Astaxanthin accumulation in response to stress by microalgae *Chromochloris zofingiensis*

Experimentelle Phykologie und Sammlung von Algenkulturen der Universität Göttingen (EPSAG)

Dorian Leger

April 17th, 2019

Supervisor: Dr. Tatyana Darienko

Table of Contents

Introduction

The microalgae *Chromochloris zofingiensis* has gained attention in recent years thanks to its production of a valuable carotenoid known as astaxanthin. Astaxanthin is widely used for pigmentation in aquaculture and in human healthcare due to its deep red coloration and strong antioxidant properties. While both synthetic and natural versions of astaxanthin exist, the USD 550 million astaxanthin market is currently met mostly by synthetic production [1]. However, natural astaxanthin produced from microorganisms is a superior antioxidant compared to its synthetic counterpart [2, 3]. As a result there is growing commercial interest in natural astaxanthin production. Natural astaxanthin uses microaglae *Haematococcus pluvialis*, or the yeast *Xanthophyllomyces dendrorhous (*formerly *Phaffia rhodozyma*) [2]. However, these organisms are not ideal biotechnological systems due to several limitations, such as slow growth, low cell density, or infection prone cultivation [4, 5]. Thus, the alternative organism *C. zofingiensis,* which is not characterized by these limitations, is emerging as a contender for mass production of astaxanthin. Nevertheless, C. *zofingiensis* faces several of its own challenges, for instance, though it grows faster and more densely than *H. pluvialis* and *X. dendrohous,* it also achieves much lower cellular concentrations of astaxanthin [6]. In addition, the astaxanthin product derived from *C. zofingiensis* is mixed with similar ketocarotenoids, which are difficult to separate, and currently unapproved for healthcare applications in major western markets [7]. Yet genetic manipulation of *C. zofingiensis* in the last two decades has demonstrated potential to increase cellular concentration and purity of the astaxanthin product [6]. Therefore, *C. zofingiensis* appears well suited to become a major cellular factory for natural astaxanthin production in the future. Roth and team (2018) recently published a full genome assembly and transcriptome of the *C. zofingiensis* type strain SAG 211-14 to accelerate gene editing for astaxanthin production [3]. Yet the decision to use SAG 211-14

simply because it is the type strain may not be optimal for commercial applications. Multiple *C. zofingiensis* strains are kept in culture collections, and thus, it is worth considering whether SAG 211-14 is the best starting point for genetic engineering. Indeed, while SAG 211-14 has received considerable attention, there has been comparatively little research on alternative strains of this species [8].

The algae culture collection with which this study was conducted, Experimentelle Phykologie und Sammlung von Algenkulturen der Universität Göttingen (EPSAG), possesses several strains of C. *zofingiensis* which have yet to be tested for their astaxanthin productivity. Therefore, it is of interest to cultivate and compare the alternative strains with the type strain. The strains tested for astaxanthin productivity in this experiment were SAG 4.80, SAG 31.80, SAG 34.80, SAG 211-14, and SAG 221-2. Various algae respond differently to elevated CO_2. Therefore, a good way to reveal differences between the five strains considered was to test their productivity in a range of CO_2 concentrations. Furthermore, this helps us understand the thresholds beyond which CO_2 concentrations begins to stifle productivity. To this end, the five *C. zofingiensis* strains were compared under 5%, 10%, and control CO_2. All algae were subjected to a light and nutrient stress regime known to stimulate carotenoid production. The results of this study indicated that the five *C. zofingiensis* strains present individual advantages and disadvantages depending on the growth conditions, and may be uniquely suited to certain biotechnological applications.

Genetic variation among the strains

The initial impetus for this study was previous phylogenetic investigations of *C. zofingiensis* conducted by Darienko et al. Those investigations combined rRNA sequencing and methylation sensitive Amplified Fragment Length Polymorphism (AFLP) methods to expose genetic distance between the five strains considered. Relatively low sequence variation was found

in the ribosomal Small Sub Unit (SSU) (see Diagram 1) and Internal Transcriber Spacer 2 region (ITS-2) sequences. The ITS-2 results indicated one hemi-CBC found between SAG 221-2 and the other strains in Helix II (Darienko, personal communication, 2018). Furthermore, "SAG 221-2 differed strongly from the other five strains in the PstI AFLP dataset (see Diagram 2). Thus, strains SAG 211-14, SAG 211-14 (genome), SAG 31.80, SAG 34.80, and SAG 4.80 seemed to be conspecific, whereas SAG 221-2 could possibly be another species" (Darienko, personal communication, 2018). In essence the phylogenetic analysis reveals that SAG 221-2 is relatively genetically distant from the other strains based on AFLP. However, the lack of a complete CBC in SAG 221-2 adds little weight to the AFLP findings. The study of CBC in sexually reproducing organisms by Müller et al. revealed that presence of CBC indicates a 93% chance of being different species, while lack of a CBC indicates a 76% chance of being the same species [9]. Based on the interpretation that CBC can proxy speciation in asexual organisms, this suggests SAG 221-2 is the same species as the other four strains considered. Nonetheless the phylogeny results are puzzling. It is therefore of considerable interest to probe how genetic differences would be reflected in physiology and productivity in this experiment.

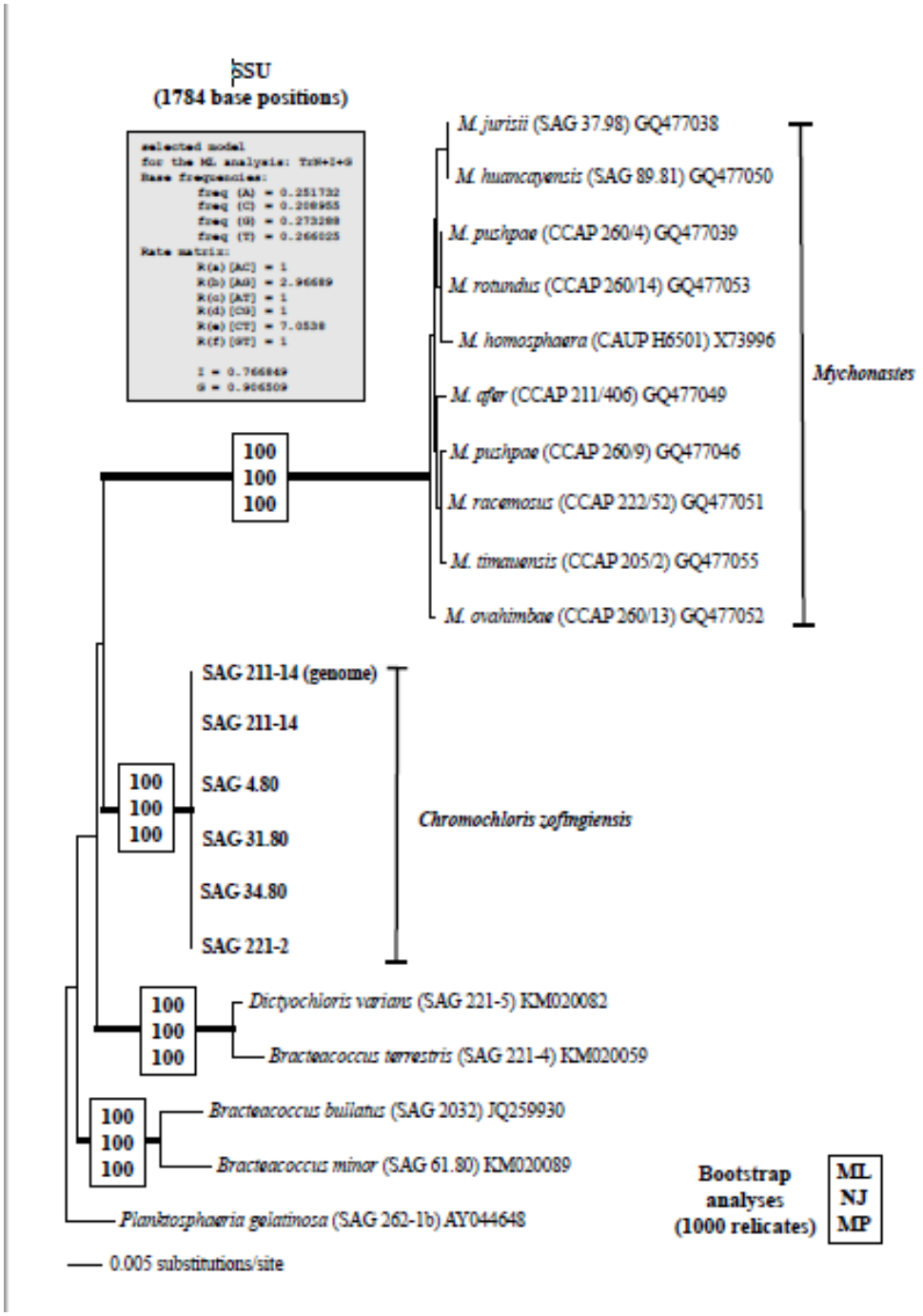


Diagram 1. Phylogeny of *C. zofingiensis* and related taxa based on the SSU.

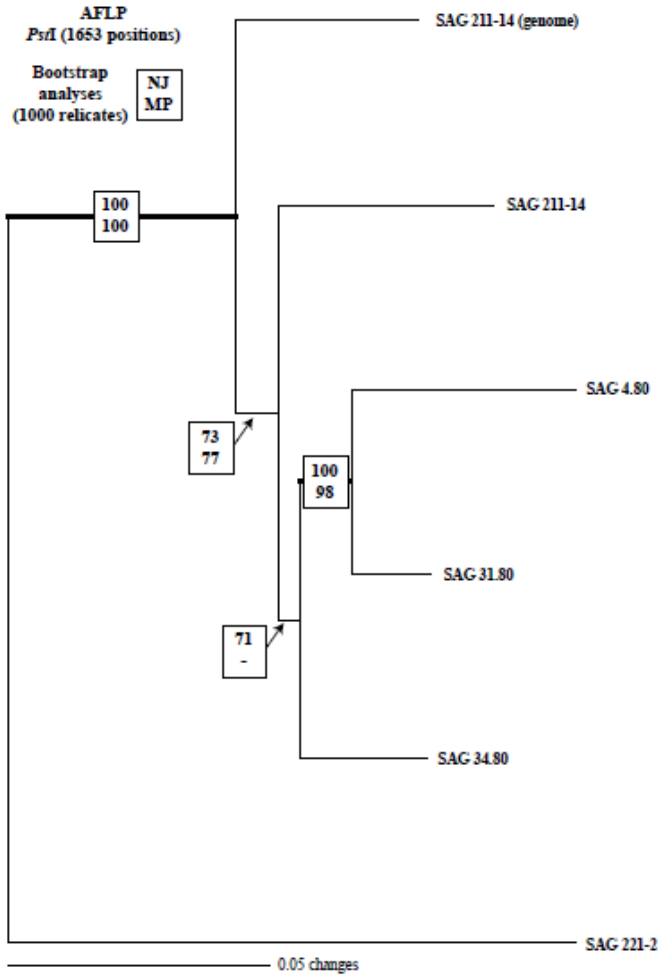


Diagram 2. Relationships among the five strains of *C. zofingiensis* based on AFLP (enzyme PstI).

Astaxanthin Background

Carotenoids are accessory photosynthetic pigments. Their role is to capture and transfer high energy light, or to dissipate excess energy as heat under stress conditions. They also play essential functions in scavenging reactive oxygen species (ROS). Over six hundred carotenoids have been found in nature, and these are broadly classified in two main categories; xanthophylls and carotenes [10]. Xanthopylls contain oxygen, while carotenes are non-oxygen containing hydrocarbons.

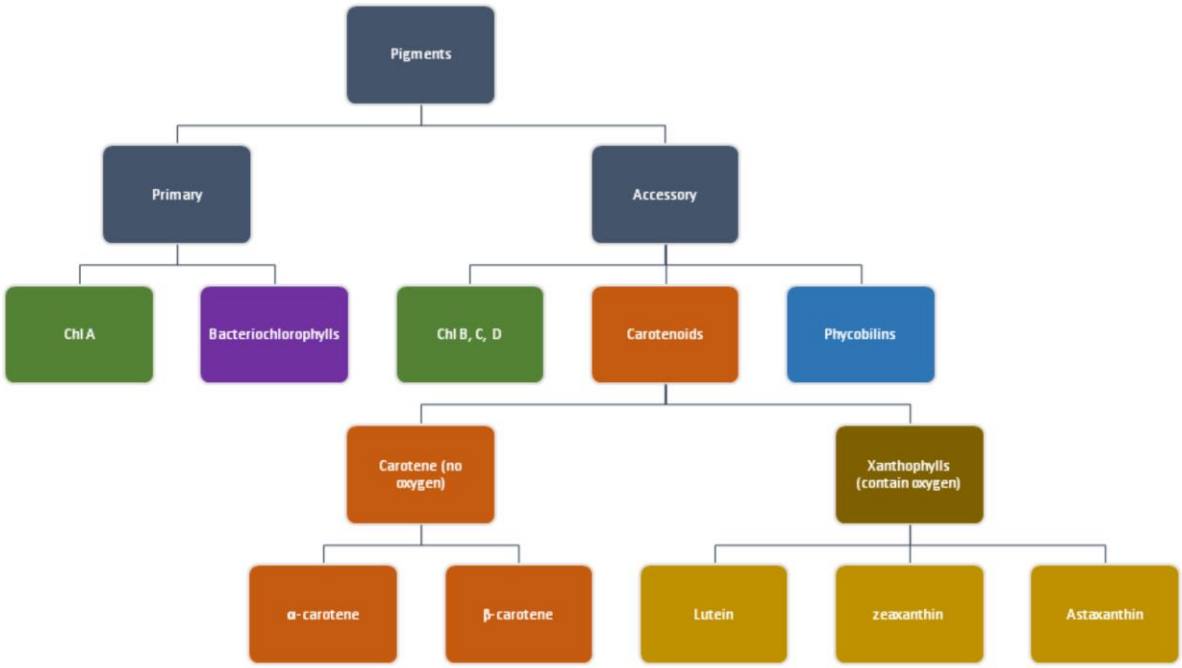


Figure 1. Classification system of major photosynthetic pigments

The major photosynthetic pigments are divided into primary and accessory pigments. Carotenoids are further divided into oxygen containing xanthophylls and non-oxygenated hydrocarbon carotenes. This diagram presents a non-exhaustive list of pigments.

Astaxanthin's chemical formula is $C_{40}H_{52}O_4$, and the molecule occurs in either a *trans* configuration and rarely in *cis* configuration [10]. Given two rotating functional groups, the *trans*

configuration can occur in three stereoisomers (see Figure 2). The stereoisomers are two enantiomers (3R, 3'R, and 3S, 3'S) and a meso form (3R, 3'S) [11]. Natural astaxanthin is over 90% mono- or diesterified with fatty acids (palmatic, stearic, linolenic, or oleic) at one or both hydroxyl groups [2]. Importantly, the ROS scavenging capacity of natural astaxanthin has been found to be 14-65 times more effective than other antioxidants, such as pycnogenol, B-carotene, Vitamin E, and Vitamin C [2].

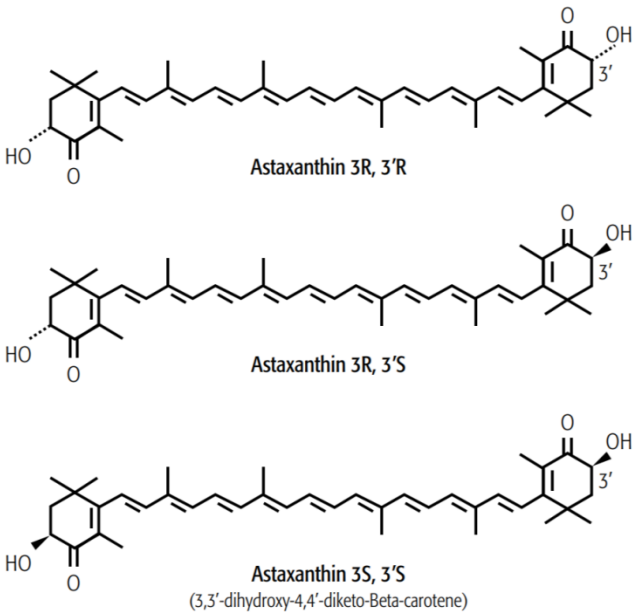


Figure 2. Astaxanthin stereoisomers

Three *trans* stereoisomers of astaxanthin are shown. The esterified forms would attach fatty acids to the hydroxyl groups. From, Capelli, B., Bagchi, D., & Cysewski, G. R. (2013). Synthetic astaxanthin is significantly inferior to algal-based astaxanthin as an antioxidant and may not be suitable as a human nutraceutical supplement. Nutrafoods, 12(4), 145-152. doi:10.1007/s13749-013-0051-5

Natural astaxanthin differs from synthetic astaxanthin in two ways, the stereoisomer configuration and the esterification. Natural astaxanthin is typically produced by organisms in only

one stereoisomer, though the specific stereoisomer varies by species. Microalgae such as *H. pluvialis* and *C. zofingiensis* produce only 3S, 3'S; yeast *X. dendrorhous* produces 3R, 3'R; while synthetic astaxanthin results in a 1:2:1 ratio of (3R, 3'R):(3R, 3'S):(3S, 3'S) [2]. Furthermore, synthetic astaxanthin is exclusively in non-esterified, free form [2]. Astaxanthin is the most abundant carotenoid found in marine animals, and gives crabs and salmon their reddish hue. The red coloration is considered a primary indicator of quality by consumers, which is perhaps not entirely superficial since astaxanthin is proven to benefit growth and survival of growing fish and shrimp [7]. This is why salmonid aquacultures heavily employ astaxanthin in feed supplementation.

Astaxanthin Biosynthesis

Astaxanthin biosynthesis well described, though several steps remain to be fully elucidated. Biosynthesis begins with the molecule isopentenyl diphosphate (IPP), which is converted to geranylgeranyl diphosphate (GGPP) (see Figure 3). GGPP is converted to phyotene by phytoene synthase (PSY), which is converted to ζ-carotene by phytoene desaturase (PDS). ζ-carotene is converted to lycopene by ζ-carotene desaturase (ZDS). Lycopene is then converted into either α-carotene (leading to lutein), or to β-carotene. If β-carotene is produced, two pathways which ultimately lead to astaxanthin can be followed. However, intermediates in *C. zofingiensis* that accumulate in these pathways such as adonixanthin and canthaxantin, are considered impurities in the final astaxanthin product. Because these ketocarotenoids are similar to astaxanthin, separation is costly [7]. Thus, metabolic engineering efforts are underway to genetically increase the final astaxanthin product purity in *C. zofingiensis*. It is worth noting that a point mutation in a stable *C. zofingiensis* mutant occurring in the PDS gene has led to over 36% more astaxanthin production than wild type [12]. Furthermore, PSY has also been overexpressed leading to higher astaxanthin

production [13]. These early initiatives combined with the full genome sequence produced by Roth et al. in 2017, suggest much more productivity and purity enhancements are imminent.

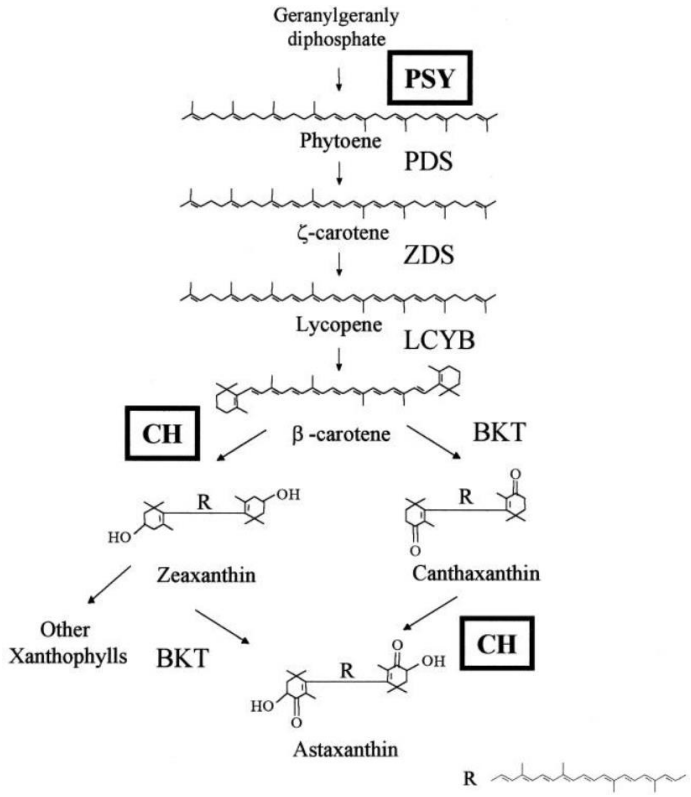


Figure 3. Astaxanthin biosynthesis

Major steps in the biosynthesis of Astaxanthin are presented. Starting from lycopene, β-carotene can be synthesized by preferential expression of lycopene β-cyclase over lycopene ε-cyclase. Several branches lead to astaxanthin from β-carotene, and future metabolic engineering will attempt to reduce the concentration of adonixanthin and canthaxanthin in purified astaxanthin product. From, Steinbrenner, J. and H. Linden. (2001). Regulation of two carotenoid biosynthesis genes coding for phytoene synthase and carotenoid hydroxylase during stress-induced astaxanthin formation in the green alga Haematococcus pluvialis.

Microalgae Cultivation

C. zofingiensis are non-motile, asexually reproducing, unicellular, spherical shaped, freshwater algae, with cell sizes from 2 to 15 μm in diameter that can grow photoautotrophically, mixotrophically or heterotrophically [7]. Cultivation of *C. zofingiensis* follows the same principals as *H. pluvialis*. Three main types of cultivation are open pond, photobioreactors (PBR), and fermenters. Open pond systems have very low maintenance costs but achieve low cell density, are easily contaminated, and subject to constant water loss [10]. Closed photobioreactors (PBR) achieve higher cell density and higher specific metabolite yields, but cost up to ten times more than open systems [14]. PBRs can include tubular or panel form, and in all cases it has been found that a short light path increases astaxanthin accumulation [14]. Fermenters implementing heterotrophic growth appear promising, and fed-batch systems that avoid substrate inhibition in these can reach volumetric astaxanthin yields as high as 56mg/L [7]. The economic factors leading to optimal choice of cultivation methods are multivariate and complex, and will continue evolving with developing technology and the genetic engineering of *C. zofingiensis*.

Materials and Methods

Experimental Set-up

Five *C. zofingiensis* strains were tested for their biomass and carotenoid accumulation under varied CO_2 treatments. The five strains chosen were SAG 4.80, SAG 31.80, SAG 34.80, SAG 211-14, and SAG 221-2 (see Table 1 and Table 2).

SAG Strain Number	Isolation From	Isolator (Date)	General Habitat
SAG 4.80	N/A	M. B. Allen (<1952)	Soil - temperate
SAG 31.80	N/A	N/A (<1977)	Soil
SAG 34.80	Germany, Ortenberg near Marburg/Lahn, on bark of deciduous tree	F. C. Czygan (1963)	Freshwater - temperate
SAG 221-2	Germany, Ortenberg near Marburg/Lahn, on bark of deciduous tree	E. Kol & F. Chodat (1934)	Soil - temperate
SAG 211-14	Switzerland, soil from Ramooswald near Zofingen, colors trail embankment red-brownish	O. C. Dönz (1933)	Soil - temperate

Table 1. Five *Chromochloris zofingiensis* strains considered in this study.

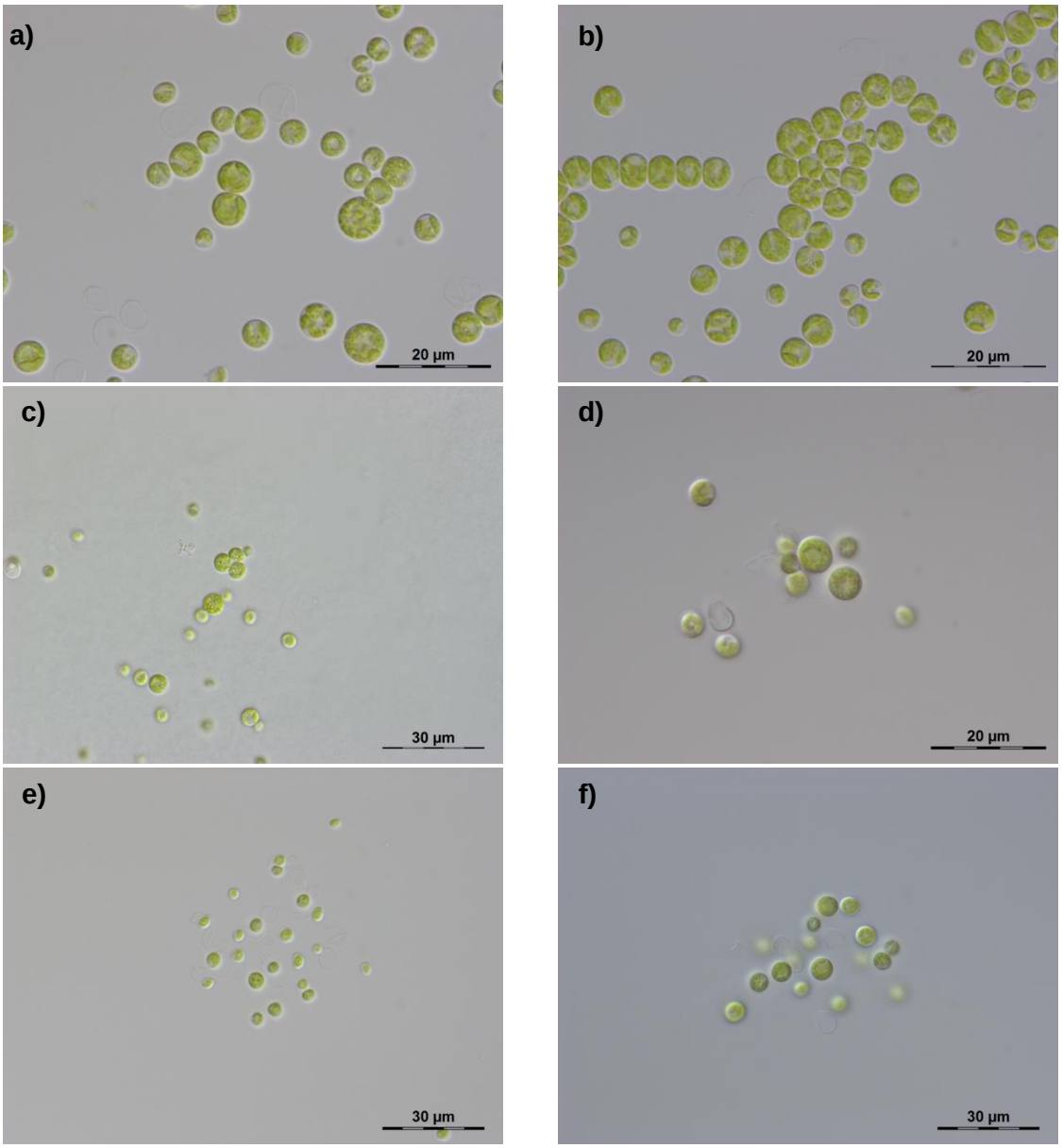


Table 2. Microscopy of *C. Zofingiensis* strains.

C. zofingiensis forms spherical adult cells with diameter approximately 10 µm, and does not contain pyrenoids. Cells contain several nuclei and chloroplasts. Image description: a) SAG 31.80 2 weeks on ESP b) SAG 211-14 3 weeks, c) SAG 211-14 (genome) 3 weeks, d) SAG 4.80 6 weeks, e) SAG 34.80 6 weeks, f) SAG 221-2 6 weeks. Images and descriptions courtesy of Dr. Tatyana Darienko.

All strains were pre-cultivated in EPSAG's standard culture conditions, 20°C and a 16-8 light-dark cycle, using 50ml of Kuhl growth medium [15]. 200 ml of additional Kuhl medium was prepared for each sample. Cultures were transferred with fresh Kuhl medium into 250 ml Kniese tubes. Starting optical density (OD) was approximately 0.06-0.09 at 567 nm on Genesys 20 (Thermo Scientific, Waltham, Massachusetts). Kniese tubes were set in aquariums based on design developed by Kuhl and Lorenzen [15]. Each strain was grown photoautotrophically with air supplementation that varied in CO_2 concentration. Two elevated CO_2 treatments and a control CO_2 were compared. Air was supplemented by bubbling at approximately 0.5L/min, and CO_2 concentrations were 5%, 10%, and ambient air CO_2 (~0.04%). Each treatment had three replicates. Thus, five strains, three treatments, and three replicas led to 45 samples in total. Following conventional practice, strains were cultured in two stages: first a 9 day growth-phase for biomass accumulation, followed by a 7 day stress-phase for carotenoid production. The temperature was maintained at 20°C in aquariums throughout the experiment. Small amounts of growth medium were replenished every day to counteract evaporation and maintain a medium volume of 250ml in the Kniese.

The growth-phase used Kuhl medium and progressive lighting from 70µmol/m^2/s (day 0 through day 1) until 140µmol/m^2/s (day 2 through day 9) [15]. The stress-phase used an N-depleted Kuhl medium (with 1/10th nitrogen content of normal Kuhl medium) and lighting at 600µmol/m^2/s (day 10 through day 16). Both growth and stress phase used a day night 16-8 cycle. To replace growth medium on day 9, Kniese tubes were emptied into large flasks and centrifuged until a pellet formed, supernatant was removed, then cells were resuspended in N-depleted medium, and transferred back to their Kniese tubes.

Total carotenoids (Car), chlorophyll A (Chl A), chlorophyll B (Chl B), pH, and biomass, were measured on days 0, 2, 5, 7, 9, and 16. On each of these measurement days, Kniese tubes

were disconnected briefly from air supplementation, and a 5 ml aliquot was syringed via the air intake opening of the Kniese. On day 9 an additional 45 ml aliquot was taken for storage and future analysis. Due to the additional aliquot on day 9, biomass of all samples was reduced by 20% at that stage.

Dry Weight

Dry weight was directly measured on day 9 and 16 by freeze drying and indirectly proxied with OD correlations on days 0, 2, 5, and 7. For direct measurements, a 5 ml aliquot was taken from each sample, OD at 567nm was recorded, and then aliquots were placed into Eppendorf tubes whose weight was predetermined. The samples were centrifuged and supernatant was discarded. The remaining pellet was frozen on liquid nitrogen briefly at -80 °C, and then freeze dried for 24-48 hours. Dry weight was measured by subtracting the empty Eppendorf weight from the pellet plus Eppendorf weight. Each strain exhibited a strong correlation R^2>0.96 between dry weight and OD at 567nm, thus allowing OD to serve as a reliable proxy for dry weight on days 0, 2, 5, and 7 (correlation provided in the supplemental). Note that concentration comparisons between day 9 and day 16 presented in results, use the day 9 (-N) value, which is to say, the values which account for a 20% reduction in biomass.

Pigments

The pigment concentrations Chl A, Chl B, and Car were estimated by a common spectrophotometry procedure which relates the absorbance of samples at different wavelengths to a relative concentration of pigments. The pigment extraction method and correlation equations were based on EPSAG's protocol. To estimate pigments on each measurement day, a 1 ml aliquot was taken from samples, pelleted (14000g x 30 min) in a 2 ml tube, and supernatant was removed. Glass beads were added to tubes. Samples were frozen to enhance cell lyses. Cell walls were broken

using glass beads and powerlyzer (5000 rpm for 1 min). Lysed samples were centrifuged and cell debris partially pelleted (max rpm for 10 seconds). Subsequently, samples were mixed with 1 ml extraction buffer (60 methanol: 40 acetone: 0.5 ammonia) and incubated on thermomixer for 20 minutes at 70 °C. After incubation, the samples were centrifuged (14000g x 20 min) and supernatant was transferred to a spectrophotometer cuvette. The absorption at three wavelengths (473 nm, 650 nm, and 665 nm) was measured after blanking with medium on the same Genesys 20 used for OD. The following equations provided by EPSAG were used to quantify pigment concentrations in mg/L.

$$\text{Chl A} = 11.24\ (\text{A365nm}) - 2.044\ (\text{A350nm}) \quad \text{(i)}$$

$$\text{Chl B} = 20.13\ (\text{A350nm}) - 4.19\ (\text{A365nm}) \quad \text{(ii)}$$

$$\text{Total Carotenoids} = \frac{1000\ (\text{A1073nm}) - 1.9[\text{Chl A}] - 63.14\ [\text{Chl B}]}{214} \quad \text{(iii)}$$

$$\text{Total Chlorophyll} = [\text{Chl A}] + [\text{Chl B}] \quad \text{(iv)}$$

The cellular concentration of pigments in mg per gDW biomass was determined by dividing total Chl, or total Car (mg/L) by dry weight (gDW/L). Hereafter, total chlorophyll and total carotenoids, will be referred to as Chl and Car.

pH

pH was measured with a standard pH probe on every measurement day.

Statistics

All statistics used to assess groupings and significant differences in means were done using Tukey test with 95% confidence intervals in Minitab 18, Minitab 18 Statistical Software (2018). [Computer software]. State College, PA: Minitab, Inc. (www.minitab.com)

Results

The majority of the results described compare the final characteristics of samples in different treatments on the last day of the stress phase, which was day 16. Furthermore, comparisons are made between day 9 and day 16, which elucidate changes in samples after stress induction. Note that all values used for day 9 are noted as day 9(-N) in the supplemental, and are post 20% biomass reduction.

Dry Weight Biomass Density on Day 16

All strains achieved their highest biomass density in 5% CO_2 treatment, except for SAG 4.80, which was highest in 10% CO_2 (see Figure A1). No significant difference was found between biomass achieved in 5% and 10% CO_2. However, for all strains, elevated CO_2 led to significantly higher biomass than control CO_2. The highest biomass achieved was by SAG 221-2 in 5% CO_2 at 5.85 gDW/L, the second highest was SAG 221-2 in 10% CO_2 at 4.44 gDW/L, and third highest was SAG 211-14 in 5% CO_2 at 3.96gDW/L.

Volumetric Pigment Concentration on Day 16

All strains achieved significantly higher pigment concentrations in elevated CO_2 than control. In control CO_2, no significant difference between strains was found with regards to Chl (average 8.92 mg/L) or Car 9 (average 3.15 mg/L). In 5% CO_2, SAG 211-14 exhibited significantly

higher Car accumulation than other strains, with a mean of 19.37 mg/L, and significantly less Chl than other strains (see Figure 4**).** In 10% CO_2, no significant differences in means were found between strains with regard to Car, however, SAG 211-14 exhibited significantly less Chl.

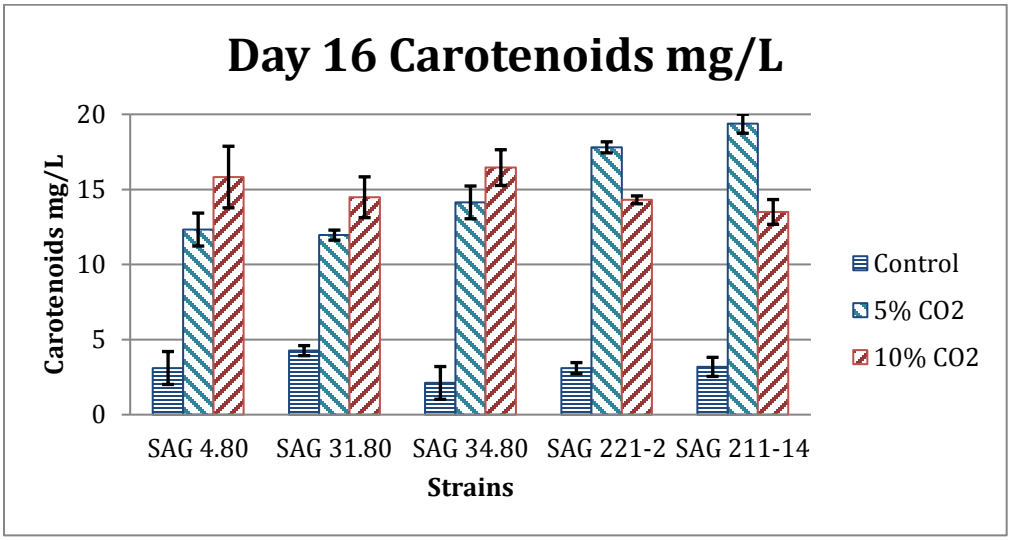


Figure 4. Volumetric carotenoid concentrations on day 16.

All strains and all treatments are compared for their volumetric carotenoid concentrations. SAG 211-14 in 5% CO_2 treatments outperforms in total carotenoid yield, followed by SAG 221-2 in 5% CO_2 treatment. Error bars are 1 standard deviation.

Cellular Carotenoid Concentration on Day 16

The effect of CO_2 on cellular carotenoid concentrations varied by strain. SAG 4.80, SAG 31.80 and SAG 221-2 exhibited significantly higher cellular Car concentration in control CO_2, followed by 10% CO_2 and lowest in 5% CO_2 (see Figure A3). Within the control CO_2 treatments, all strains performed similarly, though SAG 31.80 had the highest mean, reaching 8.24 mg/gDW. Within both the 5% and 10% CO_2 treatments, SAG 221-2 had significantly lower cellular Car than other strains, reaching only 3.04 mg/gDW (5% CO_2), and 3.22 mg/gDW (10% CO_2).

Car/Chl Ratio

For all strains, the Car/Chl ratio was higher in 10% CO_2 than control, except for SAG 221-2 (see Figure A4). SAG 31.80, SAG 34.80, and SAG 211-14 followed a trend, whereby the highest Car/Chl ratio observed was in 10% CO_2, followed 5% CO_2, followed by control. The ratio was at its lowest in control CO_2 SAG 34.80 at 0.23, and was at its highest in 10% CO_2 SAG 211-14 at 2.00.

pH

The initial pH for growth mediums prepared for SAG 4.80, SAG 31.80, SAG 34.80 were recorded as 6.10, 6.16, and 6.22, respectively. However, the initial pH of Kuhl medium prepared for SAG 221-2 and SAG 211-14, was not recorded. Furthermore, the pH of N-depleted Kuhl medium was not recorded for all strains on day 9. Therefore, the trends for pH are not clear. However, all measured values from day 2 to day 16 ranged between 5.10 to 7.28. Nonetheless, the day 16 pH values were recorded and followed a trend of highest in Control CO_2, followed by 5% CO_2, and lowest in 10% CO_2, except for SAG 211-14, where 5% CO_2 was the lowest final pH (see figure A5).

Change in Dry Weight Biomass Density after Stress Induction

For all strains in all treatments, the dry weight biomass increased significantly after stress induction between day 9 and day 16 (see Figure A6). The largest change on a percentage basis for each treatment was: in control CO_2, SAG 31.80 (127%); in 5% CO_2, SAG 211-14 (196%), and in 10% CO_2, SAG 211-14 (305%)

Change in Volumetric and Cellular Carotenoid Concentration after Stress Induction

For all strains in all treatments, with the exception of SAG 34.80 in control CO_2, volumetric carotenoid concentration increased significantly after stress induction (see Figure A7**).** The largest change occurred in SAG 211-14 in 10% CO_2 which exhibited a 684% increase in volumetric Car concentration between day 9 and day 16 measurements.

The effect of stress induction on cellular carotenoid concentration varied between strains and treatments (see Figure A8). However, all strains always reacted by consistently increasing or decreasing after stress in all treatments, with the exception of SAG 31.80, which increased in control CO_2 but decreased in elevated CO_2 treatments.

Discussion

Astaxanthin Induction

ROS comprise a group of unstable compounds whose propensity to oxidize biological material is so strong as to make them harmful. ROS are produced via several mechanisms, which include radiolysis of water by ionizing radiation, oxidative bursts by macrophages, electron leaks in electron transport chains, and photosensitization of dioxygen by chlorophylls [16-19]. The latter reaction is a constant source of stress in photosynthetic organisms.

Photosensitization occurs when Chl A molecules remain in long-lived excited states, such that after light absorption they are neither able to rapidly funnel energy to a photosystem reaction center, nor able to distribute excess energy to nearby carotenoids [20]. In this case, it becomes possible that the Chl A will undergo intersystem crossing (ISC). ISC is a molecular relaxation process where the highest occupied molecular orbital electrons go from a paired configuration (one spin up and one spin down – called singlet) to an unpaired configuration (i.e both spin up – called triplet). Typically molecules must match electronic configuration to react with each other; meaning

that singlets will not react with triplets [21]. The ground state of dioxygen is triplet, whereas the ground state of most other compounds is singlet [22]. This explains why although combustion is thermodynamically favored, electronic configuration limitations (otherwise known as kinetic limitations) prevent spontaneous combustion of biological material at the ~20% atmospheric oxygen concentrations [22]. After ISC, the triplet Chl A now has two unpaired electrons and can react with similarly configured ground-state dioxygen. The reaction transfers energy from triplet Chl A to dioxygen and yields the harmful ROS called singlet oxygen [23]. This reaction, called photosensitization, is summarized in Diagram 3.

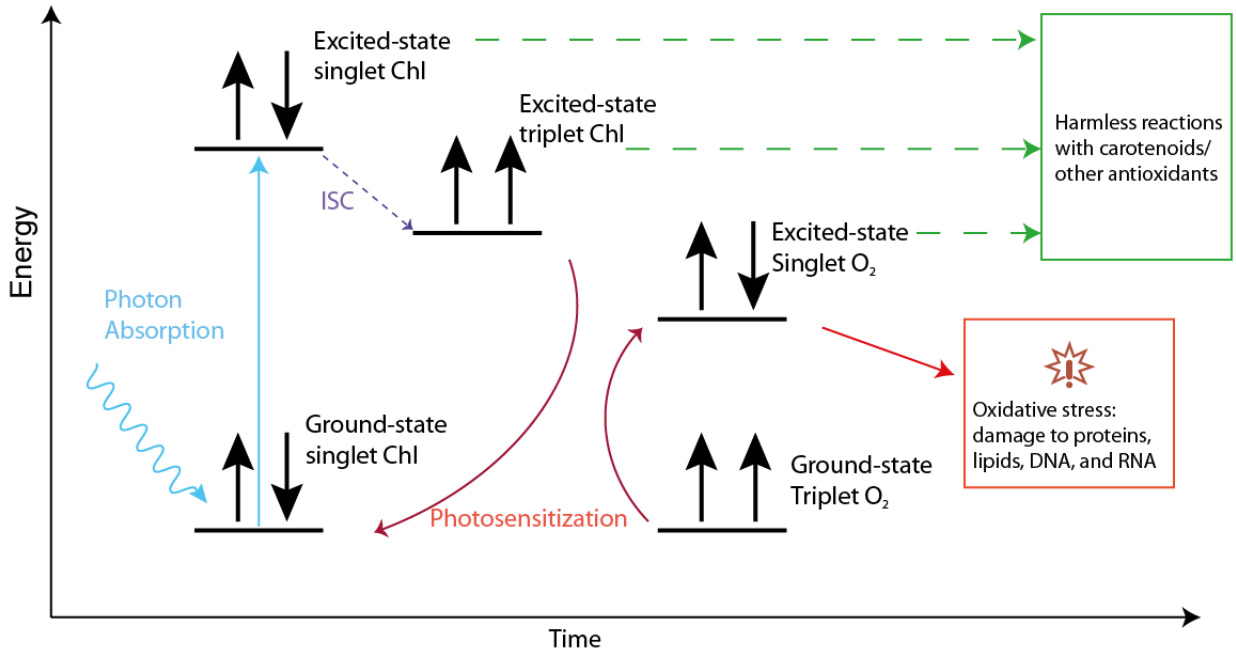


Diagram 3: Formation of the ROS singlet oxygen by photosensitization.

A ground-state Chl (left) absorbs a photon and becomes excited. After sufficient time elapses without having released its energy, the Chl undergoes intersystem crossing (ISC), and becomes a triplet Chl. The triplet Chl can then react with ground-state dioxygen in a photosensitization

reaction. In photosensitization, the Chl returns to ground-state, and the dioxygen absorbs energy by exchanging the spin of one of its electrons, becoming singlet oxygen. Singlet oxygen can then be quenched harmlessly by antioxidants, or will cause oxidative damage.

Once produced, photosynthetic organisms must cope with singlet oxygen by synthesizing carotenoids and other antioxidants to quench it. When singlet oxygen is not quenched, it will damage nearby proteins, lipids RNA and DNA. Under conditions of high light, long-lived excited chlorophylls are more common, leading to increased singlet oxygen production, which induces the cell to increase biosynthesis of antioxidant carotenoids like astaxanthin [19].

Several factors are known to stimulate astaxanthin in *C. zofingiensis.* Nitrogen limitation alters the cells C/N ratios. Given that algal cells are approximately 50% C and 10% N, excess carbon above this ratio will find its way into several carbon sinks in the cell's metabolism, including carotenoid synthesis [24]. Metal ions, such as Fe^{2+}, which produces hydroxyl radicals, and acts as cofactors in astaxanthin synthesis, is an important astaxanthin inducer [7]. Artificial addition of ROS and reactive nitrogen species, inhibition of an organism's endogenous antioxidative enzymes, and salt stress were also all found to enhance astaxanthin productivity [24]. More exotic measures to increase growth and productivity have also been described, such as standing magnetic fields [14]. However, the most prominent astaxanthin induction technique to-date remains light stress [6]. Each induction technique, in conjunction with cultivation techniques, offer trade-offs between specific metabolite yield, rate of production, cost of production, and purity of product. It is unlikely that a one-size-fits-all best solution can be described for astaxanthin production. More likely the biotechnology goals of individual producers should be assessed on a case-by-case basis.

Dry Weight Biomass Density on Day 16

The highest biomass observed was achieved by SAG 221-2 which reached 5.85 gDW/L in the 5% CO_2 treatment (see Figure A1). This biomass is similar to previous work by Campo which achieved 7 gDW/L for SAG 211-14, in a fed batch system, and by Chen which reached 3.2 gDW/L with ATCC30412 [8, 25]. It should be noted that the biomasses achieved in the present study likely underestimated the potential of the strains, given the 20% reduction in biomass that occurred on day 9. However, accurately quantifying the underestimation is not possible; given samples do not grow linearly.

SAG 211-14 achieved slightly higher biomass than SAG 221-2 in control CO_2, 0.64 gDW/L compared to 0.59gDW/L, respectively. However, SAG 221-2 significantly outperformed SAG 211-14 in the elevated CO_2 treatments. In the 5% CO_2 treatment, SAG 221-2 achieved 32% higher biomass than SAG 211-14, 5.85 gDW/L compared to 3.96 gDW/L. In the 10% CO_2 treatment, SAG 221-2 achieved 46% higher biomass than SAG 211-14, 4.44 gDW/L compared to 2.40 gDW/L. Importantly, the disparity in biomass between the two strains does not appear to be the effect of growth medium acidification, since the pH of these two strains remained similar in all treatments (see Figure A5). Therefore, the results indicate that SAG 221-2 achieves significantly higher biomass levels than SAG 211-14 in elevated CO_2 treatments.

Furthermore, while in control CO_2 treatment, SAG 211-14 achieved the highest biomass of all strains, in 5% CO_2, SAG 211-14 achieved the second highest biomass, and in 10% CO_2, SAG 211-14 achieved the lowest biomass. This result suggests that SAG 211-14 does make as efficient use of elevated CO_2 for carbon assimilation compared to the other four strains tested. Therefore, in elevated CO_2 conditions, SAG 211-14 is not the best candidate strain for biomass yield. Instead, in elevated CO_2, SAG 221-2 has more promising biomass yield. However, biomass alone is not the desired output of *C. zofingiensis* cultivation.

Volumetric Carotenoids Concentration on Day 16

Volumetric carotenoid concentration, which is the yield of a system, is a major component in biotechnological evaluation. Volumetric Car accumulation responded differently towards CO_2 in different strains. SAG 4.80, SAG 31.80, and SAG 34.80 all accumulated more volumetric carotenoids in 10% CO_2 treatment than they did in 5% CO_2. However, SAG 221-2 and SAG 211-14 accumulated more in 5% CO_2 than in 10%.

The highest volumetric carotenoid accumulation was observed in SAG 211-14 at 5% CO_2, reaching 19.4 mg/L (see Figure A9). SAG 221-2 performed only marginally lower in 5% CO_2, achieving 17.8 mg/L. Meanwhile, SAG 221-2 slightly outperformed SAG 211-14 in 10% CO_2, reaching 14.3mg/L compared to 13.5 mg/L, respectively. SAG 221-2's volumetric Car outperformance in 10% CO2 is due to entirely to the effect of its heightened biomass yield, since its cellular Car concentration was lower than SAG 211-14 in this treatment (see Figure A10).

Surprisingly, the best performing strains in 5% CO_2, were outperformed in 10% CO_2. In 10% CO_2, the highest performers were SAG 34.80 (16.5 mg/L), followed by SAG 4.80 (15.8mg/L), and followed by SAG 31.80 (14.5mg/L).

It is difficult to compare these results with literature because the measurement of carotenoids is done differently across various authors. Some of the differences include: pigment extraction buffer, spectrophotometers, wavelengths and correlation equations, and use of HPLC. Furthermore, these results are for PBR, and different results should be expected for open pond or fermenters. Nonetheless, a preliminary comparison reveals similarities of these results with literature. Campo reported Car content of 40 mg/L in batch culture with strain SAG 211-14 [8]. Thus, the results of the present study appear to qualitatively fit in these ranges, but it is difficult to offer a quantitative comparison.

The question of how much astaxanthin is present within the carotenoid pool is important for this study. However, literature review for a ratio of astaxanthin content in carotenoids offered mixed results. Chen reported 60% astaxanthin content in total pigments, meanwhile Campo reported 40% astaxanthin content in carotenoids. Thus, Chen's estimate is substantially higher. Furthermore, Chen and team found a Car/Chl ratio of 5 after 288 hours, whereas, the Car/Chl ratio in this study only exceeded 1.0 in two cases, for SAG 211-14 5% CO_2, and SAG 211-14 10% CO_2, respectively 1.05 and 2.00. A deeper investigation would be needed to clarify which authors' results best apply to the present study. However, it should be noted that work by Campo with *C. zofingienis,* and work by Chekanov with stressed *H. pluvialis,* both found much lower Car/Chl ratios than Chen, which were more in line with results found here [8, 26].

Therefore, for a conservative estimate using Campo's 40% of carotenoids ratio, it can be approximated that this study found a maximum yield of ~8.5mg/L astaxanthin with SAG 211-14 in 5% CO_2. This result is comparable with previous results with *C. zofingiensis* in fermentation cultivation (SAG 211-14, CCAP 211-14 and ATC30412) which ranged from 10.3 mg/L to 56 mg/L (Campo 2003). Once again, results of the present study likely underestimate the potential of the strains due to the 20% biomass reduction on day 9.

From the point of view of yield, in 5% CO_2 treatment the highest performance is achieved by SAG 211-14. However, in 10% CO_2 the highest performance is achieved by SAG 34.80.

Carotenoids Cellular Concentration on Day 16

The cellular concentration of the desired metabolite is an important factor in purification and processing costs for biotechnological applications. Cellular Car concentration was lowest for all strains in the 5% CO_2 treatments (see Figure A3), and responded differently to CO_2 treatment

according to strains. The highest cellular Car concentration observed was for SAG 31.80 in control CO_2 at 8.2 mg/gDW.

SAG 221-2 had similar cellular Car concentration as SAG 211-14 in control CO_2 (approx. 5.4mg/gDW), but performed significantly worse in elevated CO_2. This counterweights the previous findings that SAG 221-2 performed better than SAG 211-14 in both biomass density, and volumetric carotenoids under elevated CO_2. In elevated CO_2, SAG 211-14 outperforms SAG 221-2 in cellular concentration of metabolites.

However, the highest cellular Car concentrations in 10% CO_2, were achieved in SAG 4.80 (5.9 mg/gDW), followed by SAG 34.80 (5.8 mg/gDW), and only then followed by SAG 211-14 (5.6 mg/gDW). These results explain why SAG 34.80 and SAG 4.80 exhibited the highest volumetric carotenoid production in 10% CO_2. The results found here were similar to work by Campo, whose SAG 211-14 cultures reached a maximum total cellular Car concentration of 7mg/gDW [8].

In conclusion, in 5% and 10% CO_2 SAG 211-14 outperforms SAG 221-2 on the cellular Car concentration basis. However, in 10% CO_2 both strains are outperformed by SAG 4.80 and SAG 34.80. Since these latter two strains also exhibited the highest Car volumetric yields in 10% CO_2, they emerge as the most suitable candidates for 10% CO_2 growth conditions.

It is worth noting that although 5% CO_2 appears to deliver the best yields in this experiment, there may external economic incentives to use higher CO_2 levels. Algae biotechnology which uses industrial flue gases would ostensibly receive CO_2 concentrations as high as 30%, for example from the cement industry [27]. Since industry may pay for, and governments may subsidize biotechnology with high carbon dioxide sink rates, this may offset reduced yield. Thus, the study of highest yielding strains under 10% CO_2 is also important.

Car/Chl Ratio

The Car/Chl ratio exhibited no significant differences between CO_2 treatments. However, groupings were formed by strains (see Figure A11). It should be noted that SAG 211-14 in 10% CO_2 treatment was substantially higher than all other samples, with a ratio of 2.00. As described previously, given the extensive parameters that contribute to it, more research would be needed to verify and compare the Car/Chl with literature.

pH

It was expected that higher CO_2 would lead to higher acidification of growth medium, and a retardation of growth. For this reason, Liu suggests that air enriched between 1-5% CO_2 should support efficient photosynthesis, without causing harmful pH decrease in culture medium [7]. In this experiment, elevated CO_2, appears to have contributed to some but not extensive acidification of growth mediums, and this only in certain strains. Strains SAG 4.80, SAG 31.80, and SAG 34.80 exhibited decreasing pH on day 16 with higher CO_2 levels, however, the relationship did not hold for SAG 221-2 and SAG 211-14. It should be noted that several days of pH measurements are missing from this experiment: the starting Kuhl medium pH for SAG 221-2 and SAG 211-14, and all starting pH measurements for N-depleted Kuhl medium. However, the medium follows set ingredients, and results in pH close to 6.10. While, the mentioned measurements were missing, the final pH data retrieved on day 16 gives a sufficient indication of cultivation conditions.

Change in Dry Weight Biomass Density after Stress Induction

All strains were able to grow well under stress conditions in all treatments (see Figure A6). This, may indicate that a longer growth-phase should have been used to reach higher standing cell

densities before stress induction. The smallest change observed was in SAG 221-2 in Control CO_2, while the largest change observed occurred in SAG 211-14 in 10% CO_2, which increased 305%.

Change in Carotenoids after Stress Induction

The largest response in volumetric carotenoid concentration by percentage occurred in SAG 211-14 in 10% CO_2, with a 684% change, from 1.7 to 13.5 mg/L (see Figure A7). The smallest change occurred in SAG 34.80 control CO_2, with only 8% increase in volumetric carotenoid concentration. The results indicate that stress induction was successful in all strains and all treatments, but on a percentage basis, had a significantly greater effect in elevated CO_2.

Cellular carotenoid concentration increased for SAG 221-2 and SAG 211-14 after stress induction in all treatments, and decreased for SAG 4.80, SAG 34.80, in all treatments. SAG 31.80 showed mixed results, increasing in control CO_2, but decreasing in both elevated CO_2. The results indicate that stress induction causes different effects on cellular carotenoid concentration in different strains, but that these effects tend to be consistent across CO_2 treatments.

Nitrogen deprivation leads to reallocation of carbon away from N-dependent cell replication towards carbon storage in starch and lipids [24]. Samples which increased in biomass after stress induction but did not increase carotenoid concentration (which are lipids), must have allocated carbon fixation towards starch. The extent to which this undesirable effect occurs is a target for future genetic manipulation.

Since astaxanthin plays a photoprotection role, which is also indirectly achieved by high cell densities, it was interesting to search for a correlation between cell density and carotenoid concentration. As expected a negative relationship was found for all strains, though with varying R^2 values (see Figure A12). The negative relationship is clearly counterproductive for biotechnological purposes. Ideally, photobioreactors would prefer to achieve high cell density, and

high cellular carotenoid concentration. Thus, based on these results, it can be argued that the strain with the weakest negative correlation, which was SAG 211-14, is supported as the most biotechnological valuable object.

Change in Cellular Car/Chl After Stress Induction

For all strains and all treatments the Car/Chl ratio increased substantially after stress induction (see Figure A13). This followed expectations since cells upregulate photoprotection mechanisms after light stress. It is important to note that the effect is a result of two factors, a decrease in cellular Chl concentration (which are photosensitizers causing ROS) and an increase in cellular Car concentration (which are photoprotectors). However, on average Chl decreased 48% (see Figure A14) after stress induction, while on average Car only increased 15%, but decreased in several strains (see Figure A8). Therefore, the increase in Car/Chl observed in all strains after stress induction was often more a factor of decreasing Chl than increasing Car. Notably, because Chl content increased after stress induction, the Car/Chl ratio did not increase for SAG 31.80. Furthermore, the Car/Chl ratio changed so dramatically for SAG 211-14 in 10% CO2 because while Car increased 93%, Chl also fell 75%. No significant trends were observed between Chl A/ Chl B ratios after stress induction (data not shown). The increasing Car/Chl ratio of strains led to an observable progressive darkening and reddening of samples after stress induction.

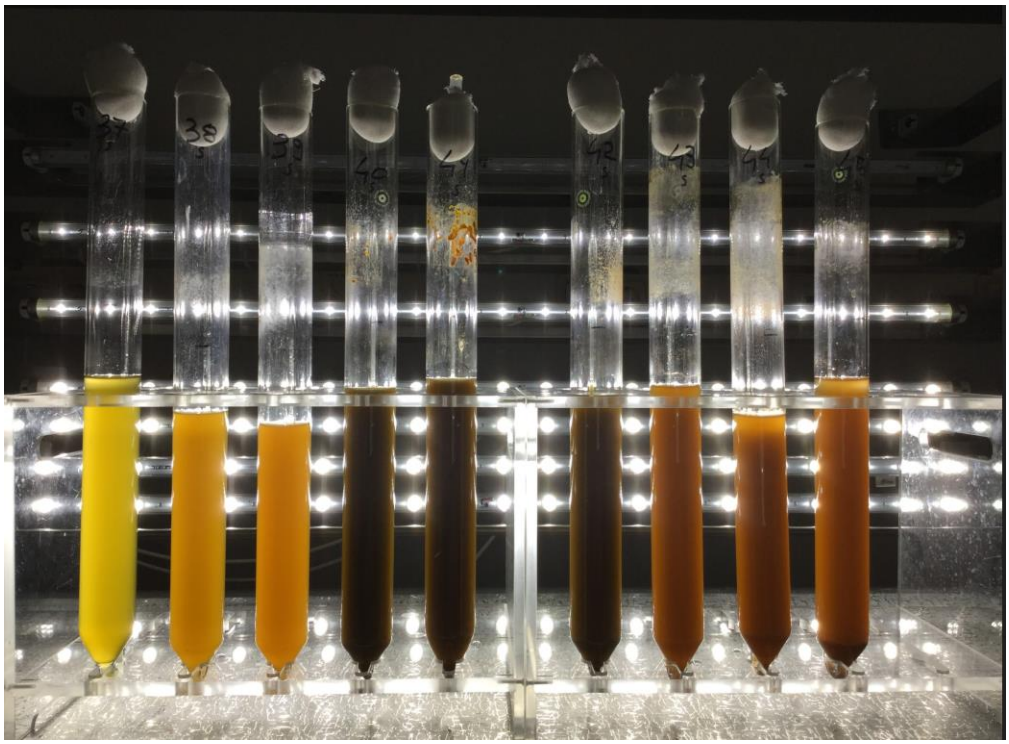


Figure 3. C. zofingiensis strain SAG 211-4 on day 16

SAG 211-14 on last day of measurement after stress induction grown in varied CO_2 treatments. From left to right, triplicate control CO_2, triplicate 5% CO_2, and triplicate 10% CO_2. The effect of stress induction caused reddening of samples. Furthermore, CO_2 supplementation on dry weight biomass is visible in these cultures, where 5% CO_2 achieves visibly denser standing culture on day 16.

Productivity of strains

All strains were significantly more productive in elevated CO_2 than in control CO_2 with regards to biomass and carotenoids (see Figure A15). The highest biomass productivity observed in 5% CO_2 was SAG 221-2 which achieved 0.36 gDW/L/day. The highest volumetric carotenoid production rate observed was with SAG 211-14 in 5% CO_2, which achieved 1.2 mg/L/day, followed by SAG 221-2 in 5% CO_2, which achieved 0.36 mg/L/day. For comparison of values see Appendix Table 1. Once again, these results likely underestimate the potential of the strains

because of the 20% reduction of biomass on day 9. Nonetheless, the results can be compared to literature review by Liu, which found biomass productivity with CCAP 211-14, SAG 211-14, and ATCC30412 to be between 0.74 to 4.7 gDW/L/day using fermentor reactors, and astaxanthin productivity to be between 0.8 to 5.6 mg/L/day [7]. Using Chen's 40% Astaxanthin/Car ratio, the latter literature results would translate to an approximate carotenoid productivity range of 2 to 14 mg/L/day. Thus, the results of the present study fit into previous work with *C. zofingiensis*.

Conclusion

This study compared the biomass and carotenoid productivity of five *C. zofingiensis*. strains in elevated CO_2 treatments. Productivity was significantly enhanced in elevated CO_2 supplementation treatments than in control for all strains. In 5% CO_2, the most productive strains for carotenoids was SAG 211-14 (1.2 mg/L/day) followed closely by SAG 221-2 (1.1 mg/L/day). However, in the same treatment SAG 211-14 showed markedly superior cellular carotenoid concentration (approx. 60%), which has important ramifications on product processing costs. It is reasonable to assume that astaxanthin yield is directly linked to carotenoid productivity and concentration. Therefore, in 5% CO_2, SAG 211-14 outperforms all other strains, on a combined volumetric astaxanthin yield and cellular concentration basis. However, for other biotechnological applications, such as biofuels, SAG 221-2 may be a more valuable candidate than SAG 211-14, since it achieved nearly 50% higher growth rates in elevated CO_2. In 10% CO2, SAG 4.80 and SAG 31.80 outperform all strains including SAG 211-14, on a combined volumetric carotenoid yield and cellular concentration basis. In conclusion, the results of this experiment indicate that SAG 211-14 is the best strain for astaxanthin production under 5% CO_2 conditions, but that higher CO_2 conditions should use SAG 4.80 and SAG 31.80.

A logical consequence of these results is the possibility of increasing yields of PBRs by connecting various strains in series, such that the output air of one strain supplements into the intake of another. In such a scenario, 10% CO_2 may first be supplemented to SAG 34.80 or SAG 31.80, and the output air, which would have lower CO_2 could be supplemented to SAG 211-14. This would allow the use of high concentration flue gases from industry without excessive loss of yield. In such a bioreactor system, oxygen concentrations may increase towards the tail end of the series. The effect of this increase could cause higher incidence of reactive oxygen species, the effect of which could either stimulate carotenoid production, or may reduce biomass yields.

Acknowledgements

I would like to thank Dr. Tatyana Darienko for supervising this project, and sharing her wisdom, passion, and years of experience with the wonderful world of microalgae. I would like to thank Birgit Olberg for technical advice and help conducting measurements. I would like to thank Dr. Maike Lorenz and Dr. Thomas Pröschold for their feedback on data analysis. Finally, I would like to thank the other three students who worked on the experiment: Woojean Kang, Aleksander Orzechowski, and Paolo Ruffino.

References

1. Global Market Insights., *Astaxanthin Market size By Application.* 2018.
2. Capelli, B., D. Bagchi, and G. Cysewski, *Synthetic astaxanthin is significantly inferior to algal-based astaxanthin as an antioxidant and may not be suitable as a human nutraceutical supplement*. Vol. 12. 2013.
3. Roth, M.S., et al., *Chromosome-level genome assembly and transcriptome of the green alga Chromochloris zofingiensis illuminates astaxanthin production.* 2017(1091-6490 (Electronic)).
4. Gutman, J., A. Zarka, and S. Boussiba, *The host-range of Paraphysoderma sedebokerensis, a chytrid that infects Haematococcus pluvialis.* European Journal of Phycology, 2009. **44**(4): p. 509-514.
5. Strittmatter, M., et al., *A new flagellated dispersion stage in Paraphysoderma sedebokerense, a pathogen of Haematococcus pluvialis*. Vol. 27. 2015.
6. Panis, G. and J.R. Carreon, *Commercial astaxanthin production derived by green alga Haematococcus pluvialis: A microalgae process model and a techno-economic assessment all through production line.* Algal Research, 2016. **18**: p. 175-190.
7. Liu, J., et al., *Chlorella zofingiensis as an alternative microalgal producer of astaxanthin: biology and industrial potential.* 2014(1660-3397 (Electronic)).
8. Campo, J.A., et al., *Accumulation of astaxanthin and lutein in Chlorella zofingiensis (Chlorophyta)*. Vol. 64. 2004. 848-54.
9. Müller, T., et al., *Distinguishing species.* 2007(1355-8382 (Print)).
10. Singh, A., S. Ahmad, and A. Ahmad, *Green extraction methods and environmental applications of carotenoids-a review.* RSC Advances, 2015. **5**(77): p. 62358-62393.
11. Moretti, V.M., et al., *Determination of astaxanthin stereoisomers and colour attributes in flesh of rainbow trout (Oncorhynchus mykiss) as a tool to distinguish the dietary pigmentation source.* 2006(0265-203X (Print)).
12. Liu, J., et al., *One amino acid substitution in phytoene desaturase makes Chlorella zofingiensis resistant to norflurazon and enhances the biosynthesis of astaxanthin.* 2010(1432-2048 (Electronic)).
13. Steinbrenner, J. and H. Linden, *Regulation of two carotenoid biosynthesis genes coding for phytoene synthase and carotenoid hydroxylase during stress-induced astaxanthin formation in the green alga Haematococcus pluvialis.* 2001(0032-0889 (Print)).
14. P Small, D., N. Huner, and W. Wan, *Effect of static magnetic fields on the growth, photosynthesis and ultrastructure of Chlorella kessleri microalgae*. Vol. 33. 2012.
15. Kuhl, A. and H. Lorenzen, *Chapter 10 Hmdling and Culturing of Chlorella,* in *Methods in Cell Biology*, D.M. Prescott, Editor. 1964, Academic Press. p. 159-187.
16. Forman, H.J. and M. Torres, *Reactive Oxygen Species and Cell Signaling.* American Journal of Respiratory and Critical Care Medicine, 2002. **166**(supplement_1): p. S4-S8.
17. Li, X., et al., *Targeting mitochondrial reactive oxygen species as novel therapy for inflammatory diseases and cancers.* 2013(1756-8722 (Electronic)).
18. Ma, J., F. Wang, and M. Mostafavi, *Ultrafast Chemistry of Water Radical Cation, H_2O•+, in Aqueous Solutions*. Vol. 23. 2018.
19. Pospíšil, P., *Production of Reactive Oxygen Species by Photosystem II as a Response to Light and Temperature Stress.* Frontiers in plant science, 2016. **7**: p. 1950-1950.
20. Lazar, T., *Taiz, L. and Zeiger, E. Plant physiology. 3rd edn*. Vol. 91. 2003.

21. Clayden, J., N. Greeves, and S.G. Warren, *Organic chemistry*. 2012, Oxford; New York: Oxford University Press.
22. Borden, W.A.-O., et al., *Dioxygen: What Makes This Triplet Diradical Kinetically Persistent?* 2017(1520-5126 (Electronic)).
23. Krieger-Liszkay, A., *Singlet oxygen production in photosynthesis.* Journal of Experimental Botany, 2004. **56**(411): p. 337-346.
24. Tan, K.W.M., et al., *Nitrogen-induced metabolic changes and molecular determinants of carbon allocation in Dunaliella tertiolecta.* Scientific Reports, 2016. **6**: p. 37235.
25. Chen, J.H., L. Liu, and D. Wei, *Enhanced production of astaxanthin by Chromochloris zofingiensis in a microplate-based culture system under high light irradiation.* 207(1873-2976 (Electronic)).
26. Chekanov, K., et al., *Effects of CO2 enrichment on primary photochemistry, growth and astaxanthin accumulation in the chlorophyte Haematococcus pluvialis.* 2017(1873-2682 (Electronic)).
27. Metz, B., et al., *IPCC Special Report on Carbon dioxide Capture and Storage*. 2005.

Appendix

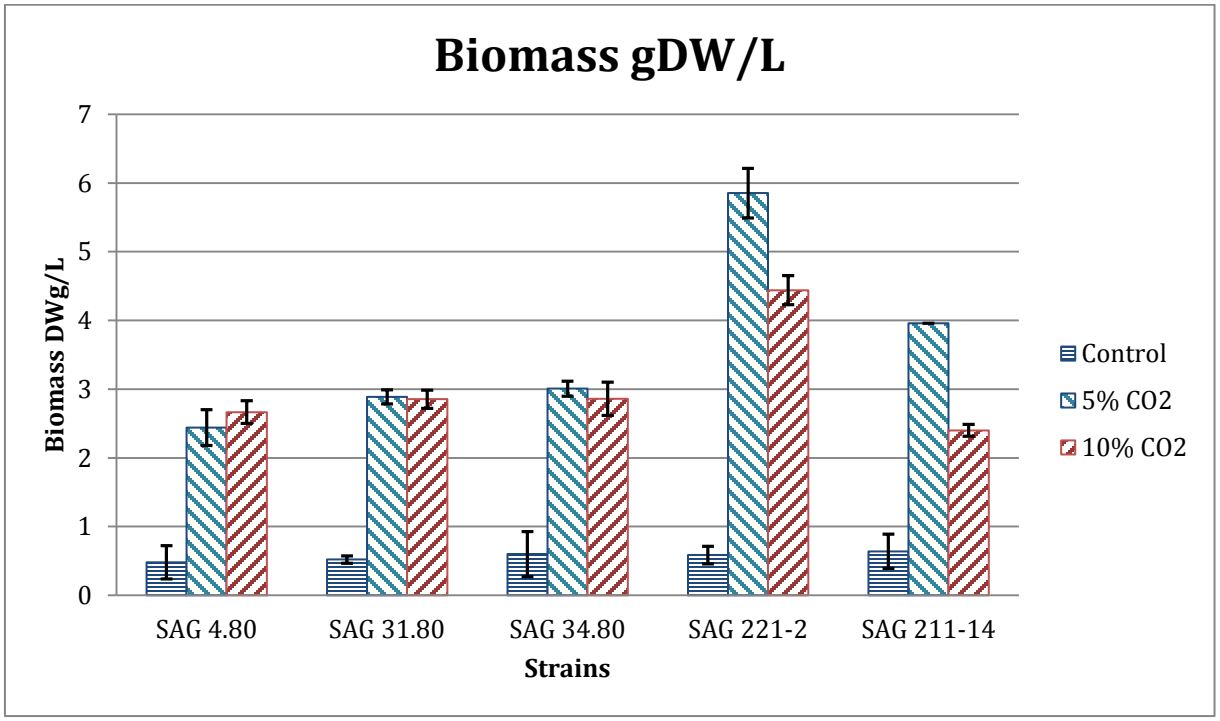


Figure A1. Dry weight biomass on Day 16 in all treatments.

Dry weight biomass on day 16 is shown for all strains and treatments. Elevated CO_2 increase biomass accumulation significantly for all strains. All strains performed best in 5% CO_2 treatment except for SAG 4.80, which performed best in 10% CO_2 treatment. The highest biomass was observed in SAG 221-2 under 5% CO_2, which reached 5.85 gDW/L.

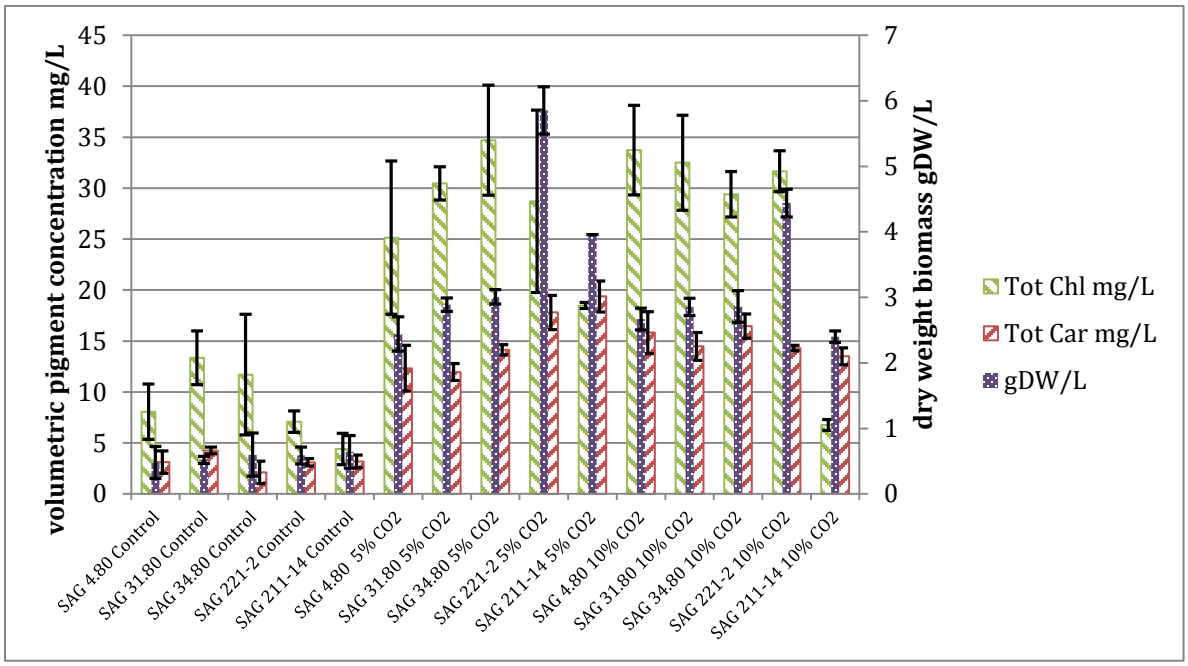


Figure A2. Volumetric pigment concentrations and biomass dry weight on day 16.

Total chlorophyll (A+B), total carotenoids, and dry weight biomass on a volumetric basis are compared in all strains and treatments. SAG 211-14 is notable for its relatively high Car/Chl ratio and SAG 221-2 is notable for its low Car content compared relative its high biomass. Error bars are 1 standard deviation.

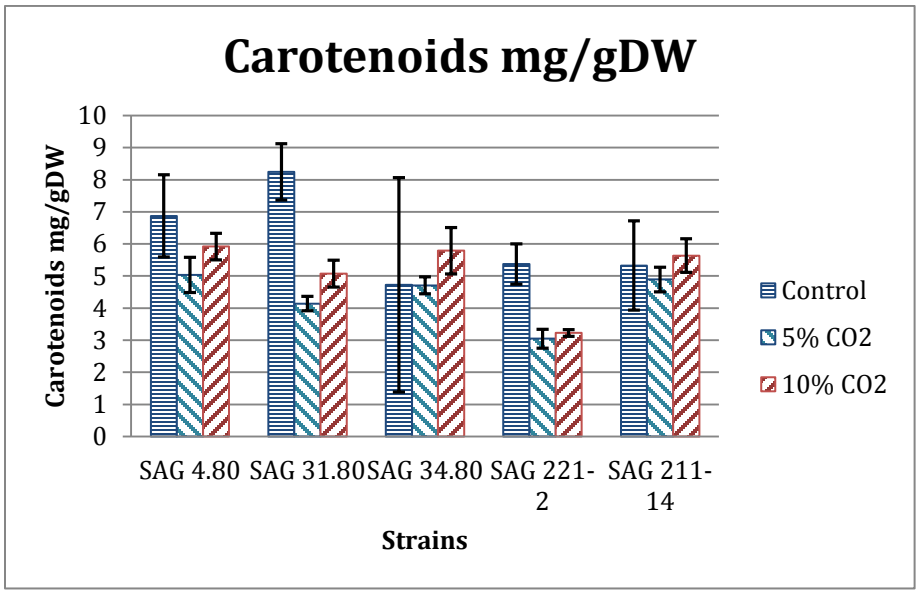


Figure A3. Cellular carotenoid concentrations on day 16.

All strains and all treatments are compared for their cellular carotenoid concentrations. SAG 31.80 in control CO2 outperforms in cellular carotenoid concentration, followed by SAG 4.80 in control CO2 treatment. SAG 221-2 exhibits low cellular concentrations. The standard deviation for SAG 34.80 is control treatment is large due to one sample having experienced a differentiated pigment profile early in its cultivation. Error bars are 1 standard deviation.

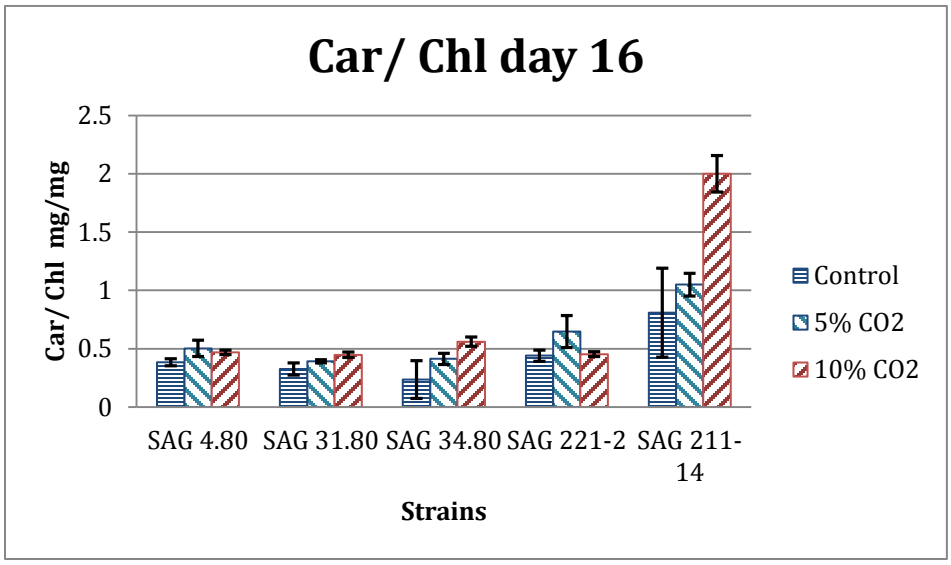


Figure A4. Car/Chl ratio on day 16.

The Car/Chl ratio behaved differently with different treatments depending on strain. It was always higher in elevated CO2 treatments compared to control, except for strain SAG 221-2. Exceptionally, SAG 221-2 exhibited its lowest Car/Chl ratio in 10% CO2. Error bars are 1 standard deviation.

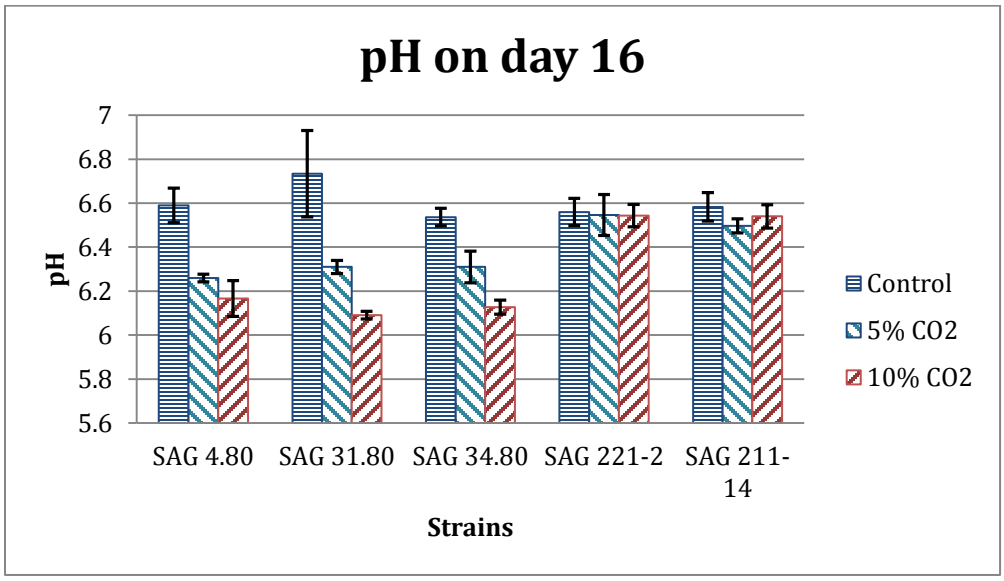


Figure A5. pH on day 16.

pH tended to decreased with increasing CO2 concentration, except with SAG 211-14. The lowest observed pH on day 16 was in SAG 31.80, which had a mean of 6.09. Error bars are 1 standard deviation.

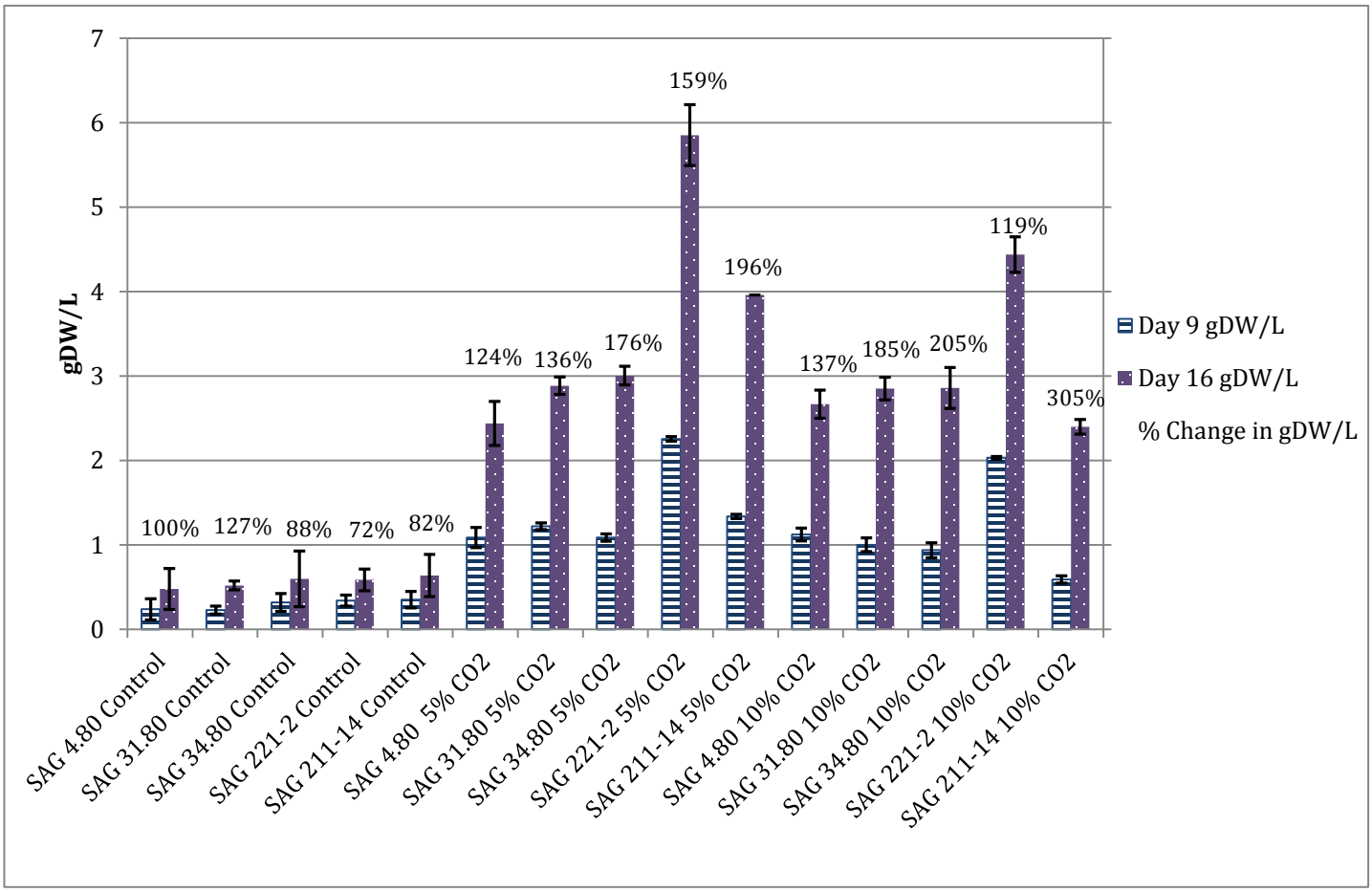


Figure A6. Dry weight biomass change after stress induction.

Dry weight biomass is compared between day 9 and day 16. All strains continued growth during stress phase. The percentages show the amount of increase. Error bars are 1 standard deviation.

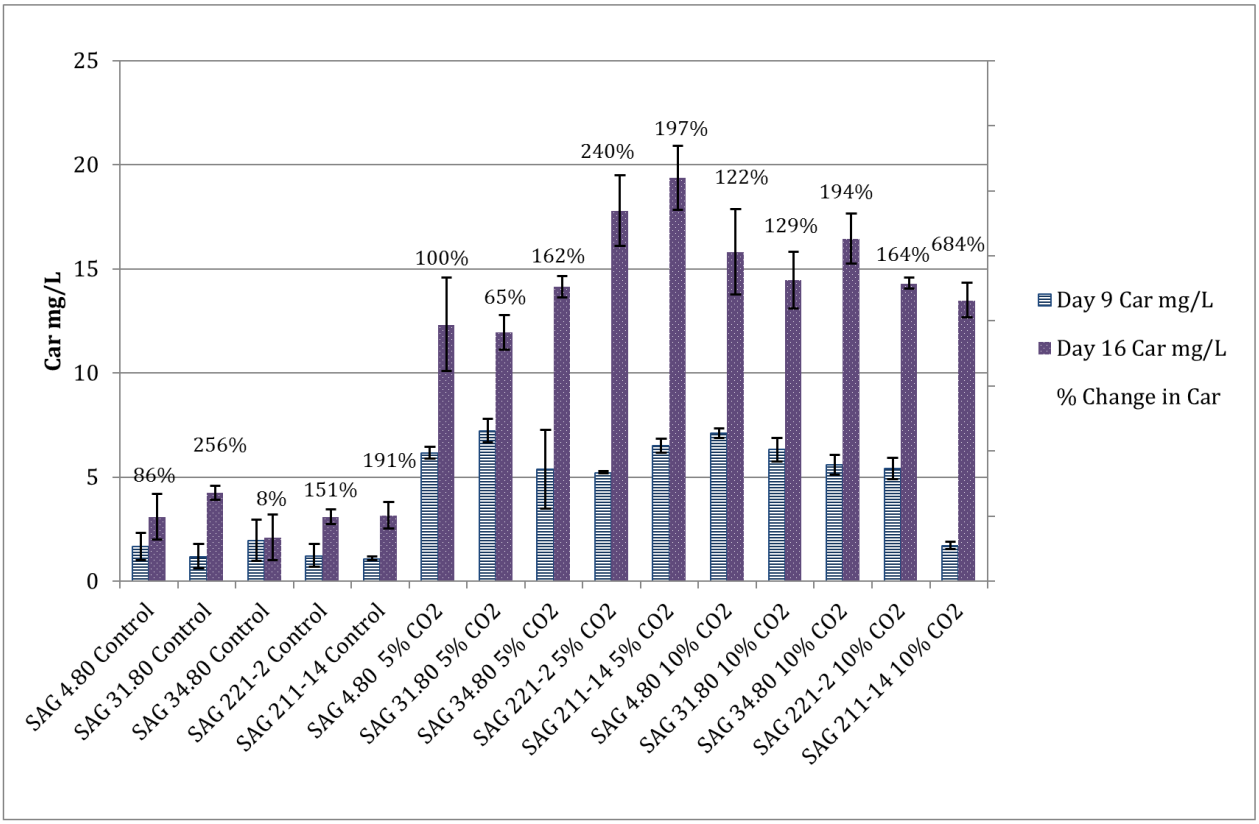


Figure A7. Carotenoid volumetric concentrations change after stress induction.

Carotenoid volumetric concentrations on day 9 and day 16. Percentages show magnitude of increase. All strains substantially increased their carotenoid concentrations after stress induction. Error bars are 1 standard deviation.

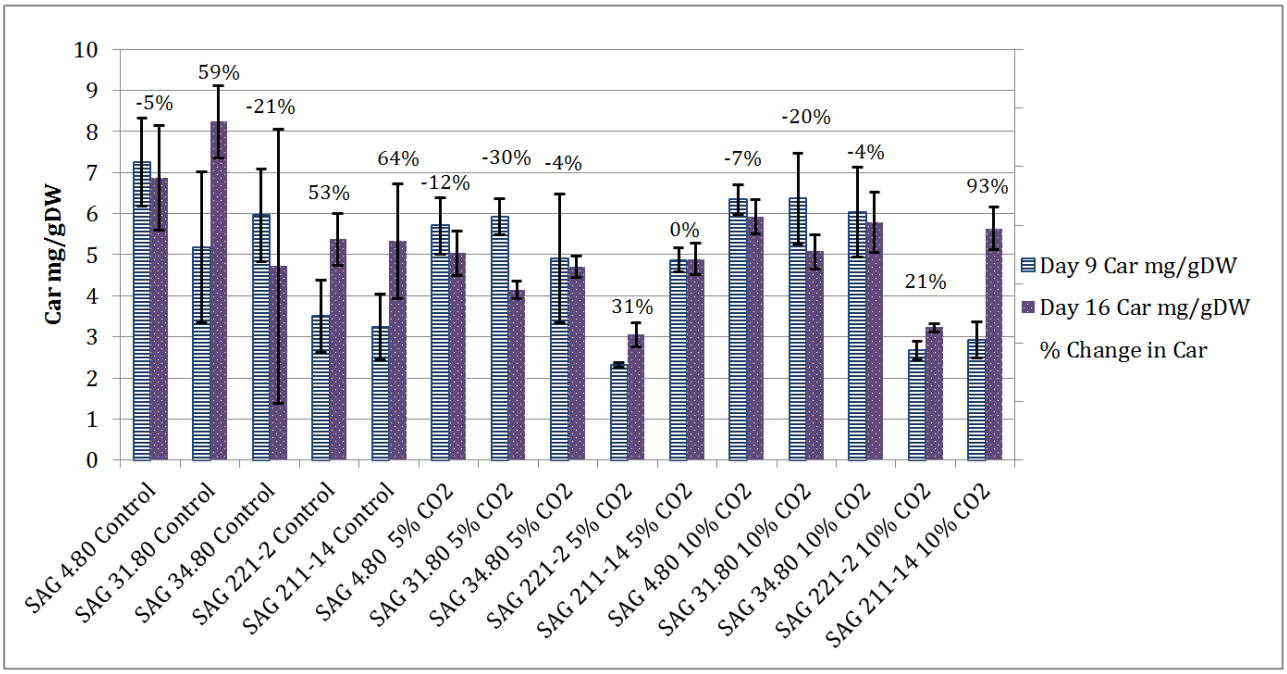


Figure A8. Carotenoid cellular concentration change after stress induction.

Carotenoid cellular concentrations are compared between day 9 and day 16. Percentages show size of increase. SAG 31.80 in control CO2 and SAG 211-14 in 10% CO2 are notable for relatively large increases in cellular carotenoid concentration increases after stress. Error bars are 1 standard deviation.

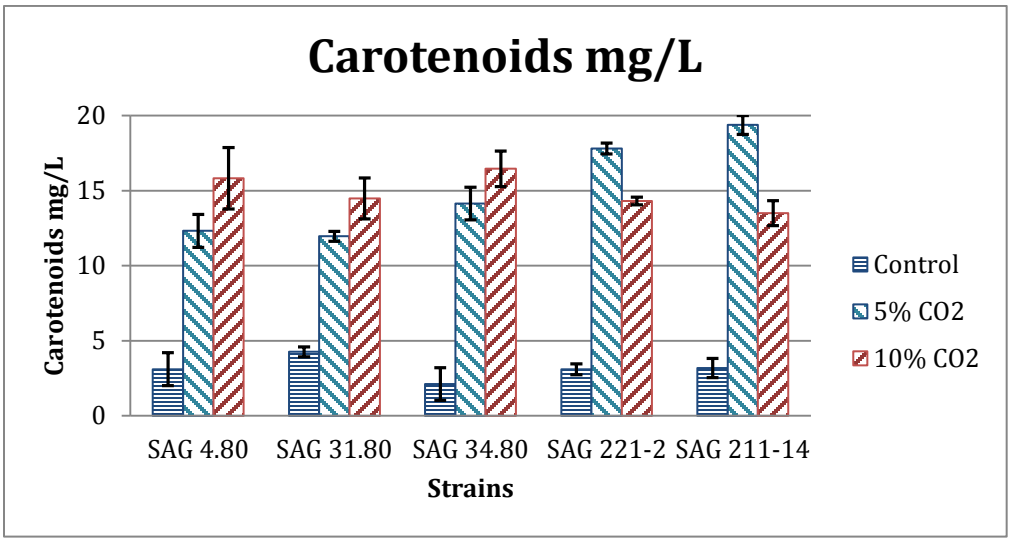


Figure A9. Volumetric carotenoid concentrations on day 16.

All strains and all treatments are compared for their volumetric carotenoid concentrations. SAG 211-14 in 5% CO_2 treatments outperforms in total carotenoid yield, followed by SAG 221-2 in 5% CO_2 treatment. Error bars are 1 standard deviation.

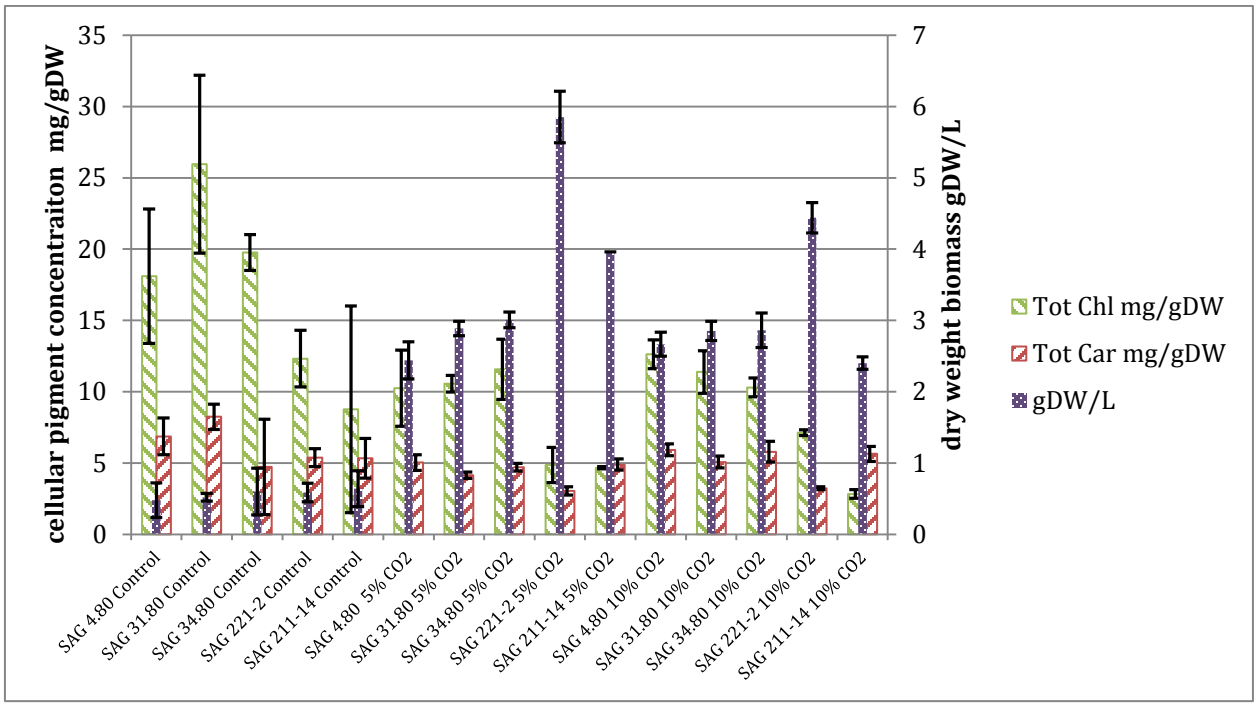


Figure A10. Cellular pigment concentrations and biomass dry weight on day 16

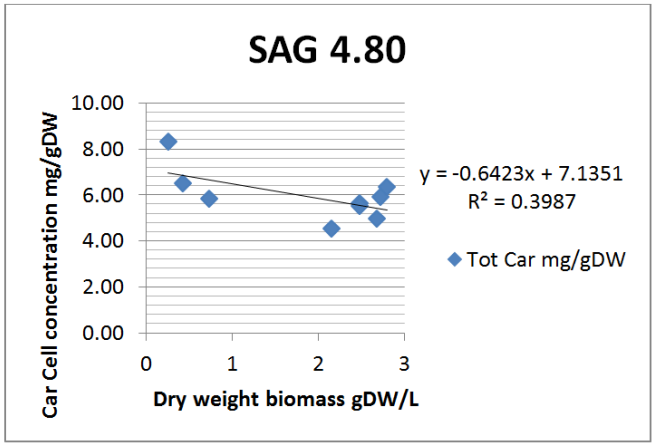
Cellular pigment concentrations and biomass dry weight on day 16 are compared. SAG 221-2 exhibits poor cellular carotenoid concentration, making it relatively less valuable for biotechnological applications. Error bars are 1 standard deviation.

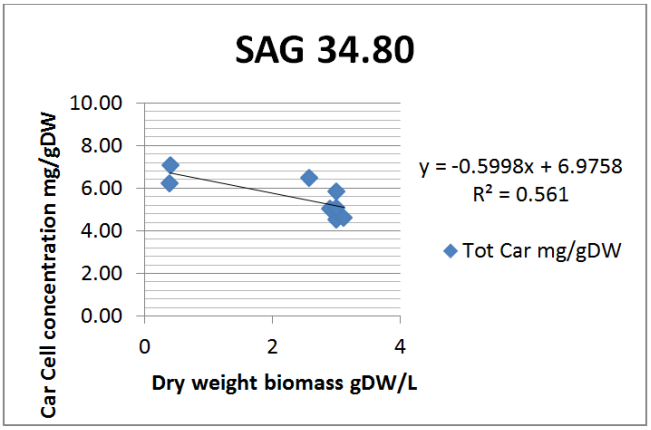
Strain	N	Mean	Grouping	
SAG 211-14	3	1,255	A	
SAG 221-2	3	0,5032	A	B
SAG 4.80	3	0,4483	A	B
SAG 31.80	3	0,3858		B
SAG 34.80	3	0,383		B

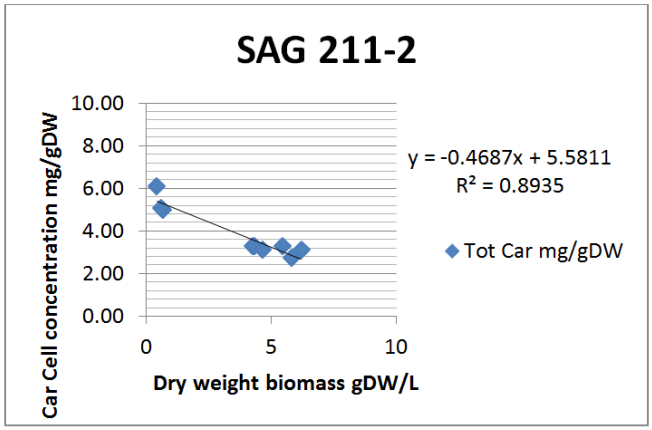
Means that do not share a letter are significantly different.

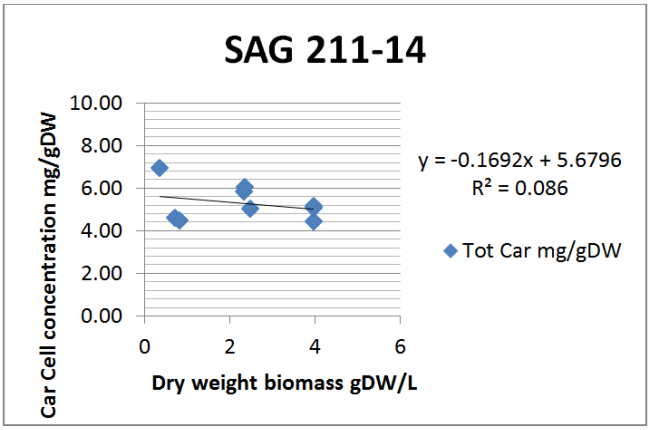
Figure A11. Groupings for Car/Chl ratio with Tukey test 95% confidence intervals.

SAG 211-14 exhibited the highest Car/Chl ratio, while SAG 34.80 exhibited the lowest. N=number of replicas Grouping shows statistically significant groups.









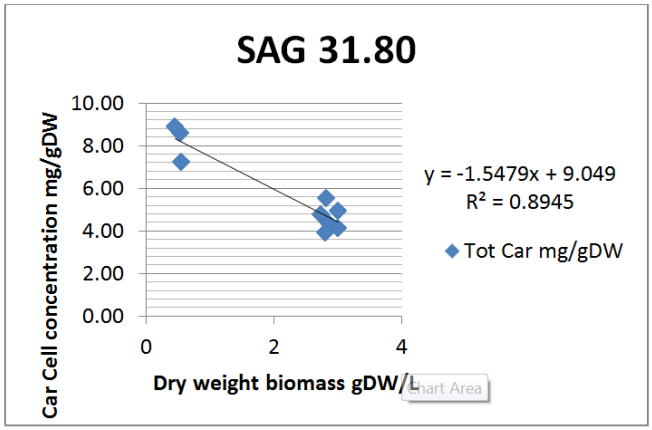


Figure A12. Dry weight biomass to carotenoid cellular concentration.

Relationships between biomass and cellular carotenoids on day 16 are all negative. Although the R^2 does not support a statistical correlation between the two factors in each case, a trend is visible. SAG 211-14 is notable for the least negative relationship and lowest R^2

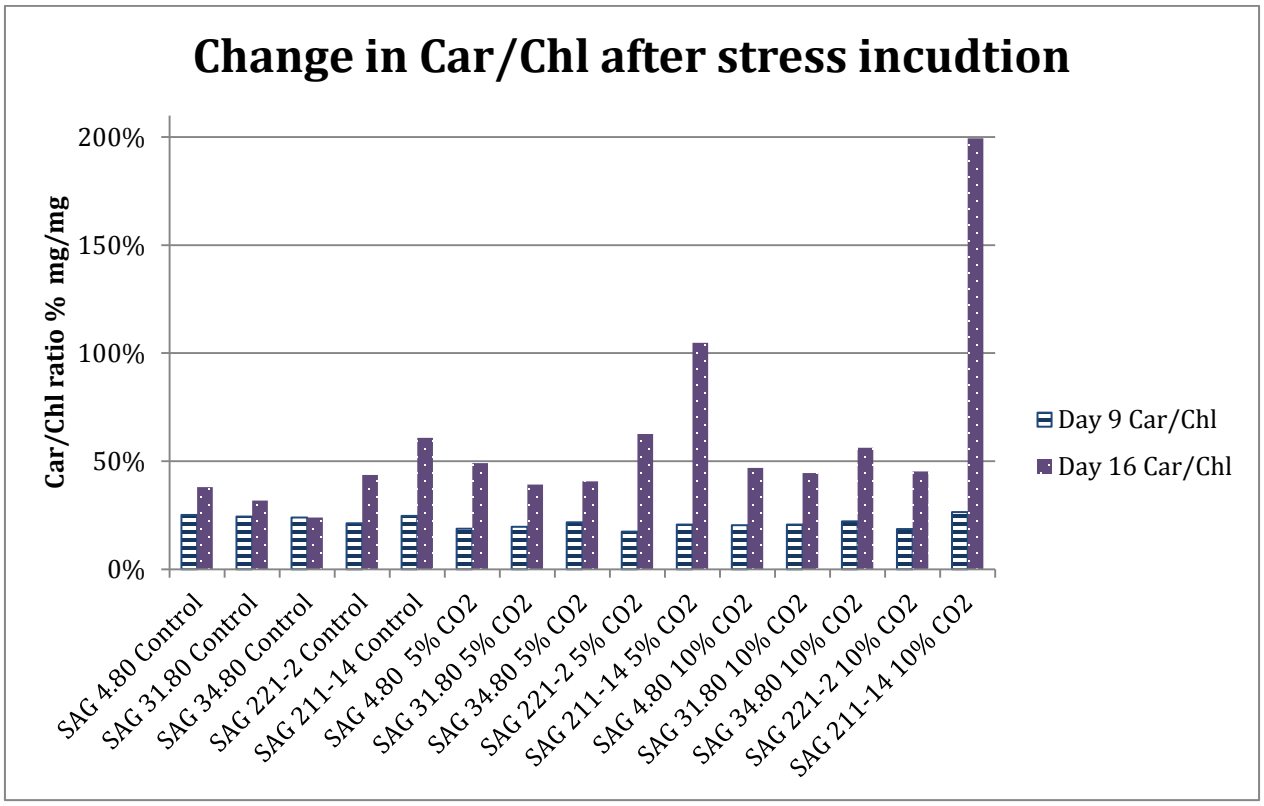


Figure A13: Change in Car/Chl ratio after stress induction.

The Car/Chl ratio is compared before and after stress. Except for SAG 34.80, all strains significantly increased their Car/Chl ratio after stress induction. SAG 211-14 showed consistently higher ratio in all treatments and reached 199% Car/Chl in 10% CO2 treatment.

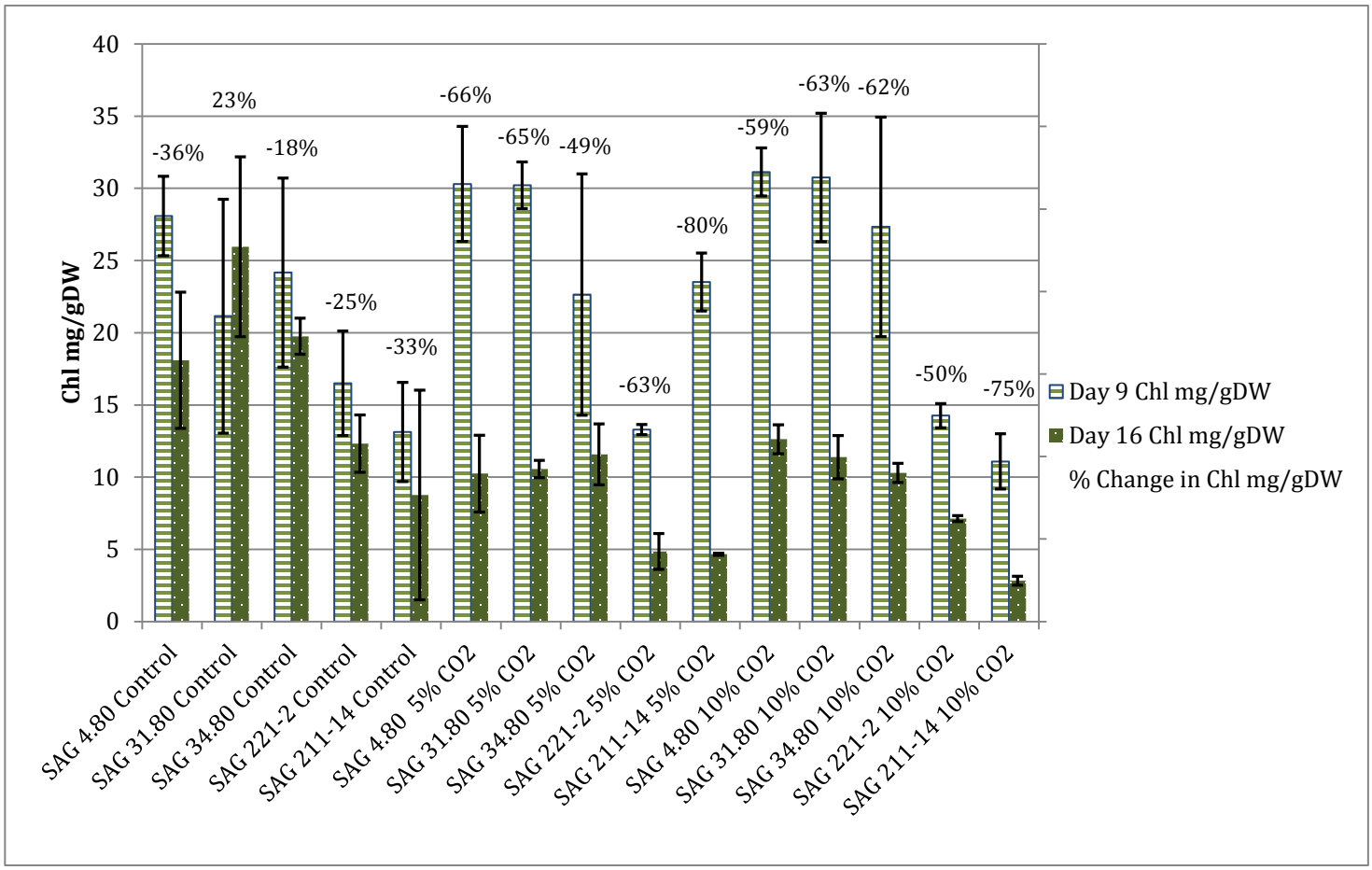


Figure A14: Chlorophyll cellular concentration change after stress induction.

Chlorophyll cellular concentrations are compared between day 9 and day 16. Percentages show size of increase. All strains decreased cellular Chl concentration after stress induction, except SAG 31.80 in control CO2. Although the standard deviation is high for SAG 31.80 Control, the same increase Chl mg/gDW trend was observed in all three replicas. Error bars are 1 standard deviation.

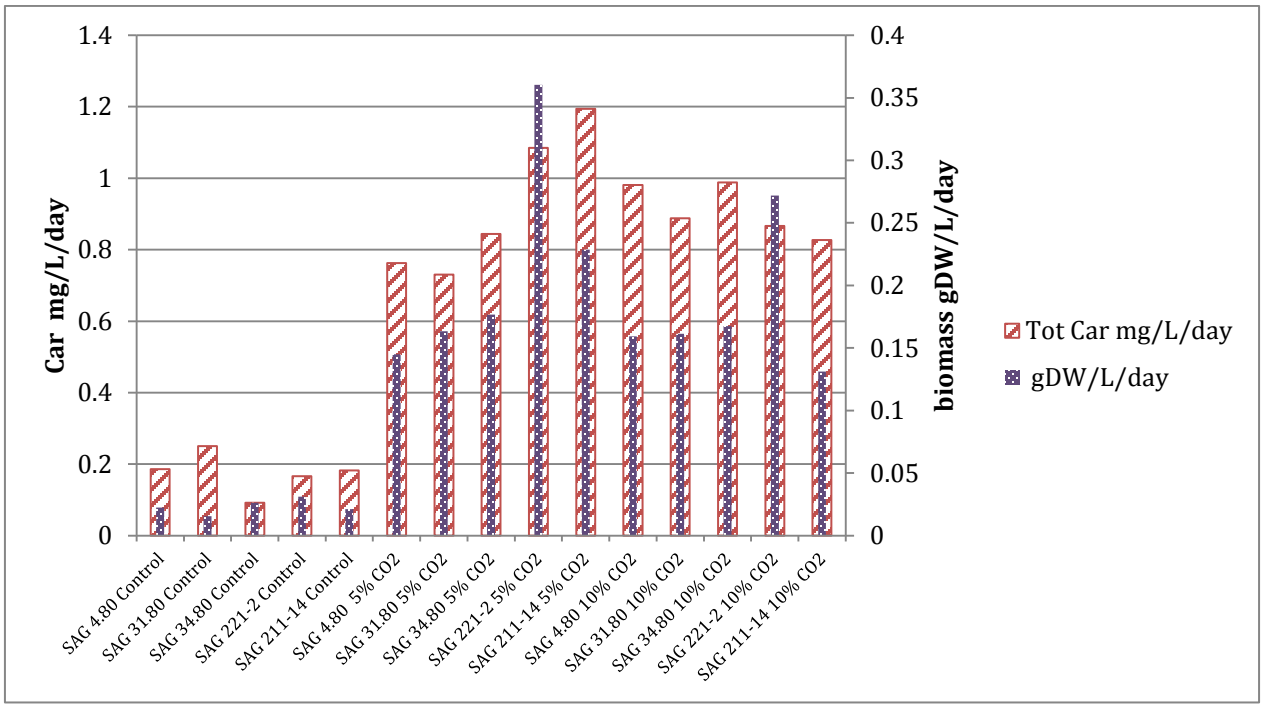


Figure A15: Carotenoids and dry weight biomass produced per liter per day.

Productivity of each strain is compared in all treatments with regards to dry weight biomass and mg carotenoids. The fastest biomass growth was in SAG 221-2 in 5% CO2, while the fastest carotenoid productivity was in SAG 211-14 also in 5% CO2. Notably, SAG 34.80 in 10% CO2 exhibited the third fastest carotenoid productivity despite relatively lackluster biomass growth.

Table 3. Production of biomass and Carotenoids.

		Biomass Density	Biomass Productivity	Carotenoid Yield	Carotenoid Productivity	Carotenoid Content	Pigment Ratio
Strain	**Treatment**	**gDW/L**	**gDW/L/day**	**Car mg/L**	**Car mg/L/day**	**Car mg/gDW**	**Car/Chl**
SAG 4.80	Control	0.48 (±0.24)	0.02	3.10 (±1.09)	0.19	6.86 (±1.28)	0.38 (±0.08)
	5% CO2	2.44 (±0.26)	0.15	12.3 (±2.24)	0.76	5.03 (±0.54)	0.50 (±0.03)
	10% CO2	2.66 (±0.16)	0.16	15.8 (±2.05)	0.98	5.91 (±0.41)	0.46 (±0.02)
SAG 31.80	Control	0.52 (±0.05)	0.02	4.26 (±0.33)	0.25	8.24 (±0.88)	0.32 (±0.05)
	5% CO2	2.88 (±0.10)	0.16	11.9 (±0.83)	0.73	4.14 (±0.22)	0.39 (±0.01)
	10% CO2	2.85 (±0.13)	0.16	14.4 (±1.35)	0.89	5.07 (±0.41)	0.44 (±0.02)
SAG 34.80	Control	0.6 (±0.32)	0.03	2.11 (±1.09)	0.09	4.72 (±3.33)	0.23 (±0.20)
	5% CO2	3.00 (±0.11)	0.18	14.1 (±0.50)	0.84	4.70 (±0.26)	0.41 (±0.01)
	10% CO2	2.86 (±0.24)	0.17	16.4 (±1.19)	0.99	5.78 (±0.72)	0.56 (±0.04)
SAG 221-2	Control	0.58 (±0.12)	0.03	3.09 (±0.36)	0.17	5.37 (±0.62)	0.44 (±0.03)
	5% CO2	5.85 (±0.36)	0.36	17.8 (±1.69)	1.09	3.04 (±0.29)	0.64 (±0.01)
	10% CO2	4.44 (±0.21)	0.27	14.3 (±0.26)	0.87	3.22 (±0.10)	0.45 (±0.00)
SAG 211-14	Control	0.64 (±0.24)	0.02	3.18 (±0.63)	0.18	5.32 (±1.39)	0.80 (±0.08)
	5% CO2	3.96 (±5.43)	0.23	19.3 (±1.52)	1.19	4.89 (±0.38)	1.04 (±0.02)
	10% CO2	2.4 (±0.08)	0.13	13.4 (±0.82)	0.83	5.63 (±0.52)	2.00 (±0.03)