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Article

Potential of new isolates of *Dunaliella salina* for natural β-carotene production

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Abstract: The halotolerant microalga *Dunaliella salina* has been widely studied for natural β-carotene production. This work shows biochemical characterization of three newly isolated *Dunaliella salina* strains DF15, DF17 and DF40 compared with *D. salina* CCAP 19/30 (confirmed to be *D. tertiolecta*) and *D. salina* UTEX 2538 (also known as *D. bardawil*). Although all three new strains have been genetically characterized as *Dunaliella salina* strains, their ability to accumulate carotenoids and their capacity for photoprotection against high light stress are different. DF15 and UTEX 2538 reveal great potential for producing large amount of β-carotene and maintained a high rate of photosynthesis under light of high intensity; however, DF17, DF40 and CCAP 19/30 showed increasing photoinhibition with increasing light intensity, and reduced contents of carotenoids, in particular β-carotene, suggesting that the capacity of photoprotection is dependent on the cellular content of carotenoids, in particular β-carotene. Strong positive correlations were found between the cellular content of each of *all-trans* β-carotene, *9-cis* β-carotene, *all-trans* α-carotene and zeaxanthin but not lutein in the *D. salina* strains. Lutein was strongly correlated with respiration in photosynthetic cells and strongly related to photosynthesis, chlorophyll and respiration, suggesting an important and not hitherto identified role for lutein in co-ordinated control of the cellular functions of photosynthesis and respiration in response to changes in light conditions, which is broadly conserved in *Dunaliella* strains. Statistical analysis based on biochemical data revealed a different grouping strategy from the genetic classification of the strains. The significance of these data for strain selection for commercial carotenoid production is discussed.

Keywords: *Dunaliella salina*, new isolates, characterization, light intensity, β-carotene, carotenoids, correlations, lutein, classification

1. Introduction

Natural carotenoids have gained increasing attention in recent years because of their health benefits compared to synthetic carotenoids [1]. These lipophilic compounds comprise a range of carotenes and xanthophylls and their health benefits generally derive from their ability to quench oxygen radicals and absorb potential damaging visible light [2-4]. Carotenoids predominantly occur in their *trans* configuration but are also naturally found in their *cis* configuration [5]. Methods for producing synthetic carotenoids especially β-carotene and zeaxanthin are well-established [1], however synthetic carotenoids are predominantly *all-trans* compounds and are of questionable benefit [6]. By contrast, intake of food supplements enriched with natural β-carotene containing both *cis-* and *trans-* stereoisomers is linked with mitigation of a range of diseases including atherosclerosis, diabetes, psoriasis and ophthalmologic diseases [7-9]. *9-cis* β-carotene is of particular nutritional and medical interest as a retinoid precursor and is associated with therapeutic effects in a number of diseases as well as possessing a good adverse effect profile [10]. This stereoisomer is difficult to synthesise chemically, it is not produced biologically by heterotrophs such as bacteria or yeasts, through fermentation, and it is present in only low amounts in fruits and vegetables [10]. *9-cis* β-carotene, along with the *13-cis* and *15-cis* isomers found in food and naturally-occurring

47 substances, may serve an important function in human physiology that cannot be replaced by
48 synthetic β-carotene.
49 Microalgae are considered the richest sources of natural carotenoids, especially strains of the
50 chlorophyta such as *Dunaliella salina*, *Haematococcus pluvialis* and various *Chlorella* species [3]. The
51 content of lutein in marigold flowers for example is commonly reported to be 0.3 mg g^{-1}, but in
52 microalgae, the content can be over 4 mg g^{-1} [11]. *Dunaliella* strains are well known for being rich in
53 lutein, zeaxanthin and β-carotene [12] and *D. salina* has been particularly widely studied as it is the
54 richest source of natural β-carotene [13] and contains high content of the 9-cis isomer (~50% of the
55 total β-carotene) [14,15]. Other valuable carotenoids with potential medical value are also present in
56 *D. salina* including violaxanthin, antheraxanthin, zeaxanthin, α-carotene and lycopene [16]. The
57 genus *Dunaliella* contains a number of species and many strains which have been identified under
58 the same species possess various carotenogenic abilities and carotenoid compositions [17].
59 In this study, as part of the D-Factory, an EU funded project, three new strains of *Dunaliella*
60 species, DF15, DF17 and DF40 isolated from salt ponds in Israel and Spain were characterised. The
61 strains have been genetically identified as strains of *Dunaliella salina* but under different subgroups
62 (MBA, www.mba.ac.uk/culture-collection/). The biochemical properties of the strains were
63 examined in this study in comparison to the known carotene hyperaccumulator, *D. salina* UTEX
64 2538, also classified as *D. bardawil* in some studies [4] and *D. salina* CCAP19/30, which has been
65 found to be identical to a *D. tertiolecta* strain and does not accumulate β-carotene under stress [18], in
66 order to assess their potential for the commercial production of carotenoids and provide further
67 insight into carotenoid metabolism.

68 **2. Materials and Methods**

69 *2.1. Algal strains and cultivation*

70 *D. salina* UTEX 2538 was obtained from the UTEX Culture Collection (Austin, TX 78712 USA)
71 and CCAP 19/30 was obtained from the Culture Collection of Algae and Protozoa (CCAP, Scotland,
72 UK). D-Factory strains DF15 and DF17 were isolated from a salt pond in Israel, and DF40 was
73 isolated from a salt pond in Monzon, Spain. The new isolates were identified as strains of or closely
74 related to *Dunaliella salina (bardawil)* by The Marine Biological Association, UK (MBA) and are now
75 deposited at the MBA culture collection (www.mba.ac.uk/culture-collection/). Algae were cultured
76 in Modified Johnsons Medium [19] containing 10 mM $NaHCO_3$ with the pH value adjusted to 7.5
77 with 10 mM Tris-buffer, and 1.5 M NaCl, which has been tested as the optimal salinity for cell
78 growth of the strains. Cultures were maintained in a temperature controlled growth chamber at (20 ±
79 2) °C with illumination provided under a 12 h light, 12 h dark cycle (12/12 LD) by white LED lights
80 with a light intensity of ~200 μmol photons m^{-2} s^{-1}. Small stock cultures were grown to mid-log phase
81 and diluted 1 in 50 (v/v) as inoculum for larger cultures in each experiment.
82 For algal cultivation, Erlenmeyer flasks containing 500 ml culture each were maintained at 25
83 °C in an ALGEM Environmental Modeling Labscale Photobioreactor (Algenuity, UK) with strictly
84 controlled conditions of light, temperature and mixing level. Under 12/12 LD conditions, cell growth
85 under a range of light intensities (200, 500, 1000 and 1500 μmol m^{-2} s^{-1}) of white LED light were
86 compared. Each growth condition was set up at least in triplicate. Cell growth was monitored
87 automatically in the bioreactor by recording the value obtained for light scatter at 725 nm in OD
88 units. Cell concentration was determined by counting the cell number in culture broth using a
89 haemocytometer after fixing the cells with 2 % formalin. The maximum specific growth rate of all
90 cultures was calculated to compare cell growth under different conditions.

91 *2.2. Microscopy observations*

92 The Eclipse Ti-U inverted research microscope (Nikon, Japan) with a Nikon Digital Sight DS-Fi1
93 camera system was used to take brightfield microscopy photographs of cells of each *Dunaliella*
94 strain. The objective lens used was Nikon SPlan Fluor ELWD 60x/0.7 and the ocular lens was Nikon
95 CFI 10x/22. The NIS-Elements software was used to acquire the photos. Differential interference

contrast (DIC) microscopy photographs were also obtained using a confocal microscope system ZEISS LSM 880 (Carl Zeiss Microscopy, US). The ZEISS Plan Apochromat 63x/1.4 oil DIC objective lens and the Carl Zeiss PI 10x/23 ocular lens were used. Images were acquired and analysed through the ZEN 2.1 LSM software.

2.3 Algal biomass analysis

Algae grown under different light intensities were harvested during mid log phase of growth at the end of the light period. Pigments were extracted from the biomass harvested from 1 ml samples of the cultures using 1m of 80 % (v/v) acetone. The absorbance of the acetone extract after clarification at the centrifuge was measured at 480 nm for total carotenoids using a UV/Vis spectrophotometer. The content of total carotenoids was calculated according to Strickland & Parsons [20]. Chlorophyll a, b and total Chlorophyll were evaluated by measuring absorbance of the acetone extract at 664 nm and 647 nm and calculated according to Porra et al. [21].

The compositions of pigments extracted from different strains were analysed using HPLC with diode array detection (DAD). Carotenoid standards of *all-trans* α-carotene, *all-trans* β-carotene and zeaxanthin were obtained from Sigma-Aldrich. Lutein and 9-cis β-carotene were obtained from Dynamic Extractions (UK). Carotenoids and chlorophylls were extracted from freshly harvested cells using methyl tert-butyl ether (MTBE) and Methanol (20:80) as extraction solvent. 15 ml of algal culture was centrifuged at 3,000 g at 18 °C for 5 min and the pellet was extracted with 10 ml MTBE-MeOH (20:80) and sonicated for 20 s. The sample was clarified by centrifugation at 3,000 g at 18 °C for 5 min, then 1-2 ml of the supernatant was filtered through 0.45 µm syringe filter into amber HPLC vials. It was then analysed using a YMC30 250 X 4.9 mm I.D S-5µ HPLC column with DAD at 25 °C, and isocratic elution with 80 % methanol: 20 % MTBE, flow rate of 1 mL min^{-1}, pressure of 90 bar. The quantities of *9-cis* and *all-trans* β-carotene, *all-trans* α-carotene, lutein and zeaxanthin in the biomass were determined from the corresponding standard curves. Glycerol, known to be regulated by salinity, was determined according to the procedures described in a previous study [18].

2.4 Oxygen evolution and dark respiration

Cells were harvested during the exponential phase and $NaHCO_3$ was added to a final concentration of 10 mM 5 minutes before the start of each measurement. The rate of net O_2 evolution and dark respiration were measured as described by Brindley et al. [22] at 25 °C using a Clark-type electrode (Hansatech) [23]. O_2 evolution was induced with 1500 µmol photons · m^{-2} · s^{-1} actinic light. After initial 30 minutes of dark adaption, O_2 evolution was measured for 5 minutes followed by dark respiration for 20 minutes. The average net rate of photosynthesis was then determined from the oxygen concentration gradient recorded over 5 minutes, dO_2/dt. Dark respiration was determined by following the same procedure, except that oxygen uptake was calculated from data recorded during the last 5 minutes of the 20-min experiment. Sodium dithionite was used to calibrate the oxygen electrode.

2.5 Statistical analysis

The data generated in this study was analysed in R. A two-way ANOVA analysis was performed to study the relationships of a series of variables measured with two factors in this work: strain and light intensity. The two-way ANOVA tests three omnibus effects: the main effect of strain or light intensity, and the interaction effect between these two factors. Correlation analysis was used to evaluate the association between each pair of the variables and the Pearson correlation method was chosen to measure the linear dependence between two variables. In correlation analysis, a correlation coefficient (the Pearson Product Moment correlation coefficient) was estimated for each pair of the variables studied. Whether or not an observed correlation is statistically significant or not was evaluated by P values. Hierarchical cluster analysis is based on the strength of the correlations and the distance in the clustering dendrogram reflects the dissimilarity among these parameters.

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Traits examined with strong correlations are grouped as a cluster. A principle component analysis was carried out using the whole data set to reveal the relatedness between the examined traits.

3. Results

3.1 Cell growth

The work presented here shows biochemical and biophysical characterisation of the three newly isolated *D. salina* strains: DF15, DF17 and DF40 compared with *D. tertiolecta* CCAP 19/30 and *D. salina (bardawil)* UTEX 2538, cultured under a series of light intensities. Cultures of five *Dunaliella* strains: CCAP 19/30, DF15, DF17, DF40 and UTEX 2538, were each maintained under identical conditions of light of 100~200 µmol m^{-2} s^{-1} in the incubator until stationary phase, and cells were photographed using a light microscope and a confocal microscope. The five strains differed in cell shape, volume and colour from each other (Figure 1(**a**), (**b**)) and the cultures of each strain were differently coloured (Figure 1(**c**)). The four *D. salina* strains were orange in colour, while CCAP 19/30 maintained a green colour throughout.

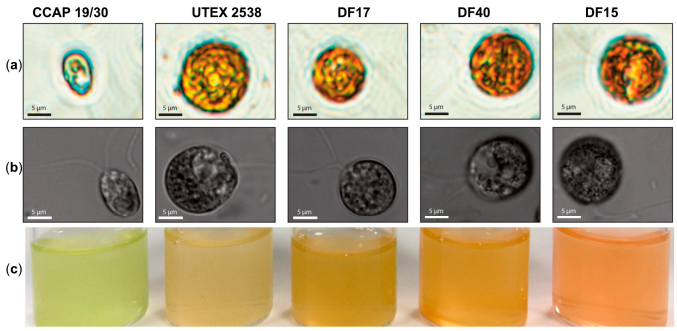
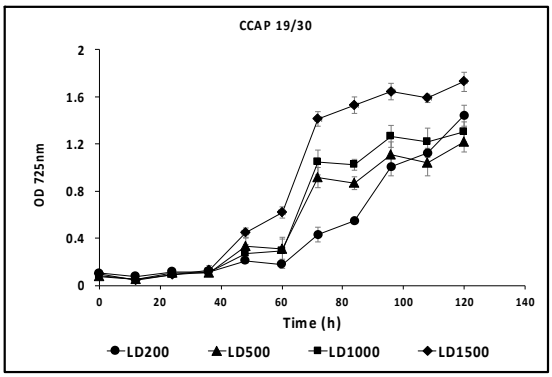
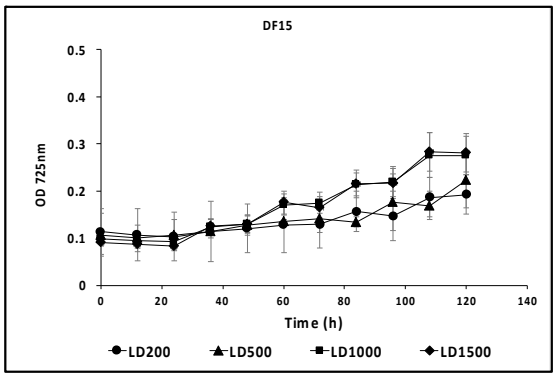


Figure 1. Microscopy observation of *Dunaliella* cells and photographs of stationary phase cultures of CCAP 19/30, UTEX 2538, DF17, DF40 and DF15 grown under a light intensity of 100~200 µmol m^{-2} s^{-1} at 20 °C. (**a**) Microscopy photographs taken through a light microscope (Nikon Eclipse Ti-U) with a magnification of 600x; (**b**) Differential interference contrast (DIC) microscopy photographs taken through a confocal microscope (ZEISS LSM 880) with a magnification of 630x. (**c**) Photographs of the cultures obtained for each *Dunaliella* strain grown under identical conditions.

Growth curves for the five strains cultivated under the same conditions of different light intensities of 200, 500, 1000 and 1500 µmol m^{-2} s^{-1} are shown in Figure 2, from which the maximum specific growth rate was calculated for each growth condition. Generally, these strains grew at a faster rate under higher light intensities. This is clearly shown for CCAP 19/30 and DF17. All strains showed the slowest growth rates under 200 µmol m^{-2} s^{-1} light intensity. In DF15 and UTEX 2538, when increasing the light intensity from 1000 µmol m^{-2} s^{-1} to 1500 µmol m^{-2} s^{-1}, no further improvement in cell growth rate was observed. It is likely that the optimal light intensity for fastest growth of DF15 or UTEX 2538 is around 1000 µmol m^{-2} s^{-1}, while 1500 µmol m^{-2} s^{-1} or higher is optimal for the other three strains. DF15 had the slowest growth rate and CCAP 19/30 the fastest.



(**a**)



(**b**)

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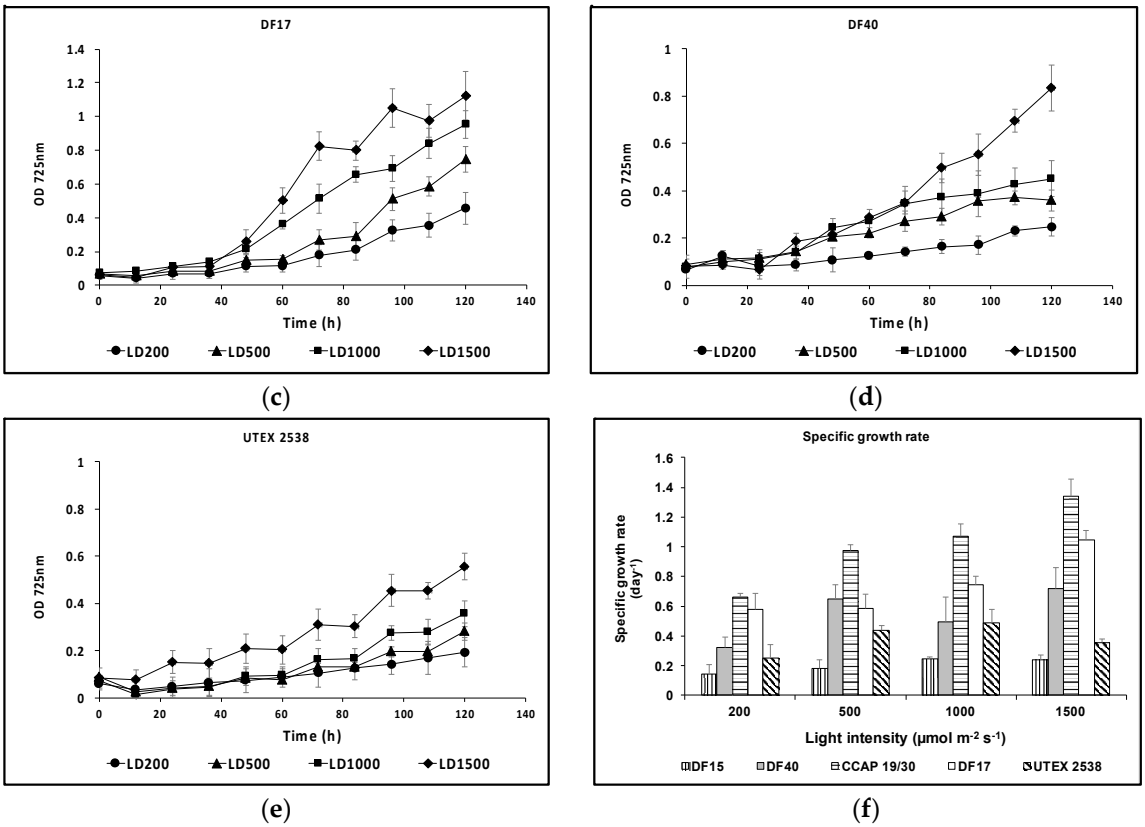
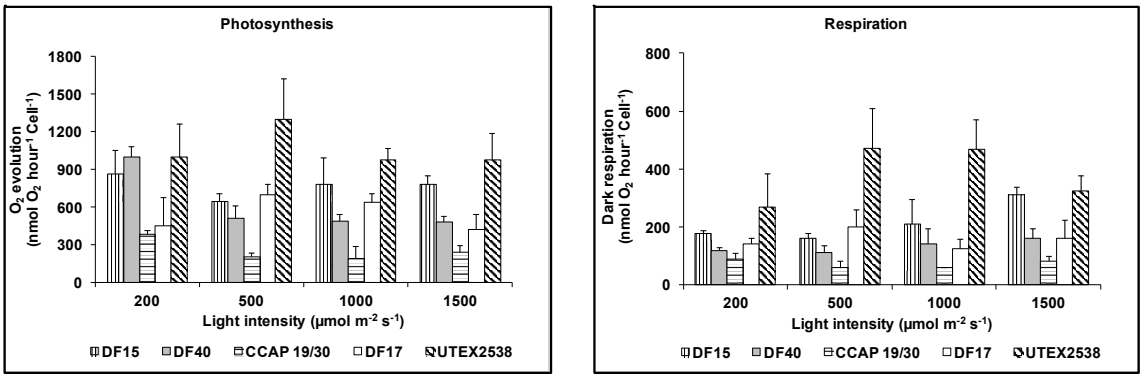


Figure 2. Growth curves for the five *Dunaliella* strains: (**a**) CCAP 19/30; (**b**) DF15; (**c**) DF17; (**d**) DF40; (**e**) UTEX 2538 each grown under four identical light intensities of 200, 500, 1000 and 1500 µmol m^{-2} s^{-1}; (**f**) specific growth rates of each strain grown under the four light intensities. Each culture condition was set up in triplicate.

3.2. Photosynthesis and respiration

Figure 3(**a**) shows that as the light intensity increased, the rate of photosynthesis decreased for DF17, DF40 and CCAP 19/30, indicating that these three strains are susceptible to photoinhibition. However, DF15 and UTEX 2538 did not exhibit photoinhibition with increase in light intensity, suggesting that these two strains have a more robust photoprotection mechanism. Figure 3(**b**) shows that the dark respiration rate patterns were similar for DF17, DF40 and CCAP 19/30. These three strains showed a slight decrease or no change in dark respiration rate with the increase in light intensity. DF15 and UTEX 2538 had a similar pattern to each other and their respiration rate increased slightly with increase in light intensity. From statistical analysis using two-way ANOVA, both strain difference and light intensity are significant factors affecting photosynthesis; less significant is the interaction between them. However, light intensity showed no significant impact on dark respiration but strain played a major role in the observed differences in dark respiration (Table 1).



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(**a**) (**b**)

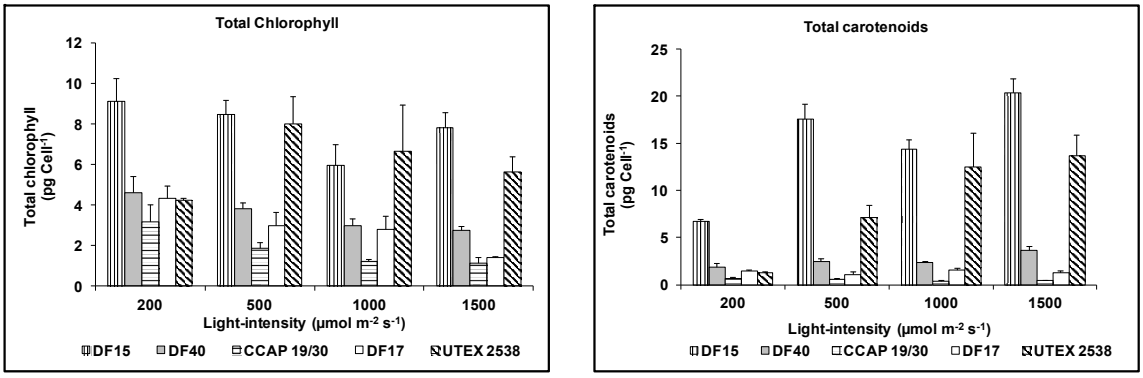
Figure 3. Photosynthesis (**a**) and respiration (**b**) of the five *Dunaliella* strains cultivated under four light intensities of 200, 500, 1000 and 1500 µmol m^{-2} s^{-1}. Samples were taken at the mid log phase and all culture conditions were repeated at least in triplicates.

Table 1. Two-way ANOVA analysis of the responses of all examined variables (photosynthesis, respiration, doubling time, *all-trans* β-carotene, *9-cis* β-carotene, glycerol, lutein, zeaxanthin, *all-trans* α-carotene, total carotenoids, total chlorophyll) to strain and light intensity and their interaction (Light intensity*Strain). The values of all observations were transformed by taking log function, square root function or reciprocal to fit linear models.

Response	Light intensity				Strain				Light intensity * Strain			
	Df	F	P		Df	F	P		Df	F	P	
Photosynthesis	3	8.1825	0.0002	***	4	71.2528	<2.2e-16	***	12	2.7966	0.0073	**
Respiration	3	1.7925	0.1641		4	52.7992	1.96e-15	***	12	2.4328	0.0176	*
Total carotenoids	3	2.9403	0.0446	*	4	693.560	< 2.2e-16	***	12	7.9749	2.52e-07	***
Total chlorophyll	3	36.529	1.55e-11	***	4	161.782	< 2.2e-16	***	12	10.285	8.41e-09	***
All-trans β-carotene	3	88.922	< 2.2e-16	***	4	474.255	< 2.2e-16	***	12	3.6878	0.0009	***
9-cis β-carotene	3	28.119	6.02e-10	***	4	730.574	< 2.2e-16	***	12	6.8407	1.67e-06	***
Lutein	3	7.3679	0.0005	***	4	118.762	< 2.2e-16	***	12	6.4955	3.08e-06	***
Zeaxanthin	3	35.542	2.31e-11	***	4	83.0526	< 2.2e-16	***	12	5.2669	3.13e-05	***
All-trans α-carotene	3	113.39	< 2.2e-16	***	4	408.180	< 2.2e-16	***	12	5.9987	7.64e-06	***
Glycerol	3	2.1170	0.1132		4	95.5589	< 2.2e-16	***	12	5.0858	4.50e-05	***

3.3 Pigment composition

Cellular contents of total chlorophyll and total carotenoids were determined for the five *Dunaliella* strains grown under the four light intensities (200, 500, 1000 and 1500 µmol m^{-2} s^{-1}) using UV/Vis spectrometry (Figure 4). Generally, the cellular content of total chlorophyll decreased while total carotenoids increased with the increase in light intensity for all five *Dunaliella* strains. Statistical analysis showed that strain difference significantly affected total carotenoids and total chlorophyll content, although total carotenoids and total chlorophyll content also responded significantly to light intensity (Table 1).



(**a**) (**b**)

Figure 4. Cellular content of total chlorophyll (**a**) and total carotenoids (**b**) of the five *Dunaliella* strains grown under four light intensities of 200, 500, 1000 and 1500 µmol m^{-2} s^{-1}. Samples were taken at the mid log phase and all culture conditions were repeated at least in triplicates.

HPLC-DAD was used to quantify the contents of major carotenoids, namely lutein, zeaxanthin, *all-trans* β-carotene, *9-cis* β-carotene, and *all-trans* α-carotene, in each strain acclimated in response to four light intensities, to understand the effect of light in carotenoid metabolism. Figure 5 shows HPLC chromatograms of the pigment extracts from the five *Dunaliella* strains grown under the light intensity of 1500 µmol m^{-2} s^{-1}. It is clear that CCAP 19/30 does not accumulate β-carotene even under

high light intensity. DF15, DF40 and UTEX 2538 have a similar pigment profile and β-carotene dominates the carotenoid composition. DF17 produced a higher relative amount of zeaxanthin under high light stress compared with the other strains, indicating the important role of zeaxanthin in DF17 for photoprotection.

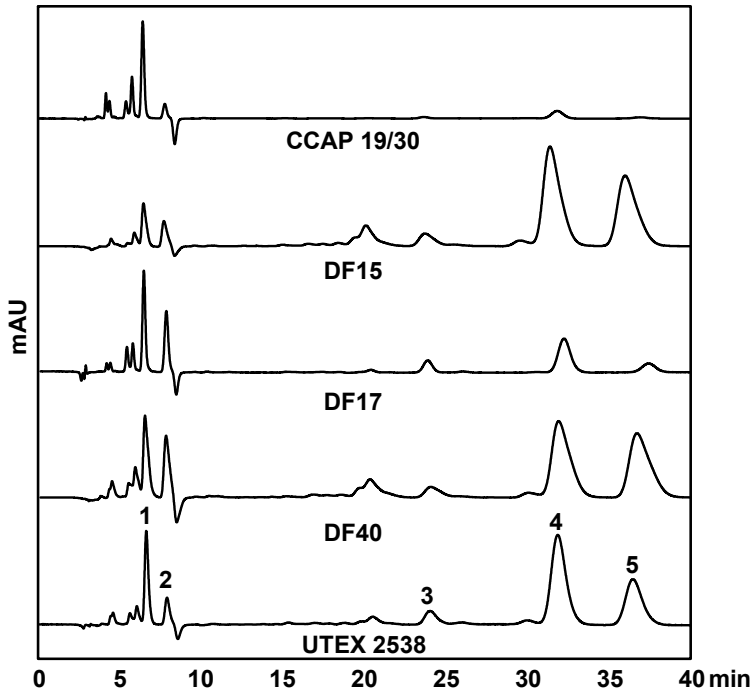


Figure 5. HPLC chromatograms of MTBE/ethanol extracts of the five *Dunaliella* strains cultivated under 1500 μmol m^{-2} s^{-1}. The major peaks shown are: (1) lutein, (2) zeaxanthin, (3) *all-trans* α-carotene, (4) *all-trans* β-carotene and (5) *9-cis* β-carotene.

The major difference between the strains was their ability to accumulate β-carotene. As shown in Figure 6(**a**) and 6(**b**), the contents of *all-trans*-β- and *9-cis* β-carotene increased with increasing light intensity in all five strains apart from UTEX 2538, which produced the highest cellular amount of *all-trans* or *9-cis* β-carotene under 1000 μmol m^{-2} s^{-1}. From the two-way ANOVA analysis, the cellular contents of *all-trans-* or *9-cis* β-carotene were found to vary significantly among strains and under different light intensities (Table 1). CCAP 19/30, DF17 and DF40 had similar responses to increasing light with a mild β-carotene accumulation, while DF15 and UTEX 2538 significantly increased β-carotene content with increasing light (Figure 6(**a**), (**b**)). DF15 and UTEX 2538 have significantly higher cellular contents of *all-trans-* or *9-cis* β-carotene than the other three strains and DF15 produces more β-carotene than UTEX 2538 under most of the light conditions. UTEX 2538, already known to be a massive carotene-accumulating strain [4], had a slightly faster growth rate than DF15 shown in Figure 2. On the other hand, DF15 accumulated a high carotene content even under low light intensity. In *Dunaliella*, variation in β-carotene content has been reported to correlate with the integral irradiance received during a division cycle and to be a specific mechanism of photoprotection [24], which may explain why DF15 has a slightly higher cellular content of β-carotene than UTEX 2538. DF15 has the advantage of accumulating a large amount of β-carotene even without light stress (Figure 5), therefore has great potential for the commercial production of β-carotene with less light energy input required.

The cellular content of lutein in the five *Dunaliella* strains grown under various light intensities is shown in Figure 6(**c**). Lutein, the most abundant xanthophyll in higher plants, is found in the light harvesting complexes in higher plants and also protects against photodamage. Its most important function is thought to be in quenching triplet chlorophyll ($^3Chl^*$) to prevent energy transfer to molecular oxygen and consequent formation of singlet oxygen $^1O_2^*$ [25], but it also quenches excited $^1Chl^*$ (NPQ) to prevent the formation of reactive oxygen species (ROS) under high light [26]. It also

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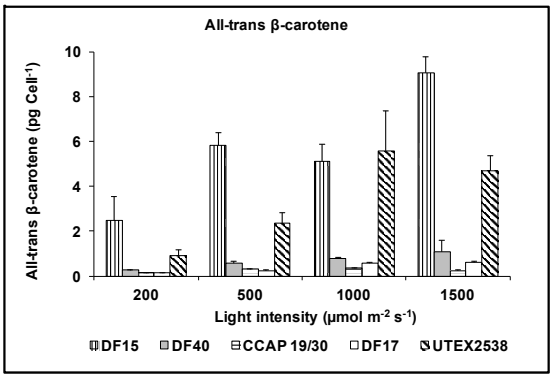
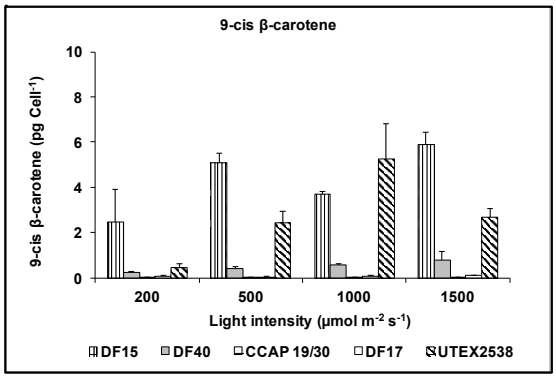
contributes to light harvesting, by transferring excitation energy to chlorophyll, and has a structural role associated with the antenna system [25], consequently the changes in antenna size due to photodamage under high light may also affect lutein content. Lutein in *D. salina* has been previously reported as a growth-coupled primary metabolite with a strong correlation with chlorophyll synthesis, but interestingly, not with light [27]. Shown in Figure 6(**c**), all strains accumulated considerably different amounts of lutein and the response to increasing light intensities varied among different strains. Lutein increased with light intensity from 200 μmol m^{-2} s^{-1} to 1000 μmol m^{-2} s^{-1} and then decreased when light increased to 1500 μmol m^{-2} s^{-1} in UTEX 2538. In DF15, lutein content did not change with light intensity from 200 μmol m^{-2} s^{-1} to 1000 μmol m^{-2} s^{-1} and only increased from 1000 to 1500 μmol m^{-2} s^{-1}. Both DF15 and UTEX 2538 accumulated significantly larger amounts of lutein under high light compared with the other strains. DF17 had the highest lutein content at 1000 μmol m^{-2} s^{-1} and lowest at 1500 μmol m^{-2} s^{-1}. Two-way ANOVA shows the cellular content of lutein is significantly affected by both the strain and light intensity.

Figure 6(**d**) shows that zeaxanthin content in all strains increased with light intensity. DF15 accumulated the highest amount of zeaxanthin, followed by DF17, UTEX 2538, DF40 and CCAP 19/30 accumulated the lowest amount. Zeaxanthin is linked to energy dissipation when excess light is absorbed via the xanthophyll cycle [25,28]. Zeaxanthin receives excess excitation energy from excited-state singlet chlorophyll ($^1Chl^*$) and dissipates it harmlessly and rapidly as heat in a process that is commonly assessed as non-photochemical quenching (NPQ) of chlorophyll fluorescence [25,28]. The carotenoids participating in this cycle are the only carotenoids present in the photosynthetic membrane that undergo very rapid, light-triggered concentration changes. High light induces de-epoxidation of violaxanthin and converts it into zeaxanthin, leading to its accumulation. This process is reversed in low light conditions. The accumulation of zeaxanthin in *Dunaliella* has been shown to parallel the accumulation of photodamaged PSII centers in the chloroplast thylakoids and decays with chloroplast recovery from photoinhibition [29]. In the present work, the increase in zeaxanthin content in high light, therefore shows that these strains have an efficient photoprotective mechanism also based on the xanthophyll cycle. Two-way ANOVA analysis (Table 1) shows that the factors of strain and light intensity determined the accumulation of zeaxanthin. Zeaxanthin accumulation was significantly different among strains and at different light intensities. Among the different strains, DF17 and UTEX 2538 had similar responses in terms of zeaxanthin accumulation.

The cellular content of *all-trans* α-carotene of the five strains grown under different light intensities is shown in Figure 6(**e**) and the cellular content of glycerol is shown in Figure 6(**f**). The content of *all-trans* α-carotene in DF15 or UTEX 2538 is much higher than that in the other three strains. α-carotene is the precursor of lutein but surprisingly α-carotene did not respond to light stress in the same way as lutein. *All-trans* α-carotene increased with the light intensity in all strains examined, and its response to increasing light intensity was very similar to the pattern of accumulation obtained for *all-trans* and *9-cis* β-carotene.



(**a**)



(**b**)

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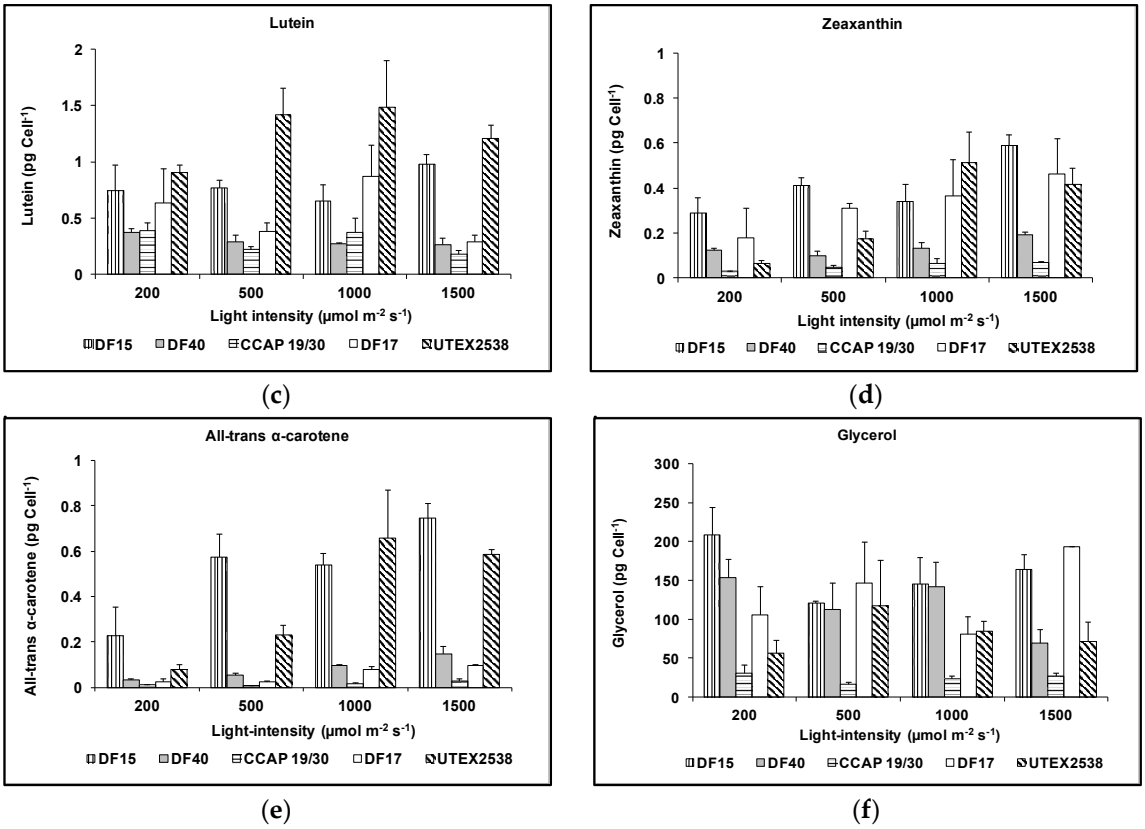

Figure 6. Cellular contents of (**a**) *all-trans* β-carotene, (**b**) *9-cis* β-carotene, (**c**) lutein, (**d**) zeaxanthin, (**e**) *all-trans* α-carotene and (**f**) glycerol in the five *Dunaliella* strains cultivated under four light intensities of 200, 500, 1000 and 1500 µmol m^{-2} s^{-1}. Samples were taken at the mid log phase and all culture conditions were repeated at least in triplicate.

3.4. Statistical analysis

Whilst the accumulated data permit elucidation of strain differences for carotenoid production, they also provided the opportunity to explore the use of statistical analysis to provide new insights into carotenoid metabolism coupled to the interdependent metabolic functions of photosynthesis and respiration. This was possible with the large set of data generated across five strains and four light intensities combined with tools of ANOVA analysis, correlation analysis and principal component analysis used in this study. With the quantitative data obtained for the five *Dunaliella* strains, statistical analysis was used as a tool in order to assess the strength of the correlations among the carotenoids and other cell growth parameters and examine the differences among the five strains. A correlation and clustering analysis was performed on the growth, photosynthesis and pigment data presented, to all five strains grown under four light conditions. The analysis was performed for each strain using all variables examined in this study (*all-trans* β-carotene, *9-cis* β-carotene, glycerol, lutein, zeaxanthin, *all-trans* α-carotene, photosynthesis, respiration, total carotenoids, total chlorophyll, and specific growth rate). Among them, glycerol is known to maintain osmotic balance in *Dunaliella* strains and as expected, the cellular content of glycerol would not respond to changes in light intensity, as shown in Figure 6(**f**). Glycerol content therefore was used to index the analysis.

The clustering dendrograph of the examined traits for each strain is shown in Figure 7 and depicts graphically several features of note amongst the strains. First, it shows that the individual carotenoids of *all-trans* β-carotene, *9-cis* β-carotene, zeaxanthin and *all-trans* α-carotene in the four *D. salina* strains are strongly correlated with each other but significantly not with lutein, except in CCAP 19/30. From this, it is clear that there is greater similarity between the four *D. salina* strains than with the CCAP19/30 strain, consistent with CCAP 19/30 not being a *D. salina* strain but instead, a *D. tertiolecta* strain [18]. Second, the dendrograph shows that accumulation of both *9-cis*

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β-carotene and *all-trans* β-carotene is positively correlated with photosynthesis over all light intensities for the *D. salina* strains, signifying a role for β-carotene in photoprotection. Third, lutein is not correlated closely with the other carotenoids but correlates more strongly with photosynthesis and respiration. This result suggests an important and not hitherto identified role for lutein in co-ordinated control of the cellular functions of photosynthesis and respiration in response to changes in light conditions, which is moreover broadly conserved in *Dunaliella* strains. Glycerol, which was not expected to change with light intensity, is weakly correlation with the different carotenoids in the *Dunaliella* strains as anticipated, but also correlates more closely with either photosynthesis or respiration.

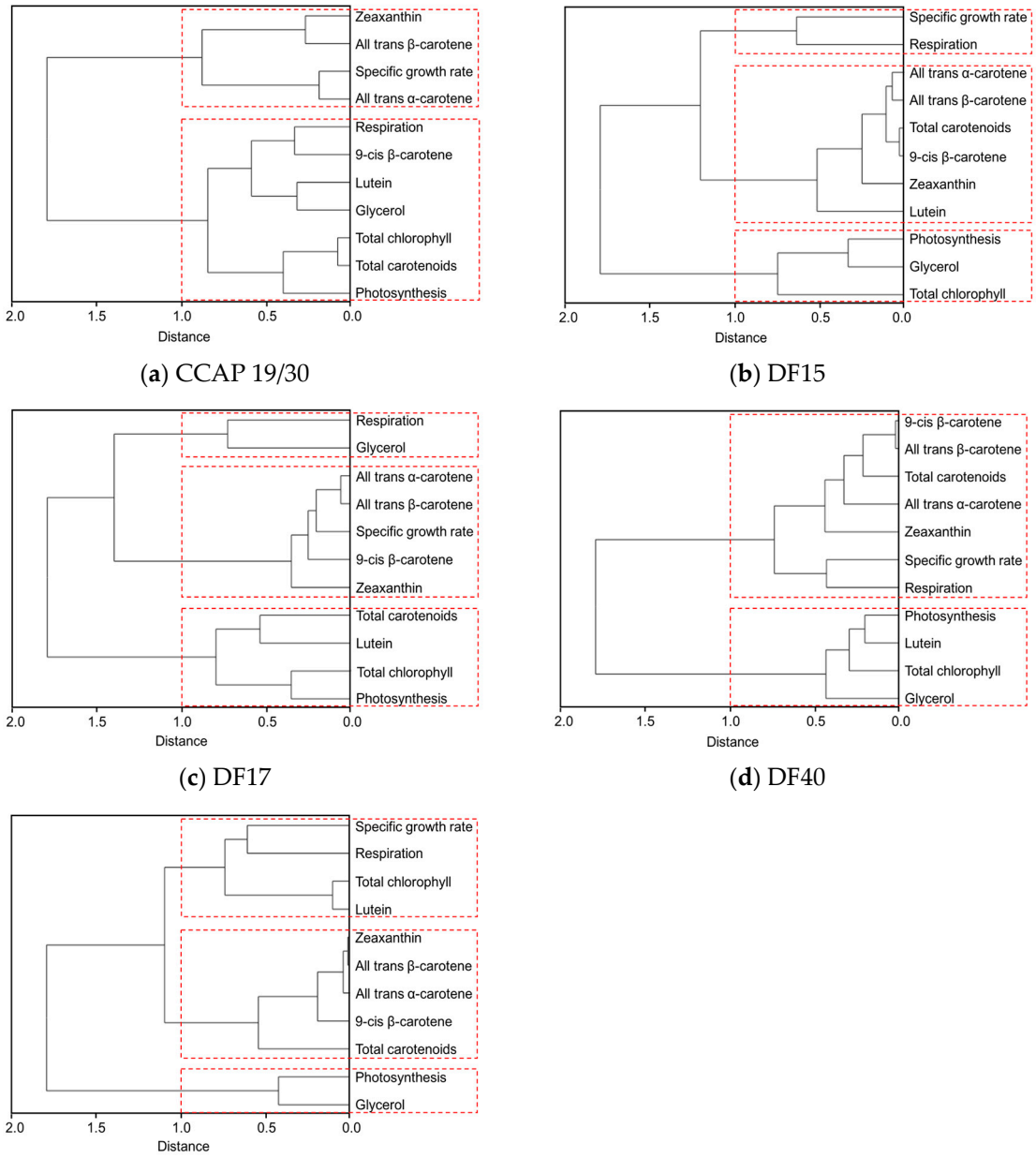

Figure 7. Cluster dendrograms of all-trans β-carotene, 9-cis β-carotene, glycerol, lutein, zeaxanthin, all-trans α-carotene, photosynthesis, respiration, total carotenoids, total chlorophyll for all five *Dunaliella* strains cultivated at four light intensities.

A principle component analysis was performed with all strains growing at all tested conditions as shown in Figure 8. The examined 11 traits can be roughly grouped into 4 groups as shown in the

graph, where *all-trans* β-carotene, *all-trans* α-carotene, *9-cis* β carotene and zeaxanthin were clustered closely, lutein, respiration and total chlorophyll were found in a second cluster, glycerol and photosynthesis were closely correlated, and the specific growth rate stands separately. The formation of two separate clusters of the carotenoids indicates two functionally distinct mechanisms for co-ordinated adaptation to changes in light conditions, broadly conserved between DF15, DF40, CCAP 19/30, DF17 and UTEX 2538. More importantly, it shows that DF17 and DF40 performed similarly under the tested environmental conditions; that DF15 is closely related to UTEX 2538, and that CCAP 19/30 is different compared to all the other strains.

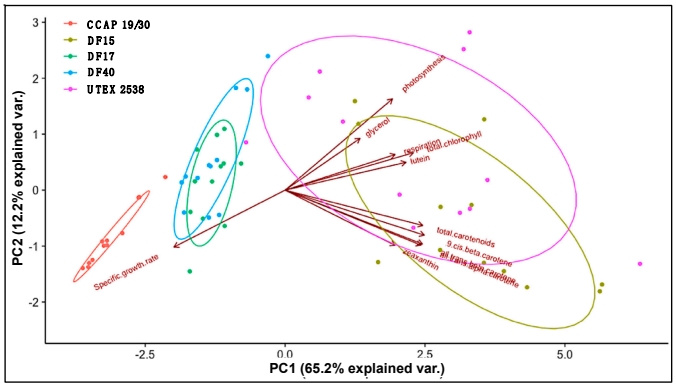

Figure 8. Principle component analysis of 11 traits (all-trans β-carotene, 9-cis β-carotene, glycerol, lutein, zeaxanthin, all-trans α-carotene, photosynthesis, respiration, total carotenoids, total chlorophyll and specific growth rate) for all five *Dunaliella* strains cultivated at four light intensities.

4. Discussion

In photosynthesis, light energy absorbed by the chlorophyll- and carotenoid-binding complexes of photosystem II is transferred to reaction centres to drive photochemistry. Excess light energy will cause light-induced damage of photosynthetic apparatus or photo-oxidative damage. Photosynthetic organisms have evolved a robust repair mechanism to replace the photodamaged photosystems; however, when the rate of photodamage exceeds the repair cycle, photosynthetic efficiency will be impaired [30]. Based on this study, it is apparent that CCAP 19/30, DF17 and DF40 are susceptible to photoinhibition, while photosynthetic efficiency of DF15 and UTEX 2538 was not affected by high light and was maintained high over all light intensities studied, suggesting they have developed better photoprotective mechanisms against light stress.

Carotenoids are variously involved in harvesting light for photosynthesis as well as preventing photoinhibition under high light stress. Exposure to white light is associated with generation of ROS, which have been shown to replace light in the induction of hyper-accumulation of carotenoids [31]. β-carotene is also associated with photoprotection and most of the beneficial effects of β-carotene is attributed to its ability to prevent oxidation processes by quenching $^1O_2$* once formed, or terminating free radical chain reactions as a result of the presence of the polyene chain, with *9-cis* β-carotene being a better scavenger of free radicals than *all-trans* β-carotene [32]. DF15 and UTEX 2538, which showed no evidence of photoinhibition with increase in light intensity, also accumulated large amounts of carotenoids, especially β-carotene, compared to the other strains.

The fact that DF15 and UTEX 2538 accumulated very large amounts of β-carotene over all light intensities is noteworthy. The accumulation of β-carotene in *D. salina* when exposed to high light mainly occurs in the β-carotene plastoglobuli, while the thylakoidal β-carotene content remains relatively unchanged [33,34]. These plastoglobuli have also been shown to contain many enzymes found in the eyespot of other flagellate algae [35,36]. However, most of the proteins that are required for the eyespot function are no longer found in *Dunaliella* and no eyespot structural elements could be found in *Dunaliella* [37]. This suggests that the plastoglobuli were once components of a functional eyespot of *Dunaliella*. The β-carotene in the eyespot probably played a crucial role in perception of light, but once it lost its function, the non-functional eyespot acted as a β-carotene

storage compartment. It is possible therefore that both DF15 and UTEX 2538 accumulated very large amounts of β-carotene in a vestigial eye-spot.

Statistical analysis tools used here have been able to reveal the correlative relationships between different carotenoids (lutein, zeaxanthin, *all-trans* and *9-cis* β-carotene and α-carotene) and the relationships between carotenoids and photosynthesis and respiration. In particular, they have identified a strong positive correlation of lutein with photosynthesis and respiration (Figure 7 and Figure 8). In humans, lutein influences brain function through a variety of mechanisms that are not well understood, but its accumulation in brain mitochondria has been proposed to protect these organelles from oxidative damage [38]. Lutein also specifically accumulates in the retina of the eye and has been linked with protection against mitochondrial stress and with mitochondrial biogenesis [39]. In plants there is a close interdependence between respiration and photosynthesis for the flow of ATP, NAD(P)H and carbon skeletons such that excess photosynthetic reducing equivalents formed by photosynthesis in light can be removed in mitochondrial respiration to reduce the tendency for ROS accumulation and photoinhibition [40] and thereby regulate the NAD(P)H: oxygen ratio to avoid cell death [41]. The clustering of lutein, photosynthesis, chlorophyll and respiration reported here attests to the strong interdependence between respiration and photosynthesis to regulate the redox state of the cell [40], and in *Dunaliella* both are linked to lutein accumulation.

Figure 9 shows the pathway for the synthesis of key carotenoids in this study. Significantly the data presented here show that α-carotene is linearly correlated with β-carotene but not lutein, although α-carotene is the precursor of lutein. This finding points to additional interactions involved in the synthesis of lutein, which are linked specifically to chlorophyll synthesis. Moreover, the positive correlation between β-carotene and zeaxanthin may suggest a proportional partitioning of β-carotene into the xanthophyll cycle and the β-carotene plastoglobuli, which is consistent with the idea that two complete pathways for β-carotene biosynthesis exist in *D. barwawil*, one in the chloroplast membranes for the biosynthesis of β-carotene and one in the plastoglobuli for the accumulation of β-carotene [34]. It is possible to conclude that *D. salina* strains have evolved coordinated universal photoprotection mechanisms for the maintenance of high efficiency under high light stress by accumulating carotenoids, in particular β-carotene. However, the effectiveness of these mechanisms varies greatly between strains and therefore the potential for β-carotene production varies among strains.

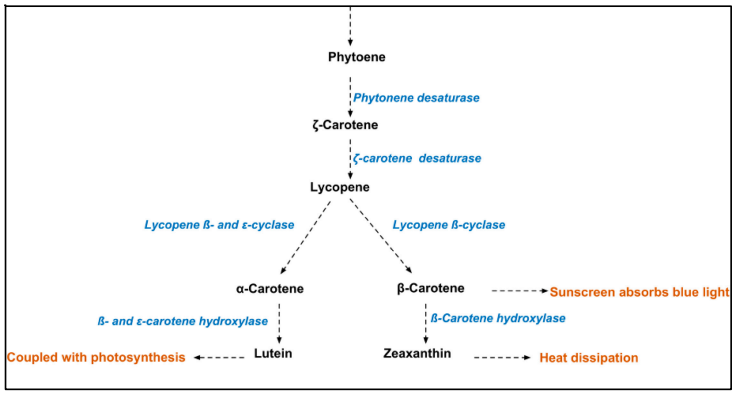

Figure 9. Carotenoids pathway showing synthesis of lutein, β-carotene, α-carotene and zeaxanthin from phytoene in *D. salina* [42]. Metabolites of carotenoids are shown in black bold and enzymes are displayed in italics.

Finally, it is noteworthy that the statistical analysis based on the data obtained from the biochemical characterisation suggests a grouping of the five strains into three different groups: (1) DF15 and UTEX 2538; (2) DF17 and DF40; and (3) CCAP 19/30 as shown in Figure 8. However, genetic classification using the approaches of bar coding shows a higher similarity between DF40 and UTEX 2538, and therefore groups the five strains into four different groups: (1) DF40 and UTEX 2538, (2) DF17, (3) DF15 and (4) CCAP 19/30 as shown in the phylogenetic tree provided by Dr.

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Declan Schroeder at The Marine Biological Association, UK [43] (Figure 10). This indicates the complicity of strain classification in *Dunaliella* by using a single classification method and the importance of strain selection for the commercial production of *Dunaliella* biomass and natural β-carotene.

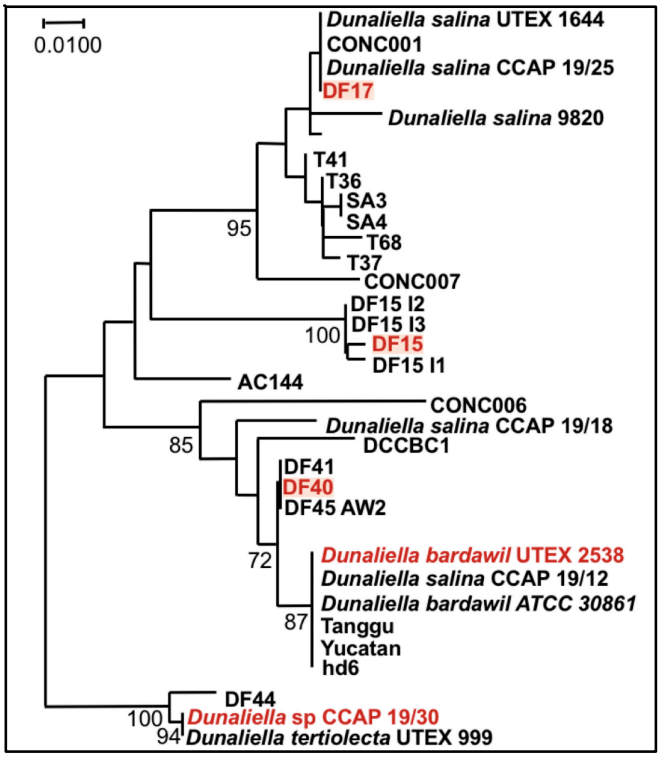

Figure 10. Phylogenetic tree showing the location of the three newly isolated *Dunaliella* strains (DF15, DF17 and DF40) compared to CCAP 19/30 and UTEX 2538 used in this study [43].

5. Conclusion

This study shows how strain difference plays a significant role in the accumulation of carotenoids in *D. salina*. Carotenoid content increased with the increase of light intensity and contributed to photoprotection against photodamage. Cellular contents of *all-trans* β-carotene, *9-cis* β-carotene, *all-trans* α-carotene and zeaxanthin, but not lutein, were closely correlated with each other, signifying synthesis of these carotenes and zeaxanthin along a metabolic pathway that is under common control. Significantly a strong correlation between lutein and respiration in photosynthetic cells was identified; there was also a strong relationship between lutein, photosynthesis, chlorophyll and respiration. Among the three newly isolated *D. salina* strains, DF15 produced a significantly higher (> 5-fold) content of β-carotene over different light intensities compared to DF17 or DF40, despite the fact that they are all strains of *D. salina*. Physiological study on the biochemical performance of the new isolated strains shows a different grouping strategy to that obtained from genetic classification. The data demonstrate the importance of strain selection from a number of *Dunaliella* strains based on their biochemical performance for the commercial production of β-carotene.

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Author Contributions: Y.X., I.I., A.B and P.H conceived and designed the experiments; Y.X., I.I. and C.W performed the experiments; Y.X. and I.I. analyzed the data; P.H contributed reagents/materials/analysis tools; Y.X. I.I. and P.H. wrote the paper.

Conflicts of Interest: The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

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