Manuscript Number: ALGAL-D-16-00320R4

Title: Microalgae for oil. Assessment of fatty acid productivity in continuous culture by two high-yield strains, *Chlorococcum oleofaciens* and *Pseudokirchneriella subcapitata*

Article Type: Full Length Article

Section/Category: Algal Biotechnology

Keywords: Microalgae; continuous culture; photochemostat; fatty acid productivity; biodiesel.

Corresponding Author: Mrs. Esperanza Del Rio, Doctor

Corresponding Author's Institution: Instituto de Bioquímica Vegetal y Fotosíntesis

First Author: Esperanza Del Rio, Doctor

Order of Authors: Esperanza Del Rio, Doctor; Elena García Gómez; José Moreno; Miguel G.Guerrero, Professor; Mercedes García-González

Abstract: The influence of nitrate supply on the performance of *Chlorococcum oleofaciens* and *Pseudokirchneriella subcapitata* has been assessed in bubble-column photochemostats operating in continuous regime. Limitation in nitrate availability resulted in both increased fatty acid levels and diminished biomass productivity, although a disparity in the specific response of the strains was apparent. Evaluation of fatty acid productivity under different levels of nitrate supply revealed the superiority of *P. subcapitata*, with maximal values of 160 mg fatty acids L⁻¹ d⁻¹, in comparison to 110 for *C. oleofaciens*. Under conditions of maximal oil yield, entailing moderate nitrate limitation, the biomass of both microalgae was enriched in saturated and monounsaturated fatty acids, thus displaying a more suitable profile as raw material for biodiesel. An analysis of the relationship between nitrogen content of the biomass and fatty acid levels reaffirmed the differential response of the strains to the restriction in nitrate availability. Differently from *C. oleofaciens*, accumulation of fatty acids in *P. subcapitata* was triggered with moderate nitrogen limitation, which had a mild effect on biomass production. The results support the adequacy of *Pseudokirchneriella subcapitata* as a high-yield source of fatty acids for biodiesel production.

Chief Editor of Algal Research

Seville, April 22nd, 2016

Dear Sir,

Please find attached the manuscript entitled “Microalgae for oil. Assessment of fatty acid productivity in continuous culture by two high-yield strains, *Chlorococcum oleofaciens* and *Pseudokirchneriella subcapitata*” to be considered for publication in Algal Research.

All the authors agree to submit this work, which it is the original work of the authors and it has not been published previously and it is not under consideration for publication elsewhere. All authors have materially participated in the research (especially E. Del Río, E. García-Gómez and J. Moreno) and article preparation (E. Del Río, M. García-González and M.G. Guerrero). We believe that this paper represents a relevant contribution to the field of Biotechnology of Microalgae, and specifically to the evaluation of the potential of high oil yield strains of microalgae for biodiesel production.

The present research attempts to establish conditions that maximize fatty acid productivity in continuous cultures of two microalgae, namely *P. subcapitata* and *C. oleofaciens*, through the in-depth characterization of the influence of nitrate supply on growth and accumulation of fatty acids. Evaluation of fatty acid productivity under different levels of nitrate supply revealed the superiority of *P. subcapitata*, with maximal values of 160 mg fatty acids L⁻¹ d⁻¹, in comparison to 110 mg L⁻¹ d⁻¹ for *C. oleofaciens*, and clearly superior to levels reported in the literature for microalgae in continuous in photochemostat. These results, together with a suitable fatty acid profile, highlight the potential of *P. subcapitata* as a high-yield source of fatty acids for biodiesel production. In the present work, continuous culture with daylight cycle illumination was used, conditions which are closer to those in which outdoor systems operate, so that the information herein reported could be easier to apply to the scaling up of the corresponding production process.

The above considerations underline (in our view) the significance of the findings reported in the manuscript, and prompt us to kindly ask you to consider it appropriate for publication.

Yours sincerely,

Dr. Esperanza del Río
Instituto de Bioquímica Vegetal y Fotosíntesis
Universidad de Sevilla/CSIC
Americo Vesputio, 49
41092 Sevilla, Spain

Dear Prof. Lammers,

Many thanks for your communication of October 16, concerning the evaluation of manuscript ALGAL-D-16-00320R3. We hope that the following explanations will properly address the Reviewer comments:

Following recommendation of the Reviewer, additional data on the yield of biomass and fatty acids on supplied PAR photons have been included in the text. These calculations have been made on the basis of measurements with a planar sensor of maximal impinging irradiance at reactor's surface ($600 \mu\text{E m}^{-2}\text{s}^{-1}$), and hence calculating the mean irradiance received over the illuminated period ($425 \mu\text{E m}^{-2}\text{s}^{-1}$). The yield of biomass and fatty acids on supplied PAR photons has been calculated strictly following the indications of the Reviewer, as also described in Breuer et al 2013 (added to the list of References).

The new obtained values for biomass quantum yield ($Y_{\text{x}}E$) for 1 and 10 mM, based on planar sensor measurements, are as follows:

<i>P. subcapitata</i>			
	$\text{NO}_3^-(\text{mM})$	P biomass (g/l)	$Y_{\text{x}}E(\text{g/mol})$
	1	0.13	0.12
	10	0.48	0.46
<i>C. oleofaciens</i>			
	$\text{NO}_3(\text{mM})$	P bio (g/l)	$Y_{\text{x}}E(\text{g/mol})$
	1	0.25	0.24
	10	0.58	0.55

Maximal values determined for fatty acids quantum yield ($Y_{\text{FA}}E$) were 0.153 and 0.105 g mol^{-1} for *P. subcapitata* and *C. oleofaciens*, respectively. Thus, the modified version of the manuscript add these yield values to those already standing, calculated according to Molina Grima et al 1997 (identical results as following Klok et al 2013), indicating in each case the type of sensor used for the measurement of irradiance. The inclusion of both sets of values (which do not differ much) would certainly facilitate comparison to readers that use either a planar or a quantum scalar sensor.

With regard to the maximum irradiance value stated in the text (measured with a quantum scalar sensor), we insist that it corresponds to an experimental value repetitively obtained for the set-up used, measured with a 4π sensor recognized and widely used in the field of microalgae, and that calibration of both the light sensor and the data acquisition system has been carefully verified. Obviously, we assume full responsibility for this information, as for the full content of the manuscript.

Thanking you very much for help and attentions, I remain,

Yours sincerely,

Esperanza del Río

Microalgae for oil. Assessment of fatty acid productivity in continuous culture by two high-yield strains, *Chlorococcum oleofaciens* and *Pseudokirchneriella subcapitata*

Esperanza Del Río, Elena García-Gómez, José Moreno, Miguel G. Guerrero and Mercedes García-González

Instituto de Bioquímica Vegetal y Fotosíntesis, Universidad de Sevilla-Consejo Superior de Investigaciones Científicas, Centro de Investigaciones Científicas Isla de la Cartuja, Avenida Américo Vespucio 49, 41092 Sevilla, Spain.

Author for correspondence:

Dr. Esperanza Del Río

Instituto de Bioquímica Vegetal y Fotosíntesis

Centro de Investigaciones Científicas Isla de la Cartuja

Avenida Américo Vespucio 49, 41092 Sevilla, Spain

Phone: +34 954489520

Fax: +34 954460065

Email: esperanza@ibvf.csic.es

Abstract

The influence of nitrate supply on the performance of *Chlorococcum oleofaciens* and *Pseudokirchneriella subcapitata* has been assessed in bubble-column photochemostats operating in continuous regime. Limitation in nitrate availability resulted in both increased fatty acid levels and diminished biomass productivity, although a disparity in the specific response of the strains was apparent. Evaluation of fatty acid productivity under different levels of nitrate supply revealed the superiority of *P. subcapitata*, with maximal values of 160 mg fatty acids L⁻¹ d⁻¹, in comparison to 110 for *C. oleofaciens*. Under conditions of maximal oil yield, entailing moderate nitrate limitation, the biomass of both microalgae was enriched in saturated and monounsaturated fatty acids, thus displaying a more suitable profile as raw material for biodiesel. An analysis of the relationship between nitrogen content of the biomass and fatty acid levels reaffirmed the differential response of the strains to the restriction in nitrate availability. Differently from *C. oleofaciens*, accumulation of fatty acids in *P. subcapitata* was triggered with moderate nitrogen limitation, which had a mild effect on biomass production. The results support the adequacy of *Pseudokirchneriella subcapitata* as a high-yield source of fatty acids for biodiesel production.

Keywords: Microalgae, continuous culture, photochemostat, fatty acid productivity, biodiesel

1. Introduction

Microalgae have several characteristics that make them an interesting feedstock for biodiesel. Among other features, stand their high productivities, the ability of some species to accumulate large amounts of triacylglycerides, or the lack of requirement of high quality agricultural land to raise the biomass [1]. Nevertheless, up to now, scarcity of scientific and technical knowledge, as well as limited practical experience, determines a high price for microalgal biomass and the biofuel thereof. Significant R&D efforts are currently being addressed to the development of viable processes able to massively generate microalgal biofuels at prices that can compete with those of established fuels [2].

From the biological point of view, selection of the most appropriate microalgal strains represents the first key step towards the development of an oil production process from microalgae. The selection must consider not only the lipid or fatty acid content, but also the biomass generation capacity, as to define the oil productivity of the microalga considered [3], [4], [5] and [6].

Nitrogen limitation is one the most common and effective strategies to trigger lipid accumulation in microalgae, although it leads to a concomitant reduction of growth. When nitrogen (N) becomes limiting, the excess of carbon fixed during photosynthesis may be diverted into storage compounds, such as carbohydrate (starch) or lipids, which serve as reservoir of carbon and energy [7], [8] and [9]. The preferential accumulation of either starch or lipids seems to be strain dependent and may be also associated with the degree of stress [8], [10] and [11]. The fatty acid profile also varies under nitrogen limiting conditions. In many microalgae, enrichment in saturated and monounsaturated fatty acids takes place under nitrogen stress. In general, a raw material rich in saturated and monounsaturated fatty acids is more suitable as biodiesel feedstock [1], [7] and [12], thus N-limited microalgal biomass becomes more adequate for this purpose.

85

86 Most of the information regarding lipid (oil) production by microalgae derives from studies
87 performed in batch cultures and is based on the lipid content in the stationary phase, which is
88 characterized by severe limitation in nutrients and light availability, with growth rate
89 dramatically affected. Values as high as 70% lipids in the dry biomass have been reported,
90 although 20-50% are more common [1] and [7].

91

92 A reliable assessment of the oil yield should only be made on the basis of an accurate
93 measurement of actual lipid/fatty acid productivity. The latter would only be precisely achieved
94 in the steady state conditions of a continuous culture, in which productivity and composition of
95 biomass keep stable. Fatty acid accumulation under moderate nitrate limitation in continuous
96 culture has been proposed as an alternative approach for the purpose of biodiesel production [3],
97 [13] and [14], as it allows growth and lipid accumulation simultaneously in a one step process.
98 This approach resembles the one step system described for astaxanthin accumulation by
99 *Haematococcus pluvialis* in continuous culture [15]. Through the right balance between growth
100 and accumulation, sustained high productivity levels of secondary metabolites can be obtained
101 [3] and [16] under moderate nitrate limitation.

102

103 In a previous work [3], a screening of 10 strains of microalgae in continuous culture was
104 performed in order to evaluate their biomass and fatty acids productivity under nitrate
105 sufficiency and limitation. Through the in-depth characterization of the influence of nitrate
106 supply on growth and accumulation of fatty acids, the present research attempts to establish
107 conditions that maximize fatty acid productivity in continuous cultures of two microalgae,
108 namely *P. subcapitata* and *C. oleofaciens* that, according to Del Río et al. [3], behaved as
109 outstanding potential source of oil for biodiesel.

110

111

2. Materials and methods

2.1. Microalgae and culture conditions

The microalgae used were *Chlorococcum oleofaciens* (SAG 213-11) and *Pseudokirchneriella subcapitata* (SAG 61-81). Cells were grown photoautotrophically on the medium described by Arnon et al. [17] modified to contain 4 mM K₂HPO₄. The amount of NaNO₃ in the feed medium was adjusted as to reach the final concentrations indicated in each case. Continuous culture was performed in six 2.0-L capacity (0.07 m diameter, 0.50 m height) jacketed sterilized photochemostats (bubble columns) containing 1.8 L of cell suspension, continuously sparged with air (33 L (L culture⁻¹) h⁻¹). Optimal temperature for growth, 25° C for *P. subcapitata* and 30°C for *C. oleofaciens*, was maintained by circulating thermostated water through the photochemostat jacket. The pH was kept at 7.5, the optimal value for growth, by on-demand CO₂ injection into the air stream entering the culture. The photochemostats were illuminated following a solar daylight cycle (12 h light:12 h dark) (Fig. S1, Supplementary material). Each bubble column was surrounded by white panels holding six vertically placed fluorescent tubes (Phillips Master TL5 HO 24W/840, white-light lamps) (Fig S2, Supplementary material), which provided maximal incident irradiance of 3500 μE m⁻² s⁻¹ in the center of the reactor. Initially, the reactors were inoculated from batch-grown cells and operated on batch mode for about 3-4 d, with maximal incident irradiance being progressively increased, until becoming close to stationary phase. Then, they were switched to operate on continuous mode, fresh medium being continuously fed at a dilution rate (flow rate) of 0.2 d⁻¹, unless otherwise stated, with withdrawal of culture at the same rate. Once steady state conditions were achieved, analytical determinations were performed. The data presented correspond to stabilized situations, being average values of 4 to 8 determinations of each steady state. Standard deviation bars (SD) are shown.

Measurement of photosynthetically active irradiance (PAR) was carried out using a 4π quantum scalar irradiance sensor QSL-100 (Biospherical Instrument, San Diego, CA), inside the photochemostat filled with water, but without cells. Alternatively, a planar sensor (Li-190SA, LI-COR, USA) was used for PAR irradiance impinging on the reactor surface. In the latter case, the maximal irradiance measured was $600 \mu\text{E m}^{-2} \text{s}^{-1}$.

2.2. Analytical procedures

Biomass concentration was determined by dry weight measurement as described previously [3]. For lipid determination, gravimetric method was employed [18] on freeze dried biomass. The lipid fraction was obtained by mechanical disruption of 20 mg of biomass employing a Mini Bead Beater (Biospec Products), using chloroform:methanol (2:1, v/v) as extracting solvent and 0.5 mm diameter glass beads. After centrifugation (5 min, $12100 \times g$), the supernatant containing the lipid fraction was collected. The pellet was washed with chloroform:methanol (2:1, v/v) and the supernatants combined. To the lipid fraction, 3 mL of HCl 0.1N and 0.3 mL Cl_2Mg 0.5% were added and the mixture shaken in a vortex mixer. After centrifugation (5 min, $1500 \times g$), the lower phase was carefully recovered into a pre-weighed vial. After solvent evaporation under vacuum, dry weight of the lipid fraction was measured in a precision balance. Fatty acid content (% DW) and profile (% of total FA) were analyzed by transesterification and gas chromatography, according to Del Río et al. [3]. For protein and carbohydrate determination, an extraction of 4 mg of freeze dried biomass was performed through incubation in NaOH 0.1M at 70°C , during 45 min. In the extract obtained, protein was estimated by the Lowry method [19], using bovine serum albumin as a standard. Carbohydrate was measured by the phenol sulfuric acid method [20] with glucose as standard. Elemental analysis of washed and dried samples was carried out using a CHNS-O elemental analyzer (FlashEA 1112 Series, Thermo).

2.3. Productivity calculation

Productivity of biomass (P_b) and that of fatty acids (P_{FA}) were estimated in steady state situations of continuous cultures: $P_b = C_b * D$; and $P_{FA} = P_b * X_{FA}$; where C_b is the concentration of biomass; D is the dilution rate; and X_{FA} the mass fraction of fatty acid in the biomass.

3. Results and discussion

3.1. Effect of nitrate availability on biomass and fatty acid productivity

In order to analyze the influence of nitrogen limitation on the productivity of biomass and fatty acids by the microalgae *Chlorococcum oleofaciens* and *Pseudokirchneriella subcapitata*, photochemostats were fed with media containing nitrate at concentrations between 1 and 10 mM, at a constant dilution rate, D , of 0.2 d^{-1} . Data on biomass concentration of the cultures can be seen in Fig S3 (Supplementary material).

Biomass productivity of the two microalgae varied in response to nitrate availability (Fig. 1A). The highest values were recorded when nitrate concentration in the feed medium was 10 mM, with biomass productivity becoming progressively lower as nitrate availability decreased. In contrast to the positive correlation between nitrate availability and biomass productivity, the cellular fatty acid content increased in response to nitrate limitation, to reach levels of 41 and 35% of the dry biomass for *P. subcapitata* and *C. oleofaciens*, respectively, at 1 mM nitrate (Fig. 1B). The comparative analysis of the two species with regard to fatty acid accumulation in response to limitation in nitrate availability revealed significant differences between them. Reducing nitrate concentration in the feed medium from 10 to 5 mM resulted in the virtual doubling of the fatty acid content in *P. subcapitata*, whereas a similar enhancement of fatty acid levels in *C. oleofaciens* was only patent at much lower nitrate availability (2 mM). A most relevant parameter for establishing the potential of a microalga as a source of oil for biodiesel is

fatty acid productivity, which combines biomass productivity and fatty acid content. As shown in Fig. 1C, *P. subcapitata* was the most productive microalgae, with 160 mg fatty acids L⁻¹ d⁻¹ when nitrate concentration in the feed medium was 3-5 mM. In *C. oleofaciens*, maximum fatty acid productivity (111 mg L⁻¹ d⁻¹) was reached at 2 mM nitrate.

The yield of biomass and fatty acids on supplied PAR photons has been calculated as described by Molina Grima et al 1997 [21] and Ación Fernández et al. 1998 [22], on the basis of average irradiance (I_{av}), and measuring incident light with a quantum scalar sensor. Identical values were obtained by calculating the yields according to Klok 2013 [14]. In *P. subcapitata*, values for Y_xE (grams of biomass produced per mol of photons absorbed) decreased in response to a reduction in nitrate concentration, being of 0.31 and 0.08 g mol⁻¹ at 10 mM and 1 mM, respectively. For *C. oleofaciens*, corresponding values of 0.38 and 0.16 g mol⁻¹ were obtained. In order to facilitate comparison of these data with those derived from PAR measurements using a planar sensor, the corresponding yields using the latter device and calculated according to Breuer et al 2013 [23], have also been derived. For *P. subcapitata*, Y_xE values of 0.46 and 0.12 g mol⁻¹ were so obtained for 10 mM and 1 mM nitrate, respectively. For *C. oleofaciens*, the corresponding values were 0.55 and 0.24 g mol⁻¹. Nitrogen limitation led to a decrease in biomass productivity, and thus in the yield of biomass on light, as also described [14] for *N. oleoabundans*. Maximal values determined for fatty acids yield on light (Y_{FA}E) were 0.104 g mol⁻¹ and 0.071 g mol⁻¹ for *P. subcapitata* and *C. oleofaciens*, respectively. The corresponding Y_{FA}E values calculated based on planar sensor measurements were 0.153 g mol⁻¹ for *P. subcapitata* and 0.105 g mol⁻¹ for *C. oleofaciens*. Regardless the approach used for the calculations, *C. oleofaciens* exhibited higher yield of biomass on light (Y_xE), whereas the yield of FA on light (Y_{FA}E) was higher in *P. subcapitata*. The capacity to maintain a high Y_xE value under nitrogen limitation can vary among species, and also the extent to which excess of energy can be channeled into storage material as fatty acids [14].

The comparison of oil yield values reported for microalgae in continuous culture in photochemostat could illustrate on the suitability of different strains for oil production. Under N sufficiency, yields of about 10 mg fatty acids L⁻¹ d⁻¹ have been reported for *Chlorella minutissima* and *Dunaliella tertiolecta* [24], and of 126 mg lipids L⁻¹ d⁻¹ for *Neochloris oleoabundans* [25]. Values of 118 mg fatty acids L⁻¹ d⁻¹ were reported for *N. oleoabundans* in continuous cultures operated in turbidostat mode [14]. Under controlled N limitation, Wen et al. [13] have reported a productivity of 145 mg lipids L⁻¹ d⁻¹ for *Chlorella pyrenoidosa*. Productivity values established in the present work speak of the high capacity for oil production of *P. subcapitata* (160 mg fatty acids L⁻¹ d⁻¹, corresponding to 180 mg lipids L⁻¹ d⁻¹) and *C. oleofaciens* (111 mg fatty acids L⁻¹ d⁻¹, corresponding to 160 mg lipids L⁻¹ d⁻¹). Moreover, the highest fatty acid productivity of *P. subcapitata* was maintained in the range 3-5 mM nitrate, which could facilitates the operation of the cultures, as possible fluctuations within that range would not affect the yield. The superiority of *P. subcapitata* (and *C. oleofaciens*) becomes even more patent when considering that the productivity values above quoted for *N. oleoabundans* and *C. pyrenoidosa* were obtained under continuous illumination at constant irradiance, whereas in the present work daylight cycle illumination was used. The latter conditions are, moreover, closer to those in which outdoor systems operate, so that the information herein reported could be easier to apply to the scaling up of the corresponding production process.

3.2. Effect of nitrate availability on biochemical composition of the biomass

The various N-limiting conditions imposed resulted in differences in the biochemical composition of the microalgae (Fig. 2). A typical response of microalgae under nitrogen limitation is the redirection of metabolism from protein synthesis towards storage compounds, like carbohydrate or lipids. A reduction in protein content was observed for both species in response to reduced nitrate availability, although in different degree. *P. subcapitata* presented higher protein content than *C. oleofaciens* in almost all nitrate supply conditions considered (Fig. 2A). Lipid was the main storage material in *P. subcapitata*, while it was carbohydrate in *C.*

oleofaciens (Fig. 2B, 2C). It is worth noting that high lipid content coexisted with a substantial protein level in *P. subcapitata*, while *C. oleofaciens* presented a remarkable combination of lipid and carbohydrate content. This should be taken into account when considering the exploitation of each biomass.

Fatty acid profile was also affected by nitrogen availability (Table 1). Biodiesel composition must satisfy with standards (European Standard EN 14214 and ASTM D6751 in Europe and USA, respectively) that regulate among others, the level for unsaturated methyl esters. In general, a raw material rich in saturated and monounsaturated fatty acids is more suitable as biodiesel feedstock [1], [7] and [12]. Actually, nitrate limitation in the feed medium led to an increase in saturated and monounsaturated fatty acids (mainly palmitic and oleic acid) in the two species, to the detriment of polyunsaturated (Table 1).

Fig. 3 shows the fatty acid profile for each species under conditions of maximal fatty acid productivity. In *P. subcapitata* and *C. oleofaciens*, the sum of C16:0 and C18:1 accounted for more than 68% of total fatty acids, in parallel with a linolenic acid content below 10%, thus being in accordance with the limit set by standard to this fatty acid methyl ester (12%). This highlights the adequacy of the biomass obtained in these conditions as biodiesel feedstock.

3.3. Relationship between fatty acids and N content of the biomass

For a detailed assessment of the relationship between fatty acid levels and N content of the biomass, it was pertinent to consider the information derived from a substantial number of samples corresponding to a variety of growth conditions. Therefore, the full analysis also included samples from experiments addressed to evaluate the response of *C. oleofaciens* and *P. subcapitata* to factors other than nitrate in the feed medium, such as temperature, pH and dilution rate (Figs. 4 and 5).

Nitrogen supply to the cells can be defined by the parameter SNI (specific nitrate input, mmol $\text{NO}_3^- \text{ g}^{-1} \text{ d}^{-1}$) [15], which considers dilution rate, nitrate concentration in the feed medium and biomass concentration. The incorporation of nitrogen into the biomass exhibited a hyperbolic response to increasing SNI (Fig. S4, Supplementary material). Reduction of SNI led to fatty acid accumulation over basal level. Whereas for triggering fatty acid accumulation in *P. subcapitata* a SNI level of about 2 mmol $\text{NO}_3^- \text{ g}^{-1} \text{ d}^{-1}$ was sufficient, a more stringent limitation in nitrate input (SNI, 0.5 mmol $\text{NO}_3^- \text{ g}^{-1} \text{ d}^{-1}$) was required in the case of *C. oleofaciens* (Fig. 4).

A negative correlation did exist between nitrogen content and fatty acid level in the biomass of the two species, although differences in the shape of the relationship were obvious (Fig. 5).

The nitrogen content at which fatty accumulation was induced was also different for each species. Accumulation of fatty acids in *P. subcapitata* was apparent even when the nitrogen content was as high as 8% of the dry biomass with an inverse linear relationship existing between nitrogen and fatty acid content. Induction of fatty acid accumulation in *C. oleofaciens* corresponded to a much lower nitrogen level, of about 5%. Adams et al. [26] analyzed the effect of nitrogen limitation in six microalgae in batch culture, concluding that the level of N stress at which lipid accumulation was triggered was unique for every species, ranging from minimal (*N. oleabundans*, 7.5% N in the biomass) to severe N stress (*S. naegelii*, 3% N). In the present work, performed in continuous culture, differences between strains were also apparent, being worthy to underline the ability of *P. subcapitata* to accumulate fatty acids in response to minimal N stress.

4. Conclusions

The capacity of two microalgal species (*Chlorococcum oleofaciens* and *Pseudokirchneriella subcapitata*) for fatty acid production has been assessed in continuous culture. The reduction in nitrate availability resulted in an increase in fatty acid content in both strains, but the overall

evaluation of fatty acid productivity revealed the remarkably high capacity of *P. subcapitata*: 160 mg fatty acids (or 180 mg lipids) L⁻¹ d⁻¹. Such a productivity value is clearly superior to that of the oleaginous strain *C. oleofaciens* and to those reported in the literature for microalgae in continuous culture. A remarkable peculiarity of *P. subcapitata* is the low threshold for nitrogen stress that triggers accumulation of fatty acids in its biomass. These results, together with a suitable fatty acid profile, highlight the potential of *Pseudokirchneriella subcapitata* as an adequate source of fatty acids for biodiesel production.

Author contributions

E. Del Río, M.G. Guerrero and M.García-González designed research; E. Del Río, E. García-Gómez and J.Moreno performed research and analyzed data; E. Del Río, M.G. Guerrero and M.García-González wrote the paper. All authors read and approved the final manuscript.

Acknowledgements

Research supported by Spanish Ministerio de Ciencia e Innovación (Project CTQ2008-06741-CO2-02/PPQ) and ACCIONA Energía S.A.

References

1. Chisti Y (2007) Biodiesel from microalgae. *Biotechnol Adv* 25: 294-306.
2. Sing SF, Isdepsky A, Borowitzka MA, Moheimani NR (2013). Production of biofuels from microalgae. *Mitig Adapt Strateg Glob Change* 18: 47-72.
3. Del Río E, Armendáriz A, García-Gómez E, García-González M, Guerrero MG (2015) Continuous culture methodology for the screening of microalgae for oil. *J Biotechnol* 195: 103-107.
4. Griffiths MJ, van Hille RP, Harrison STL (2012) Lipid productivity, settling potential and fatty acid profile of 11 microalgal species grown under nitrogen replete and limited conditions. *J Appl Phycol* 24: 989–1001.

- 334 5. Pruvost J, Van Vooren G, Le Gouic B, Couzinet-Mossion A, Legrand J (2011). Systematic
335 investigation of biomass and lipid productivity by microalgae in photobioreactors for biodiesel
336 application. *Bioresour Technol* 102: 150–158.
- 337 6. Rodolfi L, Zittelli GC, Bassi N, Padovani G, Biondi N, Bonini G, Tredici MR (2009).
338 Microalgae for oil: strain selection, induction of lipid synthesis and outdoor mass cultivation in
339 a low-cost photobioreactor. *Biotechnol Bioeng* 102: 101-112.
- 340 7. Hu Q, Sommerfeld M, Jarvis E, Ghirardi M, Posewitz M, Darzins A (2008) Microalgal
341 triacylglycerols as feedstocks for biofuel production: perspectives and advances. *The Plant J* 54:
342 621-639.
- 343 8. Procházková G, Brányiková I, Zachleder V, Brányik T (2014) Effect of nutrient supply status
344 on biomass composition of eukaryotic green microalgae. *J Appl Phycol* 26: 1359-1377.
- 345 9. Solovchenko AE (2012). Physiological role of neutral lipid accumulation in eukaryotic
346 microalgae under stresses. *Russ J Plant Physiol* 59 (2): 167–176.
- 347 10. Fernandes B, Teixeira J, Dragone G, Vicente AA, Kawano S, Bišová K, Přibyl P, Zachleder
348 V, Vítová M (2013) Relationship between starch and lipid accumulation induced by nutrient
349 depletion and replenishment in the microalga *Parachlorella kessleri*. *Bioresour Technol* 144:
350 268–274.
- 351 11. Li Y, Han D, Sommerfeld M, Hu Q (2011) Photosynthetic carbon partitioning and lipid
352 production in the oleaginous microalga *Pseudochlorococcum* sp. (Chlorophyceae) under
353 nitrogen-limited conditions. *Bioresour Technol* 102: 123-129.
- 354 12. Stansell GR, Gray VM, Sym SD. (2012). Microalgal fatty acid composition: implications
355 for biodiesel quality. *J Appl Phycol* 24: 791-801.
- 356 13. Wen X, Geng Y, Li Y (2014). Enhanced lipid production in *Chlorella pyrenoidosa* by
357 continuous culture. *Bioresour Technol* 161: 297-303.
- 358 14. Klok AJ, Martens DE, Wijffels RH, Lamers PP (2013). Simultaneous growth and neutral
359 lipid accumulation in microalgae. *Bioresour Technol* 134: 233-243.

15. Del Río E, Acien FG, García-Malea MC, Rivas J, Molina-Grima E, Guerrero MG (2005) Efficient one-step production of astaxanthin by the microalga *Haematococcus pluvialis* in continuous culture. *Biotechnol Bioeng* 91: 808-815.
16. Del Río E, Acien FG, García-Malea MC, Rivas J, Molina-Grima E, Guerrero MG (2008) Efficiency assessment of the one-step production of astaxanthin by the microalga *Haematococcus pluvialis*. *Biotechnol Bioeng* 100 (2): 397-402.
17. Arnon DI, McSwain BD, Tsujimoto HY, Wada K (1974) Photochemical activity and components of membrane preparations from blue-green algae. I. Coexistence of two photosystems in relation to chlorophyll a and removal of phycocyanin. *Biochim Biophys Acta* 357: 231-245.
18. Kochert G (1978) Quantitation of the Macromolecular Components of Microalgae. In: Hellebust JA, Craigie JS (eds) *Handbook of Phycological Methods. Physiological and Biochemical Methods*, Cambridge University Press, Cambridge, pp 189.
19. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* 193: 265-275.
20. Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F (1956) Colorimetric method for determination of sugars and related substances. *Anal Chem* 28: 350-356.
21. Molina Grima E, Camacho FG, Sánchez Pérez JA, Acien Fernández FG, Fernández Sevilla JM (1997) Evaluation of photosynthetic efficiency in microalgal cultures using averaged irradiance. *Enz Microb Technol* 21: 375-381
22. Acien Fernández FG, García Camacho F, Sánchez Pérez JA, Fernández Sevilla JM, Molina Grima E (1998) Modeling of biomass productivity in tubular photobioreactors for microalgal cultures: effects of dilution rate, tube diameter and solar irradiance. *Biotechnol Bioeng* 58 (6): 605-616.
23. Breuer G, Lamers PP, Martens DE, Draaisma RB, Wijffels RH (2013) Effect of light intensity, pH, and temperature on triacylglycerol (TAG) accumulation induced by nitrogen starvation in *Scenedesmus obliquus*. *Bioresour Technol* 143: 1-9

24. Tang H, Chen M, Simon Ng KY, Salley SO (2012). Continuous microalgae cultivation in a photobioreactor. *Biotechnol Bioeng* 109: 2468-2474.
25. Pruvost J, Van Vooren G, Cogne G, Legrand J (2009) Investigation of biomass and lipids production with *Neochloris oleoabundans* in photobioreactor. *Bioresour Technol* 100: 5988–5995.
26. Adams C, Godfrey V, Wahlen B, Seefeldt L, Bugbee B (2013) Understanding precision nitrogen stress to optimize the growth and lipid content tradeoff in oleaginous free microalgae. *Bioresour Technol* 131: 188-194.

Table 1. Influence of nitrate concentration in the feed medium on major fatty acids profile (% of total) in the biomass of *C. oleofaciens* and *P. subcapitata* in continuous culture.

	Fatty acid	1 mM	2 mM	3 mM	5 mM	10 mM
<i>C. oleofaciens</i>	C16:0	24.25	24.69	24.62	22.28	17.27
	C16:1(9)	3.82	4.09	3.55	3.52	2.74
	C16:1(7)	4.12	3.80	4.48	5.66	8.99
	C16:2 (9,12)	0.21	0.26	0.40	0.75	1.47
	C16:3(7,10,13)	1.95	1.71	1.27	1.39	1.45
	C16:4(4,7,10,13)	1.53	1.99	2.03	2.94	5.71
	C18:0	1.40	1.59	1.99	1.54	1.42
	C18:1(9)	42.11	43.30	41.18	35.93	23.61
	C18:2(9,12)	11.35	9.84	11.70	12.83	16.25
	C18:3 (5,9,12)	3.22	2.77	3.00	3.34	4.10
	C18:3(9,12,15)	5.10	5.45	5.14	6.55	11.64
	C18:4(6,9,12,15)	0.36	0.41	0.38	0.49	0.85
<i>P. subcapitata</i>	C16:0	21.43	22.69	21.86	22.31	22.13
	C16:1(9)	0.54	0.61	0.50	0.59	1.54
	C16:3(7,10,13)	0.82	0.79	0.75	0.73	1.01
	C16:4(4,7,10,13)	2.46	2.89	2.85	3.56	10.71
	C18:0	2.93	2.80	2.61	3.10	1.71
	C18:1(9)	55.91	52.61	53.97	48.92	23.05
	C18:2(9,12)	1.79	1.97	1.91	2.22	4.43
	C18:3(9,12,15)	8.43	9.25	8.72	10.51	23.11
	C18:4(6,9,12,15)	3.72	3.86	3.56	3.69	4.79

A

B

C

Fig. 1 Influence of nitrate availability on biomass productivity (A), fatty acid content (B) and fatty acid productivity (C) of *C. oleofaciens* and *P. subcapitata* in continuous culture.

436

437

A_i

438

B_i

439

C_i

440

441 **Fig. 2** Influence of nitrate availability on the biochemical composition of the biomass of *C.*
 442 *oleofaciens* and *P. subcapitata* in continuous culture: protein (A), lipid (B) and carbohydrate
 443 content (C).
 444

Fig. 3 Main fatty acids profile under conditions of maximal fatty acid productivity in the biomass of *C. oleofaciens* and *P. subcapitata* in continuous culture. Nitrate concentration in feed medium: *C. oleofaciens*, 2 mM, *P. subcapitata*, 3 mM.

Fig. 4 Influence of specific nitrate input on fatty acid content of the biomass of *C. oleofaciens* and *P. subcapitata* in continuous culture. The data correspond to experiments performed to evaluate the effect of the following factors: temperature: 20, 25, 30°C (at pH 7.5 and D=0.3 d⁻¹); pH: 6.5, 7,7.5, 8, 8.5 (D=0.3 d⁻¹ and 25°C for *P. subcapitata* and D=0.3 and 30°C for *C. oleofaciens*); dilution rate: 0.1, 0.2, 0.3,0.45, 0.6 d⁻¹ (pH 7.5 and 25°C for *P. subcapitata* and pH 7.5 and 30°C for *C. oleofaciens*); Nitrate concentration in feed medium: 1, 2, 3 ,5, 10 mM (D= 0.2 d⁻¹, pH 7.5 and 25°C for *P. subcapitata* and D= 0.2 d⁻¹, pH 7.5 and 30°C for *C. oleofaciens*).

481

482

A

483

B

484

485 **Fig. 5** Relationship between nitrogen content and fatty acid content of the biomass of *C.*
 486 *oleofaciens* (A) and *P. subcapitata* (B) in continuous culture. For information on the data, see
 487 the legend of Fig. 4.

488

489

Acknowledgements

Research supported by Spanish Ministerio de Ciencia e Innovación (Project CTQ2008-06741-CO2-02/PPQ) and ACCIONA Energía S.A.

Supplementary Material

[Click here to download Supplementary Material: Supplementary material.doc](#)

Highlights

1. Fatty acid production has been assessed under nitrogen limitation in continuous culture
2. *P. subcapitata* highlights by its fatty acid productivity ($160 \text{ mg L}^{-1} \text{ d}^{-1}$)
3. FA accumulation in *P. subcapitata* was triggered as a strong response to low N stress
4. *P. subcapitata* stands out as a high-yield source of FA for biodiesel production

Microalgae for oil. Assessment of fatty acid productivity in continuous culture by two high-yield strains, *Chlorococcum oleofaciens* and *Pseudokirchneriella subcapitata*

Esperanza Del Río, Elena García-Gómez, José Moreno, Miguel G. Guerrero and Mercedes García-González

Instituto de Bioquímica Vegetal y Fotosíntesis, Universidad de Sevilla-Consejo Superior de Investigaciones Científicas, Centro de Investigaciones Científicas Isla de la Cartuja, Avenida Américo Vespucio 49, 41092 Sevilla, Spain.

Author for correspondence:

Dr. Esperanza Del Río

Instituto de Bioquímica Vegetal y Fotosíntesis

Centro de Investigaciones Científicas Isla de la Cartuja

Avenida Américo Vespucio 49, 41092 Sevilla, Spain

Phone: +34 954489520

Fax: +34 954460065

Email: esperanza@ibvf.csic.es

Abstract

The influence of nitrate supply on the performance of *Chlorococcum oleofaciens* and *Pseudokirchneriella subcapitata* has been assessed in bubble-column photochemostats operating in continuous regime. Limitation in nitrate availability resulted in both increased fatty acid levels and diminished biomass productivity, although a disparity in the specific response of the strains was apparent. Evaluation of fatty acid productivity under different levels of nitrate supply revealed the superiority of *P. subcapitata*, with maximal values of 160 mg fatty acids L⁻¹ d⁻¹, in comparison to 110 for *C. oleofaciens*. Under conditions of maximal oil yield, entailing moderate nitrate limitation, the biomass of both microalgae was enriched in saturated and monounsaturated fatty acids, thus displaying a more suitable profile as raw material for biodiesel. An analysis of the relationship between nitrogen content of the biomass and fatty acid levels reaffirmed the differential response of the strains to the restriction in nitrate availability. Differently from *C. oleofaciens*, accumulation of fatty acids in *P. subcapitata* was triggered with moderate nitrogen limitation, which had a mild effect on biomass production. The results support the adequacy of *Pseudokirchneriella subcapitata* as a high-yield source of fatty acids for biodiesel production.

Keywords: Microalgae, continuous culture, photochemostat, fatty acid productivity, biodiesel

1. Introduction

Microalgae have several characteristics that make them an interesting feedstock for biodiesel. Among other features, stand their high productivities, the ability of some species to accumulate large amounts of triacylglycerides, or the lack of requirement of high quality agricultural land to raise the biomass [1]. Nevertheless, up to now, scarcity of scientific and technical knowledge, as well as limited practical experience, determines a high price for microalgal biomass and the biofuel thereof. Significant R&D efforts are currently being addressed to the development of viable processes able to massively generate microalgal biofuels at prices that can compete with those of established fuels [2].

From the biological point of view, selection of the most appropriate microalgal strains represents the first key step towards the development of an oil production process from microalgae. The selection must consider not only the lipid or fatty acid content, but also the biomass generation capacity, as to define the oil productivity of the microalga considered [3], [4], [5] and [6].

Nitrogen limitation is one the most common and effective strategies to trigger lipid accumulation in microalgae, although it leads to a concomitant reduction of growth. When nitrogen (N) becomes limiting, the excess of carbon fixed during photosynthesis may be diverted into storage compounds, such as carbohydrate (starch) or lipids, which serve as reservoir of carbon and energy [7], [8] and [9]. The preferential accumulation of either starch or lipids seems to be strain dependent and may be also associated with the degree of stress [8], [10] and [11]. The fatty acid profile also varies under nitrogen limiting conditions. In many microalgae, enrichment in saturated and monounsaturated fatty acids takes place under nitrogen stress. In general, a raw material rich in saturated and monounsaturated fatty acids is more suitable as biodiesel feedstock [1], [7] and [12], thus N-limited microalgal biomass becomes more adequate for this purpose.

85

86 Most of the information regarding lipid (oil) production by microalgae derives from studies
87 performed in batch cultures and is based on the lipid content in the stationary phase, which is
88 characterized by severe limitation in nutrients and light availability, with growth rate
89 dramatically affected. Values as high as 70% lipids in the dry biomass have been reported,
90 although 20-50% are more common [1] and [7].

91

92 A reliable assessment of the oil yield should only be made on the basis of an accurate
93 measurement of actual lipid/fatty acid productivity. The latter would only be precisely achieved
94 in the steady state conditions of a continuous culture, in which productivity and composition of
95 biomass keep stable. Fatty acid accumulation under moderate nitrate limitation in continuous
96 culture has been proposed as an alternative approach for the purpose of biodiesel production [3],
97 [13] and [14], as it allows growth and lipid accumulation simultaneously in a one step process.
98 This approach resembles the one step system described for astaxanthin accumulation by
99 *Haematococcus pluvialis* in continuous culture [15]. Through the right balance between growth
100 and accumulation, sustained high productivity levels of secondary metabolites can be obtained
101 [3] and [16] under moderate nitrate limitation.

102

103 In a previous work [3], a screening of 10 strains of microalgae in continuous culture was
104 performed in order to evaluate their biomass and fatty acids productivity under nitrate
105 sufficiency and limitation. Through the in-depth characterization of the influence of nitrate
106 supply on growth and accumulation of fatty acids, the present research attempts to establish
107 conditions that maximize fatty acid productivity in continuous cultures of two microalgae,
108 namely *P. subcapitata* and *C. oleofaciens* that, according to Del Río et al. [3], behaved as
109 outstanding potential source of oil for biodiesel.

110

111

2. Materials and methods

2.1. Microalgae and culture conditions

The microalgae used were *Chlorococcum oleofaciens* (SAG 213-11) and *Pseudokirchneriella subcapitata* (SAG 61-81). Cells were grown photoautotrophically on the medium described by Arnon et al. [17] modified to contain 4 mM K₂HPO₄. The amount of NaNO₃ in the feed medium was adjusted as to reach the final concentrations indicated in each case. Continuous culture was performed in six 2.0-L capacity (0.07 m diameter, 0.50 m height) jacketed sterilized photochemostats (bubble columns) containing 1.8 L of cell suspension, continuously sparged with air (33 L (L culture⁻¹) h⁻¹). Optimal temperature for growth, 25° C for *P. subcapitata* and 30°C for *C. oleofaciens*, was maintained by circulating thermostated water through the photochemostat jacket. The pH was kept at 7.5, the optimal value for growth, by on-demand CO₂ injection into the air stream entering the culture. The photochemostats were illuminated following a solar daylight cycle (12 h light:12 h dark) (Fig. S1, Supplementary material). Each bubble column was surrounded by white panels holding six vertically placed fluorescent tubes (Phillips Master TL5 HO 24W/840, white-light lamps) (Fig S2, Supplementary material), which provided maximal incident irradiance of 3500 μE m⁻² s⁻¹ in the center of the reactor. Initially, the reactors were inoculated from batch-grown cells and operated on batch mode for about 3-4 d, with maximal incident irradiance being progressively increased, until becoming close to stationary phase. Then, they were switched to operate on continuous mode, fresh medium being continuously fed at a dilution rate (flow rate) of 0.2 d⁻¹, unless otherwise stated, with withdrawal of culture at the same rate. Once steady state conditions were achieved, analytical determinations were performed. The data presented correspond to stabilized situations, being average values of 4 to 8 determinations of each steady state. Standard deviation bars (SD) are shown.

Measurement of photosynthetically active irradiance (PAR) was carried out using a 4π quantum scalar irradiance sensor QSL-100 (Biospherical Instrument, San Diego, CA), inside the photochemostat filled with water, but without cells. Alternatively, a planar sensor (Li-190SA, LI-COR, USA) was used for PAR irradiance impinging on the reactor surface. In the latter case, the maximal irradiance measured was $600 \mu\text{E m}^{-2} \text{s}^{-1}$.

2.2. Analytical procedures

Biomass concentration was determined by dry weight measurement as described previously [3]. For lipid determination, gravimetric method was employed [18] on freeze dried biomass. The lipid fraction was obtained by mechanical disruption of 20 mg of biomass employing a Mini Bead Beater (Biospec Products), using chloroform:methanol (2:1, v/v) as extracting solvent and 0.5 mm diameter glass beads. After centrifugation (5 min, $12100 \times g$), the supernatant containing the lipid fraction was collected. The pellet was washed with chloroform:methanol (2:1, v/v) and the supernatants combined. To the lipid fraction, 3 mL of HCl 0.1N and 0.3 mL Cl_2Mg 0.5% were added and the mixture shaken in a vortex mixer. After centrifugation (5 min, $1500 \times g$), the lower phase was carefully recovered into a pre-weighed vial. After solvent evaporation under vacuum, dry weight of the lipid fraction was measured in a precision balance. Fatty acid content (% DW) and profile (% of total FA) were analyzed by transesterification and gas chromatography, according to Del Río et al. [3]. For protein and carbohydrate determination, an extraction of 4 mg of freeze dried biomass was performed through incubation in NaOH 0.1M at 70°C , during 45 min. In the extract obtained, protein was estimated by the Lowry method [19], using bovine serum albumin as a standard. Carbohydrate was measured by the phenol sulfuric acid method [20] with glucose as standard. Elemental analysis of washed and dried samples was carried out using a CHNS-O elemental analyzer (FlashEA 1112 Series, Thermo).

2.3. Productivity calculation

Productivity of biomass (P_b) and that of fatty acids (P_{FA}) were estimated in steady state situations of continuous cultures: $P_b = C_b * D$; and $P_{FA} = P_b * X_{FA}$; where C_b is the concentration of biomass; D is the dilution rate; and X_{FA} the mass fraction of fatty acid in the biomass.

3. Results and discussion

3.1. Effect of nitrate availability on biomass and fatty acid productivity

In order to analyze the influence of nitrogen limitation on the productivity of biomass and fatty acids by the microalgae *Chlorococcum oleofaciens* and *Pseudokirchneriella subcapitata*, photochemostats were fed with media containing nitrate at concentrations between 1 and 10 mM, at a constant dilution rate, D , of 0.2 d^{-1} . Data on biomass concentration of the cultures can be seen in Fig S3 (Supplementary material).

Biomass productivity of the two microalgae varied in response to nitrate availability (Fig. 1A). The highest values were recorded when nitrate concentration in the feed medium was 10 mM, with biomass productivity becoming progressively lower as nitrate availability decreased. In contrast to the positive correlation between nitrate availability and biomass productivity, the cellular fatty acid content increased in response to nitrate limitation, to reach levels of 41 and 35% of the dry biomass for *P. subcapitata* and *C. oleofaciens*, respectively, at 1 mM nitrate (Fig. 1B). The comparative analysis of the two species with regard to fatty acid accumulation in response to limitation in nitrate availability revealed significant differences between them. Reducing nitrate concentration in the feed medium from 10 to 5 mM resulted in the virtual doubling of the fatty acid content in *P. subcapitata*, whereas a similar enhancement of fatty acid levels in *C. oleofaciens* was only patent at much lower nitrate availability (2 mM). A most relevant parameter for establishing the potential of a microalga as a source of oil for biodiesel is

fatty acid productivity, which combines biomass productivity and fatty acid content. As shown in Fig. 1C, *P. subcapitata* was the most productive microalgae, with 160 mg fatty acids L⁻¹ d⁻¹ when nitrate concentration in the feed medium was 3-5 mM. In *C. oleofaciens*, maximum fatty acid productivity (111 mg L⁻¹ d⁻¹) was reached at 2 mM nitrate.

The yield of biomass and fatty acids on supplied PAR photons has been calculated as described by Molina Grima et al 1997 [21] and Ación Fernández et al. 1998 [22], on the basis of average irradiance (I_{av}), and measuring incident light with a quantum scalar sensor. Identical values were obtained by calculating the yields according to Klok 2013 [14]. In *P. subcapitata*, values for Y_xE (grams of biomass produced per mol of photons absorbed) decreased in response to a reduction in nitrate concentration, being of 0.31 and 0.08 g mol⁻¹ at 10 mM and 1 mM, respectively. For *C. oleofaciens*, corresponding values of 0.38 and 0.16 g mol⁻¹ were obtained. In order to facilitate comparison of these data with those derived from PAR measurements using a planar sensor, the corresponding yields using the latter device and calculated according to Breuer et al 2013 [23], have also been derived. For *P. subcapitata*, Y_xE values of 0.46 and 0.12 g mol⁻¹ were so obtained for 10 mM and 1 mM nitrate, respectively. For *C. oleofaciens*, the corresponding values were 0.55 and 0.24 g mol⁻¹. Nitrogen limitation led to a decrease in biomass productivity, and thus in the yield of biomass on light, as also described [14] for *N. oleoabundans*. Maximal values determined for fatty acids yield on light (Y_{FA}E) were 0.104 g mol⁻¹ and 0.071 g mol⁻¹ for *P. subcapitata* and *C. oleofaciens*, respectively. The corresponding Y_{FA}E values calculated based on planar sensor measurements were 0.153 g mol⁻¹ for *P. subcapitata* and 0.105 g mol⁻¹ for *C. oleofaciens*. Regardless the approach used for the calculations, *C. oleofaciens* exhibited higher yield of biomass on light (Y_xE), whereas the yield of FA on light (Y_{FA}E) was higher in *P. subcapitata*. The capacity to maintain a high Y_xE value under nitrogen limitation can vary among species, and also the extent to which excess of energy can be channeled into storage material as fatty acids [14].

The comparison of oil yield values reported for microalgae in continuous culture in photochemostat could illustrate on the suitability of different strains for oil production. Under N sufficiency, yields of about 10 mg fatty acids L⁻¹ d⁻¹ have been reported for *Chlorella minutissima* and *Dunaliella tertiolecta* [24], and of 126 mg lipids L⁻¹ d⁻¹ for *Neochloris oleoabundans* [25]. Values of 118 mg fatty acids L⁻¹ d⁻¹ were reported for *N. oleoabundans* in continuous cultures operated in turbidostat mode [14]. Under controlled N limitation, Wen et al. [13] have reported a productivity of 145 mg lipids L⁻¹ d⁻¹ for *Chlorella pyrenoidosa*. Productivity values established in the present work speak of the high capacity for oil production of *P. subcapitata* (160 mg fatty acids L⁻¹ d⁻¹, corresponding to 180 mg lipids L⁻¹ d⁻¹) and *C. oleofaciens* (111 mg fatty acids L⁻¹ d⁻¹, corresponding to 160 mg lipids L⁻¹ d⁻¹). Moreover, the highest fatty acid productivity of *P. subcapitata* was maintained in the range 3-5 mM nitrate, which could facilitates the operation of the cultures, as possible fluctuations within that range would not affect the yield. The superiority of *P. subcapitata* (and *C. oleofaciens*) becomes even more patent when considering that the productivity values above quoted for *N. oleoabundans* and *C. pyrenoidosa* were obtained under continuous illumination at constant irradiance, whereas in the present work daylight cycle illumination was used. The latter conditions are, moreover, closer to those in which outdoor systems operate, so that the information herein reported could be easier to apply to the scaling up of the corresponding production process.

3.2. Effect of nitrate availability on biochemical composition of the biomass

The various N-limiting conditions imposed resulted in differences in the biochemical composition of the microalgae (Fig. 2). A typical response of microalgae under nitrogen limitation is the redirection of metabolism from protein synthesis towards storage compounds, like carbohydrate or lipids. A reduction in protein content was observed for both species in response to reduced nitrate availability, although in different degree. *P. subcapitata* presented higher protein content than *C. oleofaciens* in almost all nitrate supply conditions considered (Fig. 2A). Lipid was the main storage material in *P. subcapitata*, while it was carbohydrate in *C.*

oleofaciens (Fig. 2B, 2C). It is worth noting that high lipid content coexisted with a substantial protein level in *P. subcapitata*, while *C. oleofaciens* presented a remarkable combination of lipid and carbohydrate content. This should be taken into account when considering the exploitation of each biomass.

Fatty acid profile was also affected by nitrogen availability (Table 1). Biodiesel composition must satisfy with standards (European Standard EN 14214 and ASTM D6751 in Europe and USA, respectively) that regulate among others, the level for unsaturated methyl esters. In general, a raw material rich in saturated and monounsaturated fatty acids is more suitable as biodiesel feedstock [1], [7] and [12]. Actually, nitrate limitation in the feed medium led to an increase in saturated and monounsaturated fatty acids (mainly palmitic and oleic acid) in the two species, to the detriment of polyunsaturated (Table 1).

Fig. 3 shows the fatty acid profile for each species under conditions of maximal fatty acid productivity. In *P. subcapitata* and *C. oleofaciens*, the sum of C16:0 and C18:1 accounted for more than 68% of total fatty acids, in parallel with a linolenic acid content below 10%, thus being in accordance with the limit set by standard to this fatty acid methyl ester (12%). This highlights the adequacy of the biomass obtained in these conditions as biodiesel feedstock.

3.3. Relationship between fatty acids and N content of the biomass

For a detailed assessment of the relationship between fatty acid levels and N content of the biomass, it was pertinent to consider the information derived from a substantial number of samples corresponding to a variety of growth conditions. Therefore, the full analysis also included samples from experiments addressed to evaluate the response of *C. oleofaciens* and *P. subcapitata* to factors other than nitrate in the feed medium, such as temperature, pH and dilution rate (Figs. 4 and 5).

Nitrogen supply to the cells can be defined by the parameter SNI (specific nitrate input, mmol $\text{NO}_3^- \text{ g}^{-1} \text{ d}^{-1}$) [15], which considers dilution rate, nitrate concentration in the feed medium and biomass concentration. The incorporation of nitrogen into the biomass exhibited a hyperbolic response to increasing SNI (Fig. S4, Supplementary material). Reduction of SNI led to fatty acid accumulation over basal level. Whereas for triggering fatty acid accumulation in *P. subcapitata* a SNI level of about 2 mmol $\text{NO}_3^- \text{ g}^{-1} \text{ d}^{-1}$ was sufficient, a more stringent limitation in nitrate input (SNI, 0.5 mmol $\text{NO}_3^- \text{ g}^{-1} \text{ d}^{-1}$) was required in the case of *C. oleofaciens* (Fig. 4).

A negative correlation did exist between nitrogen content and fatty acid level in the biomass of the two species, although differences in the shape of the relationship were obvious (Fig. 5).

The nitrogen content at which fatty accumulation was induced was also different for each species. Accumulation of fatty acids in *P. subcapitata* was apparent even when the nitrogen content was as high as 8% of the dry biomass with an inverse linear relationship existing between nitrogen and fatty acid content. Induction of fatty acid accumulation in *C. oleofaciens* corresponded to a much lower nitrogen level, of about 5%. Adams et al. [26] analyzed the effect of nitrogen limitation in six microalgae in batch culture, concluding that the level of N stress at which lipid accumulation was triggered was unique for every species, ranging from minimal (*N. oleabundans*, 7.5% N in the biomass) to severe N stress (*S. naegelii*, 3% N). In the present work, performed in continuous culture, differences between strains were also apparent, being worthy to underline the ability of *P. subcapitata* to accumulate fatty acids in response to minimal N stress.

4. Conclusions

The capacity of two microalgal species (*Chlorococcum oleofaciens* and *Pseudokirchneriella subcapitata*) for fatty acid production has been assessed in continuous culture. The reduction in nitrate availability resulted in an increase in fatty acid content in both strains, but the overall

evaluation of fatty acid productivity revealed the remarkably high capacity of *P. subcapitata*: 160 mg fatty acids (or 180 mg lipids) L⁻¹ d⁻¹. Such a productivity value is clearly superior to that of the oleaginous strain *C. oleofaciens* and to those reported in the literature for microalgae in continuous culture. A remarkable peculiarity of *P. subcapitata* is the low threshold for nitrogen stress that triggers accumulation of fatty acids in its biomass. These results, together with a suitable fatty acid profile, highlight the potential of *Pseudokirchneriella subcapitata* as an adequate source of fatty acids for biodiesel production.

Author contributions

E. Del Río, M.G. Guerrero and M.García-González designed research; E. Del Río, E. García-Gómez and J.Moreno performed research and analyzed data; E. Del Río, M.G. Guerrero and M.García-González wrote the paper. All authors read and approved the final manuscript.

Acknowledgements

Research supported by Spanish Ministerio de Ciencia e Innovación (Project CTQ2008-06741-CO2-02/PPQ) and ACCIONA Energía S.A.

References

1. Chisti Y (2007) Biodiesel from microalgae. *Biotechnol Adv* 25: 294-306.
2. Sing SF, Isdepsky A, Borowitzka MA, Moheimani NR (2013). Production of biofuels from microalgae. *Mitig Adapt Strateg Glob Change* 18: 47-72.
3. Del Río E, Armendáriz A, García-Gómez E, García-González M, Guerrero MG (2015) Continuous culture methodology for the screening of microalgae for oil. *J Biotechnol* 195: 103-107.
4. Griffiths MJ, van Hille RP, Harrison STL (2012) Lipid productivity, settling potential and fatty acid profile of 11 microalgal species grown under nitrogen replete and limited conditions. *J Appl Phycol* 24: 989–1001.

- 334 5. Pruvost J, Van Vooren G, Le Gouic B, Couzinet-Mossion A, Legrand J (2011). Systematic
335 investigation of biomass and lipid productivity by microalgae in photobioreactors for biodiesel
336 application. *Bioresour Technol* 102: 150–158.
- 337 6. Rodolfi L, Zittelli GC, Bassi N, Padovani G, Biondi N, Bonini G, Tredici MR (2009).
338 Microalgae for oil: strain selection, induction of lipid synthesis and outdoor mass cultivation in
339 a low-cost photobioreactor. *Biotechnol Bioeng* 102: 101-112.
- 340 7. Hu Q, Sommerfeld M, Jarvis E, Ghirardi M, Posewitz M, Darzins A (2008) Microalgal
341 triacylglycerols as feedstocks for biofuel production: perspectives and advances. *The Plant J* 54:
342 621-639.
- 343 8. Procházková G, Brányiková I, Zachleder V, Brányik T (2014) Effect of nutrient supply status
344 on biomass composition of eukaryotic green microalgae. *J Appl Phycol* 26: 1359-1377.
- 345 9. Solovchenko AE (2012). Physiological role of neutral lipid accumulation in eukaryotic
346 microalgae under stresses. *Russ J Plant Physiol* 59 (2): 167–176.
- 347 10. Fernandes B, Teixeira J, Dragone G, Vicente AA, Kawano S, Bišová K, Přibyl P, Zachleder
348 V, Vítová M (2013) Relationship between starch and lipid accumulation induced by nutrient
349 depletion and replenishment in the microalga *Parachlorella kessleri*. *Bioresour Technol* 144:
350 268–274.
- 351 11. Li Y, Han D, Sommerfeld M, Hu Q (2011) Photosynthetic carbon partitioning and lipid
352 production in the oleaginous microalga *Pseudochlorococcum* sp. (Chlorophyceae) under
353 nitrogen-limited conditions. *Bioresour Technol* 102: 123-129.
- 354 12. Stansell GR, Gray VM, Sym SD. (2012). Microalgal fatty acid composition: implications
355 for biodiesel quality. *J Appl Phycol* 24: 791-801.
- 356 13. Wen X, Geng Y, Li Y (2014). Enhanced lipid production in *Chlorella pyrenoidosa* by
357 continuous culture. *Bioresour Technol* 161: 297-303.
- 358 14. Klok AJ, Martens DE, Wijffels RH, Lamers PP (2013). Simultaneous growth and neutral
359 lipid accumulation in microalgae. *Bioresour Technol* 134: 233-243.

15. Del Río E, Acien FG, García-Malea MC, Rivas J, Molina-Grima E, Guerrero MG (2005) Efficient one-step production of astaxanthin by the microalga *Haematococcus pluvialis* in continuous culture. *Biotechnol Bioeng* 91: 808-815.
16. Del Río E, Acien FG, García-Malea MC, Rivas J, Molina-Grima E, Guerrero MG (2008) Efficiency assessment of the one-step production of astaxanthin by the microalga *Haematococcus pluvialis*. *Biotechnol Bioeng* 100 (2): 397-402.
17. Arnon DI, McSwain BD, Tsujimoto HY, Wada K (1974) Photochemical activity and components of membrane preparations from blue-green algae. I. Coexistence of two photosystems in relation to chlorophyll a and removal of phycocyanin. *Biochim Biophys Acta* 357: 231-245.
18. Kochert G (1978) Quantitation of the Macromolecular Components of Microalgae. In: Hellebust JA, Craigie JS (eds) *Handbook of Phycological Methods. Physiological and Biochemical Methods*, Cambridge University Press, Cambridge, pp 189.
19. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* 193: 265-275.
20. Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F (1956) Colorimetric method for determination of sugars and related substances. *Anal Chem* 28: 350-356.
21. Molina Grima E, Camacho FG, Sánchez Pérez JA, Acien Fernández FG, Fernández Sevilla JM (1997) Evaluation of photosynthetic efficiency in microalgal cultures using averaged irradiance. *Enz Microb Technol* 21: 375-381
22. Acien Fernández FG, García Camacho F, Sánchez Pérez JA, Fernández Sevilla JM, Molina Grima E (1998) Modeling of biomass productivity in tubular photobioreactors for microalgal cultures: effects of dilution rate, tube diameter and solar irradiance. *Biotechnol Bioeng* 58 (6): 605-616.
23. Breuer G, Lamers PP, Martens DE, Draaisma RB, Wijffels RH (2013) Effect of light intensity, pH, and temperature on triacylglycerol (TAG) accumulation induced by nitrogen starvation in *Scenedesmus obliquus*. *Bioresour Technol* 143: 1-9

24. Tang H, Chen M, Simon Ng KY, Salley SO (2012). Continuous microalgae cultivation in a photobioreactor. *Biotechnol Bioeng* 109: 2468-2474.
25. Pruvost J, Van Vooren G, Cogne G, Legrand J (2009) Investigation of biomass and lipids production with *Neochloris oleoabundans* in photobioreactor. *Bioresour Technol* 100: 5988–5995.
26. Adams C, Godfrey V, Wahlen B, Seefeldt L, Bugbee B (2013) Understanding precision nitrogen stress to optimize the growth and lipid content tradeoff in oleaginous free microalgae. *Bioresour Technol* 131: 188-194.

Table 1. Influence of nitrate concentration in the feed medium on major fatty acids profile (% of total) in the biomass of *C. oleofaciens* and *P. subcapitata* in continuous culture.

	Fatty acid	1 mM	2 mM	3 mM	5 mM	10 mM
<i>C. oleofaciens</i>	C16:0	24.25	24.69	24.62	22.28	17.27
	C16:1(9)	3.82	4.09	3.55	3.52	2.74
	C16:1(7)	4.12	3.80	4.48	5.66	8.99
	C16:2 (9,12)	0.21	0.26	0.40	0.75	1.47
	C16:3(7,10,13)	1.95	1.71	1.27	1.39	1.45
	C16:4(4,7,10,13)	1.53	1.99	2.03	2.94	5.71
	C18:0	1.40	1.59	1.99	1.54	1.42
	C18:1(9)	42.11	43.30	41.18	35.93	23.61
	C18:2(9,12)	11.35	9.84	11.70	12.83	16.25
	C18:3 (5,9,12)	3.22	2.77	3.00	3.34	4.10
	C18:3(9,12,15)	5.10	5.45	5.14	6.55	11.64
	C18:4(6,9,12,15)	0.36	0.41	0.38	0.49	0.85
<i>P. subcapitata</i>	C16:0	21.43	22.69	21.86	22.31	22.13
	C16:1(9)	0.54	0.61	0.50	0.59	1.54
	C16:3(7,10,13)	0.82	0.79	0.75	0.73	1.01
	C16:4(4,7,10,13)	2.46	2.89	2.85	3.56	10.71
	C18:0	2.93	2.80	2.61	3.10	1.71
	C18:1(9)	55.91	52.61	53.97	48.92	23.05
	C18:2(9,12)	1.79	1.97	1.91	2.22	4.43
	C18:3(9,12,15)	8.43	9.25	8.72	10.51	23.11
	C18:4(6,9,12,15)	3.72	3.86	3.56	3.69	4.79

A

B

C

Fig. 1 Influence of nitrate availability on biomass productivity (A), fatty acid content (B) and fatty acid productivity (C) of *C. oleofaciens* and *P. subcapitata* in continuous culture.

436

437

A₁

438

B₁

439

C₁

440

441 **Fig. 2** Influence of nitrate availability on the biochemical composition of the biomass of *C.*
 442 *oleofaciens* and *P. subcapitata* in continuous culture: protein (A), lipid (B) and carbohydrate
 443 content (C).

444

Fig. 3 Main fatty acids profile under conditions of maximal fatty acid productivity in the biomass of *C. oleofaciens* and *P. subcapitata* in continuous culture. Nitrate concentration in feed medium: *C. oleofaciens*, 2 mM, *P. subcapitata*, 3 mM.

Fig. 4 Influence of specific nitrate input on fatty acid content of the biomass of *C. oleofaciens* and *P. subcapitata* in continuous culture. The data correspond to experiments performed to evaluate the effect of the following factors: temperature: 20, 25, 30°C (at pH 7.5 and D=0.3 d⁻¹); pH: 6.5, 7,7.5, 8, 8.5 (D=0.3 d⁻¹ and 25°C for *P. subcapitata* and D=0.3 and 30°C for *C. oleofaciens*); dilution rate: 0.1, 0.2, 0.3,0.45, 0.6 d⁻¹ (pH 7.5 and 25°C for *P. subcapitata* and pH 7.5 and 30°C for *C. oleofaciens*); Nitrate concentration in feed medium: 1, 2, 3 ,5, 10 mM (D= 0.2 d⁻¹, pH 7.5 and 25°C for *P. subcapitata* and D= 0.2 d⁻¹, pH 7.5 and 30°C for *C. oleofaciens*).

481

482

A

483

B

484

485 **Fig. 5** Relationship between nitrogen content and fatty acid content of the biomass of *C.*
 486 *oleofaciens* (A) and *P. subcapitata* (B) in continuous culture. For information on the data, see
 487 the legend of Fig. 4.

488

489