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Effect of temperature and nitrogen concentration on the growth and lipid content of *Botryococcus braunii* and biodiesel production

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ABSTRACT

The green microalga strain, *Botryococcus braunii* Kützing, was provided by the Culture Collection of Algae at the University of Texas (Austin, USA). The effects of nitrogen and temperature on growth and lipid production were studied for 18 days. Cultures of *B. braunii* were incubated in five different nitrogen concentrations: 1 g L^{-1} (group 1), 0.5 g L^{-1}(group 2), 0.25 g L^{-1} (group 3), 0.125 g L^{-1}(group 4) and free nitrogen (group 5). The highest biomass concentration (0.564±0.200 g L^{-1}) and lipid content (33.31±0.014%) were obtained by cultivation with an initial nitrogen concentration of 0.50 g L^{-1} in these experiments. In free nitrogen, the maximum lipid content was 56.31±0.026% of dry weight, while the biomass concentration was 0.270±0.063 g L^{-1} at 20 °C. The density of the biodiesel obtained from *B. braunii* was calculated as 0.8263 kg.L^{-1}, its viscosity as 4.97 $mm^2.s^{-1}$ at 40 °C and its flash point as 109 °C. According to these results, nitrogen-starved *Botryococcus braunii* increased the production of lipids and could be made more suitable for biodiesel production. This study suggests that it is possible to utilize *Botryococcus braunii* for biodiesel production.

1. Introduction

Microalgae are unicellular photosynthetic organisms that use light energy and carbon dioxide, with higher photosynthetic efficiency than plants, for the production of biomass (Benemann 1997, Miao & Wu 2006). They can be used in different applications, such as biofuel production, purification of wastewater under either autotrophic or mixotrophic conditions, extractions of high added value foods and pharmaceutical products or as food for aquaculture (Spolaore et al. 2006). The lipids from microalgae could be used in different processes for energy exploitation, including simple combustion in a boiler or a diesel engine. However, the best possible use of this oil is certainly its transformation to a biofuel, especially biodiesel (Chisti 2007). Biodiesel is currently produced commercially from plant and animal oils, but not from microalgae. This is likely to change, as several companies are attempting to commercialize microalgal biodiesel. Biodiesel is a proven fuel. Technology for producing and using biodiesel has been known for more than 50 years (Barnwal & Sharma, 2005, Fukuda et al. 2001, Knothe et al. 1997). The idea of using microalgae as a source of fuel is not new, but it is now being taken seriously because of the escalating price of petroleum and, more significantly, the emerging concern about global warming that is associated with burning fosil fuels (Chisti 2007). Microalgae biomass contains approximately 50% of carbon on a dry weight basis, and all the carbon present in the cell is usually derived from carbon dioxide. With the production of 100 tons of microalgae biomass, approximately 180 tons of CO_2 can be disposed of using natural or artificial light. Microalgae can potentially use part of the carbon dioxide from industrial plants for biodiesel production. From this point of view, microalgae can also be seen as simple CO_2 sequestrants for use in plants for greenhouse gas emissions control (Sawayama 1996, Yun et al. 1997).

Given the high productivity of microalgae, justified by the shorter generation time and higher oil content compared to crops (up to 80% on dry weight) (Metting 1996), it is clear that microalgae can contribute to large-scale fuel oils production. A further benefit is that microalgae may also be grown on arid lands unsuitable for conventional agriculture, such as desert areas, or in large reservoirs of saline water (Converti 2009).

Botryococcus braunii is a green, colonial, slow-growing microalga, and it is widespread in freshwater, brackish lakes, reservoirs, and ponds. This species is characterized by an original organization of colonies and an unusual capacity to produce unsaturated long-chain hydrocarbons, reaching contents ranging from 15% to 75% of its dry weight. In addition, it produces extracellular polysaccharides that induce the formation of colonies, the size of which depends on the hydrodynamic stress within the bioreactor (Orpez et al. 2009). It is well known for its high lipid content and has been proposed as a renewable source of biofuel for future energy requirements (Anirban et a. 1982, Dayananda et al. 2007, Wake & Hillen 1981). As a photosynthetic organism, this alga can fix atmospheric CO_2, and burning of algal hydrocarbon is not a net contributor to CO_2, benefiting environmental protection. At present, however, the production of photosynthetic fuels from *B. braunii* is not economically competitive with petroleum-derived fuels (Sawayama et al. 1992).

Several studies have shown that the quantity and quality of lipids within the cell can vary as a result of changes in growth conditions (temperature and light intensity) or nutrient media characteristics (concentration of nitrogen, phosphates, and iron) (Illman et al. 2000, Liu et al. 2008).

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The objective of this work was to investigate the effects of temperature and nitrogen concentration on the growth and lipid content of *Botryococcus braunii*.

2. Materials and Methods

2.1. Microalgal strain and stock culture conditions

A *Botryococcus braunii* Kützing UTEX 572 strain was obtained from the Culture Collection of Algae at the University of Texas at Austin between November 2008 and May 2009. Stock cultures of *B. braunii* were maintained routinely on both liquid and agar slants of soil water extract medium by regular subculturing at 3-week intervals. The basal medium (pH 7.2) consisted of (g L^{-1}): $NaNO_3$, 0.25; $CaCl_2.2H_2O$, 0.025; $MgSO_4.7H_2O$, 0.075; K_2HPO_4, 0.075; KH_2PO_4, 0.175; NaCl, 0.025, and 40 ml soil water extract. Stock cultures were maintained at 25±1 ºC with 1.6±0.2 klux light intensity under a 16:8 light:dark cycle.

2.2. Media and culture conditions

Growth experiments were conducted at different temperatures and concentrations of nitrogen. The experiment was performed in Erlenmeyer flasks of 250 mL capacity, containing 200 mL Soil water extract (SWE) medium. The medium and flasks were sterilized in an autoclave for 15 min at 121 ºC to prevent any contamination during the early stages of growth, and the pH was adjusted before sterilization. Cultures of *B. braunii* were incubated in five different nitrogen concentrations: 1 g L^{-1}, 0.5 g L^{-1}, 0.25 g L^{-1}, 0.125 g L^{-1} and free nitrogen. Three temperatures were used in this experiment: 10, 20 and 30 °C. The temperature and the nitrogen concentration in the medium were selected as the independent variables. The culture flasks were inoculated uniformly at 10% (v/v) inoculum of 18-day-old *B. braunii* culture. The culture was not aerated. The culture medium composition is described in detail in Table 1. All experiments were performed in triplicate.

2.3. Microalgal biomass concentration

The microalgae concentration was determined daily by optical density (OD) measurement at 680 nm by a spectrophotometer, model Boeco S-20 vis (Boeco, Hamburg, Germany). Dry biomass weight was measured by filtration of aliquots on pre-weighted Whatman filters (GF/C). The filtrated algae were rinsed with 10 ml distilled water three times and dried at 100 ºC for 12 h (Lee et al. 1998). Each sample was measured twice, and the average value was used. All experiments were performed in triplicate.

2.4. Harvesting of microalgae

After 18 days of incubation, the algal cells were harvested by centrifugation at 4000 rpm for 10 min; the supernatant was discarded, and the cell pellet was rinsed with distilled water. Then, the pellet was dried at 100 ºC for 12 h (Illman et al. 2000, Lee et al. 1998).

2.5. Lipids extraction

Before the disruption of the algal cells, 5 ml of phosphate buffer (pH 7.4) was added to the algal pellets to minimize side reactions. The bead beater was used for the destruction of the algal cells. After the destruction of the algal cells, the suspensions were transferred to centrifuge tubes, and 6 ml hexan per 1 g dry wt of algal cell was added as a lipid extractant. The extract, containing the algal cells, was centrifuged at 4000 rpm for 5 min. The organic layers were decanted, and the same procedure was repeated with 6 ml of each solvent. The organic solvents were combined, evaporated by an evaporator at 60 ºC, and the total lipids were measured gravimetrically. All experiments were replicated three times.

2.6. Mass cultivation of microalgae

In light of the data obtained from the laboratory work, mass cultivation experiments were done at 20°C and 0.125 g $NaNO_3$ L^{-1} (group 4) in 10 L containers because of that lipid content and biomass concentration were higher than other groups.

2.7. Lipid analysis by GC-MS

The fatty acids compositions were analyzed by GC-MS (PerkinElmer, Turbomass Gold, Mass spectrometer) equipped with FID and an SPB-1 (poly (dimethysiloxane)) capillary column (30 m x 0.32 mm ID x 0.25 µm film thickness). In determination of the fatty acid composition, samples were obtained from group 4 at 20 °C because of that lipid content and biomass concentration were higher than other groups. The column was heated at 100 ºC, and 1 µl algal lipid was injected into the column. The column was held at 180 ºC for 10 min and then ramped to 250 ºC at 3 ºC/min; then, it was held at 240 ºC for 20.7 min. The fatty acids were identified by comparing their fragmentation pattern with authentic standards (Sigma) and with a library (Dayananda et al. 2007).

2.8. Obtaining Biodiesel from Algal Oil

The transesterification method with alkali as catalyst was used in producing biodiesel from algal oil. For this reason, 10 mL of oil extracted from algae was heated up to 60 °C. In a separate container, 0.03 g caustic (potassium hydroxide) was dissolved in 1.5 mL of methanol and the alcohol-caustic mixture was poured on the heated oil. As methanol evaporates under atmospheric pressure at 65 ^{o}C, the transesterification with alkali as catalyst was done closed to atmosphere (Fukuda et al. 2001). The density of the biodiesel obtained from *B. braunii* was measured by a pycnometer, its viscosity by Ostwald viscosimeter and its flash point by a Setaflash flash point measuring device.

2.9. Statistical analysis

Mean values of the biomass concentration and lipid content of three replications and their standard deviations were calculated. Significant differences were determined using an analysis of variance (ANOVA) with 95% confidence (probability limit of $p < 0.05$). Post-hoc comparisons between sample means were tested using Duncan's test. Because the lipid yield was calculated as the average value of lipid content multiplied by the average biomass concentration, it is reported as a single value without standard deviation.

3. Results and discussion

3.1. Effect of nitrogen concentration and different temperatures on growth and lipid content

It is known that the pigmentation, lipid, and hydrocarbon metabolism of *B. braunii* cells change with growth stage (Brown et al. 1969, Aaronson et al. 1983, Wolf et al. 1985). In particular, nitrogen is the most important nutrient for culture of *B. braunii* (Largeau et al. 1980). At the same time, temperature is one of the predominant factors affecting the growth and storage products of algae. Very low temperatures cause a reduction in biomass, while high temperatures cause lipid production (Kalacheva et al. 2002). In this study, $NaNO_3$ was used as nitrogen source. The initial concentrations of $NaNO_3$ were 1 g L^{-1}, 0.5 g L^{-1}, 0.25 g L^{-1}, 0.125 g L^{-1} and free nitrogen, and three different temperatures were used: 10, 20 and 30 °C. After 18 days of culturing *B. braunii* under the conditions of different nitrogen concentration and temperatures, the biomass yield was compared to the control group. As shown in Fig. 1, the *B. braunii* biomass accumulation in different nitrogen concentrations at 10 °C was different. Additionally, the cell

growth with 0.25 g L^{-1} $NaNO_3$ was much greater than that with other nitrogen concentrations, and the growth rates was not significantly affected by nitrogen concentration ($p>0.05$). At the end of culture, the biomass concentration at 1 g L^{-1}, 0.5 g L^{-1}, 0.25 g L^{-1}, 0.125 g L^{-1} and 0 g L^{-1} (free nitrogen) nitrogen concentration were 0.228, 0.252, 0.404, 0.270 and 0.203 g L^{-1}, respectively (Table 2).

The lipid content in microalgae varies from approximately 1-85% of the dry weight (Chisti 2007a, Chisti 2007b,] Rodolfi et al. 2009) and, among other factors, is affected by the nutritional composition of the medium. Lipid accumulation in algae typically occurs during periods of environmental stress, including growth under nutrient-deficient conditions. Lack of nitrogen restricts protein synthesis, and thus, lipid accumulation increases (Converti et al. 2009). Biomass concentration, except contol group, was not significantly affected by nitrogen concentration ($p>0.05$). The highest biomass concentration (0.404±0.142 g L^{-1}) and lipid content (19.09±0.010%) were obtained by cultivation with an initial nitrogen concentration of 0.25 g L^{-1}. At 10 °C, the lipid content (46.90±0.015%) in group 5 was higher compared to the other group while the biomass (0.076±0.017 g L^{-1}) was lower than other group (Fig 1, 4).

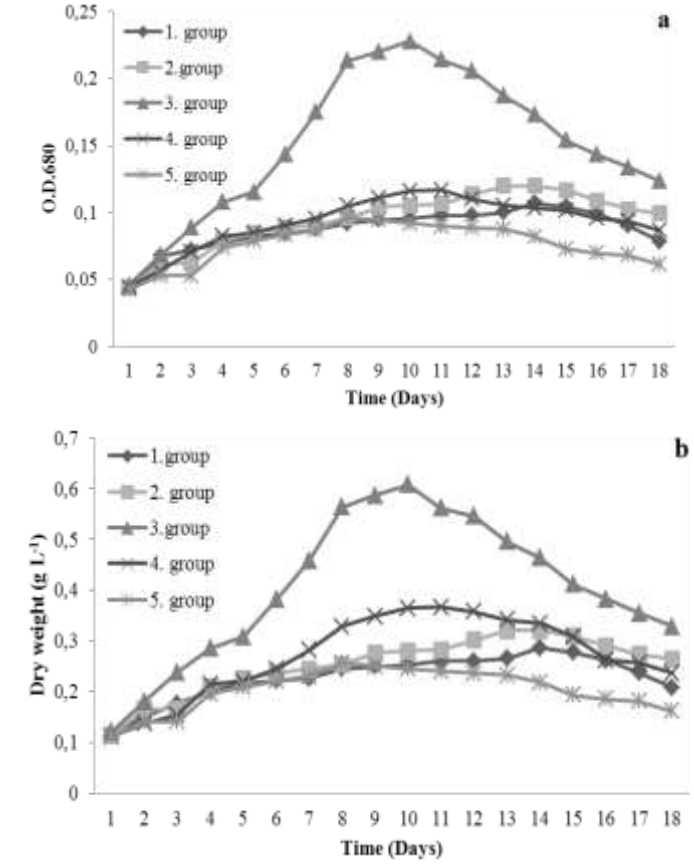

Figure 1. Growth of *B. braunii* in cultures with different nitrate concentrations at 10 °C. The (a) optical density of the *B. braunii* cultures as measured at 680 nm and (b) the dry weight of *B. braunii* g L^{-1}.

The highest biomass concentration (0.564 ±0.200 g L^{-1}) and lipid content (33.31 ± 0.014%) were found by cultivation with an initial nitrogen concentration of 0.50 g L^{-1}. With free nitrogen, the maximum lipid content and biomass concentration were 56.31 ± 0.026% and 0.270 ± 0.063 g L^{-1}, respectively, at 20 °C (Table 3, Fig 2, 4).

In the third part of this study, the highest biomass concentration (0.508 ±0.229 g L^{-1}) and lipid content (26.54±0.014%) were found by cultivation with an initial nitrogen concentration of 0.25 g L^{-1}. The maximum lipid content and biomass concentration were 55.14±0.015% and 0.250 ± 0.064 g L^{-1}, respectively, at 30 °C (Table 4, Fig. 3, 4).

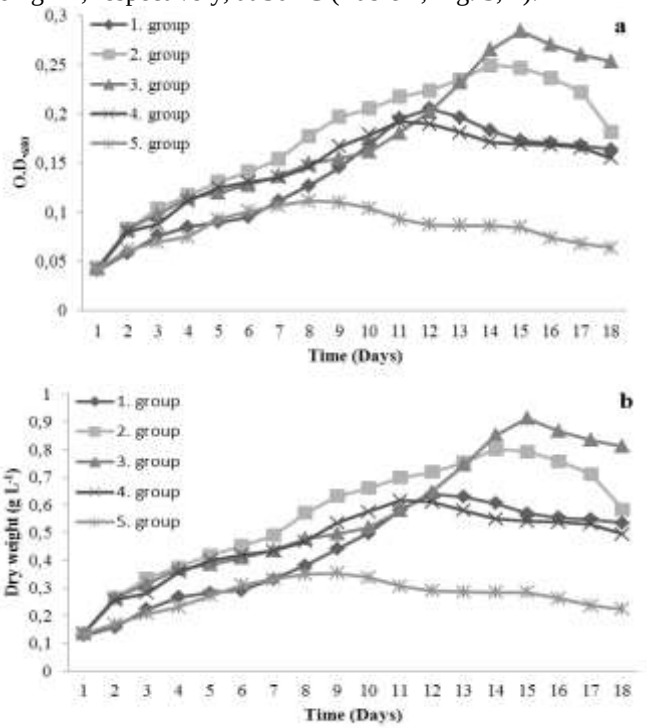

Figure 2. Growth of *B. braunii* in cultures with different nitrate concentrations at 20 °C. The (a) optical density of the *B. braunii* cultures as measured at 680 nm and (b) the dry weight of *B. braunii* g L^{-1}

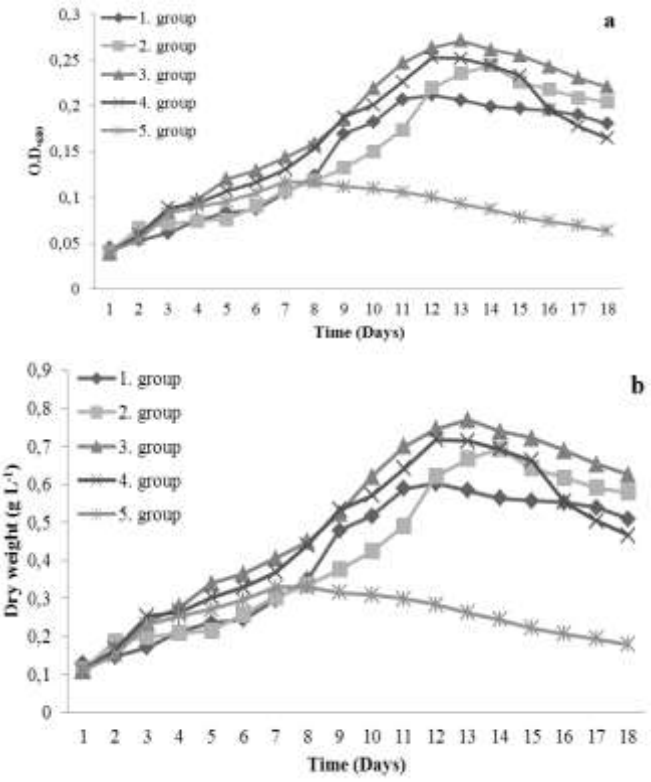

Figure 3. Growth of *B. braunii* in cultures with different nitrate concentrations at 30 °C. The (a) optical density of the *B. braunii* cultures as measured at 680 nm and (b) the dry weight of *B. braunii* g L^{-1}

Table 1. Composition of the culture media

Composition (mg L^{-1})	Experimental groups				
	Group 1	Group 2	Group 3 (control)	Group 4	Group 5
$NaNO_3$	**1000**	**500**	**250**	**125**	**0**
$CaCl_2.2H_2O$	25	25	25	25	25
$MgSO_4.7H_2O$	75	75	75	75	75
K_2HPO_4	75	75	75	75	75
KH_2PO_4	175	175	175	175	175
NaCl	25	25	25	25	25
Soil water extract	40 ml	40 ml	40 ml	40 ml	40 ml

Table 2. The biomass and lipid statistical values of *B. braunii* cultured at 10 °C

Groups	Optical density (680 nm) (N=54)	Dry weight (g) (N=54)	Lipid (dry weight %) (N=3)
1	$0.087^{bc} \pm 0.015$	$0.228^{cd} \pm 0.046$	$10.16^{e} \pm 0.039$
2	$0.094^{b} \pm 0.021$	$0.252^{bc} \pm 0.057$	$18.00^{d} \pm 0.010$
3	$0.152^{a} \pm 0.054$	$0.404^{a} \pm 0.142$	$19.09^{c} \pm 0.010$
4	$0.093^{b} \pm 0.020$	$0.270^{b} \pm 0.130$	$28.37^{b} \pm 0.085$
5	$0.076^{c} \pm 0.017$	$0.203^{d} \pm 0.045$	$46.90^{a} \pm 0.015$

Table 3. The biomass and lipid statistical values of *B.braunii* cultured at 20 °C

Groups	Optical density (680 nm) (N=54)	Dry weight (g) (N=54)	Lipid (dry weight %) (N=3)
1	$0.136^{b} \pm 0.051$	$0.426^{b} \pm 0.169$	$15.84^{e} \pm 0.517$
2	$0.176^{a} \pm 0.062$	$0.564^{a} \pm 0.200$	$33.31^{c} \pm 0.014$
3	$0.174^{a} \pm 0.072$	$0.559^{a} \pm 0.232$	$24.44^{d} \pm 0.088$
4	$0.144^{b} \pm 0.042$	$0.462^{b} \pm 0.136$	$45.30^{b} \pm 0.012$
5	$0.084^{c} \pm 0.019$	$0.270^{c} \pm 0.063$	$56.31^{a} \pm 0.026$

Table 4. The biomass and lipid statistical values of *B.braunii* cultured at 30 °C

Groups	Optical density (680 nm) (N=54)	Dry weight (g) (N=54)	Lipid (dry weight %) (N=3)
1	$0.143^{b} \pm 0.061$	$0.404^{b} \pm 0.174$	$14.56^{e} \pm 0.295$
2	$0.148^{b} \pm 0.069$	$0.418^{b} \pm 0.195$	$22.81^{d} \pm 0.014$
3	$0.179^{a} \pm 0.081$	$0.508^{a} \pm 0.229$	$26.54^{c} \pm 0.014$
4	$0.163^{ab} \pm 0.067$	$0.461^{ab} \pm 0.190$	$35.36^{b} \pm 0.010$
5	$0.089^{c} \pm 0.022$	$0.250^{c} \pm 0.064$	$55.14^{a} \pm 0.015$

Table 5. Fatty acid constituents in *Botryococcus braunii* extract and their proportional amount

Fatty acid	Amount (%)
Behenic acid	0.41
Eicosenoic acid	1.01
Linoleic acid	9.92
Linolenic acid	9.50
Margaric acid	0.28
Methil Cis 11, 14, 17 Ecosatrienoic acid	0.23
Miristic acid	0.31
Oleic acid	59.04
Palmitic acid	16.62
Pentadecanoic acid	0.18
Stearic acid	2.50

B. braunii growth appeared to be affected at 20 °C. At 10 °C, this microalga exhibited a 28% decrease in its dry weight compared to its dry weight at 20 °C. Further increases in temperature (30 °C) led to an abrupt interruption of microalgal growth, and later, the cells died. This result was easily visible because the color of the cells changed from green to brown. At the same time, a sharp drop in the microalgae growth rate was also noticed at high temperature (30 °C), as reported in the literature (Brown et al. 1998). In this study, increased lipid content was due to the reduction of the nitrogen concentration in the medium. Nitrogen-limiting conditions have been reported to significantly increase the lipid fraction of many microalgae (Illman et al. 2000). For this reason, the concentration of nitrate in the medium for *B. braunii* batch growth was reduced, while the light intensity was kept the same. The effect of reducing the $NaNO_3$ concentration on *B. braunii* growth is summarized in Tables 3 and 4. Whereas its growth rate was not significantly affected ($p<0.05$), the lipid content of *B. braunii* was increased at 20 and 30 °C. In contrast, both biomass and lipid content decreased at 10 °C compared to the experiments at 20 and 30 °C (Table 2, 3 and 4).

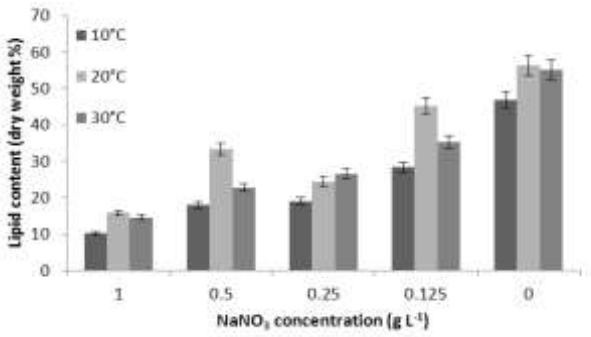

Figure 4. The total lipid content of *B. braunii* cultured at 10, 20 and 30 ºC (dry weight %).

The density of the biodiesel obtained from *B. braunii* was calculated as 0.8263 $kg.L^{-1}$, its viscosity as 4.97 $mm^2.s^{-1}$ at 40 °C and its flash point as 109 °C. To perform effective biodiesel production from microalgae, these results suggest that a balance between decreased growth and an increased lipid fraction will need to be achieved.

3.2. Determination and quantification of fatty acids from *B. braunii*

Profiles of the fatty acids are shown in Table 5. Three polyunsaturated fatty acids (linolenic, linoleic and eicosatrienoic) and two monounsaturated fatty acids (eicosenoic and oleic) were identified from the extract, representing 79-98% of the total fatty acids. Additionally, six saturated fatty acids (behenic, margaric, palmitic, stearic, pentadecanoic, and myristic acids) were present in the extract. The predominant component from the *B. braunii* extract was oleic acid, followed by palmitic acid, linolenic acid, and linoleic acid. The present results agree with an earlier finding [29]. The *B. braunii* extract showed a brownish-red color, which may indicate the existence of pigments such as carotenoids and chlorophyll, which commonly occur in *B. braunii*.

4. Conclusions

In this study, the optimal nitrogen concentration and temperature for *B. braunii* growth and neutral lipid accumulation were identified. Additionally, the fatty acid composition of the algal lipids obtained as a result of mass cultivation was determined. The variation of parameters tested (temperature and nitrogen concentration) strongly influenced algal growth and lipid accumulation. The investigated stress conditions led not only to the accumulation of lipids, but also to a reduction in microalgae growth, thereby affecting lipid production. In particular, the growth of *B. braunii* was not significantly influenced by temperature, whereas an increase in temperature from 20 °C to 30 °C decreased lipid content by 2.08%.

In this study, it can be concluded that the temperature did not significantly affect algal lipid accumulation. However, the $NaNO_3$ concentration affected algal lipid production. An increase in lipid content was observed for *B. braunii* when the nitrate concentration in the medium was reduced by 75%, although the growth remained stable. According to the results under various stress conditions, it can be said that production of algal lipids increased. Knowledge of the biochemistry and physiology of lipid synthesis, when combined with basic studies on microalgal molecular biology and genetic engineering to develop *B. braunii* strains with optimal growth and lipid production, may lead to great improvements biodiesel production and enhance the commercial viability of this alga as an optimum lipid source.

Biodiesel has been around since the invention of the automobile. At the start of the 20th century, Henry Ford planned to fuel his Model Ts with ethanol, and early diesel engines were shown to run on peanut oil (Seisel 1926). However, discoveries of huge petroleum deposits kept gasoline and diesel cheap for decades, and biodiesel was largely forgotten. However, with the recent rise in oil prices, along with growing concern about global warming caused by carbon dioxide emissions, biofuels have been regaining popularity. We expect that the simple experimental method described in this study could be very useful for the monitoring of lipid content in biodiesel produced by *B. braunii.*

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