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Two-stage cultivation of *Chlorella vulgaris* using light and salt stress conditions for simultaneous production of lipid, carotenoids, and antioxidants

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Abstract

The effects of light and salt stress (NaCl and $MgCl_2$) on the two-stage cultivation of *Chlorella vulgaris*, with or without a medium replacement, for the simultaneous production of lipid, carotenoids, and antioxidant compounds were investigated. The highest lipid productivity (15.59 ± 0.10 mg L^{-1} day^{-1}) was obtained at 5 g L^{-1} $MgCl_2$ and 140 μmol photons m^{-2} s^{-1} light intensity. The saturated fatty acids (SFA) ranged from 52.35–81.64%, monounsaturated fatty acids (MUFA) from 7.38–34.26%, and polyunsaturated fatty acids (PUFA) from 7.25–25.10%, with palmitic (C16:0), stearic (C18:0), and oleic (C18:1) acids as predominant fatty acids. Under high light intensity and nitrogen limitation in the two-stage cultivation, supplementation of 10 g L^{-1} NaCl with a medium replacement caused a marked increase in the total carotenoids (4.37 ± 0.33 μg mL^{-1}). Cultivation of *C. vulgaris* in a medium containing 5 g L^{-1} NaCl or Mg Cl_2, with or without a medium replacement step and with exposure to 140 μmol photons m^{-2} s^{-1} light intensity, led to enhanced antioxidant activities (65–79%). The different levels of antioxidant activities of the *C. vulgaris* extracts suggested the variation in the phytochemical compounds, a result of the stressed conditions.

Keywords *Chlorella vulgaris* · Two-stage cultivation · Salt stress · Lipid · Carotenoids · Phytochemical compounds

Introduction

Microalgae are the major producers of lipids, essential fatty acids, carbohydrates, hormones, recombinant proteins, and pigments such as the chlorophylls and carotenoids in the biotechnology and food industries. Therefore, microalgal cultivation is an attractive option for biochemical production with diverse applications in bioenergy, bioremediation, and nutraceutical and biopharmaceutical industries (Borowitzka and Moheimani 2013; Khan et al. 2018; Grubišić et al. 2019; Abdullah and Hussein 2020). Microalgae (Chlorophyta) are superior to the land plants (Streptophyta) as efficient sources of biofuels and bioproducts as they can produce neutral lipids in large quantities per unit area, exceeding the yield of the best oil-seed crops (Khan et al. 2018; Aratboni et al. 2019). Both sequester CO_2 through photosynthesis and reduce greenhouse gas emissions, but unlike land plants, microalgae can be cultured in high salinity or brackish water and can utilize wastewater as nutrient sources (Khan et al. 2018). The growth of microalgae can be enhanced in photobioreactors for higher biomass production (Pierre et al. 2019) by downstream processes optimized to harvest the bioproducts (Abdullah et al. 2016, 2017).

Stress involves the disruption of homeostasis as a result of a stressor application. The stress responses are the changes in the cell metabolism as the cells acclimatize and attempt to restore the homeostasis. The different stages in stress responses are alarming stage, regulation, acclimation, and adaptation (Borowitzka 2018). Stress strategies have been adopted to enhance high-value compounds production using single stress factor such as the nutritional factors (e.g., carbon source,

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nitrogen, phosphorus), environmental factors (high light intensities, temperature, pH, salinity, reactor configurations, and operating conditions) (Sun et al. 2014; Shah and Abdullah 2018; Zappi et al. 2019; Li et al. 2020). Salt stress causes several biochemical and bioenergetic alterations such as increased rates of lipid biosynthesis and enhanced biopolymer and energy production, changes in membrane permeability with ion homeostasis interruption (Alyabyev et al. 2011), and elevated level of reactive oxygen species (ROS). As a response to the increasesd ROS level, microalgae may accumulate antioxidant compounds such as polyphenols, flavonoids, and carotenoids to quench the free radicals (Edge and Truscott 2010; Wani et al. 2016). Nitrogen-limiting conditions can trigger lipid synthesis in some algal species by reducing cell division and shifting the lipid biosynthetic pathways to synthesize more neutral lipids than membrane lipids (Vitova et al. 2015). Light of suitable wavelengths and intensity can also be the key factors that affect or control the biomass and lipid production in micralgae (Sajjadi et al. 2018). The manipulation of light intensities alters the types and levels of secondary metabolites such as phenolics and flavonoids, which consequently influence antioxidant activities. There are strong correlations between the total phenolic and flavonoid contents with the antioxidant activities (Karimi et al. 2013; Ali et al. 2014).

The biomass or lipid production is significantly affected by the mode of cultivation (Ryu et al. 2019). Fed-batch and continuous mode may promote cell growth but do not necessarily lead to high lipid productivity (Ho et al. 2014) unless stressful conditions are adopted throughout the cultivation to induce product formation. However, this at the end may be at the expense of the cell growth. In plant cell culture, intermediary and production medium strategies adopted have resulted in increased hydrogen peroxide level but concomitantly with significant enhancement of cell dry weight, products of interest, and antioxidant compounds (Chong et al. 2004; Abdullah et al. 2005). The implementation of an appropriate cultivation strategy can therefore increase the biomass production with the high-value products. One of the most effective strategies is the cultivation of microalgae through the two-stage cultivation strategy (TSCS) (Aziz et al. 2020). The TSCS and semi-continuous mode could achieve high biomass with simultaneous enhanced lipid yield (Narala et al. 2016). The TSCS typically consists of the biomass production, pre-harvesting, and product induction stages, making it possible to separate the biomass growth phase (vegetative stage) from the lipid accumulation phase (stress stage) during microalgal cultivation (Aziz et al. 2020). Biomass production is enhanced under optimal culture conditions in the first phase (Johnson et al. 2018) and in the second phase microalgae may be subjected to one or more stressors including physical stimuli (temperature, light intensity) and chemical stimuli (nutrient limitations or additions).

A number of large-scale TSCSs have been successfully implemented to produce high-value metabolites (Su et al. 2017; Chew et al. 2018), such as the TSCS developed by Aquasearch to produce both oil and astaxanthin from the green microalga *Haematococcus pluvialis* in photobioreactors and open ponds (Schenk et al. 2008). However, the high costs and energy in transferring the vegetative cells from nutrient-rich (phase I) to nutrient-deficient (phase II) media are the main obstacles in attaining the commercial scale of production based on the TSCS. Hence, modifications or simplifications of this step can considerably increase the economic feasibility of the TSCS (Aziz et al. 2020). To overcome this drawback, direct addition of inducers such as NaCl to the growth medium (Xia et al. 2013), or plant hormones (Wu et al. 2018), has been investigated. The incorporation of more than one inducer may lead to an increase in the lipid accumulation and other high-value metabolites (Sun et al. 2014). However, the effects of some of these inducers in most microalgae strains are not known.

The objective of this study was to attain enhanced simultaneous production of lipid, carotenoids, and antioxidant compounds in *Chlorella vulgaris* under combined stress factors involving nutrient (nitrogen), high light intensity, and salt stress (NaCl and $MgCl_2$) in a two-stage cultivation strategy, with or without a medium replacement step. The kinetics of cell growth and product formation were evaluated, and the fatty acids profile was analyzed by the GC analysis.

Materials and methods

Microalgal culture conditions

The experimental design and culture conditions of the two-stage cultivation strategy of *Chlorella vulgaris* are shown in Fig. 1 and Table 1. The *C. vulgaris* culture was maintained in standard conditions at 25 ± 1 °C in BG-11 medium (Stanier et al. 1971), under fluorescent white light (Philips, TLD18W/54-765) at 40 μmol photons m^{-2} s^{-1}, with constant bubbling of air (filtered through a 0.22 μm microporous filter).

Stress treatments

Chlorella vulgaris cells with optical density (OD_{680} nm) of 1.6 were inoculated into BG-11 medium (10% of the total culture medium) in 1 L Erlenmeyer flasks, and incubated under standard conditions for 15 days (vegetative stage) (Table 1 and Fig. 1). In the stress stage the vegetative cultures were divided into 3 groups: (i) group 1, where the cultures were in the same medium under 40 μmol photons m^{-2} s^{-1} light for 15–20 days and designated control 1 (nutrient stress); the cultures were transferred in the same medium under 140 μmol photons m^{-2} s^{-1} for 20 days and designated control 2 (light

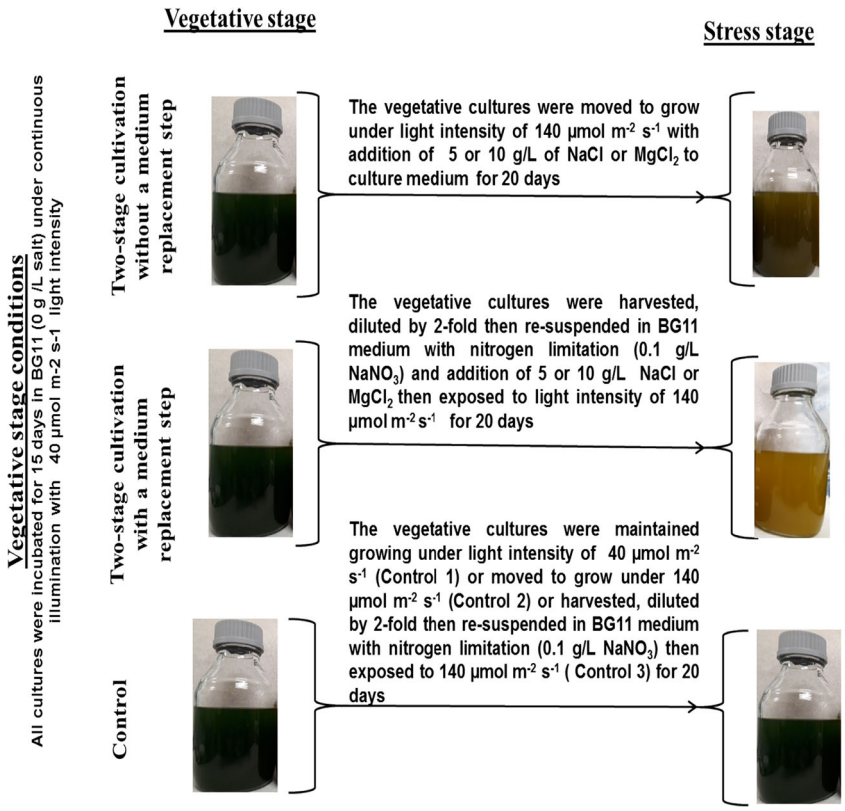

Fig. 1 Experimental design of two-stage cultivation strategy. Vegetative stage refers to *C. vulgaris* cells cultured under optimal conditions. Stress stage refers to *C. vulgaris* cells cultured under light and salt stress conditions

stress); transferred for exposure to 140 µmol photons m^{-2} s^{-1} and starvation medium for 20 days and designated control 3 (nutrient + light stress); (ii) group 2, where the flasks were exposed to 140 µmol photons m^{-2} s^{-1} with 5 or 10 g L^{-1} of NaCl or $MgCl_2$ addition and designated C1/2/5/6 (control 2 + salt); and (iii) group 3, where the cultures were harvested, washed, and diluted by 2-fold (OD_{680} = 0.76), and then re-suspended in BG-11 medium, with nitrogen limitation (0.1 g L^{-1} $NaNO_3$) and the addition of 5 or 10 g L^{-1} of NaCl or $MgCl_2$, and exposed to 140 µmol photons m^{-2} s^{-1} light intensity, designated as C3/4/7/8 (control 3 + salt). Cultures were incubated at 25 ± 1 °C for 20 days with constant bubbling of air (filtered through 0.22 µm filter).

Cell growth measurements

Cell growth was determined in both the vegetative and stress stages based on 3 mL sample removal every 5 days, by measuring the OD_{680} nm (Hsieh and Wu 2009).

The dry weight (DW) was evaluated gravimetrically. After centrifugation of 20 mL of culture, the pellets were collected and washed two times using deionized water, dried overnight at 80 °C, and then cooled and weighed. The biomass productivity (*BP*, mg L^{-1} day^{-1}) and biomass yield (*BY*, mg L^{-1}) were determined according to Hempel et al. (2012) and Vidyashankar et al. (2015), respectively as follows:

$$BP = \frac{(X_f - X_0)}{t} \quad (1)$$

$$BY = (X_f - X_0) \quad (2)$$

where X_f and X_0 are the final and initial biomass concentrations (g L^{-1}), respectively; t is the duration of the run (day).

Determination of lipid content

Lipids were extracted based on the modified Bligh and Dyer method (1959). Dried biomass (0.5 g) was added to a mixture of chloroform and methanol at the 1:1 ratio (v/v), and heated in a microwave for 1 min. Water was added to achieve the final ratio of chloroform, methanol, and water at the 1:1:0.9 ratio (v/v). The lipid-containing chloroform layer at the bottom of the separating funnel was removed, washed with 5 mL of 5% NaCl, and left to a constant weight in an oven at 60 °C. The lipid dry weight was determined:

$$L = \frac{W_L}{W_B} * 100 \quad (3)$$

where L is the lipid content (%); W_L and W_B are the weights of the extracted lipids (mg L^{-1}) and the dry biomass (mg L^{-1}), respectively.

The lipid productivity (*LP*) was calculated as follows (Hempel et al. 2012):

Table 1 Culture conditions in the two-stage cultivation strategy of *C. vulgaris*

Conditions	Stages	
	Vegetative stage	Stress stage
Control 1	The cultures were grown for 15 days in BG11 (0 g L^{-1} salt) under continuous illumination with 40 μmol photons m^{-2} s^{-1} light intensity	The cultures were continuously grown under the vegetative stage condition for 20 days
Control 2		The cultures were transferred and exposed to 140 μmol photons m^{-2} s^{-1} light intensity for 20 days
Control 3		The cultures were harvested and diluted by 2-fold, re-suspended in BG11 medium (0.1 g L^{-1} $NaNO_3$), and exposed to 140 μmol photons m^{-2} s^{-1} light intensity
C1		The cultures were transferred and exposed to 140 μmol photons m^{-2} s^{-1} light intensity, with 5 g L^{-1} NaCl addition, for 20 days
C2		The cultures were transferred and exposed to 140 μmol photons m^{-2} s^{-1} light intensity, with 10 g L^{-1} NaCl addition, for 20 days
C3		The cultures were harvested and diluted by 2 fold, re-suspended in BG11 medium (0.1 g L^{-1} $NaNO_3$) with 5 g L^{-1} NaCl addition, and exposed to 140 μmol photons m^{-2} s^{-1} light intensity
C4		The cultures were harvested and diluted by 2 fold, re-suspended in BG11 medium (0.1 g L^{-1} $NaNO_3$), with 10 g L^{-1} NaCl addition, and exposed to 140 μmol photons m^{-2} s^{-1} light intensity
C5		The cultures were transferred and exposed to 140 μmol photons m^{-2} s^{-1} light intensity, with 5 g L^{-1} $MgCl_2$ addition, for 20 days
C6		The cultures were transferred and exposed to 140 μmol photons m^{-2} s^{-1} light intensity, with 10 g L^{-1} $MgCl_2$ addition, for 20 days
C7		The cultures were harvested and diluted by 2 fold, re-suspended in BG11 medium (0.1 g L^{-1} $NaNO_3$) with 5 g L^{-1} $MgCl_2$ addition, and exposed to 140 μmol photons m^{-2} s^{-1} light intensity,
C8		The cultures were harvested and diluted by 2 fold, re-suspended in BG11 medium (0.1 g L^{-1} $NaNO_3$), with 10 g L^{-1} $MgCl_2$ addition, and exposed to 140 μmol photons m^{-2} s^{-1} light intensity

$$LP = BP*L \quad (4)$$

where LP is the lipid productivity (mg L^{-1} day^{-1}), BP is the biomass productivity (mg L^{-1} day^{-1}), and L is the lipid content (% dry weight).

The lipid yield was determined as follows (Yang et al. 2014):

$$LY = BY*L \quad (5)$$

where LY is the lipid yield (mg L^{-1}), biomass yield, BY (g L^{-1}), and lipid content, L (% dry weight).

Transesterification and fatty acid analyses

The fatty acid methyl esters (FAMEs) of the extracted lipids were analyzed according to Mandal et al. (2013). The analyses were performed using gas chromatography (Agilent 6890, Model G1530A, USA), with flame ionization detector and a DB-5 silica capillary column (60 m × 0.32 mm i.d.). The oven temperature was initially set at 45 °C to reach 60 °C at 1 °C min^{-1}, before finally programmed from 60 to 240 °C at 3 °C min^{-1}. Helium was the carrier gas at 1 mL min^{-1} flow rate. The injector temperature was 240 °C (starting at 150 °C for 1 min, ramping to 240 °C at 30 °C min^{-1}, and held at 240 °C for 30 min).

Pigment extraction and quantification

The determination of chlorophylls and carotenoids was according to Lichtenthaler and Wellburn (1983). Culture samples (10 mL) were centrifuged at 2500 rpm for 5 min and the supernatant was discarded. Ten milliliter of methanol (96%) was added and the cells were re-suspended by homogenizing at 1000 rpm for 1 min. The residue was re-extracted several times until the solvent became colorless. The homogenate was centrifuged at 2500 rpm for 10 min, the supernatants collected and finally topped up to 25 mL with 96% methanol. The absorbance between 400 and 700 nm was read where the maximum absorbance of chlorophyll *a*, chlorophyll *b*, and total carotenoids was shown at 666 nm, 653 nm, and 470 nm, respectively. The concentration of each pigment (μg mL^{-1}) was calculated as follows (Lichtenthaler and Wellburn 1983):

$$\textit{Chlorophyll a (Chl a)}\left(\mu g\ mL^{-1}\right) = 15.65\ (A_{666}) - 7.34\ (A_{653}) \quad (6)$$

$$\textit{Chlorophyll b (Chl b)}\left(\mu g\ mL^{-1}\right) = 27.05\ (A_{653}) - 11.2(A_{666}) \quad (7)$$

$$\textit{Total carotenoids}\ \left(\mu g\ mL^{-1}\right) = \frac{[1000\ (OD_{470}) - 2.86\ (Chl\ a) - 129\ (Chl\ b)]}{245} \quad (8)$$

where A_{653}, A_{666}, and A_{470} nm are the absorbance at the indicated wavelengths.

Preparation of algal extracts

One gram of freeze-dried microalgal biomass was extracted with 100 mL mixture of methylene chloride and methanol at the 1:1 ratio (v/v) for 40 min at room temperature. The extraction was repeated three times. The supernatant was collected, filtered, and evaporated using rotary vacuum evaporator (40–45 °C). The amount of extractable substances (crude extracts) was determined in mg g^{-1} DW.

Antioxidant assay

DPPH radical scavenging activity

The free radical scavenging activities of crude methanol:methylene chloride (1:1) extracts were determined (Yen and Chen 1995). Two milliliters of 2,2-diphenyl-1-picrylhydrazyl (DPPH) solution in methanol (0.16 mM), considered as a control, was mixed with 2 mL of extracts or the standard (butyl hydroxyl toluene (BHT) and vitamin C) at 200 μg mL^{-1} concentration. The mixture was kept for 30 min in the dark at room temperature. The absorbance was later measured at 517 nm, and the radical scavenging activity was calculated:

$$\text{Antioxidant activity}\ (\%) = \left(\frac{A_c - A_t}{A_c}\right) * 100 \quad (9)$$

where A_t and A_c represent the absorbance of the samples and the DPPH control, respectively.

ABTS radical cation scavenging activity

The potency of the extracts to scavenge 2,2′-azino-bis ethylbenzthiazoline-6-sulfonic acid (ABTS) radical cation was evaluated. The mixture was prepared by adding 7 mM ABTS solution with 2.45 mM potassium persulfate (1:1, v/v). The mixture was left for 4–16 h until the formation of the free radical and the absorbance became stable around 0.700 ± 0.05 at 734 nm using ethanol for dilution (Re et al. 1999). A total of 100 μL of the standard (BHT or vitamin C) or tested extracts at 200 μg mL^{-1} was mixed with 900 μL of ABTS for 45 s. The decreasing absorbance was determined after 1 min at 734 nm and the antioxidant activitiy determined:

$$\text{Antioxidant activity}\ (\%) = \left(\frac{A_c - A_t}{A_c}\right) * 100 \quad (10)$$

where A_t and A_c represent the absorbance of the samples and the ABTS control, respectively.

For IC_{50} determination, the 2-fold dilution of the sample from 500, 250, 125, 62.5, 31.25, 15.65 to 7.81 μg mL^{-1} concentrations were used.

Statistical analysis

The experiments were carried out in three replicates. The significant difference of variables was determined using one-way ANOVA with 95% confidence (probability limit of $p < 0.05$). The Tukey's test was used to identify the differences between each level of treatment. The statistical analyses were performed using Minitab software (V18, Minitab Inc., USA).

Results

Cell growth and lipid productivity

Figure 2 a and b show that all cultures exhibited no significant differences in the growth rate in the vegetative stage, but showed significant variation in the stress stage, based on the different level of salt stressors and light intensity. The control 1 and 2 cultures maintained the highest biomass growth, followed by the C5 and C6 as well as C1 conditions. The lowest cell growth was observed in C3, C4, C7, and C8, attributable to the lower inoculum density ($OD_{680} = 0.76$) from the 2-fold dilution carried out and the combined stress conditions of nitrogen deficiency (0.1 g L^{-1} $NaNO_3$), high salt stress (5 and 10 g L^{-1} NaCl or $MgCl_2$), and high light intensity (140 μmol photons m^{-2} s^{-1}). Table 2 shows that the *C. vulgaris* productivities (C1–C8) for 35 days of cultivation period were affected ($p < 0.05$) by the type and concentration of salt stress and light intensity as well as the cultivation strategy. The biomass productivity was high when the vegetative cells of *C. vulgaris* was transferred to grow under 140 μmol photons m^{-2} s^{-1} light intensity, with the addition of 5 or 10 g L^{-1} of NaCl (C1, C2) or $MgCl_2$ (C5, C6). The highest biomass productivity (67.43 ± 0.143 mg $L^{-1}day^{-1}$) and biomass yield (2.36 ± 0.05 g L^{-1}) were obtained under single-stage cultivation (control 1). Interestingly, the biomass productivity of cultures supplemented with $MgCl_2$ (C5–C6) was higher than the cultures supplemented with NaCl (C1–C4). The maximum lipid productivity (15.59 ± 0.10 mg $L^{-1}day^{-1}$) and lipid yield (560.76 ± 3.48 mg L^{-1}) were achieved with 5 g L^{-1} $MgCl_2$ addition and exposure to

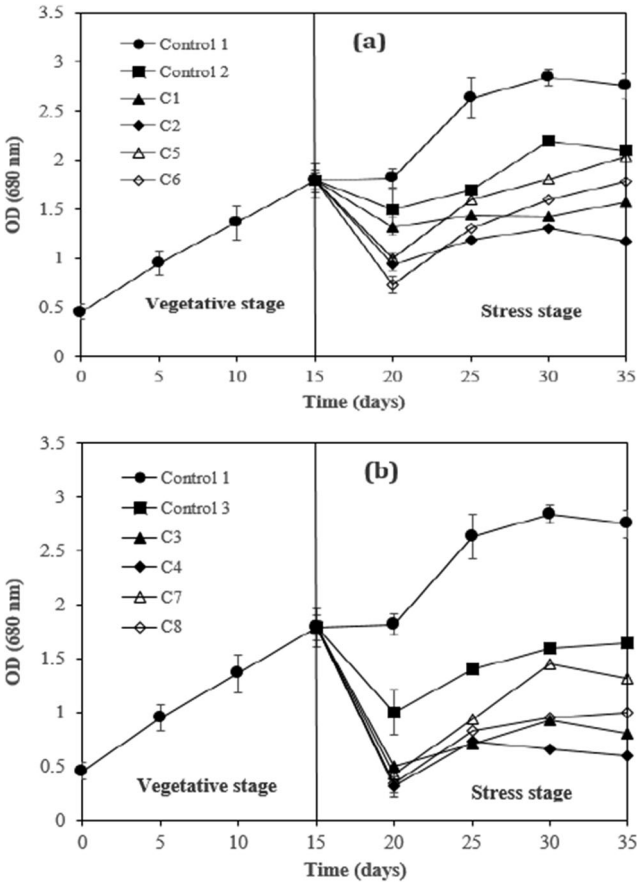

Fig. 2 Growth curve of *C. vulgaris* cultured under salt stress conditions (NaCl and $MgCl_2$), in a two-stage cultivation strategy. Error bars represent ± standard deviation of three replicates

140 μmol photons m^{-2} s^{-1} light (C5). The lipid content increased up to 37.80 ± 0.80% when the culture was re-grown in a medium with 10 g L^{-1} NaCl addition and 140 μmol photons m^{-2} s^{-1} light (C4).

Fatty acid profile

The fatty acids consisted of saturated (SFA) (52.35–81.64% of total FAME), monounsaturated (MUFA) (7.38–34.26% of total FAME), and polyunsaturated fatty acids (PUFA) (7.25–25.10% of total FAME) (Fig. 3). The highest SFA content (81.64%) was recorded in C7 when the culture was transferred to a medium containing 5 g L^{-1} $MgCl_2$ with N-limitation. High SFA was also noted in control 2 (82.05%) under light stress; control 1 (78.22%) under single stage cultivation; and control 3 (77.9%) with similar conditions to C7 but without salt stress. The other conditions exhibited SFA in the range of 52–74%. For MUFA, there was a noticeable increase in C5 (34.26%), followed by C6 (22.2%), C8 (18.57%), C3 (18.50%), C1 (16.88%), C2 (15.92%), and C4 (15.06%). In contrast to SFA, the MUFA contents were lower in C7 (7.38%) and the controls (10.07–12.96%). The PUFA in all salt stress conditions also recorded significant increase, reaching a maximum in C6 (25.10%) and others (10.1–14.15%), as compared to the controls (7.75–9.26%). Table 3 suggests that the relative percentage of fatty acids was strongly affected by the variations in the salt stress conditions. The main fatty acid component is palmitic acid (C16:0) at 11.80–41.65%, achieving the highest level under C7 (41.65%), C2 (36.4%), and C8 (26.76%). Stearic acid (C18:0) (10.70–37.90%) and oleic acid (C18:1) (3.93–17.81%) were other major components in all salt stress conditions. Optimal balance between the SFA (55.43%), MUFA (34.26%) and PUFA (10.32%) composition was attained in C5, when the cultures were supplemented with 5 g L^{-1} $MgCl_2$, under high light intensity.

Chlorophyll and carotenoid production

The chlorophyll *a* and total carotenoid concentrations were simultaneously increased in the vegetative stage (Fig. 4). The highest chlorophyll *a* content was observed in the control

Table 2 Kinetics of cell growth and lipid production of *C. vulgaris* cultured under different salt stress conditions in a two-stage cultivation strategy. Results represent mean ± standard deviation of three replicates. Different small letters indicate significant difference ($p < 0.05$)

Culture condition	Biomass productivity (*BP*) (mg L^{-1} day^{-1})	Biomass yield (*BY*) (g L^{-1})	Lipid productivity (*LP*) (mg L^{-1} day^{-1})	Lipid content (*L*) (%)	Lipid yield (*LY*) (mg L^{-1})
Control 1	$67.43 \pm 0.1.43^{a}$	2.36 ± 0.05^{a}	5.78 ± 0.12^{b}	8.58 ± 0.58^{f}	206.21 ± 4.32^{b}
Control 2	51.91 ± 1.30^{b}	1.82 ± 0.05^{b}	4.78 ± 0.12^{c}	9.20 ± 0.12^{e}	171.12 ± 4.22^{c}
Control 3	34.96 ± 1.65^{e}	1.22 ± 0.06^{e}	3.35 ± 0.16^{d}	9.60 ± 0.23^{e}	121.6 ± 5.54^{d}
C1	35.43 ± 0.82^{e}	1.24 ± 0.03^{e}	4.46 ± 0.10^{c}	12.60 ± 0.60^{d}	161.70 ± 3.63^{c}
C2	$29.06 \pm 0.1.5^{f}$	1.02 ± 0.05^{f}	4.53 ± 0.23^{c}	15.60 ± 0.60^{c}	165.36 ± 8.25^{c}
C3	17.24 ± 0.172^{h}	0.60 ± 0.03^{g}	1.42 ± 0.06^{e}	8.24 ± 0.24^{f}	53.28 ± 2.07^{e}
C4	13.81 ± 0.59^{i}	0.48 ± 0.02^{h}	$5.22 \pm 0.0.23^{b}$	37.80 ± 0.80^{a}	199.09 ± 7.89^{b}
C5	44.77 ± 0.28^{c}	1.57 ± 0.01^{c}	15.59 ± 0.10^{a}	34.83 ± 0.76^{b}	560.76 ± 3.48^{a}
C6	39.53 ± 0.71^{d}	1.38 ± 0.03^{d}	3.31 ± 0.06^{d}	8.37 ± 0.37^{f}	119.41 ± 2.29^{d}
C7	$21.72 \pm 0.0.44^{g}$	0.76 ± 0.02^{g}	3.33 ± 0.06^{d}	15.0 ± 0.50^{c}	120.50 ± 2.29^{d}
C8	19.34 ± 0.86^{g}	0.68 ± 0.03^{g}	0.56 ± 0.02^{f}	2.80 ± 0.50^{g}	20.16 ± 0.84^{f}

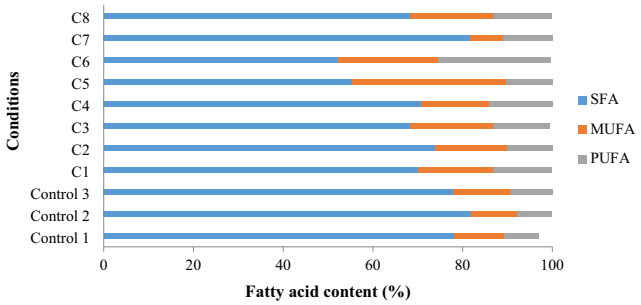

Fig. 3 Grid graph bar showing boundaries of all fatty acid components of *C. vulgaris* cultured under different salt stress conditions in a two-stage cultivation strategy. SFA saturated fatty acids (C8:0–C18:0); MUFA monounsaturated fatty acids (C16:1–C20:1); and PUFA polyunsaturated fatty acids (C18:2–C18:3)

group, having adequate nutrients in the medium but devoid of light or salt stress. During the stress stage (carotenogenesis), the total carotenoid levels increased under all salt stress and light conditions, while chlorophyll *a* started to decline until day 35, except in control 1 (Fig. 4). The C4 condition, where the culture was supplemented with 10 g L^{-1} NaCl and with a medium replacement step, recorded the lowest biomass productivity, but the highest carotenoid level (4.37 ± 0.33 μg mL^{-1}) and also the highest lipid content attained (Table 2).

Antioxidant activities

Figure 5 illustrates that the highest antioxidant activities were exhibited, based on the DPPH and ABTS methods, respectively, in C1 ($68.15 \pm 1.05\%$, $67.24 \pm 1.09\%$), C3 ($75.34 \pm 2.18\%$, $65.59 \pm 1.76\%$), and C5 ($71.27 \pm 1.8\%$, $78.76 \pm 2.35\%$). These were only slightly lower than the standards BHT ($87.63 \pm 1.55\%$, $91.82 \pm 1.68\%$) and vit. C ($88.8 \pm 1.8\%$, $93.2 \pm 1.93\%$), respectively. Based on the DPPH method, the control conditions exhibited the lowest activities (46.25 ± 1.67–$51.34 \pm 1.016\%$), and almost comparable to C2, C4, and C6–8. The C1, C3, and C5 culture extracts, respectively, exhibited the IC_{50} of 25.56, 23.12, and 24.44 μg mL^{-1} (as compared to 11.2 μg mL^{-1} for BHT, 12.9 μg mL^{-1} for vit. C) based on the DPPH; and 25.91, 26.56, and 22.12 μg mL^{-1} (as compared to 15.1 μg mL^{-1} for BHT, 14.7 μg mL^{-1} for vit. C) based on the ABTS method. Phytochemical screening showed the presence of considerable amounts of phenolic compounds, flavonoids, sterols, terpenoids, tannins, and glycosides (data not shown) in the extracts of C1, C3, and C5 cultures which were cultivated in low salt concentration at 5 g L^{-1} of NaCl and $MgCl_2$, with and without medium replacement step.

Discussion

Environmental stressors such as high salinity and light intensity are important strategies to enhance lipids and/or carbohydrates, or high-value products such as carotenoids and bioactive compounds in marine and freshwater microalgae (Ishika et al. 2017). However, high lipid production under salinity stress is often associated with cell growth retardation, resulting in lower biomass (Aziz et al. 2020). The TSCS based on salinity stress also may involve pre-harvesting step in between

Table 3 Fatty acid profile of extracted lipids from *C. vulgaris* cultured under different salt stress conditions in a two-stage cultivation strategy

Types of fatty acids	Fatty acid (%)										
	Control 1	Control 2	Control 3	C1	C2	C3	C4	C5	C6	C7	C8
Caprylic acid (C8:0)	8.30	9.02	7.90	nd	2.86	nd	1.72	Nd	nd	3.57	1.06
Capric acid (C10:0)	9.02	9.23	8.85	nd	nd	nd	2.06	Nd	nd	3.74	1.04
Undecanoic acid (C11:0)	10.50	10.20	10.63	nd	14.8	2.50	4.79	Nd	1.09	3.85	1.40
Lauric acid (C12:0)	9.53	9.98	9.29	nd	3.27	0.96	12.10	0.87	1.70	5.15	2.96
Myristic acid (C14:0)	8.02	8.33	7.90	1.68	nd	1.72	1.91	Nd	1.18	3.87	1.06
Pentanoic acid (C15:0)	4.89	4.96	4.73	1.89	1.77	1.60	2.57	Nd	1.49	3.93	nd
Palmitic acid (C16:0)	13.20	12.30	11.80	23.00	36.40	21.2	25.10	23.50	22.0	41.60	26.70
Palmetoleic acid (C16:1)	4.87	4.89	4.23	5.39	6.07	8.20	5.05	13.90	9.49	3.45	7.71
Heptadecanoic acid (C17:0)	2.83	3.33	2.50	5.63	4.18	5.12	4.53	7.16	7.89	nd	6.66
Stearic acid (C18:0)	14.70	14.60	14.30	37.90	10.70	35.10	16.00	23.90	17.00	15.8	27.5
Oleic acid (C18:1)	6.20	5.21	5.96	10.70	9.85	9.66	10.00	17.80	12.60	3.93	10.80
Linoleic acid (C18:2)	5.73	5.70	6.16	10.00	10.10	10.90	11.80	7.95	11.10	3.32	nd
Linolenic acid (C18:3)	2.02	2.06	3.10	2.95	nd	1.60	2.35	2.36	14.00	7.66	12.90
Eicosenoic acid (C20:1)	nd	nd	2.50	0.79	nd	0.65	nd	2.50	nd	nd	nd

nd not detected

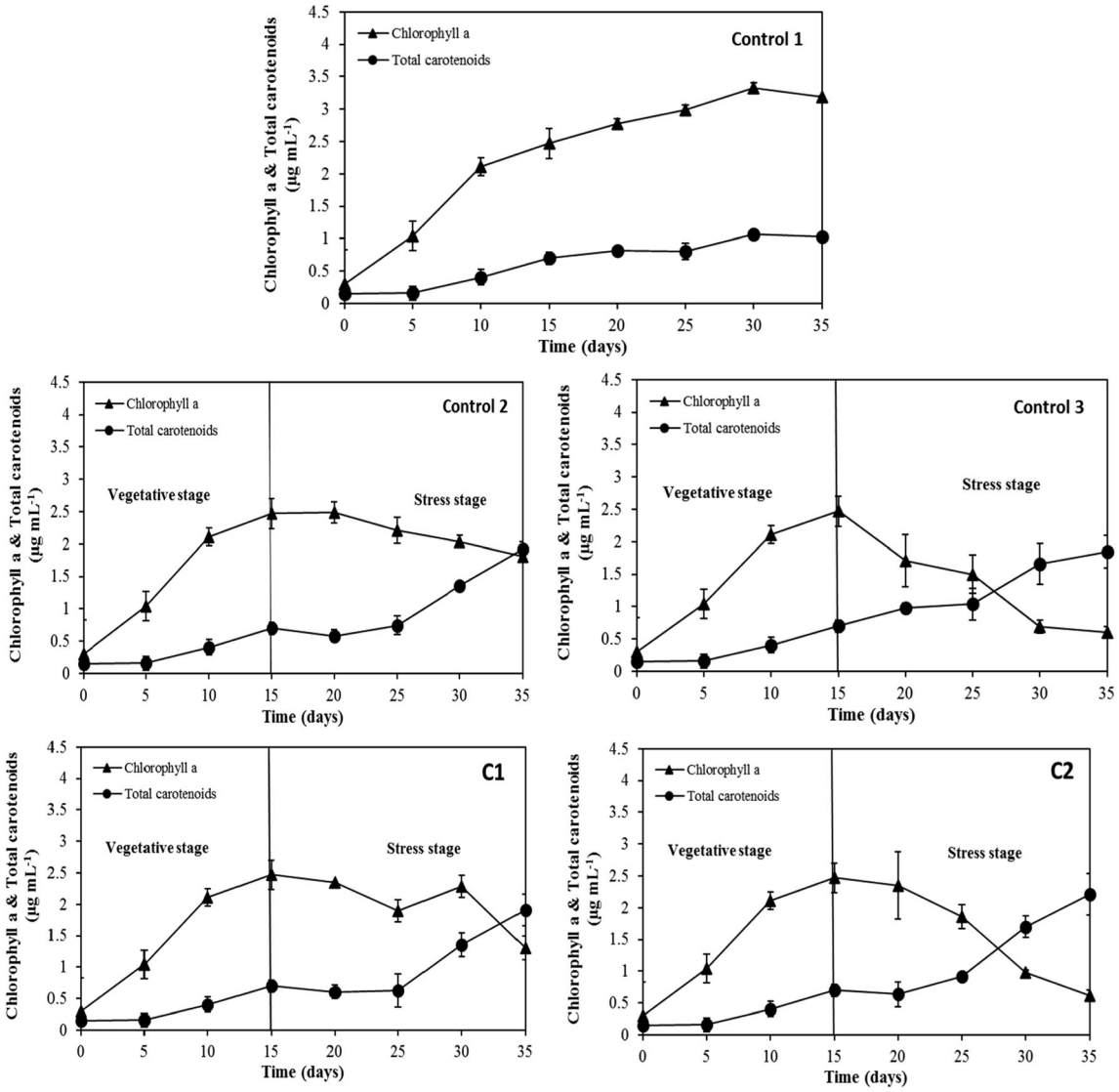

Fig. 4 Chlorophyll *a* and total carotenoid production of *C. vulgaris* under different NaCl and $MgCl_2$ stress conditions in a two-stage cultivation strategy. Error bars represent ± standard deviation of three replicates

the first and the second stages (Kakarla et al. 2018; Sajjadi et al. 2018). Eliminating or modifying this step could lower the operating cost and increase the TSCS economic feasibility (Narala et al. 2016). In our study, the effect of combined stress factors (salt stresses and light intensity) in the TSCS of *C. vulgaris* with or without a medium replacement aimed to achieve the economic viability of microalgal cultivation, with simultaneous production of lipid, carotenoids, and antioxidant compounds. The results clearly showed that the TSCS, salt stress (type and concentration), and the light intensity significantly affected the growth and lipid accumulation. The highest lipid productivity was recorded when *C. vulgaris* was cultured using TSCS without a medium replacement (C5) (Table 2). Similar results have been obtained where the lipid production in *Scenedesmus obtusus* XJ-15 is improved by direct addition of NaCl into the growth medium, which is more effective than transferring the vegetative cells from the nutrient-replete to nutrient-depleted media (Xia et al. 2013).

The lipid content in microalgal cells can be enhanced by a single inducer such as nitrogen starvation (Rehman and Anal 2019), phosphate limitation (Yu et al. 2016), high salinity (Shah and Abdullah 2018; Gour et al. 2020), high light intensities (He et al. 2018), or by combining the different stress factors (Sun et al. 2014). Hyper-accumulation of lipid content is generally achieved by combining these stimuli in the cultivation process (Guedes et al. 2011), such as combining the physical stress factors and nutrient starvation (Singh et al. 2016), but this may be at the expense of cell growth (Yu et al. 2016). The high light intensity in combination with salt stress in our study had led to an increase in the lipid yield of *C. vulgaris* (C5), higher than control 2 which was exposed to only light intensity factor in the stress stage. The TSCS of

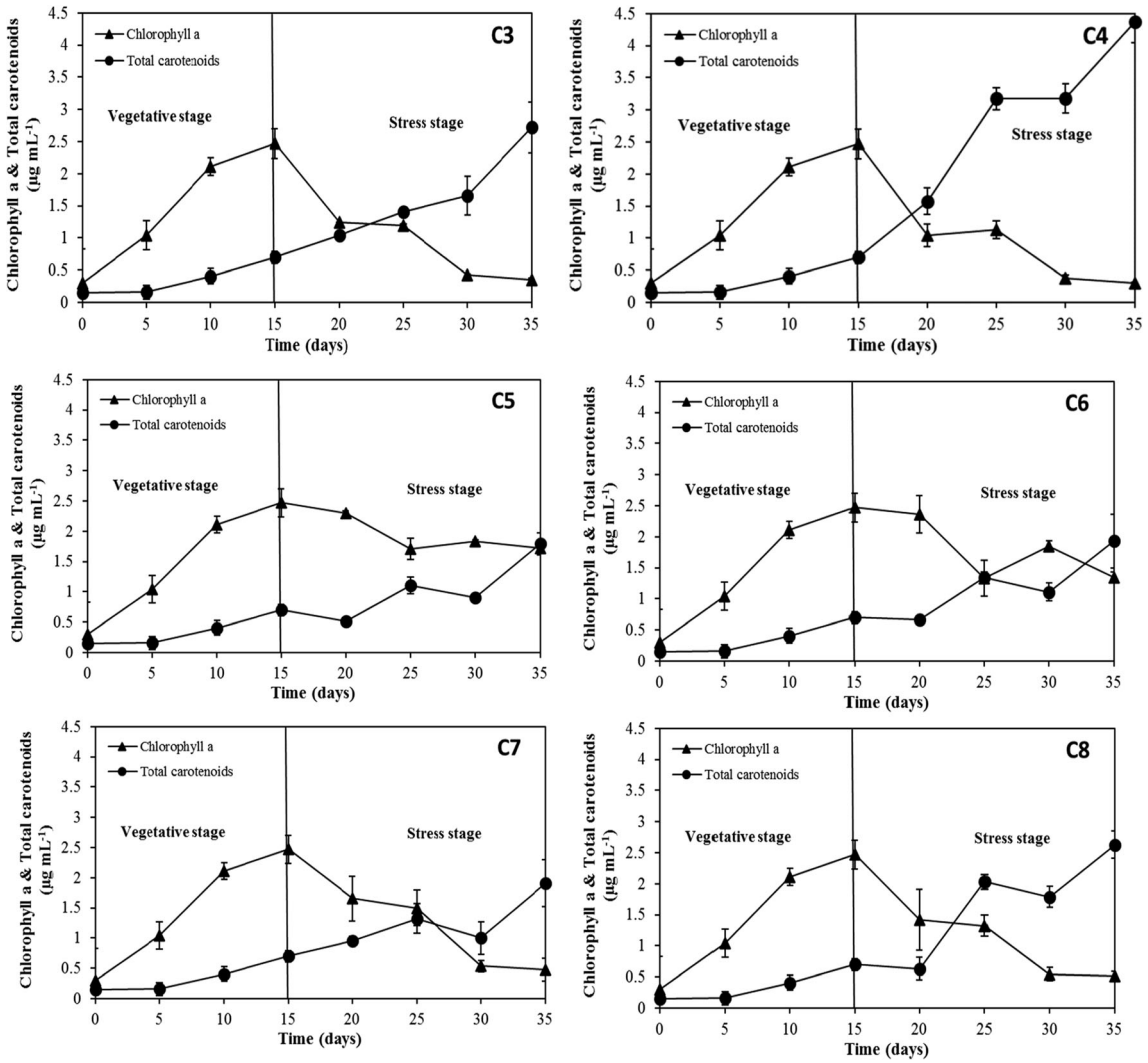

Fig. 4 (continued)

Neochloris oleoabundans HK-129 has been investigated where in the first stage, maximal biomass productivity is achieved under optimal nutrient, and in the second stage, the effects of combined nitrogen starvation, high light intensity, and high concentration of iron (Fe^{3+}) on lipid accumulation are carried out. The biomass/lipid productivities obtained under nitrogen starvation suggest that the effects of light intensities are superior to the effects of Fe^{3+} (Sun et al. 2014). Similarly, the combined two stress factors have been found to increase the lipid productivity (25–54%), higher than the

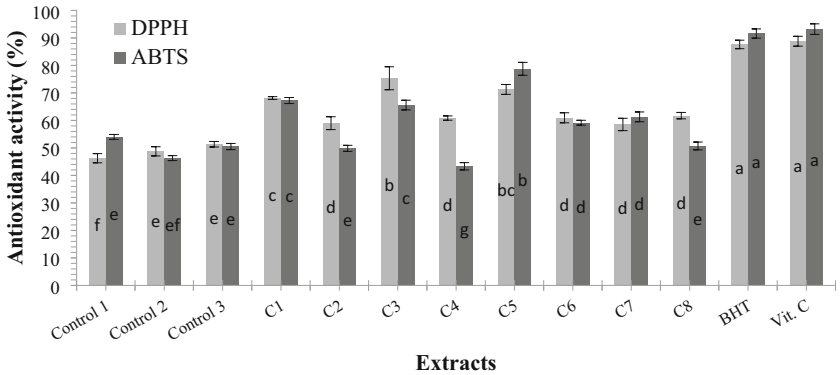

Fig. 5 Antioxidant activity of *C. vulgaris* extracts (200 μg mL^{-1}) cultured under different salt stress conditions in a two-stage cultivation strategy as measured by the DPPH and ABTS radical scavenging methods. Different small letters on the bars indicate significant difference ($p < 0.05$). Error bars represent standard deviation of three replicates

single stress conditions (Kwak et al. 2016). Nitrogen deficiency as a single stress factor may not improve the lipid productivity in all microalgal species. The combination of two or more types of stressors is not only beneficial but is also needed in some microalgal species (Ho et al. 2017).

Combining high salinity and light intensity as stressors not only affects cell growth and lipid but also the fatty acid composition (Xia et al. 2014), as similarly exhibited in our study (Table 3 and Fig. 3). The exposure of the *C. vulgaris* vegetative cells in the stress stage to the high salt stress and light intensity significantly alters the fatty acid profile. Various lipid extraction methods have been carried out for transesterification process (Mandal et al. 2013). For biodiesel production, the microalgal species should ideally have high level of lipid and triacylglycerol (TAG), and a balanced fatty acid composition. The lipid produced from green microalgae species is generally similar to that of vegetable oils, which mainly contain C16 and C18 fatty acids, and are therefore appropriate for biodiesel (Francisco et al. 2010; Mondal et al. 2017). The long chain fatty acids (C16–18) is preferable as the increase in carbon chain length leads to an increase in the biodiesel properties such as heat of combustion, cetane number, and viscosity (Francisco et al. 2010). The *C. vulgaris* culture in our study exhibits predominantly C16 and C18 fatty acids, suggesting its suitability as biodiesel feedstock, whilst at the same time can be harnessed as a source of high-value caroetenoids and antioxidants.

Under optimal conditions (vegetative stage), the level of chlorophyll *a* in the *C. vulgaris* cells was significantly high. The accumulation of chlorophyll is favored under conditions that are optimal for cell growth, which is consistent with their role in photosynthesis (Faraloni and Torzillo 2017). On the other hand, the carotenoid accumulation in *C. vulgaris* cells was induced under stressed conditions, and especially under nitrogen deficiency with high light intensity and/or salt addition (Fig. 4). *Dunaliella salina* cultivated at extremely high (232 g L^{-1} NaCl) and high salt stress (58 g L^{-1} NaCl) has the total carotenoids elevated to 9.67 ± 0.19 μg mL^{-1} and 1.54 ± 0.08 μg mL^{-1}, respectively (Gallego-Cartagena et al. 2019). Reactive oxygen species (ROS) may be generated under stressed conditions and carotenoids may be induced as a defense mechanism, in a biochemical pathway promoted by the presence of additional salt, mediated by abscisic acid (Yoshida et al. 2004). The resulting increase in abscisic acid concentration has triggered counter responses including with higher level of carotenoids produced (Pancha et al. 2015).

The combined stress conditions (salt stress and light intensity) in the TSCS had resulted in the *C. vulgaris* cell extracts exhibiting considerably different antioxidant activities as measured by the DPPH and ABTS radical scavenging methods. The C1, C3, and C5 extracts had shown the lowest IC_{50} values, suggesting the strongest antioxidant activities. These can be correlated to the presence of unsaturated bonds and the hydroxyl groups of the compound extracts which exhibits high ability for scavenging free radicals and preventing the oxidation processes (El-fayoumy et al. 2020). Out of seven phytochemicals estimated quantitatively in the methanolic extracts of *C. vulgaris*, phenols are found the highest, followed by alkaloids, terpenoids, and glycosides and the tannin are the least (Prabakaran et al. 2018).

The effects of different NaCl salts have been tested on different microalgal species where a dose-dependent upregulation of antioxidant activities have been observed with the elevation of phenolic compounds, flavonoids, and the pigments. Maximum upregulation of the total flavonoid content (71.8 ± 0.21 mg QE g^{-1} DW) is registered in *Pithophora cleveana* grown in hypersaline conditions (Mukherjee et al. 2020). The salt stressors have led to changes in the level of antioxidant compounds which could scavenge the highly toxic ROS (Noctor et al. 2016). The imbalance of the cellular ions and the osmotic pressure, under salt stress, could retard the cell growth (Shalaby et al. 2010). The increased phytochemical compounds and the radical scavenging activities of *C. vulgaris* at low and high salinities therefore indicate its response to the abiotic stressors. There is a need to strike a balance between the level of intracellular oxidant and antioxidant molecules. While stressful conditions could result in the production of oxidizing agents, the hazardous consequences could be avoided by having the cells over-producing the antioxidant molecules (Barsanti et al. 2008). The developed stressful cultivation condition is therefore an effective strategy to promote the microalgal antioxidant machinery (Skjanes et al. 2013), especially if the aim is for industrial production of the high-value antioxidant compounds.

Our study shows that the TSCS of *C. vulgaris* without a medium replacement, with direct addition of 5 g L^{-1} $MgCl_2$ to the growth medium and exposure to 140 μmol photons m^{-2} s^{-1} light intensity, is a suitable strategy for lipid and fatty acid production for biodiesel. The $MgCl_2$ stress displays a lower effect on the cell growth than the NaCl stress as magnesium is essential for microalgal growth. Magnesium not only constitutes the central atom of chlorophyll, but also as the co-factor of some enzymes in the metabolic pathways (Wang et al. 2014). The highest carotenoid level was obtained when *C. vulgaris* was cultivated using the TSCS with a medium replacement, addition of 10 g L^{-1} NaCl, and exposure to 140 μmol photons m^{-2} s^{-1} light. The TSCS, with or without a medium replacement, with an addition of 5 g L^{-1} NaCl or $MgCl_2$, and exposure to 140 μmol photons m^{-2} s^{-1} light intensity, are also the appropriate strategies for phytochemical compounds production. These results constitute a further step towards the development of potential and cost-effective technologies for the production of lipid, carotenoid, or antioxidant compound–enriched microalgal biomass.

Conclusion

The oleaginous *Chlorella vulgaris* cultivated under light and salt stress conditions using two-stage cultivation strategy, with or without a medium replacement step, had shown elevated production of lipid and high-value antioxidant compounds. Low concentrations of $MgCl_2$ enhanced the lipid productivity with high saturated, monounsaturated, and polyunsaturated fatty acids, suggesting the potential for its eventual use as a biodiesel feedstock. High NaCl concentration in combination with nitrogen deficiency and high light intensity induced the carotenoid production in stress phase, with a medium replacement step. The low salt stresses could improve the production of antioxidant phytochemicals. Salt-supplemented cultures in the two-stage cultivation exhibited a promising strategy for simultaneous production of lipids and high-value compounds for bioenergy and biopharmaceutical applications.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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