SCREENING AND CHARACTERIZATION OF A MUTANT LIBRARY OF THE MICROALGA *NANNOCHLOROPSIS OCEANICA* FOR GROWTH AND LIPID PRODUCTION AT HIGH CO_2 CONDITIONS

By

HOA THI VU

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Dr. G. Charles Dismukes

And approved by

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ABSTRACT OF THE THESIS

Screening and Characterization of a Mutant Library of the microalga Nannochloropsis oceanica for Growth and Lipid Production at High CO_2 Conditions

By HOA THI VU

Thesis Director:

Dr. G. Charles Dismukes

Nannochloropsis is a genus of microalgae that produces substantial amounts of storage lipids referred to as triacylglycerides that are derived from fatty acids. These products are precursors to important dietary lipids for use in oils and as replacements for hydrocarbon fuels. To enhance lipid production in *Nannochloropsis oceanica* CCMP 1779 we applied an insertional mutagenesis approach to produce a library of 1200 strains containing single insertions located randomly in the genome. The collection was screened for growth at high CO_2 (≤10%) and low pH, and for high lipid content. Seven mutants were selected for quantitative assays and characterization of photosynthetic efficiency. All 7 strains and the wild type (WT) grow fastest as measured by growth rate and final dry biomass at 2% CO_2. The WT had a higher growth rate and biomass production compared with the mutant strains at 2% and 10% CO_2. There was pH and CO_2-dependence of the growth rate of mutants and the WT. A level of 10% CO_2 was a stress condition for *Nannocholoropsis oceanica* CCMP 1779. One strain (G2) grown at 2% and 10% CO_2 had a higher lipid content by Nile Red fluorescence than the WT. When quantified by lipid extraction, this strain did not show a higher lipid content than the WT by day 15 of growth.

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Last but not least I want to thank all my family and friends for being by my side in good and bad moments, loving me for who I am and for their unconditional support. They know I love them ☺

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Screening and Characterization of a Mutant Library of the microalga *Nannochloropsis oceanica* for Growth and Lipid Production at High CO_2 Conditions

Hoa Vu[1,2], Yunbing Ma[2], Yuan Zhang[1,2], Gennady Ananyev[2,4], Colin Gates[2,3], Zhiyan Du[3], Christoph Benning[3], G. Charles Dismukes[2,4]

[1]Department of Biochemistry and Microbiology, Rutgers, New Brunswick, NJ 08901

[2]Waksman Institute of Microbiology, Rutgers University, Piscataway, NJ 08854

[3]Department of Plant Biology, Michigan State University, East Lansing, MI 48824

[4]Department of Chemistry and Chemical Biology, Rutgers University, Piscataway, NJ 08854

Abstract:

Nannochloropsis is a genus of microalgae that produces substantial amounts of storage lipids referred to as triacylglycerides that are derived from fatty acids. These products are precursors to important dietary lipids for use in oils and as replacements for hydrocarbon fuels. To enhance lipid production in *Nannochloropsis oceanica* CCMP 1779 we applied an insertional mutagenesis approach to produce a library of 1200 strains containing single insertions located randomly in the genome. The collection was screened for growth at high CO_2 (≤10%) and low pH, and for high lipid content. Seven mutants were selected for quantitative assays and characterization of photosynthetic efficiency. All 7 strains and the wild type (WT) grow fastest as measured by growth rate and final dry biomass at 2% CO_2. The WT had a higher growth rate and biomass production compared with the mutant strains at 2% and 10% CO_2. There was pH and CO_2-dependence of the growth rate of mutants and the WT. A level of 10% CO_2 was a stress condition for *Nannocholoropsis oceanica* CCMP 1779. One strain (G2) grown at 2% and 10% CO_2 had a higher lipid content by Nile Red fluorescence than the WT. When quantified by lipid extraction, this strain did not show a higher lipid content than the WT by day 15 of growth.

Keywords: Microalgae**,** *Nannochloropsis oceanica*, Random Mutagenesis, Library screening, Lipid Production, CO_2 tolerance.

1 Introduction

The global economy and human activity depend on fossil fuels, the depletion of which threatens the sustainability and energy security of our entire civilization [1]. Fossil fuels contribute 80% of primary energy consumed for industries and transport [2].The burning of fossil fuels contributes to numerous environmental problems: greenhouse gas emissions, climate change [3]. Coal power plants can release effluent containing up to 15% CO_2 [4]. It is therefore urgent to find a sustainable energy source to replace the demand on fossil fuels and improve CO_2 mitigation [5]. Biofuel production from renewable sources with zero carbon emission is a sustainable way to resolve the energy crisis [1].

Biofuels are sustainable because of their renewability, biodegradability, and inherent net production of virtually no greenhouse gases [6]. Biofuels are divided into three generations. The first generation uses corn starch and soy beans to make bioethanol. An unintended consequence, however, is an increase in food prices since the food supply is directly converted to bioethanol. The second generation uses non-food biomass to make liquid fuels. Using cellulose or lignin to convert to bioethanol is a costly process since technical availability for this process is lacking. The third generation uses cells as a factory for biomass from sunlight energy through photosynthesis. This generation is a promising technology for future energy. Using photosynthetic organisms to convert sunlight to biomass is a promising strategy for future energy production. Microalgae can grow in sea water and waste water, avoiding the issue of competing for available fresh water [7]. Algae accumulate high percentages of neutral lipid in the cell, reaching as much as 20-50% lipid in dry biomass [8].

Triacylglycerols (TAGs) are the main form of lipid storage in microalgal cells. Microalgae store TAGs primarily under stress conditions such as nutrient limitation, high temperatures and high light intensities [9]. The lipid composition of higher plant seeds and algae are highly similar, suggesting that lipid synthesis pathways in higher plants and algae share some analogy. Fatty acids are synthesized in the plastid and are then transferred to the cytosol and subsequently converted to TAGs by a series of biochemical reactions. TAGs can be extracted from algal cells and converted to biodiesel by the chemical process of transesterification. In choosing an algal strain for biodiesel production it is important that the strain has high lipid accumulation as well as a suitable lipid profile for biodiesel conversion. Ma et al. compared TAG production and lipid composition of nine *Nannochloropsis* strains. These results showed that the *Nannochloropsis oceanica* strain has high TAG production and high desired fatty acids composition for biodiesel production [10]. The lipid contents of *N. oceanica* strains are mainly C16-C18 fatty acids and are suitable for biodiesel conversion [10, 11] There are several established methods to enhance lipid production in algae. High irradiation can enhance lipid accumulation up to 30% [12]. Nitrogen starvation in *Nannochloropsis* shows an increase up to 60% lipid in total dry biomass, but starvation can reduce the growth rate of culture, which reduces biomass yield [12, 13]. Changing salinity, light intensity and light/dark cycle could affect lipid accumulation and lipid profile [14].

Genetic engineering has been used to direct the products of carbon fixation and photosynthesis to fatty acid synthesis and ultimately induce TAG accumulation. There are two main approaches for genetic engineering. The first one is targeting and overexpressing specific genes that participate in TAG synthesis to enhance lipid

production [15]. Vigeolas *et al.* showed that overexpression of cytosolic yeast G3PDH in the seeds of *Brassica napus* resulted in a 40% increase in TAGs [16]. Knocking down genes that compete for the carbon precursors of the lipid synthesis pathway can push those precursors to lipid synthesis as well. Besides targeted mutagenesis, random insertional mutagenesis has also been used to generate large mutant libraries and screen for desired phenotypes. Cheng *et al.* showed that gamma irradiation can enhance biomass production by more than 300% in *Spirulina* sp. under 15% CO_2 conditions [17]. *Nannochloropsis gaditana* mutants generated by chemical and insertional mutations have higher photosynthetic efficiency [18]. An enhanced lipid production of *Chlamydomonas reihardtii* by a chemical mutagenesis method by using ethyl methane sulfonate was published by Lee *et. al*. [19]. By random mutagenesis method, Ma *et al.* discovered a new gene, cia6, required for pyrenoid formation in *Chlamydomonas reinhardtii* [20].

In 2012, Vieler *et al.* published the completed draft genome of *N. oceanica* CCMP 1779. More than one-half of the 28 Mb genome is annotated, giving more insight into the metabolic mechanisms of the organism [21]. But metabolic pathways and specifically the lipid synthesis pathway remain unclear; enzymes may have several isoforms and the extent of competition or collaboration between isoforms is unknown. Our aim was to generate a mutant library of *Nannochloropsis oceanica* CCMP 1779 (hereby *N. oceanica)* by randomly inserting a plasmid with a hygromycin resistance cassette into the genome. The mutant library was generated in collaboration with Dr. Christoph Benning from Michigan State University, whose lab developed the electroporation protocol for *N. oceanica*. A library of 1200 strains was generated. After phenotype and low pH screening 7 mutants were selected and characterized at high CO_2

conditions (2% and 10%). The wild type (WT) and the mutant strains showed optimum growth at 2% CO_2. The WT had a higher growth rate and biomass accumulation than the mutants at 2% and 10% CO_2. The mutants had higher Nile Red fluorescence normalized to OD_{750} than the WT at 10% CO_2. Gene mapping and growth of mutants at higher CO_2 concentrations and lipid profile comparison between mutants and the WT in each CO_2 condition will be our future focus.

2. Materials and Methods

2.1. Cell culturing

Nannochloropsis oceanica CCMP 1779 was kindly provided by our collaborator Dr. Christoph Benning from Michigan State University and obtained from Bigelow (National Center for Marine Algae and Microbiota). The culture was grown photoautotrophically in A+ medium [22] supplemented with 10 mM of (what?) at 22 ºC and 40 μE/m^2/s under 16/8 hours light/dark cycles using LED light. The pH of the culture was set to 8.2 using Tris buffer. The cell culture was grown in liquid medium with shaking at 100 rpm in a growth chamber and maintained on 1.5% agar A+ medium. Agar plates were stored at 4 °C for later use.

2.2 Measurement of growth rate

The growth rate was measured by optical density at 750nm (OD_{750}) of 100 μL culture (dense culture might be diluted with necessary dilution factor) using a Biotek Neo2 plate reader configured for spectrophotometry. Specific growth rate and doubling time were calculated using the following equations:

$$\mu = \frac{(LnNt - LnN1)}{(Tt - T1)} \quad (1)$$

$$g = \frac{\mathrm{Ln}2}{\mu} \quad (2)$$

where μ is specific growth rate, N_t and N_0 are the cell number at time points t and 0 , and *g* is doubling time. LnN is equal to the optical density (absorbance) at 750nm under the assumption that all cells scatter light identically in all conditions (no size changes or aggregation).

2.3 Dry weight determination

A 10 mL aliquot of cell culture was filtered through pre-weighed glass microfiber filter Whatman GF/B filter paper, diameter 47 mm, (Whatman International Ltd., Maidstone, UK). The filter with cell culture was washed with deionized water to remove residual salts. The filter was then dried at 60 ^{o}C for 24 hours. The dry weight was measured as the difference between the filter weight before and after filtration and drying.

2.4 Lipid extraction method

A lipid extraction method was developed based on the method of Bligh and Dyer [23] with some modifications. In brief: 15 mL of cell culture (0.05 g dry biomass) was centrifuged at 7000 rpm for 10 minutes. The pellet was washed twice with deionized water. 0.1g of glass beads (0.5 mm in diameter) was added to the pellet, then, the sample was mixed with methanol/chloroform solution 600:300 μL (2:1, v/v) and sonicated for 1 minute. Then 240 μL of 0.73% NaCl (w/v) solution [24] was added to the mixture. The mixture was then vortexed and incubated for 30 min at room temperature. The mixture was centrifuged at 12000 rpm for 10 minutes. The chloroform phase was extracted. 200 μL chloroform was added to the leftover cell debris to obtain the remaining lipid. The chloroform phase was collected in a pre-weighed 1.5 mL Eppendorf tube and vacuum dried for 1 hour.

2.5 Measurement of the lipid content by Nile Red fluorescence spectrometry

Nile Red (9-diethylamino-5-benzo[α]phenoxazinone) is a lipid soluble dye. Nile Red fluorescence is used for in situ cellular lipid quantification in mammalian, bacterial, yeast, and algal cells [25-29]. Acetone, DMSO (Dimethyl sulfoxide), hexane and ethanol

are common solvents as the Nile Red carrier [30]. We developed a method for Nile Red staining in algal cells using acetone as the dye carrier. Briefly, Nile Red (Sigma-Aldrich) working solution was prepared from 200 μg/mL stock solution in absolute acetone mixed with deionized water to a final concentration of 8 μg/mL (in 4% acetone). The working solution was placed in the dark at room temperature for 30 minutes to reach equilibrium. The stock solution was covered with foil and stored at 4 °C. An aliquot of 100 μL of cell culture was mixed with 100 μL Nile Red working solution. The mixture was incubated in the dark at room temperature for 30 minutes. Nile Red fluorescence intensity was measured using a Biotek Neo2 microplate reader with excitation at 535 nm and emission at 570 nm.

2.6 Generation of mutant library by electroporation

A mutant library of randomly inserted cassettes was generated via transformation. The electroporation procedure for *N. oceanica* was based on the method developed by Vieler et al. [21]. Cell culture at early log phase (1-2x10^7 cells/mL) was harvested by centrifugation. The pellet was washed twice with 375 mM cold sorbitol to remove excess salt and resuspended. A total of 3-5 mg of linearized pnoc401 or pnoc401 MmeI-seq PCR fragment encoding resistance to hygromycin B (Fig. 1) was added along with 30-50 mg of salmon sperm DNA (Invitrogen) to the suspension in a 2 mm electroporation cuvette. Electroporation was conducted using a Bio-Rad GenePulser II. After pulsing, the cells were resuspended in f/2 medium [31] for 48 hours in continuous light before they were transferred on a warm top-agar f/2 media, 0.05% Phytoblend (Caisson Laboratories) in 1:1 dilution (vol:vol) with 50 μg/mL hygromycin for selection. Resistant colonies were

observed as early as 10–14 days after electroporation; colonies were usually transferred after about 3 weeks.

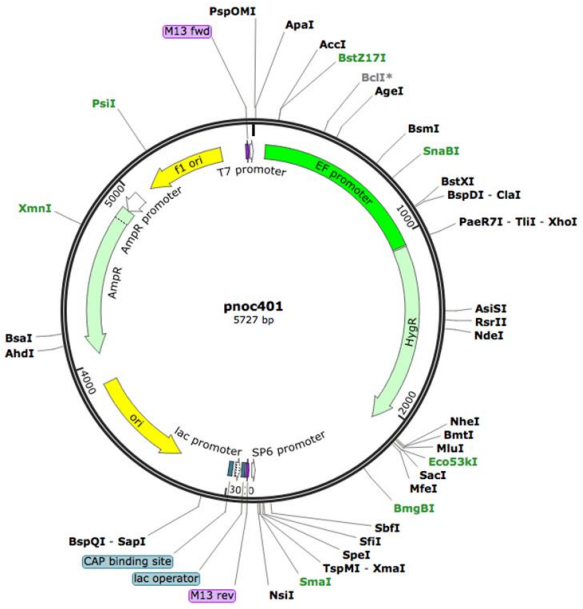


Fig. 1. Plasmid Pnoc401 construction with a Hygromycin B resistance cassette. Plasmid size is 5727 bp with hygromycin resistant gene. AhdI is the restriction site. (Courtesy of Eric Poliner)

2.7 Screening the mutant library based on growth rate and low pH tolerance

2.7.1 First round screening based on growth rate

The colonies were grown in 200 µL A+ medium in transparent 96-well, flat bottom plates (Costar) at 22 ^{o}C and 40 µE/m^2/s under 16/8 hours light/dark cycles using LED light in a 2% CO_2 atmosphere in a Caron Chamber (Caron, OH). Cell density was estimated by optical density at 750 nm (OD_{750}) which was measured daily using a Biotek Neo2 microplate reader. The growth rates and doubling times of each mutant were calculated. 10% of the mutants with the highest growth rates (shortest doubling times) were selected for the second round of screening.

2.7.2 pH tolerance screening

A second round of screening for low pH tolerance was made to account for acidification of the medium that occurs upon bubbling with CO_2, according to the equation:

$$CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons HCO_3^- + H^+$$

Hence, mutants that can proliferate at high CO_2, must also be able to propagate in acidic media. A+ growth media was made with three different buffers and five pH values. A+ media was buffered using 2-(N-morpholino)ethanesulfonic acid (MES) to maintain the pH at 5.5, 6.1, and 6.4; (3-(N-morpholino)propanesulfonic acid) (MOPS) was used to buffer the pH at 7.2; and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) was used to maintain the pH at 8.2 as sea water pH.

The mutants selected from the first round of screening were inoculated on 1.5% agar A+ plates with the aforementioned pHs. The mutants that grew in high pH (8.2, 7.2 and 6.4) and also showed growth at pH 6.1 were selected for the third round of screening. No mutants was able to grow at 5.5 pH.

2.7.3 Third round of screening-qualitative screening for pH tolerance

A third round of screening was done starting from the mutants selected from agar plates that were grown in liquid A+ medium (200 μL) in 96-well transparent, flat bottom plates (Costar) in the aforementioned range of pH values at 22 ºC, 40 μE/m^2/s under 16/8 hours light/dark cycles using LED light and in 2% CO_2 atmosphere in a Caron Environmental Chamber (Caron, OH). The optical densities OD_{750} were measured daily using a Biotek Neo2 microplate reader. The growth rates and doubling times of each

mutant were calculated. 25% of the mutants with the highest growth rates (shortest doubling times) were selected for characterization.

2.8 Characterization of "winner" strains at different CO_2 conditions

The mutants selected from the third round growth and the wild type were grown in 80 mL culture. Inoculation was done at OD_{750} = 0.1 (approx. 3.72*10^7 cells/mL) in A+ medium at 22 ^oC and 40 $\mu E/m^2/s$ under 16/8 hours light/dark cycles using LED light in a Caron environmental chamber for the 2% CO_2 experiment and a multichannel cultivator MC 1000-OD (PSI-Czech Republic) for the 10% CO_2 experiment. The flow rate was set to 0.25 vvm (vvm-volume of air per volume of culture and per min). The OD_{750}, chlorophyll fluorescence yield measured ex. 430 nm, em. 680 nm and Nile Red fluorescence yield at ex.535 nm, em. 570 nm, were measured every 48 hours.

2.9 Pulse Sequence of Fast Repetition Rate Fluorometer (FRRF)

Variable fluorescence Fv/Fm is a parameter analogous to efficiency of PSII in using light energy in photons to undergo charge separation. Fv is the difference between maximum and minimum fluorescence (Fm-Fo) where Fm corresponds to the value for the maximum number of reduced reaction centers and Fo is the dark-adapted state. In Charles Dismukes' lab, Gennady Ananyev has developed a laser based fluorometer (FRRF) to measure the functionality of photosystem II [32]. The samples of *N. oceanica* were measured by 120 trains of 50 sequential "single-turnover" flashes timed to each advance the WOC forward one oxidation state. In total of 6000 flashes with total measuring time 120 s and 100 Hz frequency between flashes, 0.5 s between trains, and 3 minutes of dark pre-incubation.

2.10 Statistical Analysis

The data analysis for standard deviation and standard error were made using the Graphpad Prism software. P-values ≤ 0.05 were considered to be significant. All displayed error bars indicate standard error (SE).

3. Results

3.1 Mutant library generation and screening process

Two pnoc401 construction plasmids were used to randomly insert into the genome by electroporation. One was a linearized whole plasmid and one was a pnoc401 MmeI-seq PCR fragment. After electroporation, the cell cultures were spread on f/2 agar plates. The plates were incubated at 22 ^{o}C and 40 μE/m^2/s under 16/8 hours light/dark cycles using LED light for 3 weeks or until green colonies appeared on the plate surface. The green *N. oceanica* colonies were selected and transferred to 96-well f/2 agar plates. 1200 colonies were selected from hygromycin treated f/2 agar plates after several electroporation cycles. The 96-well agar plates containing the hygromycin B resistant colonies were stored at 4^{o}C and propagated for future screening.

3.2 High-throughput screening of the mutant library

The collection of mutants that grew on hygromycin was further selected using a three level screen, first for growth rate, next for low pH tolerance, finally for increasing CO_2 tolerance. In the first round of screening based on growth rate, 1200 mutants were inoculated in 96 well Costar transparent plates with 200 μL A+ medium in each well. The plates were placed in a Caron chamber at 2% CO_2. The OD_{750} was measured daily and the doubling times were calculated. 10% (120) of the mutants with the fastest growth rate, and consequently shortest doubling times, were selected for the second round of screening.

The second round of screening was based on tolerance to low pH. 120 mutant strains were inoculated on A+ agar medium that was buffered at different pHs (5.5, 6.1, 6.4, 7.2 and 8.2). The mutant strains grew well on 8.2 and 7.2 pH plates. At low pH

(≤6.4) the mutant strains showed a pH dependence on growth. The lower pH the less mutants could survive. No growth was observed at pH 5.5. (Fig. 2). The 25 mutants showed better growth at pH 6.1 were selected for the third round of screening. The third round of screening was to quantify the pH-dependence of the 25 mutants in liquid A+ medium with aforementioned pHs based on growth rate.

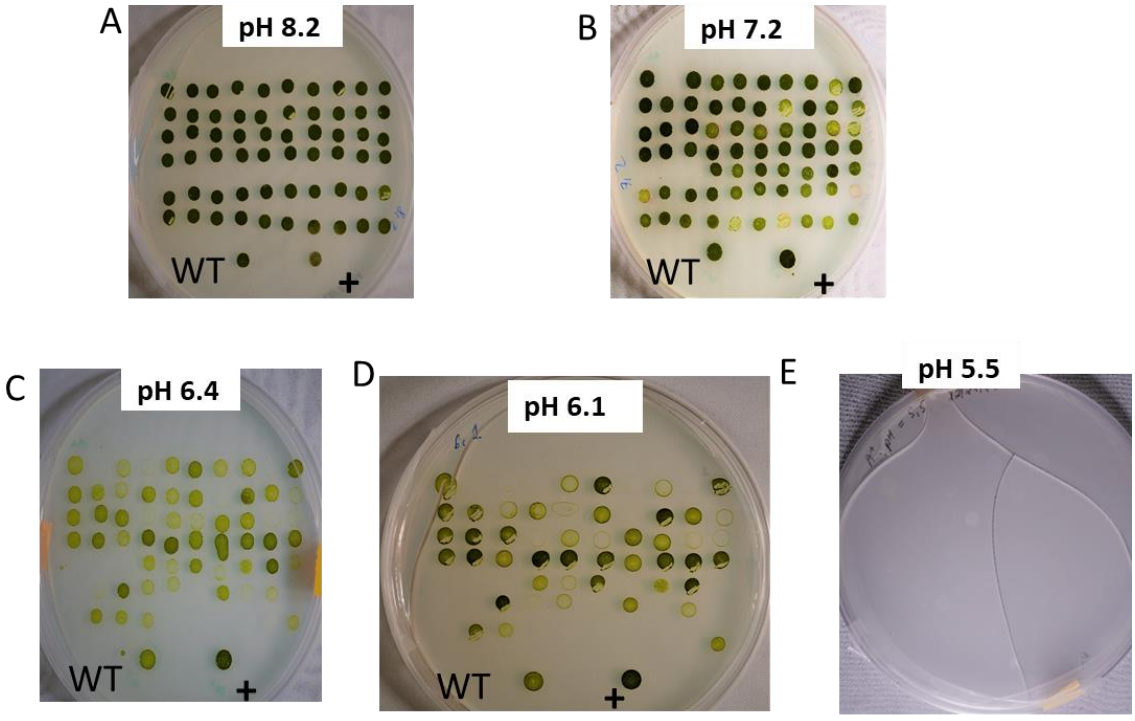
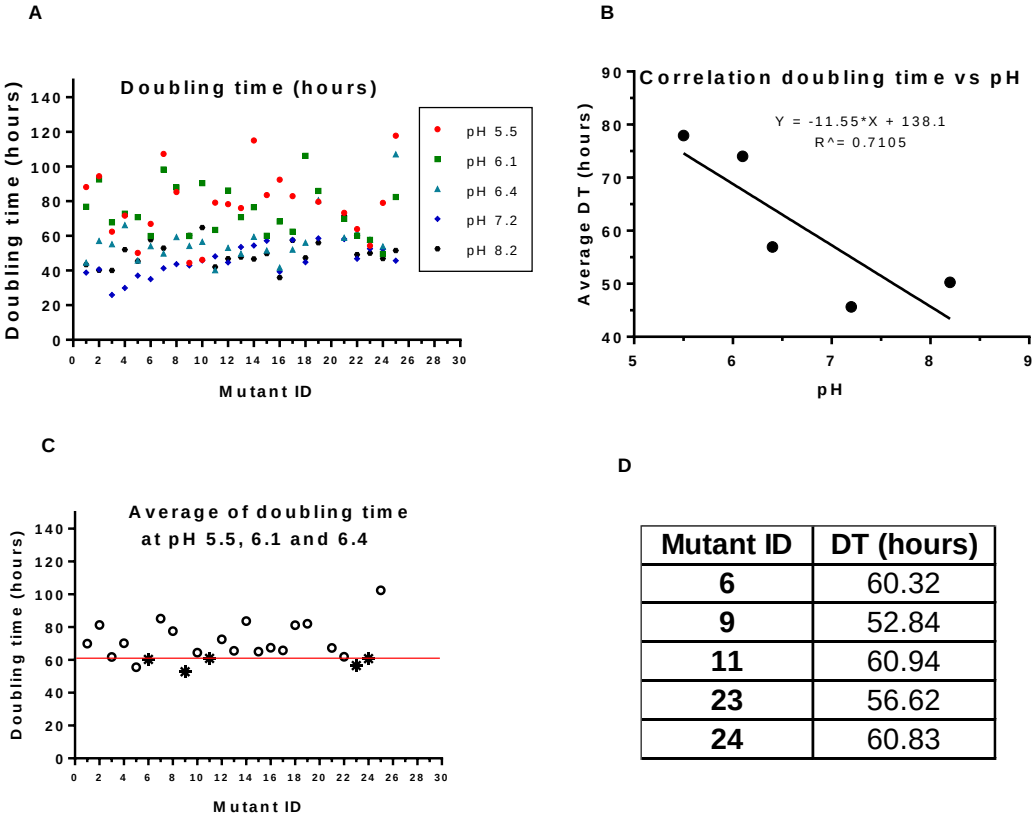


Fig. 2. Phenotypic screening based in pH tolerance of the *N. oceanica* CCMP 1779 mutants. (A-E) Growth phenotype of the mutants on 8.2, 7.2, 6.4, 6.1, 5.5 pH plates respectively.

The third round of screening was conducted based on growth rate of the selected strains in A+ liquid medium with decreasing pH gradient: 8.2, 7.2, 6.4, 6.1, and 5.5 respectively. The 25 mutants were grown on 200 µL A+ medium in 2% CO_2 atmosphere. The OD_{750} was measured during six days and the doubling time (DT) of each mutant in each pH condition was calculated (Fig. 3). At pH 8.2 and 7.2 the mutant strains had the shortest DT at around 45 hours (Fig. 3A); decreasing pH negatively affected growth rate

(Fig. 3B). The averages of the mutant DTs at pH 5.5, 6.1 and 6.4 were calculated (Fig. 3C). Five mutants with ID numbers 6, 9, 11, 23, 24 had the shortest DT compared to other mutants and were selected for the mutant characterization at high CO_2 condition.



Mutant ID	DT (hours)
6	60.32
9	52.84
11	60.94
23	56.62
24	60.83

Fig. 3. Doubling time of the 25 mutants at 2% CO_2. (A) DT at pH 8.2, 7.2, 6.4, 6.1 and 5.5. (B) Correlation between the averages DT of 25 mutants' vs pH. (C) The average DT of each mutant at pH 5.5, 6.1, 6.4. (D) The DT of the five selected mutants # 6, 9, 11, 23 and 24.

In addition to the low pH screening, an alternative screening of 96 mutant strains (one 96-well plate) was conducted based on colony size. From this plate, 8 mutants were selected: A1, B1, C1, D1, E2, F2, G2 and H2, transferred to a f/2 solid agar plate, and grown at 22 ^{o}C and 40 µE/m^2/s under 16/8 hours light/dark cycles using LED light for 2 weeks. The four healthiest colonies B1, C1, E2 and G2 were selected for the next stage of characterization (Fig. 4) and transferred to 250 mL flasks.

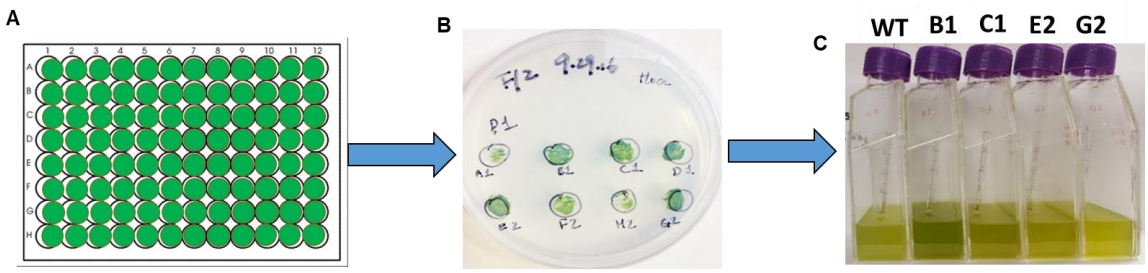


Fig. 4. Phenotype screening procedure. The 8 mutant strains selected were transferred to an agar f/2 plate for a second round of screening. The four strains B1, C1, E2 and G2 showed bigger and greener colonies. They were selected and transferred into liquid f/2 medium, air bubbling.

The doubling time during 9 days of growth for the WT and the mutants B1, C1, E2 and G2 were 49, 52, 55, 53 and 48 hours respectively (Fig. 5B). The strains B1 and G2 grew at the shortest doubling time. Measurements of chlorophyll variable fluorescence showed that the B1 and G2 strains had highest average Fv/Fm (Fig. 6). Based on growth rate and chlorophyll variable fluorescence, the strains B1 and G2 were selected to be characterized at higher CO_2 gassing, together with the five mutant strains selected previously from 1200 strains by the three-stage screening for low pH tolerance.

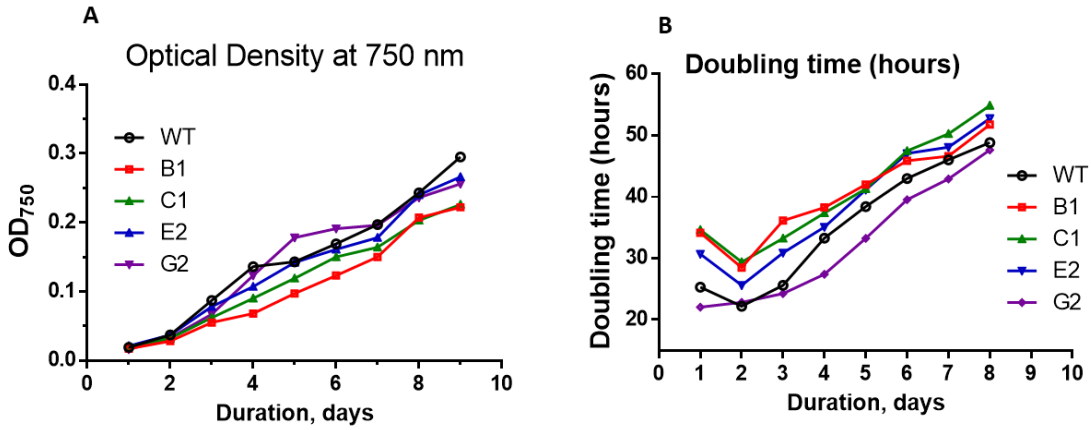


Fig. 5. Growth rate at OD_{750} and the doubling time of the mutants B1, C1, E2 and G2. (A) Growth rate by OD_{750}. (B) Doubling time of the 4 mutants and the WT. 9 days of growth, air boubling, (N=1).

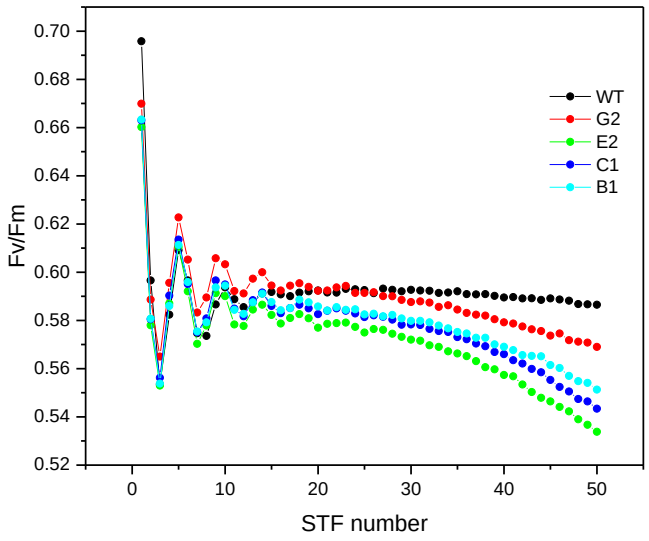


Fig. 6. *In vivo* variable chlorophyll-a fluorescence of 5 strains in response to 50 single turnover flashes (STF number) at fixed flash frequency (100 Hz) following dark adaptation for 2 minutes. FRRF was performed at 22 oC using 50 microsecond flashes. Mutant strains: B1, C1, E2, G2 and the WT.

3.3 Characterization of the 7 mutants at 2% CO_2

From the 1200 mutants containing the hygromycin plasmid, the collection was screened for low pH tolerance. After three rounds of screening, 5 mutants were selected for further characterization under different CO_2 conditions. Two other mutant strains, B1 and G2, were selected based on Fv/Fm as previously described. Two CO_2 conditions were used for screening, 2% and 10%, as described in the method. The growth rate was measured by OD_{750} every two days for 15 days. At 2% CO_2, The WT showed the highest growth rate compared with mutant strains (Fig. 7A) from initial OD_{750}~ 0.1 to 2.24 after 15 days and reached the highest dry biomass accumulation as well (Fig. 7C): 7.4 gL^{-1}. From day 13 to 15 all mutant samples display a decrease in the growth rate, suggesting accelerated CO_2 sensitivity. For this reason, we considered day 11 as the upper limit for comparison of performance prior to accelerated CO_2 sensitivity.

The fluorescence yield from Nile Red was measured for each strain every two days starting from day 3 of inoculation. The Nile Red fluorescence increased continuously during growth, and specifically when the samples reached stationary phase beginning at day 11 (Fig 7B) before accelerated CO_2 poisoning occurs. At day 15, the mutant strain G2 had highest fluorescence intensity followed by WT and B1 strains. A quantitative gravimetric assay was conducted by lipid extraction, as described on the methods. The WT and G2 strains showed the highest lipid accumulation after 15 days of growth 0.73 g/L and 0.72 g/L, respectively (Fig 7D).

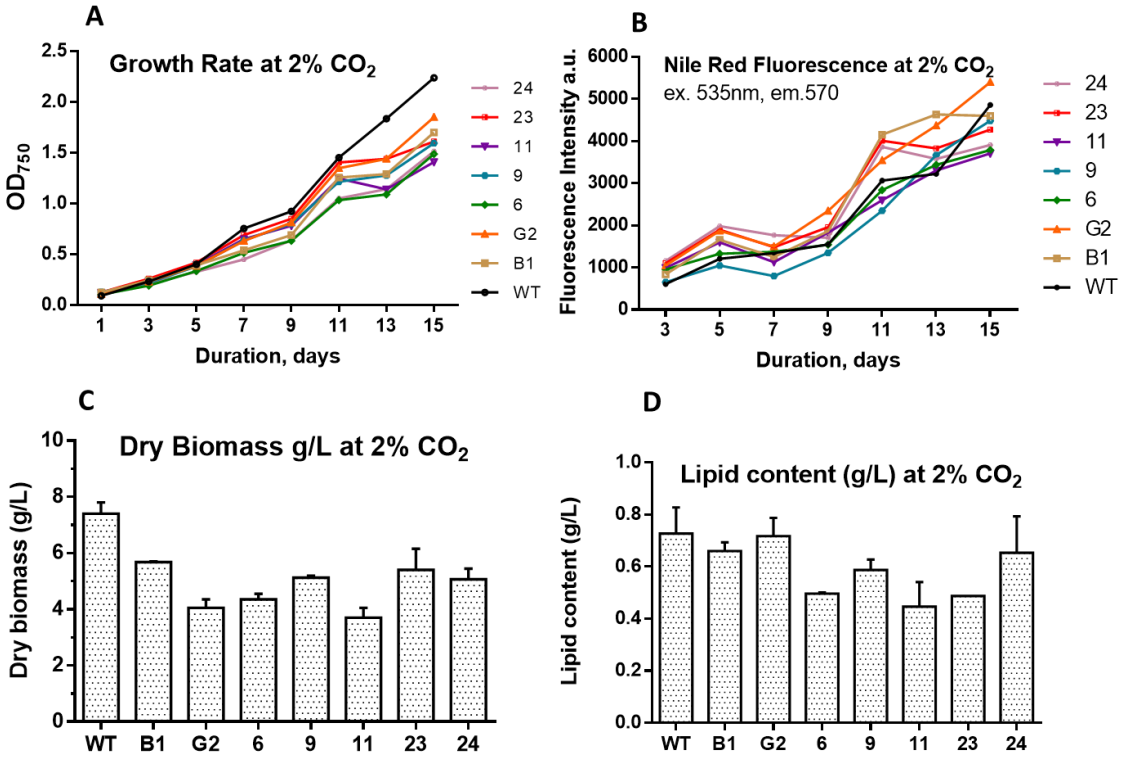


Fig. 7. Biomass and lipid accumulation of the WT compared to the mutant strains at 2% CO_2 during 15 days of inoculation**.** (A) Growth rate at OD_{750} of the mutants and the WT. (B) Nile Red fluorescence intensity. (C) Dry biomass (gL^{-1}) of the mutants and WT after 15 days of growth. (D) Extracted lipid content (g/L) by gravimetric analysis after 15 days of inoculation. The seven mutants and the WT were grown at 2% CO_2 for 15 days at initial OD_{750} = 0.1 (approx. 3.72*10^7 cells/mL), N=1 (N is the number of replicates).

Nile Red fluorescence was normalized to OD_{750} for each strain at 2% CO_2 during 15 days of incubation (Fig. 8A). As mentioned above, at day 11 the accelerated CO_2 poisoning occurs to the mutants. The strains B1, 24 had highest Nile Red normalized to OD_{750}, next group was the strains G2, 6, 23 and the WT and the mutant strains 9 and 11 had lowest Nile Red normalized to OD_{750} (Fig. 8A). At day 13 and 15 The WT showed the lowest Nile Red fluorescence per OD_{750}, while B1 and G2 strains showed the highest. The extracted lipid content per dry biomass was calculated as extracted wt lipids/wt biomass (Fig. 8B). This ratio showed that the WT content ~10% lipid, G2 contained 18% of dry biomass, #24 was 13%, and #23 had 9%. Mutant strain #23 produced the lowest lipid content compared to the other strains in both the Nile Red assay and the gravimetric assay.

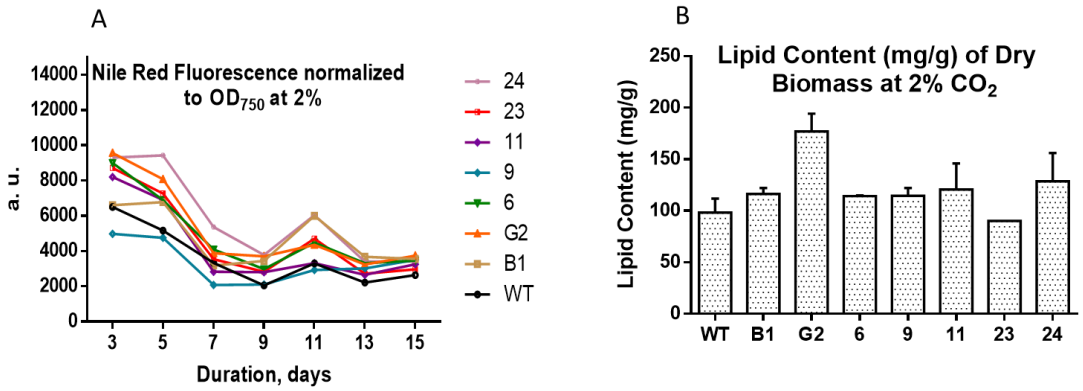


Fig. 8. Nile Red with OD_{750} and lipid content with dry biomass of the mutant strains and the WT at 2% CO_2. (A) Nile Red fluorescence normalized to OD_{750} from day 3 to day 15. (B) Lipid content (mg/g) per dry biomass at day 15 (extracted wt lipids/wt biomass). The seven mutants and the WT were grown at 2% CO_2 for 15 days at initial OD_{750} = 0.1 (approx. 3.72*10^7 cells/mL).

3.4 Characterization of the 7 mutants at 10% CO_2

The goal of the project was to domesticate and characterize the growth rate and lipid content of generated mutants at 10-15% CO_2 concentration. The seven mutant strains and the WT were grown at 10% CO_2 as described in Methods. All the strains were

previously grown at air condition and followed by 2% CO_2 gassing. Next, the strains were inoculated at an initial OD_{750}=0.1 and pre-adapted 5% CO_2 for 24 hours. Following transfer to 10% CO_2, the WT showed a higher growth rate compared to the mutant strains, reaching an optical density OD_{750}=0.43±0.08 after 15 days (Fig. 9A). The deviation at day 9 to 11 was caused by double flow rate in one of the three replicates. It means that not only the CO_2 concentration affect to the growth rate of the strains but also the flow rate. The seven mutant strains had lower growth rates, with strains B1 and G2 slightly higher than the other mutants at OD_{750}=0.29±0.03 (g/L) and 0.28±0.01 (g/L) respectively. Strain 9 had the slowest rate of growth, OD_{750}=0.20±0.01 (P values ≤0.05 at day 15, 3 biological replicates). Biomass was harvested at day 15 (Fig. 9C). The WT had the highest dry biomass, 0.86±0.14, followed by strains 6, 23, B1, and 24 with indistinguishable dry biomasses of 0.65±0.05, 0.65±0.08, 0.65±0.03, and 0.64±0.04 (g/L), respectively. Strain 9 had the lowest biomass accumulation at 0.52±0.08 (g/L). This data agrees with the growth rate measured by OD_{750}. The strains which had a higher growth rate based on OD had a corresponding higher biomass accumulation based on dry weight.

Nile Red fluorescence showed the strains B1 and G2 had highest fluorescence (Fig. 9B). Strains 6, 11, 23 and 24 had similar fluorescence intensities and slightly higher than the WT. Strain 9 had lower Nile Red fluorescence than the WT. The WT accumulated high lipid content 0.26±0.03 (g/L), while strain 23 was the one with the highest lipid content compared to the other mutants, 0.25±0.09 (g/L) (Fig. 9D).

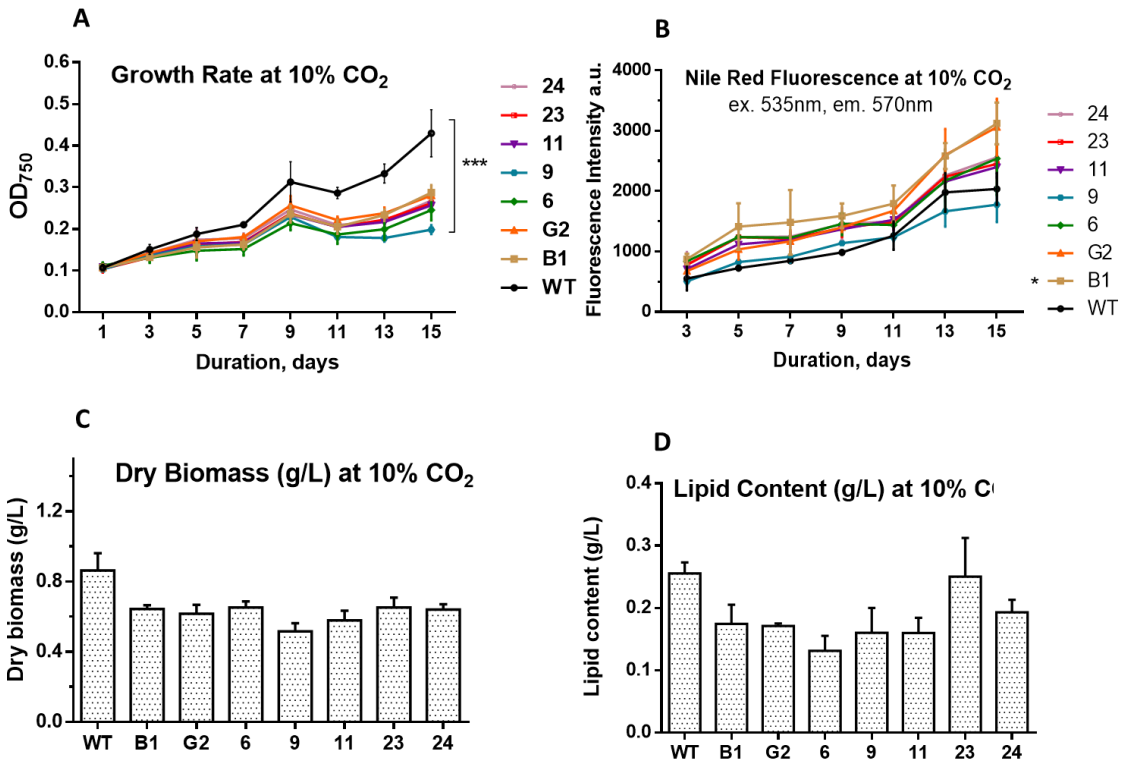


Fig. 9. Biomass and lipid accumulation of WT and 7 mutant strains at 10% CO_2 during 15 days of growth**.** (A) OD_{750}. (B) Nile Red fluorescence intensity. (C) Dry biomass (g/L) after 15 days of growth. (D) Lipid content (g/L) after 15 days of incubation. The seven mutants and the WT were pre-adapted at 2% and 5% CO_2 for 24 hours, initial OD_{750} = 0.1 (approx. 3.72*10^7 cells/mL). ANOVA analysis ($P \leq 0.05$), symbol "*" means significant compared with the WT. N=3.

Nile Red fluorescence was normalized to OD_{750} at 10% CO_2 during 15 days of incubation (Fig. 10A). The WT showed the lowest Nile Red per OD_{750}, and B1 and G2 showed the highest. It may due to the mutants having higher Nile Red fluorescence but lower cell numbers (lower OD_{750}) so the Nile Red normalized to OD_{750} was higher. Mutant strain 9 had the lowest between the mutants. The Nile Red fluorescence normalized to OD_{750} increased from day 13 to 15 because the cells were in stationary phase at 10% CO_2 and accumulated more lipid which was reflected in Nile Red. The lipid content of dry biomass (Fig. 10B) showed that the WT content was ~30±7.4 % lipid and

strains 9 and 23 had higher lipid content per percentage of dry biomass compared with the WT, 32.2±14.2 and ~ 40.4±19.5% respectively.

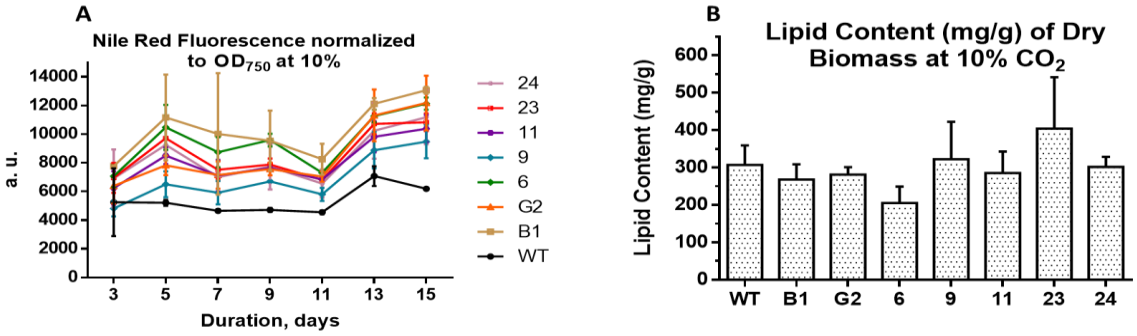


Fig. 10. **.** Nile Red per OD_{750} and Lipid content per dry biomass at 10% CO_2. (A) Nile Red fluorescence normalized to OD_{750}. (B) Lipid content (mg/g) per dry biomass (wt/wt) at day 15. The seven mutants and the WT were pre-adapted at 2% and 5% CO_2 for 24 hours, initial OD_{750} = 0.1 (approx. 3.72*10^7 cells/mL).

3.5 Comparing mutant strains and WT at different CO_2 condition

All strains grew better at 2% CO_2 than at 10% CO_2 (Fig. 11). At 2% CO_2 all the strains had similar growth rates by OD, about 5 times higher than at 10% CO_2 (Fig. 11A). They also had higher dry biomass accumulation; the WT reached ~7.4 (g/L) at 2% CO_2 and ~0.5 (g/L) at 10% CO_2 (Fig. 11C). Nile Red fluorescence normalized to OD at 2% CO_2 was 1.5-2 times greater compared with 10% CO_2. Only strain G2 had higher Nile Red fluorescence than the WT at 2% CO_2 by 11% at day 15 of growth, while the other strains were all lower. In contrast, at 10% CO_2 all strains except strain 9 had higher Nile Red fluorescence than the WT ranging from 18% to 53% higher (Fig.11B). This result differed appreciably from the results based on gravimetric determination of the extracted lipid (Fig. 11D), which was ~ 2.7 times higher at 2% vs. 10% CO_2. At 2% CO_2 the WT and G2 strains had indistinguishable lipid content, while strain 24 was slightly lower. At 10% CO_2 the WT and strain 23 had indistinguishable lipid content, while all other strains were lower lipid content, in sharp contrast to the Nile Red method.

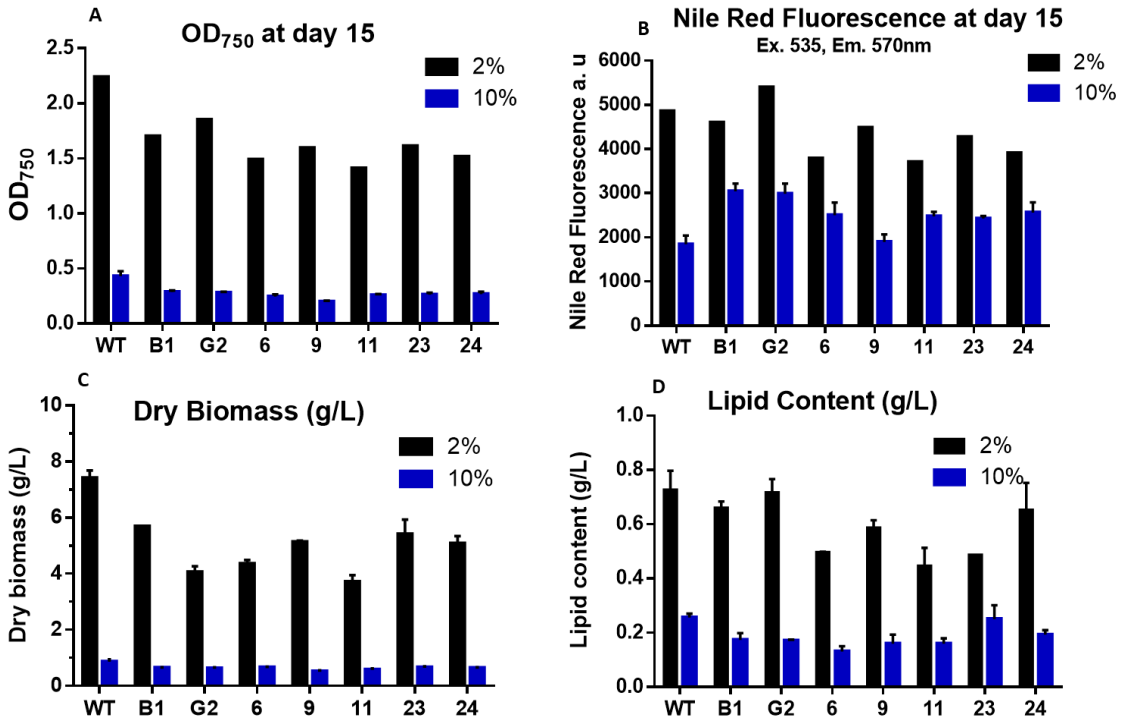


Fig. 11. Comparison of OD_{750}, Nile Red fluorescence, Dry biomass and Lipid content of the mutant strains and the WT at 2% and 10% CO_2 at day 15 of growth. (A) OD_{750}, (B) Nile Red fluorescence, (C) Dry biomass, (D) Lipid content (g/L). The seven mutants and the WT started with OD_{750} = 0.1 (approx. 3.72*10^7 cells/mL). N= 1 at 2% CO_2, N=3 at 10% CO_2.

A correlation plot of dry biomass against OD_{750} at day 15 of growth for the strains grown in 2% and 10% CO_2 is shown in Fig. 12A-B. At 2% and 10% CO_2, the WT had greater growth rate and dry biomass. The B1 had higher dry biomass but strain G2 had higher growth rate at day 15 at 2% CO_2 (Fig. 12A). The WT had lowest Nile Red per OD_{750} at day 15 at 2% and 15% CO_2, but in contrast the WT showed no higher lipid per dry biomass at 2% and 10% CO_2 compared with the mutants strains (Fig. 12C-D). Strain G2 had highest lipid content and Nile Red per OD_{750} at 2% CO_2, so the G2 could be a selected mutants for lipid production at 2% CO_2. At 10% CO_2, strain 23 had highest lipid content but the strain B1 had highest Nile Red fluorescence per OD_{750}.

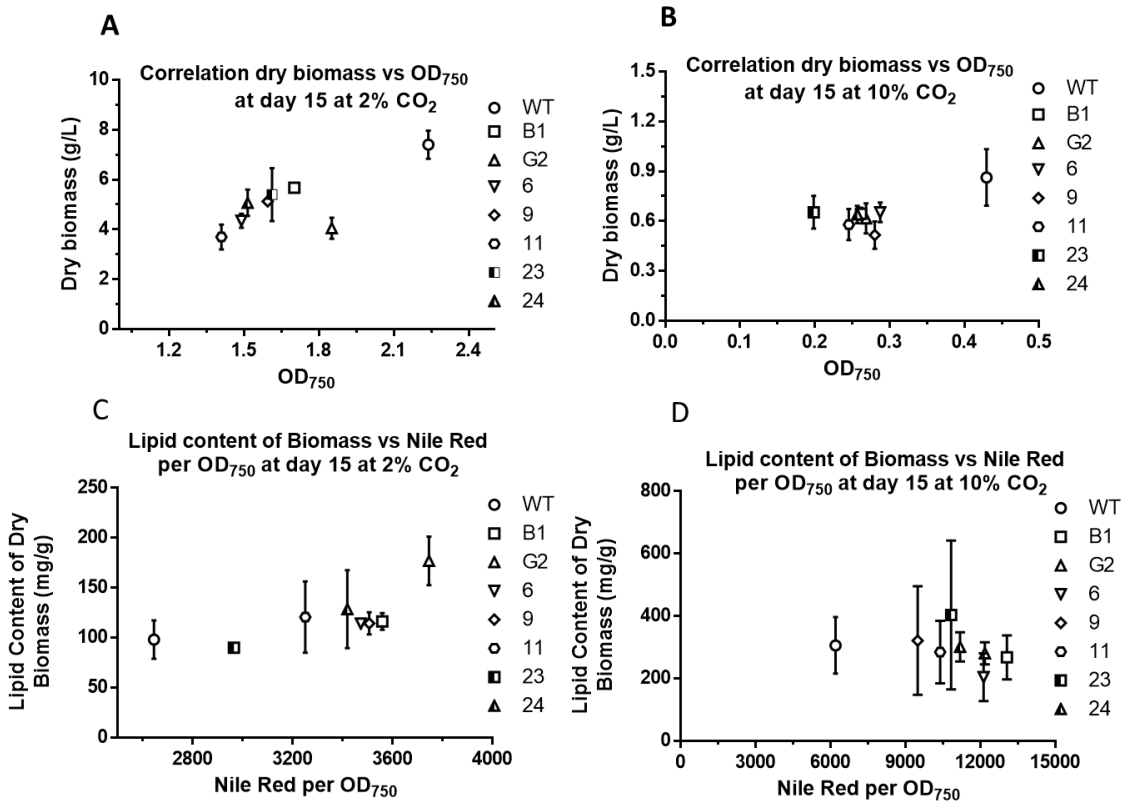


Fig. 12. Correlation between dry biomass vs OD_{750} of the mutants and the WT and Lipid content of dry biomass at 2% and 10%. (A-B) Correlation between dry biomass vs OD_{750} at 2% and 10% CO_2 respectively at day 15 of growth. (C-D) Correlation between lipid content of dry biomass vs Nile Red per OD_{750} at 2% and 10% CO_2 respectively at day 15 of growth

We sought to determine whether the cellular content of chlorophyll, measured by fluorescence intensity from intact cells (ex. 430 nm and em. 680 nm), was correlated with the cellular light scattering at OD_{750} (Fig. 13A). At 2% CO_2, a linear correlation was observed over a 10 fold OD range with good R^2 residual equal to 0.93. The data show a break between 1<OD<1, which was caused by diluting the culture when the OD_{750} greater than the threshold of the plate reader (OD threshold= ~1). At 10% CO_2 a poorer linear correlation is observed with R^2 equal to 0.77 and a slope that is 50% larger than the slope at 2% CO_2 (Fig. 13B). The lack of constant proportionality (slope) between fluorescence and OD at the two CO_2 conditions indicates that one or both methods is an unreliable

measure of cell number. Accordingly, the gravimetric method of biomass determination was adopted as the preferred method for quantification.

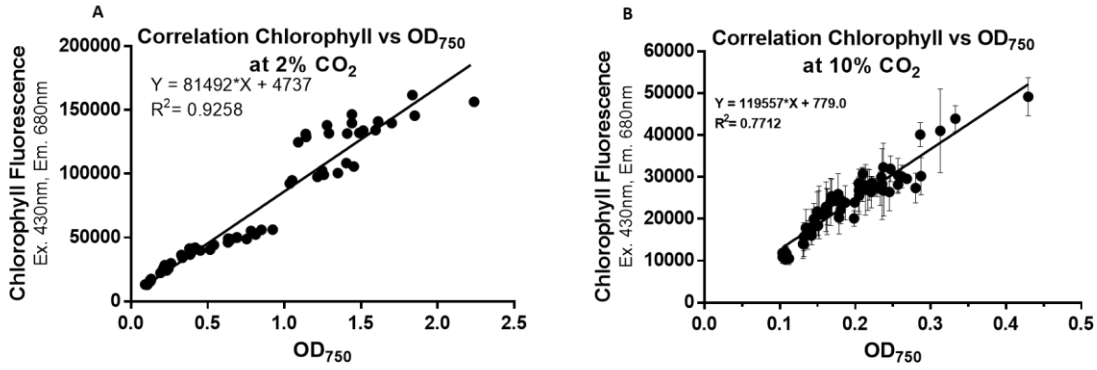


Fig. 13. Correlation between whole cell chlorophyll fluorescence vs OD_{750} of the mutants and the WT at 2% (A) and 10% (B) CO_2 on day 15 of growth. (A) Correlation between chlorophyll fluorescence and OD_{750} at 2% ~~C~~O_2, equation Y = 81492*X+4737, R^2=0.93. (B) Correlation between chlorophyll fluorescence and OD_{750} at 10% CO_2, equation Y = 119557*X+779.0, R squared =0.7712. The seven mutants and the WT started with OD_{750} = 0.1 (approx. 3.72*10^7 cells/mL).

3.6 The Chlorophyll fluorescence response of strains at 5% and 10% CO_2

Chlorophyll variable fluorescence yield (Fv), which is a measure of the PSII quantum yield, was measured for the strains and how it changes with increasing flash number, following a 2 minute dark adaptation. This experiment is a measure of how effective PSII charge separation can continue under illumination in the presence of 5% and 10% CO_2 (Fig. 14A-B). The relative PSII quantum yield (Fv/Fm = (Fm-Fo)/Fm) was measured for individual flashes as described in the legend of Figure 14, and described in Methods. An indication that the cells do adapt to the higher CO_2 by increasing PSII charge separation QY at the later times following the minimum. This time range on the order of tens of seconds is associated with the dark reactions of CBB. The small decrease in the initial value of Fv/Fm may not be noteworthy as this could reflect merely a change in the lifetime of the S state populations in the dark. Higher CO_2 adaptation may shift the populations to higher S states, decreasing the initial Fv/Fm.

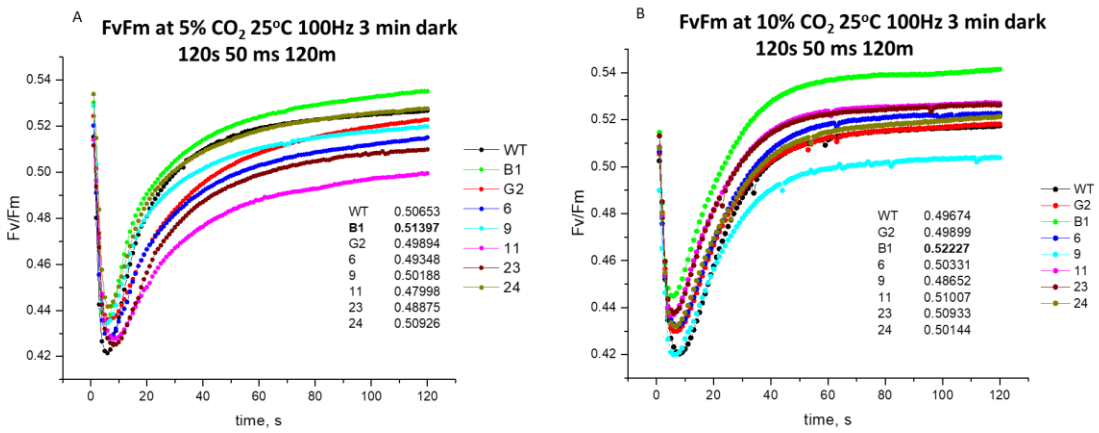


Fig. 14. The Chlorophyll fluorescence response, Fv/Fm, of strains at 5% and 10% CO_2. Each strain is dark adapted for 3 minutes followed by 120 flash trains each 1 second apart (120 s duration). Each train is comprised of 50 single turnover flashes (STF train) with each flash separated by 10 ms and each STF train separated by 2 ms (total duration 1 s per STF train). (A) The Fv/Fm response at 5% CO_2. (B) The Fv/Fm response at 10% CO_2.

4. Discussion

The goal of this project was to generate mutants from *N. oceanica* CCMP 1779 by random insertional mutagenesis, and screen them for increased lipid biomass content under CO_2 concentrations equal to that released by coal-fired power plants. A collection of 1200 mutants was generated and screened based on healthier phenotypes (larger colonies that were more green) and low pH tolerance. 7 mutants were selected for characterization under 2%, 5% and 10% CO_2 concentrations and compared to the WT.

Overall, the WT had higher growth rate and biomass and total lipid accumulation compared with the mutants in all tested conditions. Unlike WT, the mutants had a hygromycin cassette inserted in their nuclear genomes altering the normal metabolism of the cells. Coping with this abnormal metabolism might cause slower growth of the mutants compared with the WT. The metabolism alteration was also observed using Nile Red fluorescence measurement as a proxy for neutral lipid production (TAGs). The mutant strain G2 showed higher Nile Red fluorescence at all 2% and 10% CO_2 conditions. But it showed lower lipid content measured gravimetrically by lipid extraction method. The disagreement between the Nile Red fluorescence results and the lipid extraction of the strain G2 and other mutants' results keep us from being certain that the mutants had some beneficial characteristics in lipid production over the WT. The question is whether Nile Red fluorescence could *in vivo* accurately measure lipid content of a cell culture. Nile Red is hydrophobic dye, therefore it binds to a lipid body and could also bind to hydrophobic domains of non-lipid cellular components [30]. The volatility of the solvents and dye carriers might can affect the accuracy of the method as well [30].

On other hand, the gravimetrical lipid extraction method used here was developed for larger biomass samples [23] than we were able to use. Only ~ 0.11 g of dry biomass was used in the 2% CO_2 experiment and only ~0.012g.dry biomass for the 10% CO_2 experiment. An unusually complex cell wall structure of *Nannochloropsis sp*. is an important point to keep in mind for lipid extraction or any manipulation of the cell culture [33, 34].

This project demonstrated the dependence of growth and biomass accumulation on pH and CO_2 concentration of *N. oceanica* strains. The growth rate in 2% CO_2 slows below pH 7 from 43 h to 78h. pH 8.2 is a standard pH for marine algae since sea water pH is ~ 8.2. Our studies showed that the doubling time was 43 hours at pH 7.2 and 50 hours at pH 8.2. Moreover, at 2% CO_2 the pH of the cell culture was about 7.1, all the mutant strains and the WT grew 7 times faster at 2% CO_2 than at 10% CO_2 (pH 6.6). A possible explanation could be that although *N. oceanica* CCMP 1779 is a marine alga and it has the mechanism to propagate and regulate the internal cell pH with the environment, *N. oceanica* grows better at pH equal to ~7.2 which similar to its internal cell pH of 7.4 [35]. The 7.2 pH of the medium is similar to the internal pH of the algae, which could mean the cell needs less energy for pH regulation and the extra energy could be used for biomass production. The pH-dependence of growth rate of algae was confirmed in other studies [36, 37].

Based on the gravimetric data (dry biomass and lipid extraction) growth on 2% CO2 produced much more lipid ca 0.8-0.6 g/L than at 10% CO_2, ca 0.25 -0.1 g/L. However, the relative content as percent biomass, increased by 2-3 fold. At 2% CO_2, the WT only had 10% lipid accumulation, vs. 30% at 10% CO_2. This may be explained by

10% CO_2 causing a stress condition, algae accumulate more lipid under stress [13]. Pal *et. al.* reported *Nannochloropsis* sp. accumulated 15% lipid dry biomass at exponential phase and 30% at stationary phase [14]. The application for this could be to grow the culture at 2% CO_2 to stimulate cell division to optimize biomass accumulation until the culture reached stationary phase (after 11 days for the mutants), then shift to CO_2 stress condition (10%) to induce the lipid production.

The WT and mutants could not grow at 10% CO_2 without pre-adaptation and dilute starting culture. Pre-adaption at 2% and 10% CO_2 for 24 hours and dense cell culture (approx. 3.72*10^7 cells/mL) helps the strains tolerate to this condition. But the culture could have a faster growth rate at 10% CO_2 at least with a full cell cycle acclimation.

The short-term chl fluorescence transient kinetics measured at 0.5 s (50 flashes at 100 Hz flash rate), which measures the charge separation from water to the PQ pool, shows that all mutants have reduced capacity relative to the WT at 10% CO_2. This means that all mutants exhibit reduce capacity for light energy conversion by PSII (either or both PQH_2 production and ΔpH gradient, eg. *pmf*) at this CO2 concentration.

Random mutagenesis is most useful when complete coverage of the genome is possible to enhance the chance of getting a mutant with interesting characteristics (a complete library). Ma. *et. al.* used a *Chlamydomonas reinhardtii* library of 43000 mutants for screening [20]. The genome size of *N. oceanica* CCMP 1779 is ~28.7 Mb and encodes for 12,012 proteins [21]. We had access to a library of 1200 mutants, approx. 10% of the genes were insertionally disrupted. Hence, there is relatively small chance that we could obtain a desired phenotype.

To sum up, we established the platform to screen the *N. oceanica* mutant library based on low pH tolerance and colony size. G2 showed more Nile Red fluorescence than the WT. All the strains showed a dependence on pH and CO_2 concentration. A larger library of mutants is needed for a better chance of discovering a desired phenotype.

5. Conclusion

After three rounds of screening based on growth rate and pH tolerance, five mutants were selected for further characterization from different CO_2 concentrations. Two mutants were selected from the phenotype screening method. All the strains grew ~7 time better at 2% CO_2. Pre-adaptation and dense cell cultures are needed for 10% CO_2 growth. 10% CO_2 is a stress environment for *N. oceanica* to grow. The mutant strain G2 had a higher Nile Red fluorescence than the WT. But G2 did not show higher lipid content by gravimetrical method. The lipid content of the WT was 10% at 2% CO_2 and 30% at 10% CO_2.

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