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Lipids production from *Scenedesmus obliquus* through carbon/nitrogen ratio optimization

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Lipids production from *Scenedesmus obliquus* through carbon/nitrogen ratio optimization

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Abstract. Microalgae are one of the most promising sources of raw material for biofuel production and derivatives since its high yield of biomass and metabolites possess a low environmental impact. However, its implementation on large scale facilites still faces challenges such as the optimisation of lipid production (due to strain capacity and environmental factors) and downstream processes (extraction and separation of the lipidic fraction). The objective of the present investigation was to determine the potential of the carbon/nitrogen ratio as a technical tool for the improvement of total lipids on *Scenedesmus obliquus*. The carbon/nitrogen ratio was evaluated using a non-factorial design coupled with surface response methodology with sodium bicarbonate and sodium nitrate as carbon and nitrogen source. Results showed that the optimal conditions that enhanced the lipid deposition (up to 66% w/w) were 1.5 g L^{-1} sodium bicarbonate and 0,125 g L^{-1} of sodium nitrate. Finally, the results of the fatty acid profile shown the presence of stearic acid ($C_{18:0}$) with 22.63% and elaidic acid ($C_{18:1}$) with 77.38%, with the absence of fatty acids of two or more double bonds. In conclusion, the adjustment in the carbon/nitrogen ratio favours the final deposition of lipids in *Scenedesmus obliquus* which is emerging as a possible candidate for the production of lipids of interest for the generation of biodiesel.

1. Introduction

Microalgae have emerged as an environmentally sustainable alternative for obtaining fuels, due to their ability to convert CO_2 into biomass enriched with lipids which can be transformed into biodiesel (via transesterification) [1-3] or other fuels (such as bioethanol, jet-fuel and gasoline) under adverse nutritional conditions [2]. Another of its strengths is its ability to grow in wastewater [4] and not compete with agricultural processes since it does not require arable land for its production [5]. However, its use is not yet commercially extended [3], due to high costs in stages of the process [6,7]. Factors such as the specific productivity of the strain used [3], the antagonism between biomass production and lipid accumulation [8,9], as well as the replicability of the results obtained in the laboratory when carried out on a larger scale (demonstrative or industrial) [10], are the main challenges for competitive production. Therefore, the optimisation of these variables is crucial to increase the sustainability of the production of microalgae as a raw material for biofuels production. There are methods frequently used to induce the accumulation of lipids, such as the reduction in the concentration of nitrogen and phosphorus in the culture media [11-13], as well as the variation in the concentration of micronutrients (such as S, Mn, Fe, Zn and others) [14]. And the carbon source used in the media [15,16]. The objective of the present study was to determine the potential of *Scenedesmus obliquus* in the production of lipids for biodiesel in different concentrations of determining nutrients.

2. Materials and methods

2.1. Microorganism
S. obliquus was acquired from the company Nutré S.A.S (Colombia) and maintained in 0.5 L photobioreactors, with 0.2 L of bold basal medium [17] without pH adjustment, light intensity of 200 µmol m^{-2} s^{-1}, photoperiod of 12:12 and temperature of 30°C.

2.2. Experimental design
In order to improve the production of lipids, a non-factorial central experimental design 3^2 (3 levels, 2 factors) was applied with adjustment of the concentrations of nitrogen from sodium nitrate ($NaNO_3$) and carbon from sodium bicarbonate ($NaHCO_3$) through the software STATISTICA 7 [18] (Table 1). Bold basal medium was used as the control. Once the culture conditions were determined, scaling was carried out at a volume of 20 L in flat plate photobioreactors (15 x 35 x 50 cm).

Table 1. Design of experiments.

Treatment	T0 (Control)	T1	T2	T3	T4	T5	T6	T7	T8	T9
$NaHCO_3$ (g L^{-1})	0	1	0.793	1.000	1.500	1.500	1.500	2.000	2.207	2.000
$NaNO_3$ (g L^{-1})	0.250	0.075	0.125	0.175	0.054	0.125	0.195	0.075	0.125	0.175

2.3. Biomass quantification and analysis
The biomass concentration (in g L^{-1}) was obtained from dry weight [19]. Once every ten days for 30 days (four samples in triplicate) 15 mL of medium was taken and filtered using previously weighed 47 mm GF-C glass fibre filters (PALL Corporation). The filtered sample was dried overnight at 60 °C and then stored in a desiccator until constant weight was obtained.

Total lipids were quantified by adapting the protocol proposed by Folch [19]. The quantification of phosphates (PO_4^{3-}) and nitrates (NO_3^-) was carried out using the colorimetric method of molybdo-vanado-phophosphoric acid [20] and the nitrate sensor LAQUA Twin (Horiba) respectively.

The lipid profile of the microalga was determined by gas chromatography with a selective mass detector (GC-MS) by dissolving the sample in hexane, and the identified fatty acids were analysed according to the established ranges [21].

3. Results and discussion

3.1. Design of experiments and biomass analysis
Table 2 shows the biomass obtained for the different treatments after 30 days of culture; it can be seen that treatments 0 to 5 changed its colour, from green to orange (Figure 1). The final biomass for all the experiments was lower than that obtained by the control (1.46 g L^{-1}). An important factor to note is that those treatments with the highest concentrations of sodium bicarbonate (7, 8 and 9) generally had a lower concentration of biomass. According to experimental data obtained for *Scenedesmus sp* [16], *Dunaliella Salina* [22] and *Chlorella sp* [23], the reduction in the available nitrogen leads to a decrease in the final concentration of biomass; this phenomenon occurs even when a carbon source (either organic or inorganic) is added into the culture media. However, the type and concentration of the carbon source are particular for each microalgae strain, since in the case of *Scenedesmus sp*, no significant differences were found in cultures with sodium bicarbonate, whereas *D. Salina* and *Chlorella sp* showed better results with much higher concentrations. From the results obtained for the concentration of nitrates and phosphates in the different treatments, it was found that in all treatments, nitrates were highly consumed; this behaviour has been reported by [16,24] for other species of microalgae supplemented with different concentrations of bicarbonate, while phosphate was barely consumed in most treatments (Table 2).

The addition of an inorganic carbon source increases the phosphorus consumption [16,22], which counteracts the adverse effect of the absence of nitrogen on the availability of the phosphates in the medium [25,26]. Results for the concentration of lipids at day 30 (Table 2), showed that those treatments with nitrate concentration close to 0.125 g L^{-1} (T2, T5 and T8) obtained the highest

percentages of lipids (54,84%, 64,41% and 70,83% w/w respectively), were T8 recorded the highest value, which is almost as double as the control (39.73% w/w). It should be noted that the nitrate concentrations evaluated in this research are higher than those used by [27,28], where a lower nitrogen concentration corresponds to an increase in lipid accumulation. The value obtained in T8 is significantly higher than other concentrations reported for other strains of the same genus (which is usually between 10% - 15% of the biomass) [8,29], and in some cases, it can be up to 25% w/w of the biomass obtained [30]. Data presented by [16] and [31] showed values between 34% to 64% for different strains of *Scenedesmus sp*, cultivated in the absence of a nitrogen source and supplemented with sodium bicarbonate (0.6 g L^{-1} and 2.0 g L^{-1} respectively).
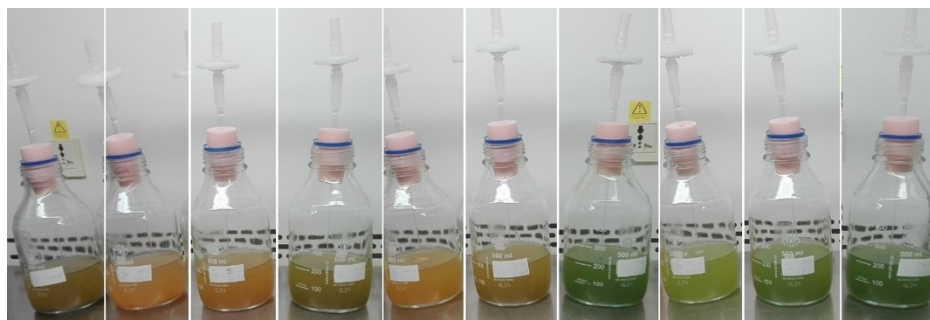

Figure 1. Treatments (0-9 from left to right) of *S. obliquus* under C/N ratio.

Table 2. Effect of different treatments on biomass and lipids production.

Treatment	Biomass		NO₃ consumed (%)	PO₄ consumed (%)	Lipid	
	g L^{-1}	g L^{-1} d^{-1}			g L^{-1}	%w/w (DW)
0	1.46	0.046	92.38	26.05	0.58	39.73
1	1.18	0.035	71.86	20.06	0.60	50.85
2	1.24	0.036	79.00	17.16	0.68	54.84
3	1.26	0.037	85.33	32.23	0.60	47.62
4	1.02	0.029	65.57	19.15	0.58	56.86
5	1.18	0.032	80.91	18.79	0.76	64.41
6	1.04	0.030	85.33	38.04	0.46	44.23
7	0.90	0.024	70.59	22.60	0.54	60.00
8	0.96	0.026	80.91	27.14	0.68	70.83
9	0.96	0.028	84.29	69.99	0.64	66.67

From the results obtained a Pareto analysis was obtained (Figure 2(a)) and a response surface (Figure 2(b)), in which it was possible to demonstrate that, by using a confidence > 99.95, the concentration of nitrate and sodium bicarbonate positively affect the final concentration of lipids. These results show that at higher concentrations of bicarbonate (> 1.5 g L^{-1}) and intermediate concentrations of sodium nitrate (0.125 g L^{-1}) were optimal for achieving the highest lipid concentration.
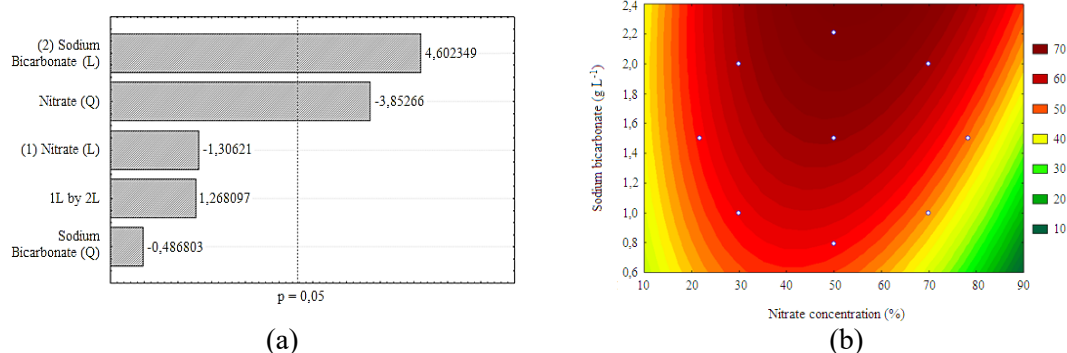

(a) (b)

Figure 2. Pareto analysis (a) and surface response (b) from the experimental data.

3.2. Culture scaling

From the conditions obtained earlier, the culture volume was scaled up to 20 litres in flat-panel photobioreactors. The final concentration of biomass and its productivity were slightly lower than those obtained in the 0.2 L experiments, going from 1.18 g L^{-1} to 1.05 g L^{-1} (Table 3). This behaviour can be justified due to the change in the geometry of the photobioreactor, since the rectangular geometry of the flat-plate reduced the turbulence, promoting the accumulation of nutrients and biomass in areas were the light and mixing are not uniform (also known as dead zones) [32] and under the air inlet [33]. The lack of homogeneity in the mixture contributes to the precipitation of biomass, so these cells will not have the same contact with the nutrients of the medium [34]. The reduction in the final concentration of biomass is in agreement with the results presented by [28], in which, increasing the volume of culture from 0.4 L to 14 L, the concentration of biomass was reduced by about 69%. However, there are results such as those obtained by [35], in which by increasing the culture volume of S. dimorphus (from 0.6 L to 10 L) the concentration of biomass increased by up to 15%.

Table 3. Effect of scaling-up on biomass and lipids production.

Vol. (L)	Biomass		NO$_3$ consumed (%)	PO$_4$ consumed (%)	Lipid	
	g L^{-1}	g L^{-1} d^{-1}			g L^{-1}	%w/w (DW)
0.2	1.18	0.032	80.91	18.79	0.76	64.41
20	1.05	0.028	73.48	9.03	0.67	66.67

The percentage of lipids obtained for the culture at 20 L (66.67%) was similar to the results for cultures in 0.2 L (64.41%), the final concentration of biomass of the former was lower, therefore, its Net lipid concentration was relatively lower than that obtained in 0.2 L (from 0.76 g L^{-1} to 0.67 g L^{-1}) (Table 3). This result differs from the ones presented by [35], who obtained a substantial reduction on total lipids (from 29% w/w to 20% w/w) when the volume of the culture was increased from 0.6 to 10 L. In the same way [28] report a reduction (from 18% w/w to 14% w/w) on the lipidic fraction after increased the culture volume from 0.4 L to 8 L. In this specific case, both biomass and lipid concentration decreased, which means that scaling was not successful or viable for production objectives. The lack of homogeneity in the mixture can also justify the similar percentages of lipids between the culture at 20 L and that of 0.2 L even when there was a difference in the concentration of biomass. The rectangular shape of the photobioreactor, as well as the location of the diffuser in the lower part, led to the accumulation of nutrients and cells forming films in dead zones such as edges and low walls. A certain amount of nutrients was trapped in the dead zones where lack of aeration prevented their correct assimilation, probably corresponding to the proportion of nitrogen and phosphorus that was never consumed compared to the smaller volume crop (Table 3). With fewer cells present in the suitably agitated medium, competition for dissolved nutrients was less. Therefore, the consumption of nitrogen and phosphorus slow down, but at the same time, their lower availability and earlier exhaustion contributed to accelerating the lipid accumulation [36] compensating for the reduction in biomass production.

3.3. Lipidic profile

The profile of fatty acids is presented in Table 4, where the presence of Stearic acid (a saturated fatty acid or SFAs) with 22.63% and Elaidic acid (a monosaturated fatty acid or MUFAs) with 77.38% is highlighted as unique presents. Also, the presence of Linolenic acid or compounds of the group of polyunsaturated fatty acids (or PUFAs) was not detected.

Table 4. Fatty acid profile of *S. obliquus*.

Molecular formula	Name	IUPAC	Area (%)	Fatty acids (%)
CH$_3$(CH$_2$)$_{16}$COOH	stearic acid	octadecanoic acid	0.27	22.63
C$_{18}$H$_{34}$O$_2$	elaidic acid	*Trans*-9-octadecanoic acid	0.93	77.38

According to [37,38] a high content of SFAs and MUFAs guarantees the oxidative stability of the fuel. On the other hand, low content of PUFAs ($\leq$1%) and linolenic acid ($\leq$12%) prevents the oxidation of the fuel components [21]. The proportions of SFA and MUFAs obtained for the lipid extract of a culture of *S. obliquus* allow proposing this strain as a promising candidate for the production of biodiesel due to the absence of fatty acids. Among the compounds detected, it is possible to highlight the presence of 2-ethylhexyl acrylate, which is used for the oxygenation of fuels, contributing to the lower production of atmospheric pollutants [39]. However, its proportion concerning the total composition of the microalgae was low, which implies a possible disadvantage. In other studies, carried out with other strains of *Scenedesmus sp*. as published by [5,31] percentages were obtained between 3-5% for C18:0 and 17-25% for C18:1. these results contrast with the percentages lower than 1% for the same fatty acids in the present study. This leads to the need to evaluate new formulations of media to obtain similar proportions of fatty acids in a higher concentration.

4. Conclusions.

The cultivation of *S. obliquus* obtained the highest lipidic content (70.83% w/w) under high concentration of sodium bicarbonate (2.0 g L^{-1}) and medium to low concentration of sodium nitrate (0.125 g L^{-1}) with a final concentration of 0.68 g L^{-1} of total lipids and 0.96 g L^{-1} of biomass in dry weight. Led to final production of 0.68 g L^{-1} of lipids. The scaling of culture at 20 L showed a reduction in the final production of lipids (0.67 g L^{-1}) attributed to the formation of dead zones due to the geometry of the photobioreactor; however, the lipidic profile indicates an adequate composition concerning the fatty acids produced by the microalga. This result shows that *S. obliquus* is a promising species for the production of lipids for biodiesel.

Acknowledgments

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