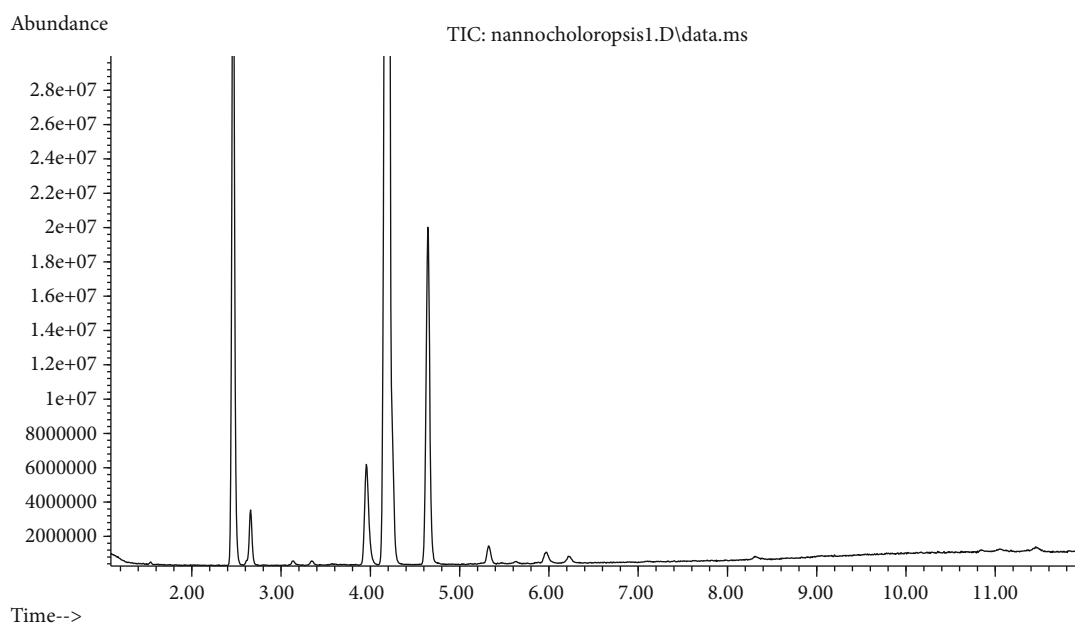
FIGURE 4: GC chromatogram depicting fatty acid composition of *Nannochloropsis* sp.

TABLE 3: Fatty acid quantification (%, $w\,w^{-1}$) of lipid extracted from *Nannochloropsis* sp.

Fatty acids	Amount (%, $w\,w^{-1}$)			
	Nitrogen limited	Nitrogen repleted	Standard nitrogen	Nitrogen free
Saturated fatty acid				
Palmitic acid (C16:0)	13.68 ± 0.14	15.73 ± 0.32	15.44 ± 0.12	14.85 ± 0.22
Stearic acid (C18:0)	4.90 ± 0.30	3.72 ± 0.1	3.34 ± 0.01	3.76 ± 0.32
Eicosanoic acid (C20:0)	0.45 ± 0.21	0.47 ± 0.12	0.76 ± 0.13	0.69 ± 0.01
Monounsaturated fatty acid				
Palmitoleic acid (C16:1)	31.24 ± 0.3	32.75 ± 0.21	33.38 ± 0.35	33.54 ± 0.54
Oleic acid (C18:1)	39.51 ± 0.6	37.81 ± 0.3	35.95 ± 0.25	36.71 ± 0.64
Eicosenoic acid (C20:1)	0.27 ± 0.2	0.22 ± 0.20	0.18 ± 0.12	0.21 ± 0.30
Polyunsaturated fatty acid				
Linoleic acid (C18:2)	9.41 ± 0.4	8.83 ± 0.51	10.53 ± 0.32	9.73 ± 0.43
Linolenic acid (C18:3)	0.54 ± 0.01	0.47 ± 0.21	0.42 ± 0.52	0.51 ± 0.34

Data reported as mean ± SD. $n = 3$ repetitions.

0.4 wt.%, surplus well the minimum requirements of 96.5 wt.% methyl ester content as per EN14103 standard.

4. Conclusion

This work evaluated the locally isolated oleaginous *Nannochloropsis* sp. by imposing nitrate starvation. Results show that N-starvation gives a positive response in accumulating lipids, especially the content of oleic acid (~71 wt.%). After optimization (light intensity $120\,\mu$mol photon m^{-2}s^{-1}, temperature $25 \pm 2°$C, and standard f/2 media with continuous air-CO_2 while shaking at 80 rpm), in 14-day cycle cultivations, the highest lipid in biomass was 68.0 ± 2.3 wt.%, while maximum biomass concentration was 850.3 ± 25.4 mg L^{-1} and biomass productivity stands at 60.73 ± 0.9 mg L^{-1} d^{-1}. The work proves that although lower biomass is produced under N-limitation, lipid content was considerably higher. It was found that nitrogen starvation has enhanced lipid production; however, it reduces the growth rate (total biomass) obtained. In order to further the next cultivation cycle, a higher (three times higher) concentration of standard media needs to be added to the culture for continuous growth. Therefore, *Nannochloropsis* sp. has great potential for biomass for biodiesel production, which facilitates exporting the produced biodiesel to cold climate regions for better cold performance due to higher unsaturated fatty acids.

Data Availability

The research data used to support the findings of this study are included in the article.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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