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# Enrichment on polyunsaturated fatty acids by *Rhodomonas salina* (Cryptophyta) following ethyl methane sulphonate induced random mutagenesis

## Enriquecimiento de ácidos grasos poliinsaturados por *Rhodomonas salina* (Cryptophyta) después de mutagénesis aleatoria inducida por etil metanosulfonato

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**ABSTRACT** | A protocol through mutagenesis with ethyl methane sulphonate and selection with herbicide Quizalofop was developed for *Rhodomonas salina*. Mutants showing resistance to Quizalofop had enriched lipid and PUFAs content. Two mutants were obtained with a significantly increased capacity for lipids (50-60% higher than WT) and PUFAs (50% higher that WT) accumulations, demonstrating the feasibility of genetically improving a species that doesn´t form discrete colonies in solid medium.

**Palabras clave**
Microalgas
Mutagénesis aleatoria
Ácidos grasos
Mejoramiento genético

**RESUMEN** | Se desarrolló un protocolo de mutagénesis con etil metanosulfonato para Rhodomonas salina. Los mutantes enriquecidos en contenido de lípidos y ácidos grasos poliinsaturados (PUFAs) se seleccionaron por su resistencia al herbicida Quizalofop. Se obtuvieron dos mutantes con una capacidad significativamente mayor de lípidos (50-60% más altos que WT) y acumulaciones de PUFA (50% más altos que WT) que demuestran la viabilidad de mejorar genéticamente una especie que no forma colonias discretas en medio sólido.

## INTRODUCTION

*Rhodomonas salina* (Cryptophyta) is a marine microalgae with several attributes of interest for aquaculture, such as: lack of a cell wall, small size, massive outdoor growth and high protein, lipid and polyunsaturated fatty acid (PUFAs) contents (Tremblay *et al*., 2007; Thoisen *et al*., 2020). In order to obtain high levels of commercially important metabolites, microalgae usually must be grown under growth-limiting conditions, which results in low biomass yield and, consequently, poor metabolites productivities (Su *et al*., 2011; González et. al., 2019).

PUFAs, such as docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA) and arachidonic acid (AA), are important membrane constituents and metabolic intermediates that can not be endogenously synthesize by several groups of animals but instead have to be obtained from their diet (Tocher *et al*., 2008). These compounds have pharmaceutical applications (Harwood and Caterson, 2006) and as important components in the diet of cultivated marine organisms (Hemaiswarya *et al*., 2010; Dourou *et al*., 2020).

It is well established that culture conditions can modulate the PUFAs content of microalgae, being low temperatures, as well as, low nitrogen and high irradiance availabilities, the most effective conditions to achieve it (Khozin *et al*., 2002; Solovchenko *et al*., 2008; Guevara *et al*., 2016; Wang *et al*., 2019).

Low productivity has limited the use of microalgae as commercial sources of PUFAs, so genetic improvement of most promising species is mandatory to accomplish economic feasibility. Random mutagenesis-selection is broadly applied in biotechnology industries that use microorganisms, including microalgae, because of a main advantage: no previous knowledge of the biochemical pathways or genes

regulating them is required for its application (Queener and Lively, 1986). This technique has been applied to improve productivity of the carotenoids astaxanthin in *Haematococus pluvialis* (Chen *et al*., 2003) and PUFAS in *Pavlova lutheri* (Meireles *et al*., 2002), *Nannochloropsis oculata* (Chaturvedi *et al*., 2004) and *Tetraselmis tetrathele* (Cortez *et al*., 2015), but there is not information on mutants of *Rhodomonas salina* with high lipid and PUFAs contents.

In order to further improve the nutritional quality of *Rhodomonas salina*, and thus make it even more attractive for aquaculture, a mutagenesis protocol with ethyl methane sulphonate (EMS) was developed for this species. Mutants enriched in lipid and PUFAs content were then selected by resistance to the herbicide Quizalofop.

## MATERIALS AND METHODS

### Algal strain and culture conditions

*Rhodomonas salina* (CS-174) was obtained from the CSIRO Algal Culture Collection, CSIRO Division of Marine Research, Hobart, Tasmania. An axenic clonal culture (wild type WT) was prepared and cultured in seawater (3.3% w/v) filtered, autoclaved, and enriched with f/2 medium (Guillard, 1975). Cultures were maintained at 25°C with continuous irradiance of 100 µmol $m^{-2}$ $s^{-1}$ and permanently bubbled with filtered (0.22 µm) atmospheric air.

### Sensitivity of *R. salina* to the herbicide Quizalofop

Microalgae cultured in 2.5 ml vials at a cell density of 500,000 cells $ml^{-1}$ were pelleted (centrifugation at 1000 x g for 10 min) and resuspended in 2.5 ml of f/2 medium containing different concentrations of Quizalofop (50, 100, 150, 200, 400, 600, 800 and 1000 µM) to be placed in a growth chamber under standard culture conditions for 24 hours. Three replicates of each treatment were established. The next day, cell densities were determined through Neubaüer counting chamber and the survival percentages at each concentration of Quizalofop, were determined. Quizalofop concentration that caused a mortality percentage above 70% was selected for screening of mutants potentially improved on their lipids content.

### Mutagenesis and screening

A volume of 20 ml of culture of *R. salina* (1.5 x $10^6$ cells $ml^{-1}$, $OD_{750nm}$= 0.415) were placed in a Petri dish and mutagenized with a 0.1 M final concentration of ethyl methane sulfonate (EMS), with gentle agitation under standard culture conditions, according to Chatuverdi *et al*. (2004). Every 15 min, to complete 120 min, 1 ml of culture was removed, centrifuged at 1000 x g for 10 min and washed twice with 1 ml of sodium thiosulfate (0.16 M) to remove EMS. Washed cells were resuspended in 5 ml of f/2 medium and maintained in a growth chamber for 24 h at 25°C and continuous irradiance of 50 µmol $m^{-2}$ $s^{-1}$. Percentage of survival for each EMS treatment was determined with respect to a control culture (not exposed to EMS) by cell counting through a Neubaüer chamber. Cultures that resulted in survival percentages below 10% were used to isolated single cells through the micropipette washing technique. The isolated cells were individually cultivated in 5 ml of f/2 medium during 10 d at 25°C under continuous irradiance of 50 µmol $m^{-2}$ $s^{-1}$.

Surviving clones were grown in 10 ml of f/2 medium containing Quizalofop concentration previously determined, at 25°C under continuous light with an irradiance of 100 µmol $m^{-2}$ $s^{-1}$ for 20 d. The fastest growing clones, as determined by the growing parameters estimated, were selected for further analysis.

### Growth parameters, total lipids content and fatty acid profiles

Wild type and selected mutant strains were cultured in batch mode, in 500 ml of f/2 medium at 25°C under continuous light of 100 µmol $m^{-2}$ $s^{-1}$ and permanently bubbled with filtered atmospheric air. Growth was monitored over a 20-day period by cell counts thought Neubaüer counting chamber and then calculated

the specific growth rate (μ, $day^{-1}$) (Guillard, 1975). Once cultures reached exponential growth phase, 5 ml of culture were harvested by centrifugation at 7000 x g at 15°C for 10 min, washed twice with ammonium formiate 0.4 M to remove salt from biomass and frozen to −70°C until lipids analyses.

Total lipids were extracted from a known dry mass of algal cells following the Bligh and Dyer (1959) method and analyzed by the lipid charring technique (Marsh and Weinstein, 1966), using tripalmitin as standard for the calibration curve.

Fatty acids were determined by gas chromatography–mass spectrometry and expressed as percentages of total fatty acids. The conversion of fatty acids (FAs) into their methyl esters was carried out according to the Sato and Murata (1988) method using a Hewlett-Packard G 1800B GCD system (Hewlett-Packard Co., Wilmington, DE), with an omegawax TM 250 fused silica capillary column (Supelco, 30 m, 0.25-mm inner diameter) equipped with a mass spectrometry detector. The injector and detector were set at 250 and 280 °C, respectively. The carrier gas was helium at a flow rate of 3 mL $min^{-1}$. The temperature program was set to increase from 100 to 180 °C at 20 °C $min^{-1}$, then from 180 to 220 °C at 15 °C $min^{-1}$, after which the temperature was held at 220 °C for 30 min. The mass spectrometer was operated in the electron impact mode with an electron energy of 70 eV, source temperature 280 °C, mass range m/z 10–450, and EM voltage, 1750. Compounds were identified by a NIST2000 mass spectra library search.

**Statistical analysis**

In order to asses statistical significance of differences among wild type and mutant strains, data of growth, lipid and fatty acid content were analyzed by one-way analysis of variance (ANOVA) with the LSD multiple comparison test (Least Significant Difference) (Zar, 1996) as *a posteriori* test. Analyses were conducted with Statgraphics Plus 4.1 software.

## RESULTS

**Sensitivity of *Rhodomonas. salina* to Quizalofop**

All Quizalofop concentrations tested negatively affected survival of *R. salina*. The effect was not as noticeable between 50 and 600 µM but survival decreased drastically from 800 µM (Fig. 1). In order to ensure that just highly resistant mutants will survive during screening phase, Quizalofop 1000 µM, which causes just 5% survival in the wild type strain (Fig. 1), was used to select strains with potentially improved lipids content.

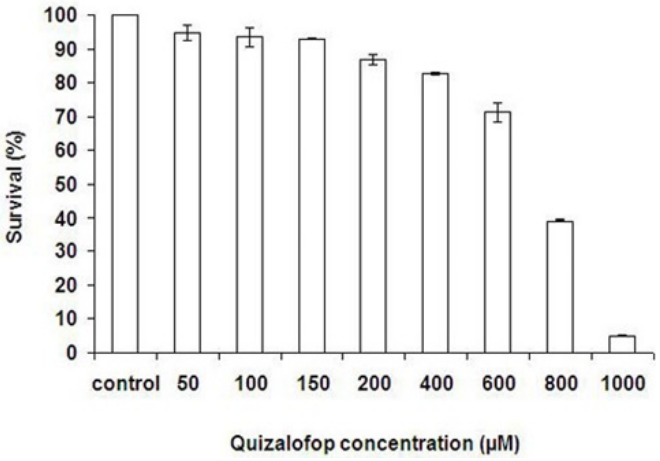


**Figure 1** Effect of Quizalofop on the survival rate of algal cells in *Rhodomonas salina* cultures. The survival rate of each treatment (mean ± SD) was counted by comparing with control cultures (100% survival).

**Mutant isolation**

The cells of *R. salina* treated with 0.1M EMS showed a significantly decrease on their survival with increasing exposure time. The lowest survival percentages were 5 and 2% at 90- and 105-min exposure, respectively (Fig. 2), so these exposure times were used to isolate mutant clones.

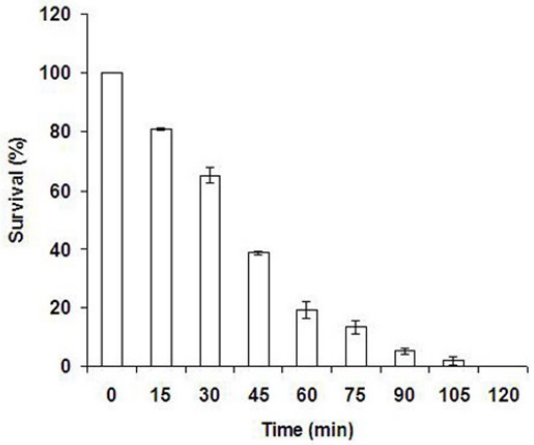


**Figure 2** Effect of EMS treatment on the survival rate of algal cells in *Rhodomonas salina* cultures. The survival rate of each treatment (mean ± SD) was counted by comparing with control cultures (100% survival).

Despite many efforts made, it was not possible to grow *R. salina* in solid medium (data not shown) so, in this study, mutants of *R. salina* must be isolated by micropipette washing technique.

Six mutants were isolated and cultured in the presence of Quizalofop (1000 µM): 90a, 90b, 90c (cells exposed to 0.1 M EMS for 90 minutes), 105a, 105b and 105c (cells exposed to 0.1 M EMS for 105 min). All of the mutant strains (and more drastically the wild type strain) were negatively affected by the presence of Quizalofop; however, strains 105b and 105c were able to grow in this condition, demonstrating an exceptionally high resistance to this herbicide (Fig. 3). These two mutant strains were selected for further analysis.

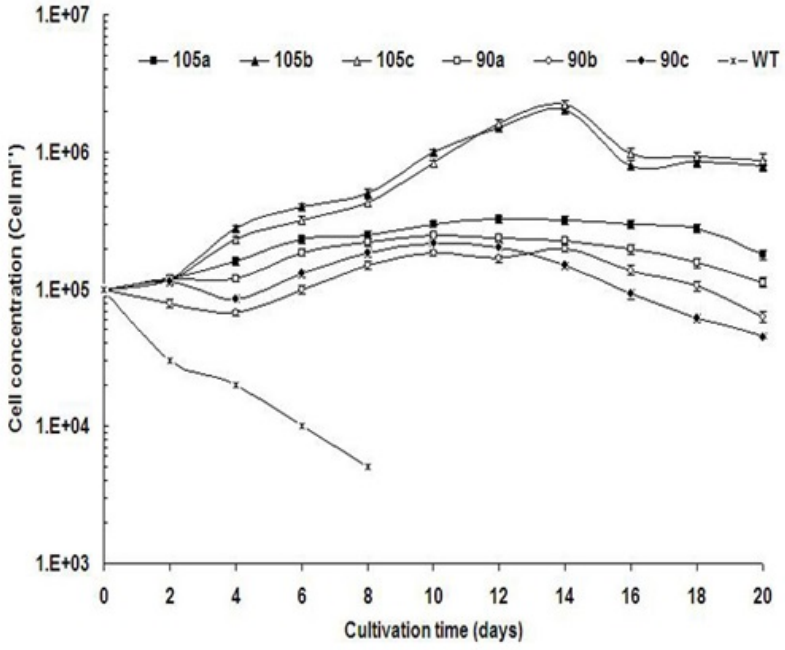


**Figure 3** The growth curves of mutants (90a, 90b, 90c, 105a, 105b and 105c) and the wild type (WT) of *Rhodomonas salina* (mean ± SD) in f/2 medium with Quizalofop (1000 µM).

### Growth parameters, total lipids content and fatty acid profiles of mutants and wild type strains

Wild type and mutant strains (105b and 105c) showed a similar growth pattern (Fig. 4). Their highest maximum cell density ($4.3\pm0.37x10^6$ cells $ml^{-1}$) and growth rate ($\mu= 0.8\pm0.08$ $day^{-1}$) did not differ between mutants and wild type strains under the culture conditions evaluated in this study ($P> 0.05$).

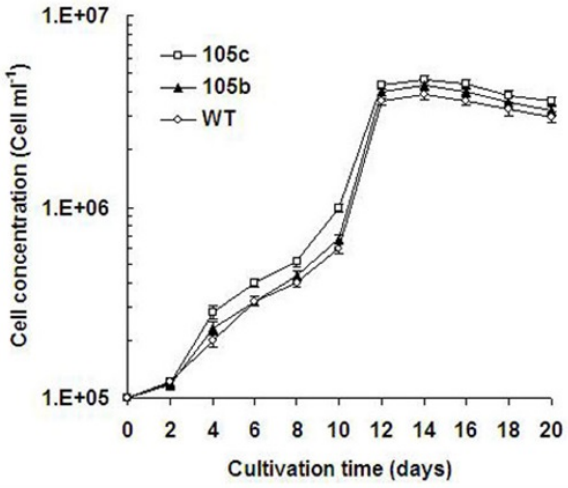


**Figure 4** The growth curves of mutants (105b and 105c) and the wild type (WT) of *Rhodomonas salina* (mean ± SD) in f/2 medium under standard culture conditions.

**Table 1** Fatty acid methyl esters and total lipid (mg/g dry matter) of wild type (WT) of *Rhodomonas salina* and two selected mutant strains (105b and 105c) cultured under identical culture conditions. *

| | Strains | | |
|---|---|---|---|
| **Fatty acid composition** | **105b** | **105c** | **WT** |
| 12:0 | 0.25 ±0.02 | 0.2 ± 0.02 | 0.2 ± 0.01 |
| 13:0 | 0.4 ± 0.02 | 0.35 ± 0.02 | 0.43 ± 0.03 |
| 14:0 | 14 ± 0.84 | 14 ± 0.98 | 13 ± 0.75 |
| 15:0 | 0.71 ± 0.04 | 0.5 ± 0.03 | 0.6 ± 0.04 |
| 16:0 | 24 ± 1.22 | 24 ± 1.9 | 21 ± 1.7 |
| 17:0 | 0.53 ± 0.02 | 0.69 ± 0.05 | 0.68 ± 0.04 |
| 18:0 | 2.5 ± 0.17 | 3.5 ± 0.17 | 3.5 ± 0.21 |
| 20:0 | 0.1 ± 0.01 | 0.11 ± 0.01 | 0.13 ± 0.01 |
| 21:0 | 0.12 ± 0.01 | 0.12 ± 0.01 | 0.19 ± 0.01 |
| 22:0 | 0.38 ± 0.02 | 0.11 ± 0.01 | 0.16 ± 0.02 |
| 23:0 | 0.26 ± 0.02 | 0.12 ± 0.04 | 0.14 ± 0.03 |
| 24:0 | 0.13 ± 0.01 | 0.15 ± 0.05 | 0.15 ± 0.02 |
| Total saturated | 43.69 | 43.59 | 39.78 |
| 16:1n-7 | 3.88 ± 0.27 | 2.25 ± 0.14 | 2.54 ± 0.2 |
| 18:1n-9 | 11.60 ± 0.7 | 12.77 ± 0.77 | 14.02 ± 0.84 |
| 18:1n-7 | 2.95 ± 0,18 | 4.53 ± 0.27 | 3.37 ± 0.2 |
| 18:1n-5 | 0.21 ± 0.01 | 0.16 ± 0.01 | 0.1 ± 0.01 |
| 20:1n-11 | 0.18 ± 0.01 | 0.12 ± 0.02 | 0.18 ± 0.02 |
| 24:1n-9 | 0.86 ± 0.05 | 0.26 ± 0.02 | 0.2 ± 0.02 |
| Total monounsaturated | 19.68 | 20.08 | 20.41 |
| 18:2n-6 | 9.57 ± 0.67 | 8.19 ± 0.66 | 7.1 ± 0.68 |
| 18:3n-6 | 0.72 ± 0.04 | 0.99 ± 0.08 | 1.32 ± 0.11 |
| 18:3n-3 | 8.63 ± 0.69 | 5.32 ± 0.48 | 5.71 ± 0.34 |
| 18:4n-3 | 2.4 ± 0.14 | 3.69 ± 0.22 | 3.55 ± 0.21 |
| 20:4n-6 (ARA) | $3.25 \pm 0.2^a$ | $3.52 \pm 0.28^a$ | $2.6 \pm 1.46^b$ |
| 20:5n-3 (EPA) | $10.2 \pm 0.61^a$ | $12.42 \pm 0.75^a$ | $4. \pm 0.36^b$ |
| 22:6n-3 (DHA) | $7.6 \pm 0.46^a$ | $7.74 \pm 0.46^a$ | $4.19 \pm 0.25^b$ |
| Total PUFA | $42.37^a$ | $41.87^a$ | 28.47b |
| Total fatty acid | $105.74 \pm 6.87^a$ | $105.54 \pm 8.44^a$ | $88.66 \pm 7.62^b$ |
| Total Lipid | $182 \pm 10.93^a$ | $194 \pm 16.52^a$ | $121 \pm 9.60^b$ |

*Results represent the mean value of experiments performed in triplicate ± SD
Equal superscripts at the same row indicate no significant differences between averages (ANOVA, LSD F-test; P>0.05).

Regarding the quantity and quality of lipids accumulated by the strains, both mutants exhibited a significantly (P>0.05) higher content of total lipids, total fatty acids (TFA) and total PUFAs than the wild type strain (Table 1). The total lipid content of the mutants was 50% (105b) and 60% (105c), higher that the wild type. The mutant strains were also significantly enriched on the main PUFAs: EPA, DHA and ARA. The EPA content of strains 105b and 105c was 2.6 and 3.1-fold higher than the wild type strain, respectively. On the other hand, DHA and ARA contents attained by both mutant strains were very similar, being 1.8 and 1.3-fold higher than the wild type's contents, respectively (Table 1).

## DISCUSSION

Interest in PUFAs i.e. DHA, EPA and AA have increased recently because of the recognition that they are important for good health and in lowering the risk of diseases where chronic inflammation plays an important role (Harwood and Caterson, 2006; Simonetto *et al*.,2019). Moreover, these compounds are essential for the growth and development of marine fish larvae, shrimp, and molluscs (Hemaiswarya *et al*., 2010; González *et al*., 2019). The latter has motivated the search for microalgae with high PUFAs content for biotechnological an aquacultural purposes. The random mutagenesis has played an important role in achieving this objective, because it has helped to increase the content of PUFAs in many species of microalgae (Meireles *et al*., 2002; Chaturvedi *et al*., 2004; Tanadul *et al*., 2018). However, it was thought that this technique could be applied only to those species of microalgae capable of forming colonies on solid medium (Williams *et al*., 1979). In this study it was shown that it is possible to apply this methodology on species that does not meet this requirement. Although much more time consuming than agar plating, isolation of mutant cells under microscope allows broaden the range of species susceptible to be genetically improved by this technique.

The application of mutagenesis with EMS as a mutagenic agent and Quizalofop as a selection tool allowed to obtain two mutants of *Rhodomonas salina* with increased contents of total lipids and PUFAs (Table 1). It is important to say that the reversions occurring spontaneously in a mutant strain can restore the wild phenotype (Van Der Meer and Zhang, 1988). So, it is essential to check that improved attributes have been fixed in the mutant´s genome. In this study, resistance of mutant strains to Quizalofop, as well as their lipids and PUFAs contents, was maintained even after one year in an inhibitor free medium (data not shown).

Genetic improvement through transgenesis, to increase the nutritional quality of *R. salina* is difficult due to the complexity that shows its chloroplast (Fraunholz *et al*., 1997), so that the mutagenesis results in an attractive approach to achieve this goal.

It has been demonstrated that mutated strains have higher growth and higher contents of total lipids and PUFAs than wild type. Dunstan *et al*. (2005) cultured *R. salina* (strain CS174) at 20°C and 70-100 µmol $m^{-2}s^{-1}$ and obtained a cell density of 360,000-370,000 cells/ml, 22% of total lipids, 6.5% of DHA, 9.3% of EPA and 2.6% of ARA. These values are lower than those obtained with mutant strains of *R. salina* in this research.

Finally, we conclude that it is possible to obtain mutants of *R. salina* by directed evolution through random mutagenesis and selection with a significantly increased capacity for lipids (50-60% higher than WT) and PUFAs (50% higher that WT) accumulations demonstrating the feasibility of genetically improving a species that doesn´t form discrete colonies in solid medium.

## CONFLICT OF INTERESTS

No conflict of interest is declared.

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