




# Improving the lipid content of *Nannochloropsis oculata* by a mutation-selection program using UV radiation and quizalofop

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## Abstract

Microalgal biomass with a high content of lipids and fatty acids is generally obtained by culture under stress conditions, limiting its growth and increasing production costs. However, it is possible to obtain strains with higher content of some of the desired biochemical component through genetic improvement strategies. Our objective was to increase the lipid content through a mutation-selection procedure in the microalga *Nannochloropsis oculata*. This procedure involved the ultraviolet radiation exposure of microalgae at different times at different densities and selecting surviving colonies. Subsequently, they were exposed to the herbicide quizalofop-p-ethyl, selecting the colonies with lower survival. An 85% mortality in the UV-exposed microalgae was recorded at 120 min for  $1 \times 10^5$  cells mL<sup>-1</sup>. Two strains surviving quizalofop-p-ethyl were obtained, with only one strain surviving in standard culture conditions. The comparison of the new and original strains shows that the growth rate of the new strain of *N. oculata* (S3) is greater than that of the original strain, and it also had a higher content of total lipids and some fatty acids such as (a) arachidonic acid (up to five times higher than original); (b) oleic and heptadecaenoic acids (more than double than the original strain); (c) elaidic, tridecanoic, and palmitic acids (slightly higher than the original strain). There were significant differences in composition profile (carbohydrates, proteins) in comparison with the original strain. In conclusion, the mutation-selection procedure for obtaining new strains with higher lipid content is suitable for the freshwater microalga *N. oculata*. It could be considered as a strategy of genetic improvement with potential for aquaculture, food, pharmaceuticals, and biodiesel.

**Keywords** Microalga · Eustigmatophyceae · Random mutagenesis · Fatty acids · Lipids

## Introduction

The microalgae are aquatic, photosynthetic, and microscopic organisms that play a key role in aquatic ecosystems

supplying organic matter and molecules such as polyunsaturated fatty acids (PUFAs) to organisms of higher trophic levels (Bellou et al. 2014). The successful commercial exploitation of microalgae is based on their nutritional content as a commercial source of PUFAs, energy, protein, vitamins, and sterols (Muller-Feuga 2000; Hemaiswarya et al. 2011; Bougaran et al. 2012), as well as by their content of antioxidants and pigments (Hemaiswarya et al. 2011; Borowitzka 2013), decreasing mortality and promoting the growth of larval fish and crustaceans of commercial interest (Muller-Feuga 2000; Hemaiswarya et al. 2011). Microalgae are an attractive group as source for a wide range of chemicals products with several applications such as dietary, nutritional, cosmetics, pharmaceuticals, and bioenergetics (Olaiola 2003; Qin et al. 2012). The great demand for biofuels and the growing market of natural products creates the necessity of incorporating strategic technologies such as genetic engineering to meet the demand for raw materials obtained from microalgae through more efficient metabolic pathways favoring sustainable applications (Qin et al. 2012).

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The manipulation of variables in culture conditions has proven that it is possible to modify the biochemical composition of microalgae (León-Bañares et al. 2004; Doan and Obbard 2012; Bellou et al. 2014; Lukes et al. 2017; Pavón-Suriano et al. 2017; Ishika et al. 2018). However, the maximum synthesis of biochemical compounds is defined by the genome of the organism (Doan and Obbard 2012). The improvement of the strains allows the optimization of the nutritional quality of microalgae, increasing their content of proteins and fatty acids (León-Bañares et al. 2004; Chatuverdi et al. 2004; Cortez et al. 2015; Pavón-Suriano et al. 2017). Currently, genetic manipulation of microalgae is a promising strategy considered as a feasible alternative for the production of compounds with commercial interest. However, despite biotechnological interest on microalgae, there are few stable methods for genetic transformation of some species (Qin et al. 2012).

The genetic improvement through random mutagenesis is optimized in terms of the type and dose of mutagen to use, as well as the method of selection of the mutants generated (Ertola et al. 1994; Cortez et al. 2015). The methods used to induce mutations include physical agents such as UV and gamma radiation (Griffiths et al. 2000; Chatuverdi et al. 2004; Feng et al. 2015; Cortez et al. 2015; Zhang et al. 2018), and X-ray (Griffiths et al. 2000; Cortez et al. 2015); even alone, UV radiation induces mutations in microalgae that favor the biosynthesis and accumulation of fatty acids (Zayadan et al. 2014; Liu et al. 2015). However, the chemical agents are the most commonly used as ethyl-methanesulfonate (EMS) (Griffiths et al. 2000; Chatuverdi et al. 2004; Chatuverdi and Fujita 2006; Cortez et al. 2015) methyl-methane-sulfate (MMS), nitrosoguanidine (GTN) and ethyl-nitrosourea (ENU) (Griffiths et al. 2000; Cortez et al. 2015) and *N*-methyl-*N*-nitrosourea (MNU) (Chatuverdi et al. 2004; Cortez et al. 2015). The type of inhibitor to be used on the mutant strain should be selected based on the desired improved characteristic (Gómez and González 2001). For example, the fatty acid synthesis inhibitors Cerulenin and Quizalofop are used for the induction of microalgal mutants with greater capacity for synthesis of polyunsaturated fatty acids (Chatuverdi et al. 2004; Chatuverdi and Fujita 2006; Cortez et al. 2015). Mutation-selection techniques used with microalgae have been applied to increase the synthesis of fatty acids and pigments with different mutagen agents and using inhibitor substances for the selection of specific characteristics (Shaish et al. 1991; Zhang et al. 1997; Yong et al. 2003; Chatuverdi et al. 2004; Chatuverdi and Fujita 2006; Gómez et al. 2013; Cortez et al. 2015).

The genus *Nannochloropsis* is a unicellular marine microalgae belonging to the class Eustigmatophyceae (Hibberd 1981, Chatuverdi and Fujita 2006; Kagan and Matulka 2015), of the family Monodopsidaceae (Hibberd 1981). Cultivation conditions include temperatures of 16–

27 °C, 12:12 h photoperiod (light/dark), illumination of 13–40  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ , and salinities of 0–35‰ (Barsanti and Gualtieri 2006). It has been reported that *Nannochloropsis* sp. has high levels of proteins and PUFAs (Chatuverdi and Fujita 2006; Kilian et al. 2011; Kagan and Matulka 2015), and antioxidant pigments (Kilian et al. 2011; Kagan and Matulka 2015); even the oil derived from *N. oculata* is reported safe as a dietary supplement (Kagan and Matulka 2015). In addition, *Nannochloropsis* sp. is used as a source of omega-3 fatty acids in mariculture (Zou et al. 2000; Sanchez et al. 2008; Kagan and Matulka 2015), because of the high PUFA content (Zou et al. 2000; Chatuverdi and Fujita 2006; Sanchez et al. 2008), especially eicosapentaenoic acid (EPA), arachidonic acid (ARA), and docosahexaenoic acid (DHA). *Nannochloropsis oculata* can be adapted to freshwater medium, thus generating a freshwater strain, which can be used for feeding freshwater zooplanktonic organisms such as rotifers and cladocerans (Pérez-Legaspi and Rico-Martinez 1998; Pérez-Legaspi et al. 2015; Rico-Martinez et al. 2016). This microalga is considered promising for industrial applications as a source of polyunsaturated fatty acids (Assaf 1989). Therefore, it is desirable to implement metabolic engineering technologies to improve some attributes of the genus *Nannochloropsis* sp. (Chatuverdi and Fujita 2006). Our goal was to achieve the genetic improvement of *N. oculata* (freshwater strain) using mutation-selection to obtain new strains with higher content of lipids.

## Materials and methods

### Test organism

*Nannochloropsis oculata* Droop (Hibberd, 1981) obtained from the algae culture collection (UTEX strain LB 2164; Austin, TX, USA) was used and adapted and cultivated in freshwater (freshwater strain) in Bold's Basal Medium (BBM) (Stein 1979), using distilled water in Erlenmeyer flasks (1 L) maintaining them at a temperature of  $25 \pm 2$  °C and continuous light at  $80 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ , with constant aeration. Once the algae reached the exponential growth phase, aeration was eliminated, allowing algal sedimentation at 4 °C in a refrigerator for its subsequent washing. The supernatant was decanted replacing with 50 mL of new and sterile BBM, suspending the algae, and transferring them into a 50-mL new, sterile centrifuge tube. The microalgae were centrifuged at  $7177 \times g$ , 15 min, and the algae pellet was resuspended in sterile distilled water. The pellet was transferred to various 1.5 mL centrifuge tubes, and centrifuged at  $20,096 \times g$ , 7 min at 10 °C. Subsequently, sterile growing medium was added to concentrated algal biomass pellets in the 50 mL tube. Cell density was determined using a Neubauer Chamber in triplicate, evaluating the purity of the strain and cellular integrity using an optical microscope (Carl Zeiss Primo Star).

## Microalgae sensitivity to the herbicide

This procedure was performed according to the protocols of Chatuverdi et al. (2004) and Cortez et al. (2015) with slight modifications. The original strain of *N. oculata* at an initial density of  $5 \times 10^3$  cells mL<sup>-1</sup> was exposed to different concentrations (100, 200, 400, 600, 800, 900, and 1000 µM) of the herbicide quizalofop-p-ethyl (Pestanal, Sigma-Aldrich) in BBM including a negative control (BBM without herbicide), in a test volume of 5 mL in triplicate. These treatments were maintained at  $25 \pm 2$  °C for 24 h at  $54 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ . After the exposure period, we estimated the cell density for each treatment, assessing the percentage of mortality; selecting the highest concentration of the herbicide (900 µM) where the microalgae showed lower survival (15%).

## Exposure to UV radiation

Once washed, algal biomass was obtained, a stock solution of *N. oculata* with a density of  $1 \times 10^7$  cells mL<sup>-1</sup> was prepared in BBM and then we obtained aliquots for performing the three treatments with the microalgae at different densities ( $1 \times 10^5$ ,  $5 \times 10^5$ , and  $2.5 \times 10^6$  cells mL<sup>-1</sup>) in 2.5 mL of culture medium in triplicate, in sterile polystyrene 24-well plates (Costar, Corning Inc.). The plates were exposed to ultraviolet radiation ( $\lambda = 354$  nm) using a UV light lamp (Philips, TUV30W/G30 T8) at a distance of 10 cm with different exposure times (30, 60, 90, and 120 min), according to the protocol of Bougaran et al. (2012) with slight modifications. Then, we estimated the percentage of mortality for each treatment using the viable count method considering the ability to form colonies on a solid medium according to Madigan et al. (2004), involving the inoculation of an aliquot (10 µL) to different decimal dilutions of each treatment on bacteriological agar (BD Bioxon™) with Bold's Basal medium in Petri dishes new and sterile, by triplicate. These plates were incubated for 3 weeks in a 12:12 h photoperiod (light/dark) at  $80 \mu\text{mol photons m}^{-2} \text{s}^{-1}$  at  $25 \pm 2$  °C. Later, we counted the colonies using a colony counter (Felisa), including only plates with 30 to 300 colonies. The CFU (colony forming units) were estimated using the following equation:  $\text{CFU mL}^{-1} = \text{No. colonies} / (\text{aliquot} * \text{dilution})$ .

Once we estimated the percentage mortality, we selected the treatment that showed the highest mortality during exposure to UV light ( $1 \times 10^5$  cells mL<sup>-1</sup> for 120 min), assuming that it suffered mutation. From this treatment, six colonies were isolated called S1, S2, S3, S4, S5, and S6; transferring them to 5 mL BBM in test tubes, including control of *N. oculata* (without UV exposure) with the same cell density ( $1 \times 10^5$  cells mL<sup>-1</sup>). All the mutant strains and control were kept in continuous light ( $54 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) at  $25 \pm 2$  °C for 15 days, assessing cell density until the end of the

exposure period. Subsequently, those strains with better growth (S2, S3, and S4) were selected to expose them to the herbicide quizalofop-p-ethyl.

## Herbicide exposure of mutant strains

This procedure was carried out according to Chatuverdi et al. (2004). After 15 days of cultivation, aliquots of each strain of *N. oculata* survivor UV radiation (S2, S3, and S4) and one strain control (microalgae without UV exposure) were collected, obtaining an initial density of  $1 \times 10^6$  cells mL<sup>-1</sup> in 1 l of BBM containing the herbicide quizalofop-p-ethyl at a 900 µM concentration in Erlenmeyer flasks, providing constant aeration and keeping them at  $25 \pm 2$  °C and  $54 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ . The growth rate of the strains exposed to the herbicide was evaluated every 2 days for 8 days in triplicate. Subsequently, we selected strains (S2 and S3) which showed greater resistance to the herbicide, transferring them to standardized culture conditions for further analysis including a control strain of *N. oculata* (not subjected to the process of mutation-selection).

## Analysis of the new strains

From an initial density of  $1 \times 10^6$  cells mL<sup>-1</sup>, the growth rate of both strains S3 and control (not subjected to mutation-selection process) were evaluated in triplicate. Strains S2 and S4 were not analyzed since they did not survive the standardized cultivation conditions. Cell size of the two strains, control and S3 ( $N = 30$ ), was measured using a micrometer reticule (10:100 Carl Zeiss) and an optical microscope at  $\times 40$ . In addition, we estimated the cell density and dry weight of the biomass from 50 mL samples of both strains in exponential and stationary phase. The microalgal dry weight was measured in triplicate according to the gravimetric method of Sorokin (1973) with slight modifications. Microfiber filters 55 mm (Whatman GF/C, nominal particle retention: 2.7 µm) were placed in a desiccator for 24 h until a constant weight was recorded using an analytical balance (Denver Instrument Co.). The biomass from 50 mL of microalgae samples filtered through filters using a vacuum pump was collected and placed in sterile Petri dishes; wet weight was recorded and subsequently placed in a culture oven (ECOSHEL 9162) at 50 °C for 24 h. The dry weight biomass was calculated from the difference in weight of the filter and the filter + algae the difference in weight. Filters containing the dry biomass in exponential phase were placed in test tubes to determine protein using the Bradford method, according to Kruger (2002), and adapted for microalgae by Arredondo et al. (2007). In brief, 1 N NaOH was added and the sample incubated for 60 min at 100 °C. The calibration curve was made with egg albumin. Carbohydrate analysis

was carried out using the sulfuric phenol-acid method (Dubois et al. 1956) using anhydrous D-glucose for the standard curve. The analysis of chlorophyll *a* was according to Jeffrey and Humphrey (1975). Extraction of chlorophyll *a* was from samples in exponential phase using acetone (99.8%) and then sonicating samples on ice for two cycles of 5 min in a sonicator (Branson 1510), standing at 4 °C for 16 h, followed by centrifugation at  $4306.2 \times g$ , 10 min, 4 °C, and recovering the supernatant to the absorbance of the supernatant was measured at  $\lambda = 664$  nm, and chlorophyll *a* concentration was calculated using the equation: chlorophyll *a* =  $11.41 E_{664}$  (Moheimani et al. 2013). The extraction of total lipids included the analysis of both exponential and stationary phases. This analysis was conducted with Soxhlet equipment using chloroform/methanol (1: 2 v/v), according to Halim et al. (2012). The quantification of total lipids was performed using the gravimetric method of Del Ángel et al. (2007).

### Fatty acid analysis

Esterification of the total lipids extracted was by the method of Lepage and Roy (1984), adding 270 mg of KOH to the sample, and then 30 mL of methanol and 10 mL of water, allowing evaporation. Subsequently, once the sample was cool, we added 30 mL of HCl 3% in methanol and heated again until the formation of salts. Then, we rinsed the sample with 20 mL of distilled water, placed it on a separation funnel, adding 30 mL of hexane ( $10 \times 10$  mL), and strongly agitating for 1 min. The supernatant was recovered to evaporate the solvent. Samples were then stored for later analysis by gas chromatography. Fatty acids were identified and quantified with a gas chromatograph (Perkin Elmer, Mod. Autosystem) with flame ionization, involving an Omegawax column (250, 30 m  $\times$  0.25 mm, 0.25  $\mu$ m) and nitrogen as the carrier gas. The temperature of the injector was 250 and 300 °C for the detector. The oven temperature was 100 °C (5 min) with a slope of 5 °C  $\text{min}^{-1}$  up to 220 °C with a ramp of 3 °C  $\text{min}^{-1}$  until 250 °C (10 min). The quantification of the fatty acids considering the lipid fraction in relation to the dry weight and the percentage of lipids obtained, according to Zavřel et al. (2018).

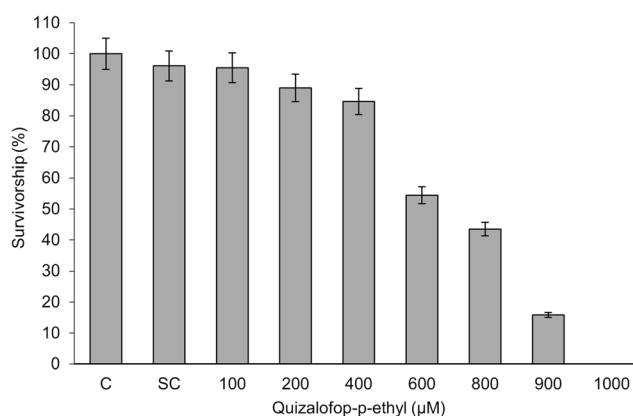
### Statistical analysis

The comparison between the control strain (not subjected to mutation-selection program) and the new strain (S3) in their size measurements and biochemical analysis such as density, dry weight, protein, carbohydrate, lipids, and chlorophyll were performed by one-way analysis of variance (ANOVA) using Statistica 7.0 (Statsoft, Inc. 2004) ( $p < 0.05$ )

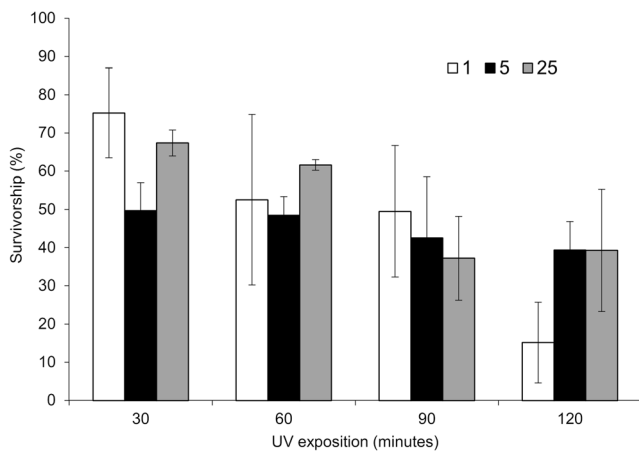
## Results

The estimation of the sensitivity of the original microalgae *N. oculata* exposed to the herbicide quizalofop-p-ethyl shows that a higher concentration of the herbicide increasingly inhibited the algal growth rate. We found 900  $\mu\text{M}$  as the maximum concentration where some surviving cells are still observed (Fig. 1). Therefore, this concentration was selected to inhibit the strains obtained after UV exposure. When the *N. oculata* was exposed to UV I, we found that the lowest rate of survival occurred at 120 min at a density of  $1 \times 10^5$  cells  $\text{mL}^{-1}$  and it is the treatment with less ability to form colonies in comparison with the other densities and times of exposure (Fig. 2). To isolate mutant strains, six isolates (S1, S2, S3, S4, S5, and S6) were taken from this treatment (120 min,  $1 \times 10^5$  cells  $\text{mL}^{-1}$ ) and cultivated in standardized conditions for 15 days, selecting those that showed better or similar growth in comparison to the control (Fig. 3). In this way, we obtained the strains S2 ( $1.3 \times 10^6 \pm 0.43 \times 10^4$  cells  $\text{mL}^{-1}$ ), S3 ( $1.6 \times 10^6 \pm 0.08 \times 10^4$  cells  $\text{mL}^{-1}$ ), and S4 ( $1.3 \times 10^6 \pm 0.24 \times 10^4$  cells  $\text{mL}^{-1}$ ).

The cultivation of the mutant strains (S2, S3, S4) in BBM with 900  $\mu\text{M}$  of quizalofop-p-ethyl, showed that only strains S2 and S3 were able to survive, showing resistance to the herbicide (Fig. 4). However, once these strains were transferred to media in standard conditions, only strain S3 survived. This strain and the control strain were cultured to assess their growth rates; we observed S3 strain grew faster than did the original strain (time to reach the exponential phase) (Fig. 5). In addition, the measurement of cells ( $N = 30$ ) showed that the new strain had larger cells (length  $3.5 \pm 0.7$   $\mu\text{m}$ , width  $3.6 \pm 0.6$   $\mu\text{m}$ ) in comparison with the original strain (length  $2.7 \pm 0.6$   $\mu\text{m}$ , width  $2.8 \pm 0.5$   $\mu\text{m}$ ), where the difference in width was significant ( $p < 0.05$ ).



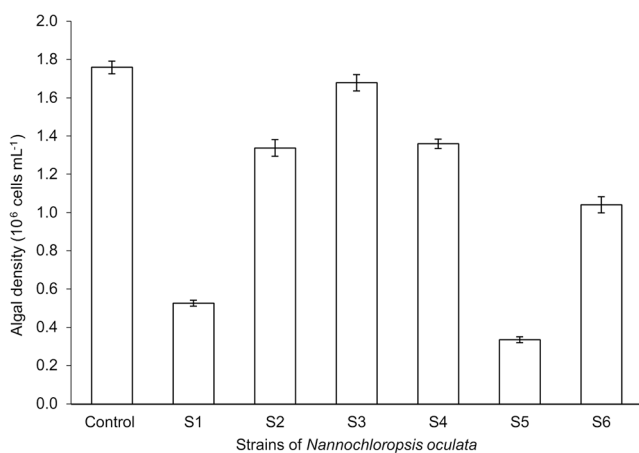
**Fig. 1** Effect of the herbicide quizalofop-p-ethyl on the survivorship of the microalgae *N. oculata* ( $5 \times 10^3$  cells  $\text{mL}^{-1}$ ) after 24 h of exposure. Mean  $\pm$  standard deviation,  $n = 3$ . Abbreviations: C control, SC solvent control



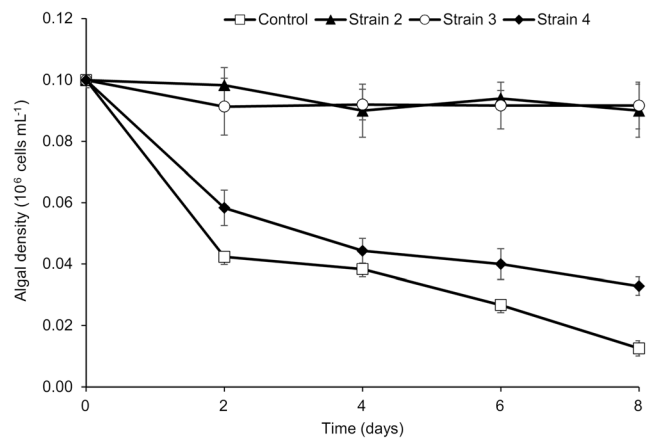
**Fig. 2** Effect of the UV radiation ( $\lambda=354\text{ nm}$ ) on the survivorship of the microalgae *N. oculata* after several exposition times at several densities ( $1, 5,$  and  $25 \times 10^5\text{ cells mL}^{-1}$ ). Mean  $\pm$  standard deviation,  $n = 3$

The composition of the original strain (not subject to the process of mutation-selection) and the new strain (S3) subjected to the process of mutation-selection is shown in Table 1. The exponential phase shows the original strain had highest cell density and dry weight, while strain S3 had greater dry weight in the stationary phase with a similar cell density than the original strain. The protein and carbohydrate content of strain S3 was slightly lower than the original strain. Chlorophyll *a* was higher in the exponential phase for the S3 strain. The evaluation of total lipids shows that S3 strain had a higher lipid content in both exponential and stationary phases compared to the original strain. Chlorophyll *a* was higher in the exponential phase for the S3 strain.

Table 2 shows the composition of fatty acids for both strains in both exponential and stationary phases. Arachidonic acid was five times greater ( $1.54 \pm 0.003\text{ mg g}^{-1}\text{ DW}$ ) in strain S3 than in the original strain ( $0.275 \pm$

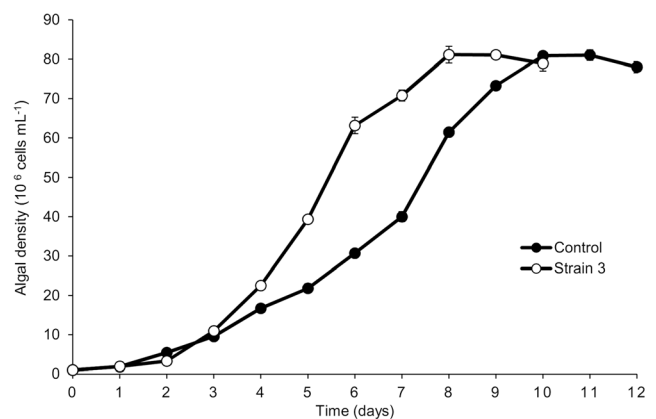


**Fig. 3** Algal density of different isolated strains of *N. oculata* obtained from the treatment  $1 \times 10^5\text{ cells mL}^{-1}$  exposed to UV radiation by 120 min. Mean  $\pm$  standard deviation,  $n = 3$



**Fig. 4** Growth rate of the mutant strains of *N. oculata* cultivated in Bold's medium with the herbicide quizalofop-p-ethyl ( $900\text{ }\mu\text{M}$ ). Mean  $\pm$  standard deviation,  $n = 3$

$0.001\text{ mg g}^{-1}\text{ DW}$ ) in the exponential phase, followed by oleic acid ( $0.913 \pm 0.001$  and  $15.441 \pm 0.53\text{ mg g}^{-1}\text{ DW}$ ), with more than double than the original strain ( $0.393 \pm 0.001$  and  $7.133 \pm 0.005\text{ mg g}^{-1}\text{ DW}$ ) at any stage of growth. Elaidic acid was slightly higher in the S3 strain ( $10.95 \pm 0.01\text{ mg g}^{-1}\text{ DW}$ ) in comparison to the original strain ( $7.86 \pm 0.02\text{ mg g}^{-1}\text{ DW}$ ) in the exponential phase, followed by linoleic acid. Similarly, the tridecanoic acid was higher in S3 strain ( $0.45 \pm 0.001\text{ mg g}^{-1}\text{ DW}$ ) than the original strain ( $0.22 \pm 0.004\text{ mg g}^{-1}\text{ DW}$ ). Palmitic acid increased slightly in the S3 strain ( $16.54 \pm 0.021\text{ mg g}^{-1}\text{ DW}$ ) compared with the original strain ( $12.23 \pm 0.031\text{ mg g}^{-1}\text{ DW}$ ) in the exponential phase and decreased when it reached the stationary phase. Heptadecaenoic acid was increased (doubled) in the S3 strain ( $4.22 \pm 0.006\text{ mg g}^{-1}\text{ DW}$ ) in comparison with original strain ( $2.4 \pm 0.009\text{ mg g}^{-1}\text{ DW}$ ) in the exponential phase and decreased in the stationary phase. The fatty acids that decreased their content in the S3 strain in contrast with the original strain were pentadecanoic, palmitoleic, and margoric acid.



**Fig. 5** Growth curve of the new *N. oculata* strain (S3) obtained by mutation-selection procedure and the original strain. Mean  $\pm$  standard deviation,  $n = 3$

**Table 1** Bromatological analysis of two strains of *Nannochloropsis oculata*

	Control (original strain)		Strain (S3)	
	Logarithmic	Stationary	Logarithmic	Stationary
Density (cells mL <sup>-1</sup> )	64.1 ± 0.7 × 10 <sup>6</sup> S	82 ± 1.3 × 10 <sup>6</sup> NS	60.1 ± 1.5 × 10 <sup>6</sup> S	81.1 ± 0.9 × 10 <sup>6</sup> NS
Dry weight (µg mL <sup>-1</sup> )	1142 ± 102 S	1034 ± 60 S	866 ± 32 S	1402 ± 28 S
Protein (%)	27.8 ± 1.8 S		23.15 ± 0.69 S	
Carbohydrate (%)	20.42 ± 0.69 S		18.18 ± 0.58 S	
Lipid (%)	44.71 ± 1.3 S	49.19 ± 1.4 S	57.05 ± 1.1 S	59.39 ± 1.0 S
Chlorophyll a (µg mL <sup>-1</sup> )	6.40 ± 0.42 NS		7.07 ± 0.34 NS	

Notes: Comparison among strains (original vs. S3):  $p < 0.05$

NS non-significant, S significant

## Discussion

Random mutation-selection using UV radiation and quizalofop-p-ethyl with *N. oculata* resulted in a mutant strain (S3) with faster growth than the original strain and also higher content of total lipids and several fatty acids: arachidonic (5×), oleic and heptadecaenoic acid (2× other fatty acids such as elaidic, tridecanoic, and palmitic acids increased slightly in comparison with the original strain). Meireles et al. (2003) and Bougaran et al. (2012) mention that it is possible to use UV radiation as a mutagen in the same way as a chemical agent, even not knowing which genes are affected. It is a simple way to induce random mutation and increase the productivity of metabolites by microalgae. Chatuverdi et al. (2004), Bellou and Aggelis (2012), and Mühlroth et al. (2013) mention that the herbicide quizalofop-p-ethyl inhibits the activity of acetyl-CoA carboxylase (ACCasa) cytosolic increasing the substrate malonyl-CoA (precursor of the synthesis of fatty acids), causing accumulation of triacylglycerols (TAGs). This mechanism has been reported

by Bellou and Aggelis (2012) for the microalgae *Chlorella* sp. and *Nannochloropsis salina*. Therefore, it is convenient to apply this herbicide as a selective agent in microalgae, inducing the production of the TAG. Cortez et al. (2015) report that up to 100 µM of quizalofop-p-ethyl can be used as a selective agent on the microalga *Tetraselmis tetrahele*. Chatuverdi et al. (2004) report that 25 µM of quizalofop-p-ethyl inhibits the growth rate of the wild strain of *N. oculata*, while the mutant strains were found to tolerate up to 75 µM of the herbicide. In our study, we found that both the wild type and the S3 mutant strain of *N. oculata* can tolerate up to 900 µM of and still obtain surviving cells which are more resistant than *T. tetrahele* and *N. oculata* to increased levels of the herbicide. Our mutant strain (S3) of *N. oculata* can grow at 900 µM quizalofop-p-ethyl.

Other studies have subjected microalgae to programs of mutation-selection for improvement of fatty acids by using a combination of different mutagenic agents and selective inhibitors (Table 3). Among them, Meireles et al. (2003) suggest that random mutagenesis can be successfully applied to in-

**Table 2** Fatty acid composition (mg g<sup>-1</sup> DW) of the new strain obtained by mutation-selection procedure and comparison with the original strain *Nannochloropsis oculata* (n = 3)

Fatty acids/stage	Control strain		Strain “S3”	
	Logarithmic	Stationary	Logarithmic	Stationary
Tridecanoic (C13:0)	0.223 ± 0.004	–	0.456 ± 0.001	–
Pentadecanoic (C15:0)	0.656 ± 0.017	–	–	0.178 ± 0.004
Palmitic (C16:0)	12.239 ± 0.031	15.593 ± 0.001	16.545 ± 0.021	15.441 ± 0.267
Palmitoleic (C16:1)	0.743 ± 0.017	1.279 ± 0.003	0.856 ± 0.003	1.010 ± 0.030
Margaric (C17:0)	–	0.787 ± 0.003	–	0.297 ± 0.005
Heptadecanoic (C17:1)	2.404 ± 0.009	3.492 ± 0.010	4.222 ± 0.006	2.791 ± 0.053
Oleic (C18:1n9c)	0.393 ± 0.001	7.133 ± 0.005	0.913 ± 0.001	15.441 ± 0.315
Elaidic (C18:1n9t)	7.868 ± 0.022	–	10.954 ± 0.011	–
Linoleic (C18:2n 6c)	18.358 ± 0.005	20.660 ± 0.005	21.109 ± 0.046	22.925 ± 0.439
Arachidonic (C20:4n6)	0.275 ± 0.001	–	1.540 ± 0.003	0.356 ± 0.012
Cis-13,16-docosadienoic (C22:2)	–	–	–	0.059 ± 0.004
Total	43.159 ± 0.107	48.945 ± 0.027	56.595 ± 0.092	58.38 ± 1.129

**Table 3** Improvement of fatty acids in some microalgae by mean of a mutation-selection program

Microalgae	Mutagen	Method selection	Improvement of mutant strains	Reference
<i>Pavlova lutheri</i>	UV		Increased production of EPA and DHA (33%)	Meireles et al. (2003)
<i>Nannochloropsis oculata</i>	MNU	Quizalofop	Higher content of n-3 PUFAs and total fatty acids such as triacylglycerol, linoleic, arachidonic, and EPA	Chatuverdi et al. (2004)
<i>N. oculata</i>	EMS	Cerulenin and erythromycin	Increase of eicosapentaenoic acid	Chatuverdi and Fujita (2006)
<i>Isochrysis affinis galbana</i>	UV	Flow cytometry	Up to 80% in the production of lipids neutral	Bougaran et al. (2012)
<i>Tetraselmis tetrahele</i>	EMS	Quizalofop	Higher content of arachidonic acid (91%).	Cortez et al. (2015)
<i>N. oculata</i>	UV	–	Higher lipid: chlorophyll ratios	Srinivas and Ochs (2012)
<i>Chlorella</i> sp.	UV	–	Improve lipid content	Liu et al. (2015)
<i>N. oculata</i>	UV	Quizalofop	Higher production lipids and fatty acids such as oleic and arachidonic acids	Our study

EMS ethyl-methane-sulfonate, MNU N-methyl-N-nitrosourea, EPA eicosapentaenoic acid, DHA docosahexaenoic acid, PUFAs polyunsaturated fatty acids

crease the production of n-3 fatty acids in *Pavlova lutheri*. Liu et al. (2015) propose that *Chlorella* sp. can be considered as a candidate for the production of biodiesel, after being subjected to a process of mutation in order to improve the lipid content. Bougaran et al. (2012) recommended the selection of cells with particular characteristics after a process of mutation-selection program without affecting the growth rate. Doan and Obbard (2012) also selected new strains of *Nannochloropsis* sp. with higher content of fatty acids by flow cytometry.

Cortez et al. (2015) obtained mutant strains of *T. tetrahele* with higher content of arachidonic acid than the wild strain, suggesting that the process of mutation-selection using EMS and quizalofop is suitable for the genetic improvement of this algal strain. Chatuverdi et al. (2004) used MNU and quizalofop to obtain mutant strains of *N. oculata* with a higher content of n-3 PUFAs and total fatty acids such as triacylglycerol, linoleic, arachidonic, and EPA indicating that this process of mutagenesis is a good tool to manipulate both PUFAs and EPA in this microalga. In our study, the process of mutation-selection by the combination of UV radiation to induce random mutagenesis combined with quizalofop-p-ethyl as a selective agent in a freshwater strain of *N. oculata* was shown to be suitable to obtain new strains with higher lipid production and higher fatty acids such as oleic and arachidonic acids in comparison with the original strain.

The marine *N. oculata* is appreciated for the cultivation on large-scale cultivation of rotifers and fish hatcheries and is recognized as a good source of EPA and PUFA for application in human health (Chatuverdi and Fujita 2006). The new strain is an enhanced alternative with better lipid and fatty acids content for application as live food in freshwater aquaculture. However, it is necessary to carry out more investigations of metabolic engineering with microalgae to have a better understanding on the fatty acid biosynthetic machinery (Bellou et al. 2014), which will produce improved microalgal strains in

their content of EPA and DHA to improve their quality for use in aquaculture and human consumption (Chatuverdi and Fujita 2006; Doan and Obbard 2012; Cortez et al. 2015). In conclusion, the mutation-selection program used in the freshwater microalga *N. oculata* using UV radiation and the herbicide quizalofop-p-ethyl is suitable to obtain strains with higher lipid content and fatty acids, without affecting its rate of growth or cell volume, offering a new alternative that can favorably contribute to applications in the aquaculture, pharmaceutical, and food industries as well as biofuels.

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