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Enhancement of neutral lipid productivity in the microalga *Isochrysis* affinis *Galbana* (T-Iso) by a mutation-selection procedure

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Abstract :

Microalgae offer a high potential for energetic lipid storage as well as high growth rates. They are therefore considered promising candidates for biofuel production, with the selection of high lipid-producing strains a major objective in projects on the development of this technology. We developed a mutation-selection method aimed at increasing microalgae neutral lipid productivity. A two step method, based on UVc irradiation followed by flow cytometry selection, was applied to a set of strains that had an initial high lipid content and improvement was assessed by means of Nile-red fluorescence measurements. The method was first tested on *Isochrysis* affinis *galbana* (T-Iso). Following a first round of mutation-selection, the total fatty acid content had not increased significantly, being 262 ± 21 mgTFA $(gC)^{-1}$ for the wild type (WT) and 269 ± 49 mgTFA $(gC)^{-1}$ for the selected population (S1M1). Conversely, fatty acid distribution among the lipid classes was affected by the process, resulting in a 20% increase for the fatty acids in the neutral lipids and a 40% decrease in the phospholipids. After a second mutation-selection step (S2M2), the total fatty acid content reached 409 ± 64 mgTFA $(gC)^{-1}$ with a fatty acid distribution similar to the S1M1 population. Growth rate remained unaffected by the process, resulting in a 80% increase for neutral lipid productivity.

Keywords : strain selection ; *Isochrysis* ; neutral lipid ; ultra violet ; flux cytometry ; biofuel

1 Introduction

Today, microalgae are at the center of a highly dynamic research field. These aquatic photosynthetic organisms were originally grown mainly in aquaculture to serve as feed for fish, shrimps and shellfish. The practical knowledge on microalgal culture acquired has enabled the development of a larger research domain in biotechnology. The commercial applications of microalgae cover a wide spectrum, including pigments, antioxidants, polysaccharides and oil for a range of different purposes (Spolaore et al., 2006). The importance of nutritional oils, in particular the polyunsaturated fatty acids (PUFA), has attracted a great deal of attention. Some microalgae have a high PUFA content, for example eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA) (Berge and Barnathan, 2005). Some species can also be a good source of triglycerides, which can be turned into biodiesel. One advantage of using microalgae for biofuel is that they have a significant higher yield in terms of biomass per surface than terrestrial species. Indeed, the annual dry biomass productivity can be 10 to 50 times higher than that of land plants. Microalgae also show much higher oil production than terrestrial oleaginous species, producing 5 to 10 times more than oil palm depending on the species, (Chisti, 2007; Wijfels et al., 2010). The use of microalgae for biofuel is growing extremely rapidly, with an unprecedented level of investment: the race for biofuel has seen the creation of a dozen private companies with funding in millions of USD for work on the microalgae-based biofuel. Starting in the 1970s with the pioneer work compiled in the NRLE report (Sheehan et al., 1998), a new wave of research expanded and has recently exploded. Paths of research now include the search for the best adapted species to produce lipids, the testing of their metabolic responses to culture conditions, the understanding of biosynthesis and regulation pathways and their genetic improvement.

The microalgae world is facing a similar challenge to that experienced by classical agriculture in the 20th century. Beyond the identification of strains for particular applications, the selection for particular traits will be an important field in the near future. However, the search for improved characters in microalgae has received little attention until now. Improvement of terrestrial plants has exploited natural bio-diversity for centuries, selecting naturally-acquired traits that offered ecological advantages. This approach was the basis for classical selection programs that led to great improvements, not only in agriculture but also in biotechnology

programs; this enabled, for example, the improvement of penicillin production rates from 0.06 to 26 $mg \cdot mL^{-1}$ (Queener and Lively, 1986).

Although methods based on the isolation of microalgal cells make it possible to select lines very efficiently (Molina Grima et al., 1995), the use of mutagenesis agents or UV stress to cause changes can lead to higher profits: earlier publication from Shaish et al. (1991) used UV irradiation to select rich beta-carotene resistant *Dunaliella bardawil* strains. Using a similar procedure, Alonso et al. (1996) were able to increase the EPA content of the diatom *Phaeodactylum tricornutum* by 37%. More recently, Meireles et al. (2003) succeeded in increasing the production of EPA and DHA by 33% in *Pavlova lutheri* using a UV-based mutagenesis process. Chemical mutagenesis agents were also used successfully to improve compound production, e.g., ethyl methane sulfonate allowed Chaturvedi and Fujita (2006) to increase the EPA production in *Nannochloropsis oculata* and Mendoza et al. (2008) to select carotenoid hyperproducing strains of *Dunaliella salina*.

Selection of cells with particular traits can be done manually by infinite dilution or micro manipulation. However, automatic sorting techniques are much faster, resulting in a higher number of sorted cells and, hence, a faster recovery of the resulting population. The combined use of fluorochrome and flow cytometry sorting was only recently described for the selection of high-lipid content strains of *Tetraselmis suecica* (Montero et al., 2010) and for the selection of carotenoid hyper-producing strains for the green algae *Dunaliella salina*(Mendoza et al., 2008).

As a method for increasing the neutral lipid content of microalgae, we propose the use of a two step mutation-selection procedure. This procedure, described in the present paper, combines UV mutagenesis with automated sorting to select the cells with the highest lipid production, based on Nile-red fluorochrome staining (Dempster and Sommerfeld, 1998; McGinnis et al., 1997) and a flow cytofluorimeter (de la Jara et al., 2003) fitted with a sorting device. This procedure was first tested with the small (4 to 6 μm) Prymnesiophyceae *Isochrysis* affinis *galbana* clone Tahiti (T-Iso), commonly grown for aquaculture. This microalga is an excellent food, particularly for bivalves and has, as such, been very well studied. Its fatty acid content is naturally relatively high and fatty acid distribution among lipid classes also matches the

requirements for biodiesel production. In addition, the possibility of enhancing the fatty acid content of *Isochrysis galbana* was already mentioned by Alonso et al. (1994), who confirmed the genetic determination of fatty acid variation and suggested that selection and mutation-selection would be likely to improve its fatty acid content. This information led us to choose this haptophyte for the present study.

2 Material and methods

2.1 Microalgal strains

Isochrysis affinis *galbana* clone Tahiti (T-Iso) was provided by the CCAP Culture Center of Algae and Protozoa and verified by rDNA 18S sequencing as being CCAP strain 927/14.

2.2 Culture conditions

All the experimental cultures were performed under nitrate-limiting conditions to enhance lipid production and were run in triplicate. Cultures were grown in 2-L flasks and bubbled with 0.22 μm filtered-air. Once filled with the culture medium, flasks were sterilized for 20 min at 120 °C. The enrichment solution consisted of 1 $\mathrm{mL \cdot L^{-1}}$ Conway (Walne, 1966) with a nitrate concentration of 0.6 mM,*i.e.*, half that of standard Conway medium. The cultures were maintained at a constant temperature of 21°C and under a constant irradiance of 80 $\mu\mathrm{mol \cdot m^{-2} \cdot s^{-1}}$. Initial cell concentration was 5.10^5 $\mathrm{cell \cdot mL^{-1}}$.

2.3 Cell counting

Cells were counted with a Malassez counting cell and image analysis (SAMBA software). Cell concentration was also assessed by absorption measurement at λ=680 nm (A_{680}) and λ= 800 nm (A_{800}) with a Bio-Tek μQuant Universal Microplate Spectrophotometer. For growth rate calculation, the Gompertz model modified by Zwietering et al. (1990) was fitted to the experimental data, according to equation 1:

$$Ln\frac{X}{X_0} = A \times exp(-exp(\frac{\mu_{max} \times exp(1)}{A} \times (l - t) + 1)) \quad (1)$$

where A is the maximum cell concentration, μ_{max} is the maximum specific growth rate, X_0

is the initial cell concentration at t_0, X is the cell concentration at t and l is the lag time defined as the t-axis intercept of the tangent at the inflection point.

2.4 Cell size analysis

Cell size measurements were made with a Coulter Counter Multisizer 3 (Beckman Coulter, High Wycombe, U.K.). Before measurement, samples were diluted to 1/50 with sterile seawater. Cell size, given as equivalent sphere diameter, was then calculated using MS-Multisizer 3 software (Beckman Coulter, High Wycombe, U.K.).

2.5 Mutation procedure

Unlike many other species, T-Iso cannot be readily cultured on gelose. Hence, irradiation must be performed in a liquid medium. Mutation was induced using a $\lambda = 254$ nm UV-C lamp (340 $\mu W \cdot cm^{-2}$, Bioblock). The exposure was carried out in a sterile Petri dish (100 mm) where 12 mL of culture were deposited (this volume made possible a complete spreading and limited self-shading). The distance between the lamp and the dish was set at 13.5 cm (distance related to the configuration of the lamp) and irradiation lasted 3 to 32 min.

A set of preliminary experiments was done to establish the correct UV dose for mutagenesis. We assumed that a 10% survival rate of irradiated cells was associated with a substantial mutagenic effect. We therefore tested different irradiation periods (3, 6, 9, 12, 16 and 32 min) and measured the cell concentration and absorption A_{680} according to the duration of UV exposure. Survival rate was computed as the ratio of the minimum cell concentration measured after UV exposure to the initial cell concentration or as the corresponding ratio for A_{680}.

Each mutation operation was carried out in triplicate on Petri dishes. After exposure, cells were transferred into a 100 mL Erlenmeyer filled with filtered sterilized Walne-enriched seawater. Cultures were subsequently maintained in the dark for 24 hours to avoid photoreactivation that would lead to photorepair of UV-induced damage (Singh, 1975). The cultures were then placed in a culture cupboard at 21 °C and under a 80 $\mu mol \cdot m^{-2} \cdot s^{-1}$ irradiance. The complete procedure is illustrated in (figure 1)

2.6 Staining with Nile-red

Nile-red (9-dietylamino-5H-benzo(a)phenoxazine-5-one) is a lipophilic fluorochrome (Greenspan et al., 1985) that provides a fast staining method for neutral lipids. In an hydrophobic medium, the stained lipid corpuscles appear yellow. Excitation wavelength was $\lambda = 525$ nm and emission wavelength was $\lambda = 580$ nm. The dye was dissolved in acetone to obtain a 250 $\mu g \cdot mL^{-1}$ concentration. This solution was added to the culture samples at a concentration of 1 $\mu L \cdot mL^{-1}$.

The correlation between the Nile-red fluorescence and total fatty acid concentration was assessed as follows. An N-limited batch culture was sampled periodically for twenty-four days, when stationary phase had been reached. Samples were analyzed for total fatty acid concentration in the culture using both gas chromatography and Nile-red fluorescence, measured with a microplate spectrofluorimeter (Tecan Safire).

2.7 mutation-selection procedure

Batch cultures in stationary phase under nitrate starvation were used and processed with a Facs Aria Cytometer, fitted with an automatic sorting device. Sheath fluid was PBS (Phosphate Buffer Saline). Laser emission wavelength and filter were set as specified above. As Nile Red is not referenced, phycoerythrine ($\lambda = 560$ nm) was taken as a reference instead. Agitation, followed by filtration through a mesh was necessary to remove the aggregates before the samples were pumped into the cytometer. Nile Red was added immediately before the cytometer step. Two runs cytometer runs were made, to verify the quality of the cell selection. The sorted cells were collected in a 3 mL sterile box. At the first appearance of recovery, the algal cells were transferred into a 50-mL Erlenmeyer until cell concentration reached 1.10^6 cell $\cdot mL^{-1}$, then transferred in a 150-mL Erlenmeyer in order to obtain the necessary biomass to proceed with the subsequent analyses.

The mutation-selection procedure (figure 1) consisted of two successive cycles. First, the wild type strain (WT) was sorted by flow cytometry: the 10 % of cells showing the strongest fluorescence intensity per cell were selected and picked out by the automatic device. The triplicate cultures that were inoculated with the sorted cells were called S0. Second, the cells were

analyzed for their TFA content once stationary phase was reached under N starvation.

Three sub-samples of the S0 culture were then exposed to UV irradiation for 32 min as described in the mutation procedure section. After a 30-day delay, needed for growth recovery, the resulting S0M1 population was sorted again using flow cytometry to select the 10% of cells showing the strongest fluorescence intensity. The resulting S1M1 population was again allowed to recover for 30 days and was analysed for cell TFA content once stationary phase under N starvation was achieved. During the second cycle, the S1M1 population was again exposed to a further 32 min UV irradiation as previously described and the resulting S1M2 population was then sorted by flow cytometry after recovery, resulting in the S2M2 population. The S2M2 TFA content was finally analyzed once stationary phase under N starvation was achieved.

2.8 Fatty acid analyses

Gas Chromatography (GC) analyses of fatty acids were carried out on triplicate cultures. Three successive samples were taken every two days from the onset of the stationary phase for the nitrogen-depleted batch cultures. Sample volume was adjusted so as to obtain a total population of 1.5 10^8 cells in the sample, which allowed GC analyses in the correct range. Each sample was filtered on a 450 °C pre-combusted GF/C filter (Whatman, diameter 47 mm), then deposited in a glass bottle filled with 6 mL Folch reagent and deep-frozen (-80 °C).

Lipid class separation was performed by column chromatography according to the method of Soudant et al. (1995), improved for microalgae. A borosilicate Pasteur pipette (0.5 cm internal diameter and 9 cm long) was plugged with silanized glass wool and dry-loaded on 4.5 cm with 600 mg of silica gel 60 (Merck 0.063-0.200 mm, 6% H_2O). The column was conditioned with 5 mL chloroform; then 3 mL lipid extract (evaporated under nitrogen) were put though column chromatography. The neutral lipids, glyco- and phospholipids were eluted with chloroform (10 ml), acetone (20 ml) and methanol (15 ml), respectively. Solvents were evaporated and the lipid classes were weighed on a microbalance (Satrorius MC210P). All fractions were kept in Folch reagent and frozen (-20 °C) until further analyses.

For fatty acid analysis, the total lipid extract (2 mL) and lipid class fractions were evapo-

rated under nitrogen and transmethylated by direct transesterification with BF3-methanol at 100°C for 10 min (Metcalfe and Schmitz, 1961). Distilled water (1 mL) and hexane (1 mL) were added and vortexed. The upper organic phases, containing fatty acid methyl esters (FAMEs), were collected and assayed by GC-FID. FAME quantification was calculated compared to the C17 internal standard (Sigma) by GC-FID, using a gas chromatograph (Auto system Gas Chromatography, Perkin-Elmer, Massachusetts, USA) equipped with an autosampler and fitted with a split/splitless injector and flame ionisation detector. The separation was carried out with a BPX-70 capillary column (60 m long, 0.25 mm internal diameter, 0.25 lm film thickness; SGE, Austin, Texas, USA), containing a polar stationary phase (cyanopropyl-siloxane).

In order to express the fatty acid content on a per-carbon basis, particulate carbon was measured using a CN elemental analyzer (Thermoelectron). Methionine, aspartic acid and nicotinamide, which have different N and C percentages, were used for calibration. Samples were collected on a precombusted GF/C filter (Whatman, 25 mm diameter). The filters were deposited in limp glass, placed in steam room and dried at 75 °C for 24 hours, then deep-frozen until analysis.

2.9 Statistics

ANOVA were conducted to test for result significance, using Statgraphics software and with $\alpha = 0.05$, unless otherwise mentioned.

3 Results and Discussion

3.1 Nile-red staining and mutation procedure

We first assessed the capacity of Nile-red to stain the microalgae. Fluorescence monitoring of the cultures showed that fluorescence increased sharply immediately after dying, remained constant for 1 to 3 minutes and then decreased (Figure 2). Hence, in the subsequent work, the fluorescence measurements were undertaken within 1 to 2 minutes after cell staining.

A positive linear correlation (n = 8 ; r = 0.99 ; critical value = 0.925, for $\alpha = 0.001$) was found between fatty acid concentration and Nile-red fluorescence for T-Iso throughout the course

of a single batch culture (Figure 3). This result demonstrated that Nile-red fluorescence could readily be used to assess fatty acid concentration for T-Iso.

Moreover, the examination of Nile-red fluorescence according to the cell concentration kinetics (Figure 4) showed that Nile-red fluorescence steadily increased until day 24, when the cell population had reached stationary phase, indicating that lipids still accumulated during the stationary phase under N starvation. This result is consistent with previous observations (Richardson et al., 1969; Sukenik and Wahnon, 1991; Alonso et al., 2000).

T-Iso cells were very sensitive to the duration of UV-irradiation (table 1). Indeed, survival rate rapidly decreased as the irradiation period lengthened. Long irradiation, up to 32 min, resulted in very low survival rates: down to 19% when estimated with A_{680} and 10% when computed on the basis of the cell number. Not all of the cultures recovered but, when they did, recovery was observed within the 20 days following irradiation. The recovery frequency displayed a general decreasing trend with irradiation duration, except for the 16 min irradiation period, which showed a very high recovery frequency. For practical reasons, (*e.g.* temperature and evaporation control) we did not investigate irradiation periods lasting more than 32 min; this duration being considered a satisfactory trade-off between the UV dose required to obtain a substantial mutagenic effect, recovery frequency and technical constraints.

3.2 Total fatty acid content

Subsequent to S0 selection at the beginning of the experimental period, TFA content was compared for the WT (WT^a) and S0 populations. Results in table 2 shows that the initial selection step did not result in a significantly different TFA content (on a per carbon or a per cell basis) for S0, when compared to WT^a.

Two years later, once the two mutant populations S1M1 and S2M2 were obtained, the WT population (WT^b) was again analysed for TFA content (table 2), in order to allow accurate comparison for phenotypic traits between the different populations. TFA content for WT^b was found somewhat higher than for WT^a, although the difference was not significant, as assessed with an ANOVA test ($\alpha = 0.01$). Therefore, results for S1M1 and S2M2 are discussed hereafter against WT^b, further referred to as WT.

The mean total fatty acid (TFA) content for the WT population was calculated as 3.9 $\pm$

0.4 pgTFA $\cdot$ cell^{-1} (table 2). This result is somewhat higher than the findings of Dunstan et al. (1993), which fall in the range 1.70 - 2.99 pgTFA $\cdot$ cell^{-1}, and even higher than the range reported for the same species elsewhere: 1.95 - 2.36 (Brown et al., 1993), 1.98 - 2.56 (Tzovenis et al., 2003 a), 1.2 (Volkman et al., 1989) and 1.00 - 1.37 (Ferreira et al., 2008). The average carbon quota for the WT strain was found to be 15.0 $\pm$ 2.2 pgC $\cdot$ cell^{-1} and was therefore higher than values previously reported for T-iso: 5.1-5.8 (Thompson et al., 1993) in turbidostat, 8.6-9.3 (Nelson et al., 1992) and 10.6 (Troedsson et al., 2005). Nevertheless, the high carbon quota reported here may be driven by the N starvation and the concomitant lipid accumulation experienced by the cells during the stationary phase. This resulted in a content of 262 $\pm$ 21 mgTFA $\cdot$ (gC)$^{-1}$, which is roughly twice the 123 mgTFA $\cdot$ (gC)$^{-1}$ reported by Troedsson et al. (2005).

The first mutation-selection step (Figure 6) did not result in a significant increase in the TFA content for the S1M1 culture (269 $\pm$ 49 mgTFA $\cdot$ (gC)$^{-1}$). After the second mutation-selection step, the TFA content increased markedly to 409 $\pm$ 64 mgTFA $\cdot$ (gC)$^{-1}$ and resulted in a significant 1.6 fold increase compared with the WT culture. Nile-red fluorescence was also compared among the 3 cultures. Figure 5 shows the adequation between the Nile Red fluorescence increase and that of the TFA content, expressed on a per-cell basis. This, in turn, confirmed our previous conclusion that the Nile-red staining was a suitable method to readily assess the TFA content of T-Iso cells.

Growth rates were not substantially affected for the mutant populations (table 3, ANOVA $\alpha = 0.05$). Neither did carbon quota vary significantly during the mutation-selection procedure, with a mean value of 17.3 $\pm$ 4.3 pgC $\cdot$ cell^{-1} (table 2). The mean cell diameter remained constant also, at 5 μm, as confirmed by ANOVA ($\alpha = 0.05$) (table 3). The biomass productivity was therefore unaffected by the mutation-selection procedure, resulting in a 1.6 fold increase for lipid productivity.

3.3 Fatty acid distribution among lipid classes

The distribution of fatty acids among the three main lipid classes was assessed for the WT, S1M1 and S2M2 populations in order to show the pattern of irradiation impact . Under N starvation, the WT strain was able to accumulate fatty acids in the neutral lipid class that represented up to 70% of the total fatty acids, while glycolipids and phospholipids accounted for less than 10% and 30%, respectively. The first mutation-selection cycle (Figure 7) resulted in a significant increase for the fatty acids in the neutral lipid class (up to 80% of the total fatty acids) and a concomitant decrease in phospholipids in the S1M1 population, while glycolipids were not significantly affected (ANOVA, $\alpha = 0.01$). Conversely, the second mutation-selection cycle resulted in a balanced increase in total fatty acids and did not further significantly alter the fatty acid distribution among lipid classes.

At the end of the second mutation-selection cycle, the increase in total fatty acid content, together with the observed modifications in the fatty acid distribution, without any significant changes in biomass productivity, resulted in an overall 1.8 fold increase for neutral lipid productivity.

3.4 Distribution of saturated, monounsaturated and polyunsaturated fatty acids

A surprisingly unbalanced fatty acid distribution among SFA, MUFA and PUFA was found for the three cultures, SFA and MUFA accounting for roughly 35 to 40% by weight, while PUFA were only 20% of the TFA (table 4). The results reported here for relative PUFA content were indeed substantially lower than those of Renaud et al. (1995) and Pernet et al. (2003) who reported a 46 to 54 % range. However, fatty acid saturation can be influenced by culture conditions, as suggested by the results of Dunstan et al. (1993) who reported a more balanced distribution of fatty acids among SFA (31%), MUFA (30%) and PUFA (36%). It is further stressed that under N starvation, accumulation of fatty acids in the neutral lipid class should markedly decrease the relative content of PUFA that are mainly located in cell membranes.

3.5 Stability of the fatty acid composition and growth characteristics *versus* time

Stability for the S2M2 population was checked 18 (S2M2-T18) months after it was created. The phenotypic characteristics for S2M2-T18 and WT were measured concomitantly. The TFA content for S2M2-T18 was measured to be 415 ± 80 mgTFA $\cdot$ (gC)$^{-1}$, being 1.7 times higher than the TFA content for WT (238 ± 69 mgTFA $\cdot$ (gC)$^{-1}$) and not significantly different from that measured for S2M2. C quota was also checked for S2M2-T18 (17.2 ± 1.7 pgC $\cdot$ cell^{-1}) and had not changed significantly with time. Finally, maximum growth rate for S2M2-T18 (0.32 ± 0.03 d^{-1}) and WT (0.30 ± 0.02 d^{-1}) was not significantly different either (ANOVA, $\alpha = 0.05$). Hence, these results demonstrated the phenotypic stability for the resulting mutant population and proved the proposed mutation-selection process to be an efficient way to increase lipid productivity for *Isochrysis* affinis *galbana*.

4 Conclusion

The combination of UV mutagenesis with flow cytometry sorting significantly enhanced the lipid content of *Isochrysis* affinis *galbana*. The two-step method presented here resulted in a gross increase in TFA from 262 mgTFA $\cdot$ (gC)$^{-1}$ to 409 mgTFA $\cdot$ (gC)$^{-1}$. A significant increase in neutral lipids relative to total lipids was also recorded, while cell maximum growth rate was not affected by the procedure. This resulted in a 1.8 fold increase for neutral lipid productivity. We were able to verify the stability of the phenotypic characteristics for the S2M2 population after a one and a half year cultivation period and showed that both maximum growth rate and fatty acid content remained stable. Our results demonstrate that this strain selection procedure applied to microalgae can substantially enhance lipid productivity. These results may open new fields of application in aquaculture (better feed properties), human nutrition (higher and better production of PUFAs) and biofuels. Although we focused on lipid productivity here, the strain selection procedure could be used for other valuable products and application fields.

It will be a difficult challenge to elucidate which genes were hit during the UV irradiation process, not only because these effects were randomly spread but also because lipid biosynthesis pathways are very intricate. However, we believe that these results are a starting point

for further genomic investigations. The construction of differential cDNA libraries will allow us to investigate the genes involved in biosynthesis pathways and hopefully identify those affected during the mutation treatment. This procedure will therefore contribute to deepening our knowledge of the lipid biosynthesis pathways in this family of microalgae.

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5 References

References

Alonso, D., Segura del Castillo, C., E., M. G., Cohen, Z., 1996. First insight into improvement of eicosapentaenoic acid content in *Phaeodactylum tricornutum* (Bacillariophyceae) by induced mutagenesis. Journal of Phycology 32 (2), 339–345.

Alonso, D., Segura del Castillo, C., Garcia Sanchez, J., Sanchez Perez, J., Camacho, F., 1994. Quantitative genetics of fatty acid variation in *Isochrysis galbana* (Prymnesiophyceae) and *Phaeodactylum tricornutum* (Bacillariophyceae). Journal of Phycology 30 (3), 553–558.

Alonso, D. L., Belarbi, E.-H., Fernandez-Sevilla, J. M., Rodriguez-Ruiz, J., Grima, E. M., 2000. Acyl lipid composition variation related to culture age and nitrogen concentration in continuous culture of the microalga phaeodactylum tricornutum. Phytochemistry 54 (5), 461–471.

Berge, J., Barnathan, G., 2005. Fatty acids from lipids of marine organisms: molecular biodiversity, roles as biomarkers, biologically active compounds, and economical aspects. Marine biotechnology I 96, 49–125.

Brown, M. R., Garland, C. D., Jeffrey, S. W., Jameson, I. D., Leroi, J. M., 1993. The gross and amino acid compositions of batch and semi-continuous cultures of *Isochrysis* sp. (clone T.ISO), *Pavlova luther* and *Nannochloropsis oculata*. Journal of Applied Phycology 5, 285–296.

Chaturvedi, R., Fujita, Y., 2006. Isolation of enhanced eicosapentaenoic acid producing mutants of *Nannochloropsis oculata* ST-6 using ethyl methane sulfonate induced mutagenesis techniques and their characterization at mRNA transcript level. Phycological Research 54 (3), 208–219.

Chisti, Y., 2007. Biodiesel from microalgae. Biotechnology Advances 25 (3), 294–306.

de la Jara, A., Mendoza, H., Martel, A., Molina, C., Nordstron, L., de la Rosa, V., Diaz, R., 2003. Flow cytometric determination of lipid content in a marine dinoflagellate, *Crypthecodinium cohnii*. Journal of Applied Phycology 15 (5), 433–438.

Dempster, T., Sommerfeld, M., 1998. Effects of environmental conditions on growth and lipid accumulation in *Nitzschia communis* (bacillariophyceae). Journal of Phycology 34 (4), 712–721.

Dunstan, G. A., Volkman, J. K., Barrett, S. M., Leroi, J.-M., Jeffrey, S., 1993. Essential polyunsaturated fatty acids from 14 species of diatom (Bacillariophyceae). Phytochemistry 35 (1), 155 – 161.

Ferreira, M., Maseda, A., Fabregas, J., Otero, A., 2008. Enriching rotifers with "premium" microalgae *Isochrysis aff. galbana* clone T-ISO. Aquaculture 279 (1-4), 126–130.

Greenspan, P., Mayer, E., Fowler, S., 1985. Nile red: a selective fluorescent stain for intracellular lipid droplets. Journal of Cell Biology 100 (3), 965–973.

McGinnis, K., Dempster, T., Sommerfeld, M., 1997. Characterization of the growth and lipid content of the diatom *Chaetoceros muelleri*. Journal of Applied Phycology 9 (1), 19–24.

Meireles, L., Guedes, A., Malcata, F., 2003. Increase of the yields of eicosapentaenoic and docosahexaenoic acids by the microalga *Pavlova lutheri* following random mutagenesis. Biotechnology and Bioengineering 81, 50–55.

Mendoza, H., de la Jara, A., Freijanes, K., Carmona, L., Ramos, A., de Sousa Duarte, V., Valera, J., 2008. Characterization of *Dunaliella salina* strains by flow cytometry: a new approach to select carotenoid hyperproducing strains. Electronic Journal of Biotechnology 11, 1–13.

Metcalfe, L., Schmitz, A., 1961. The rapid preparation of fatty acid esters for gas chromatographic analysis. Analytical Chemistry 33 (3), 363–364.

Molina Grima, E., Sanchez Perez, J., Garcia Camacho, F., Medina, A., Gimenez, A., Lopez Alonso, D., 1995. The production of polyunsaturated fatty acids by microalgae: from strain selection to product purification. Process Biochemistry 30, 711–719.

Montero, M., Aristizabal, M., Garcia Reina, G., 2010. Isolation of high-lipid content strains of the marine microalga tetraselmis suecica for biodiesel production by flow cytometry and single-cell sorting. Journal of Applied Phycology, 1–5.

Nelson, J. R., Guarda, S., Cowell, L. E., Heffernan, P. B., 1992. Evaluation of microalgal clones for mass culture in a subtropical greenhouse bivalve hatchery: growth rates and biochemical composition at 30 °c. Aquaculture 106 (3-4), 357–377.

Pernet, F., Tremblay, R., Demers, E., Roussy, M., 2003. Variation of lipid class and fatty acid composition of *Chaetoceros muelleri* and *Isochrysis* sp. grown in a semicontinuous system. Aquaculture 221 (1-4), 393 – 406.

Queener, S., Lively, D., 1986. Screening and selection for strain improvement. In: Demain, A., Solomon, N. (Eds.), Manual of industrial microbiology and biotechnology. American Society for Microbiology, Washington, DC, pp. 155–169.

Renaud, S. M., Zhou, H. C., Parry, D. L., Thinh, L., Woo, K. C., 1995. Effect of temperature on the growth, total lipid content and fatty acid composition of recently isolated tropical microalgae *Isochrysis* sp., *Nitzschia closterium*, *Nitzschia paleacea* and commercial species *Isochrysis* sp. (clone T.ISO). Journal of Applied Phycology 7, 595–602.

Richardson, B., Orcutt, D., Schwertner, H., Martinez, C., Wickline, H., 1969. Effects of nitrogen limitation on the growth and composition of unicellular algae in continuous culture. Applied Microbiology 18 (2), 245–250.

Shaish, A., Ben-Amotz, A., Avron, M., 1991. Production and selection of high beta-carotene mutants of *Dunaliella bardawi*l (Chlorophyta). Journal of Applied Phycology 27 (5), 652–656.

Sheehan, J., Dunahay, T., Benemann, J., Roessler, P., 1998. A look back at the U.S. Department of Energy's Aquatic Species program: Biodiesel from algae. Tech. rep., US Dpt of Energy.

Singh, P. K., 1975. Photoreactivation of uv-irradiated blue-green algae and algal virus lpp-1. Archives of Microbiology 103 (1), 297–302.

Soudant, P., Marty, Y., Moal, J., Samain, J., 1995. Separation of major polar lipids in *Pecten maximus* by high-performance liquid chromatography and subsequent determination of their fatty acids using gas chromatography. Journal of Chromatography B: Biomedical Sciences and Applications 673 (1), 15–26.

Spolaore, P., Joannis-Cassan, C., Duran, E., Isambert, A., 2006. Commercial applications of microalgae. Journal of Bioscience and Bioengineering 101 (2), 87–96.

Sukenik, A., Wahnon, R., 1991. Biochemical quality of marine unicellular algae with special emphasis on lipid composition. I. *Isochrysis galbana*. Aquaculture 97 (1), 61–72.

Thompson, P., Guo, M., Harrison, P. J., 1993. The influence of irradiance on the biochemical composition of three phytoplankton species and their nutritional value for larvae of the pacific oyster (*Crassostrea gigas*). Marine Biology V117 (2), 259–268.

Troedsson, C., Grahl-Nielsen, O., Thompson, E. P., 2005. Variable fatty acid composition of the pelagic appendicularian *Oikopleura dioica* in response to dietary quality and quantity. Marine Ecology Progress Series 289, 165–176.

Tzovenis, I., De Pauw, N., Sorgeloos, P., 2003 a. Optimisation of T-ISO biomass production rich in essential fatty acids I. effect of different light regimes on growth and biomass production. Aquaculture 216 (1-4), 203–222.

Volkman, J., Jeffrey, S., Nichols, P., Rogers, G., Garland, C., 1989. Fatty acid and lipid composition of 10 species of microalgae used in mariculture. Journal of Experimental Marine Biology and Ecology 128 (3), 219–240.

Walne, P. R., 1966. Experiments in the large scale culture of Ostrea edulis (L.). Vol. 25 of Fisheries investigations series II. Her Majesty's Stationery Office, London.

Wijfels, R., Barbosa, M. J., Eppink, M., 2010. Microalgae for the production of bulk chemicals and biofuels. Biofuels Bioproducts and Biorefining 4, 287–295.

Zwietering, M. H., Jongenburger, I., Rombouts, F. M., Riet, K. V. T., Jun. 1990. Modeling of the bacterial growth curve. Applied and Environmental Microbiology 56 (6), 1875–1881.

Tables

Table 1: survival rate for cells and recovery frequency for cultures exposed to UV radiation. Survival rates were estimated on the basis of A_{680} measurements. Data are presented with standard deviation given in brackets.

Irradiation period (min)	Survival rate (%)	Recovery frequency (%)	Replicates
0	100 (0)	100	10
3	53 (20)	75	8
6	50 (15)	50	8
9	40 (8)	50	8
12	28 (11)	44	9
16	21 (6)	75	3
32	19 (-)*	33	3

* Standard deviation could not be calculated since only one culture recovered

Table 2: TFA content and C quota for the three populations. Values presented are means and standard deviations in brackets. Means were compared by ANOVA ($\alpha = 0.01$) and groups with the same letter indicate no significant difference.

Strain	TFA ($\mathrm{pgTFA \cdot cell^{-1}}$)	TFA ($\mathrm{mgTFA \cdot (gC)^{-1}}$)	C quota ($\mathrm{pgC \cdot cell^{-1}}$)	Number of replicates
WT^a	2.9 ^{a,b} (1.2)	188 ^{a,b} (58)	16.0 ^{a} (5.6)	9
S0	2.1 ^{a} (0.4)	178 ^{a} (28)	11.8 ^{a} (0.2)	6
WT^b	3.9 ^{b} (0.4)	262 ^{b} (21)	15.0 ^{a} (2.2)	6
S1M1	4.6 ^{b} (0.7)	269 ^{b} (49)	17.4 ^{a} (3.7)	6
S2M2	7.4 ^{c} (1.8)	409 ^{c} (64)	18.4 ^{a} (5.1)	13

TFA analyses for WT^a and S0 were conducted subsequently to S0 selection. Two years later, WT^b was analysed for TFA content together with the two mutant populations S1M1 and S2M2

Table 3: mean cell diameter and maximum growth rate (μ_{max}) for the three populations. Values presented are means, with standard deviations in brackets. Means were compared by ANOVA ($\alpha = 0.05$) and groups with the same letter indicate no significant difference.

Culture	μ_{max} (d^{-1})	mean cell diameter (μm)	Number of replicates
WT	0.40^a (0.08)	4.76^a (0.56)	5
S1M1	0.45^a (0.09)	4.94^a (0.55)	6
S2M2	0.38^a (0.05)	4.98^a (0.52)	6

Table 4: fatty acid composition (% of total fatty acid) for the WT, S1M1 and S2M2 populations. Values presented are means and standard deviation of at least six replicates. SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids.

FA	WT	S1M1	S2M2
14:0	24.6 (1.3)	25.5 (1.1)	22.1 (1.6)
15:0	0.6 (0.0)	-	-
16:0	13.5 (0.8)	15.2 (1.6)	15.9 (1.6)
18:0	0.8 (0.2)	0.9 (0.2)	0.7 (0.1)
SFA	39.7 (2.1)	41.9 (1.2)	39.7 (0.3)
14:1 *n*-5	0.3 (0.0)	-	0.2 (0.0)
16:1 *n*-9	1.4 (0.6)	0.5 (0.3)	1.9 (1.2)
16:1 *n*-7	3.9 (0.8)	3.8 (1.6)	5.1 (1.4)
18:1 *n*-9	26.4 (0.7)	24.2 (3.7)	28.9 (1.1)
18:1 *n*-7	1.2 (0.2)	2.0 (0.5)	1.1 (0.2)
MUFA	33.4 (1.2)	30.4 (4.6)	37.9 (2.6)
16:2 *n*-6	-	0.1 (0.0)	0.1 (0.0)
16:2 *n*-4	0.3 (0.1)	0.2 (0.1)	0.2 (0.1)
18:2 *n*-6	3.3 (0.3)	4.4 (0.7)	3.8 (0.5)
18:3 *n*-3	2.8 (0.1)	2.3 (0.5)	3.1 (0.3)
18:4 *n*-3	9.0 (0.8)	5.5 (1.3)	6.8 (1.2)
18:5 *n*-3	1.0 (0.6)	0.3 (0.1)	0.4 (0.2)
20:2 *n*-6	-	-	0.1 (0.0)
20:3 *n*-6	-	-	0.1 (0.0)
20:3 *n*-3	0.1 (0.0)	-	0.2 (0.1)
20:4 *n*-6	0.2 (0.0)	0.1 (0.0)	0.1 (0.0)
20:4 *n*-3	0.3 (0.0)	-	0.3 (0.1)
20:5 *n*-3	0.3 (0.0)	-	0.2 (0.0)
22:3 *n*-3	0.4 (0.1)	-	0.2 (0.0)
22:5 *n*-6	1.0 (0.1)	1.0 (0.2)	0.8 (0.2)
22:5 *n*-3	0.8 (0.0)	0.1 (0.0)	0.9 (0.2)
22:6 *n*-3	6.8 (0.5)	5.1 (0.9)	5.0 (1.0)
PUFA	26.4 (1.8)	19.3 (3.5)	22.4 (2.8)

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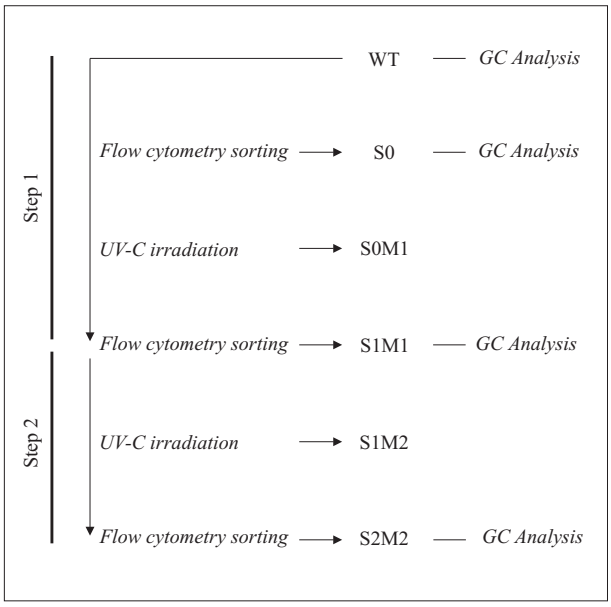

Figure 1: mutation-selection procedure used to improve the lipid content in the microalgae *Isochrysis* affinis *galbana*.

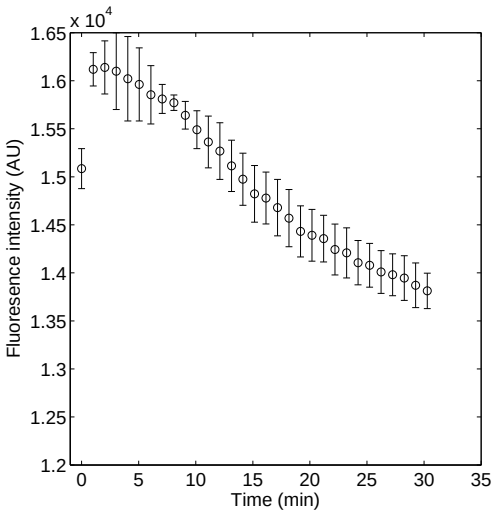

Figure 2: Nile-red fluorescence decrease over time. Error bars are standard deviation, n = 3

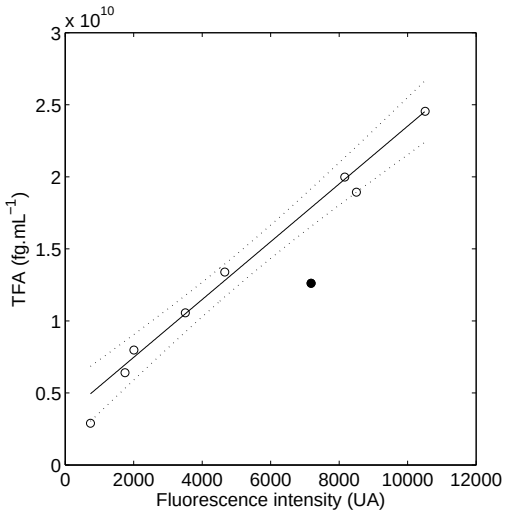

Figure 3: relationship between fatty acid concentration and fluorescence intensity. Dashed lines are the 95% confidence interval for the regression. (•) data not considered for regression

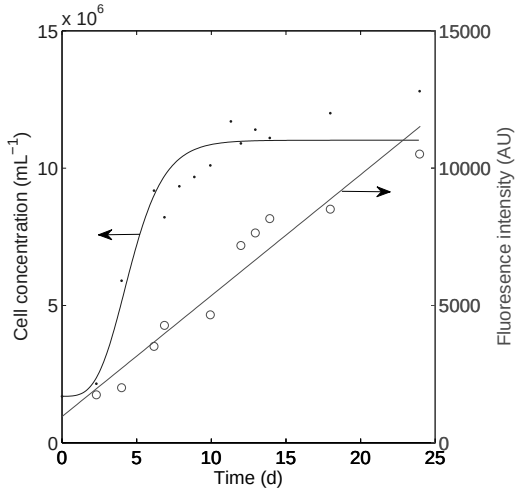

Figure 4: cell concentration and Nile-red fluorescence during a N-limited batch culture. (·) cell concentration, (o) fluorescence

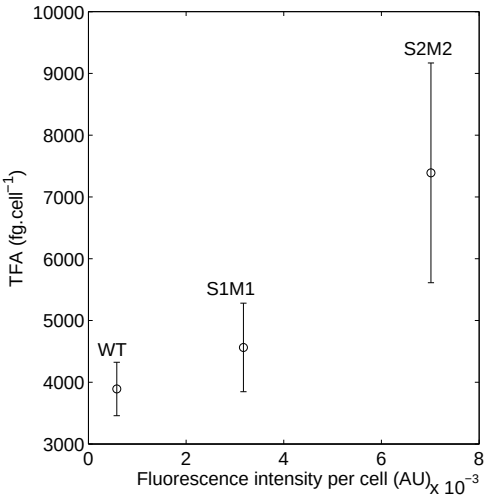

Figure 5: TFA content according to fluorescence intensity per cell for the three populations stained with Nile-red.

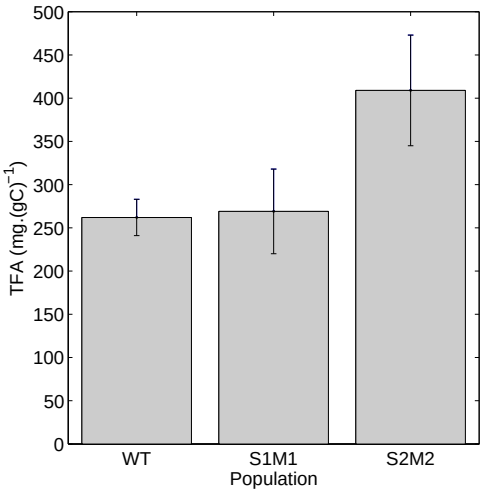

Figure 6: total fatty acids in WT, S1M1 and S2M2 populations.

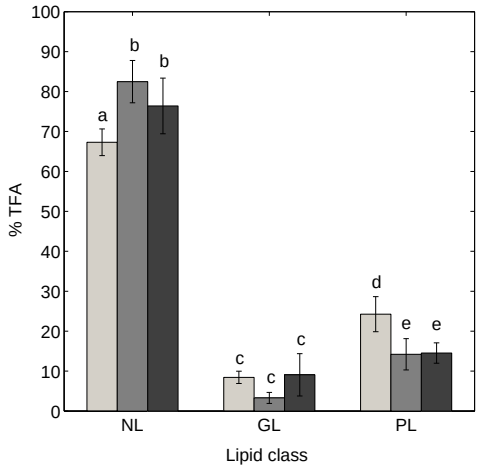

Figure 7: distribution of fatty acids among the lipid classes for the three populations (n $\geq$ 3). light grey: WT ; dark grey: S1M1 ; black: S2M2. NL: neutral lipids ; GL: glycolipids ; PL : phospholipids. data with same letters are not significantly different (ANOVA, $\alpha = 0.01$)