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RESEARCH PAPER

Enhanced lipid productivity of *Chlamydomonas reinhardtii* with combination of NaCl and $CaCl_2$ stresses

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Abstract

Salinity (NaCl) stress treatment is a strategy to induce lipid accumulation in microalgae. This study aimed to investigate the effect of a combination of two salts (NaCl/$CaCl_2$) on lipid productivity of *Chlamydomonas reinhardtii*. *C. reinhardtii* was cultured in a two-stage culture comprising 9-day active growth in C medium followed by 3-day salt stress in C medium with various concentrations of NaCl (50−200 mM)/$CaCl_2$ (100 mM). In salt stress stage, NaCl (200 mM), $CaCl_2$ (100 mM), and the NaCl/$CaCl_2$ mixture inhibited growth but increased the lipid content in *C. reinhardtii* in comparison with NaCl (0, 50, and 100 mM) conditions. Combinatorial treatment with 100 mM NaCl/100 mM $CaCl_2$ resulted in the highest lipid content (73.4%) and lipid productivity (10.9 mg/L/days), being 3.5- and 2.1-fold, respectively, in salt-free control conditions, and 1.8- and 1.5-folds, respectively, with 200 mM NaCl. Furthermore, 100 mM NaCl/100 mM $CaCl_2$ treatment markedly upregulated glycerol-3-phosphate dehydrogenase (*GPDH*), lysophosphatidic acid acyltransferase (*LPAAT*), and diacylglycerol acyltransferase (*DAGAT*), which are involved in lipid accumulation in *C. reinhardtii*. The upregulation of these genes with 100 mM NaCl/100 mM $CaCl_2$ resulted in the highest lipid content in *C. reinhardtii*. Therefore, stress treatment using two salts, 100 mM NaCl/100 mM $CaCl_2$, is a potentially promising strategy to enhance lipid productivity in microalgae.

Keywords *Chlamydomonas reinhardtii* · NaCl · $CaCl_2$ · Lipid accumulation · Biofuel production

Introduction

Microalgae are recognized as promising and sustainable feedstocks for biofuel production because of their high photosynthesis efficiency, high growth rate, short harvesting cycle, and high lipid synthesis/accumulation potential. The accumulated lipids in microalgal cells are highly valued as biofuel precursors, especially biodiesel [1–6]. However, microalgal biodiesel production is still in its infancy with respect to its commercialization.

Enhancement of lipid productivity is critical for generating a highly efficient biodiesel production system based on microalgae and for reducing the production cost of microalgal biodiesel. Production of microalgal biodiesel primarily depends on the cellular lipid content and biomass productivity of microalgae. Inducing high lipid accumulation in microalgal cells is, therefore, considered a promising strategy to enhance lipid productivity. Stress conditions such as nitrogen starvation [7–9], iron starvation [10], copper stress [11], temperature [12], pH [13, 14], high-light intensity [15], and salts [7, 15–18] effectively induce high lipid accumulation in microalgae. Among these, salt stress is an easy and low-cost strategy to achieve mass microalgal cultivation [15]. A two-stage microalgal culture system, comprising (i) an efficient growth stage in culture medium or wastewater effluent medium, and (ii) a subsequent high lipid accumulation-inducing stage owing to salt stress (salt stress stage), has been proposed for enhancing microalgal lipid productivity [19].

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Several studies have reported an enhancement in lipid accumulation in various microalgal species such as *Scenedesmus* spp., *Chlorella* spp., and *Chlamydomonas* spp. in the presence of high NaCl concentrations. *Scenedesmus* sp. CCNM 1077 displayed lipid enhancement of up to 33.1% in medium containing 400 mM NaCl [20]. *Scenedesmus obliquus* XJ002 displayed a high lipid content of 32.3% in the presence of 200 mM NaCl [21]. *Chlorella sorokiniana* SDEC-18 displayed high lipid accumulation up to 54.6% in medium containing 30 g/L NaCl [22]. *Chlorella sorokiniana* HS1 displayed a high lipid content of 44% in medium containing 60 g/L NaCl [19]. Marine microalga, *Chlamydomonas* sp. JSC4, displayed a high lipid content of 59.4% in 7% sea salt [7]. *Chlamydomonas mexicana* displayed a high lipid content of 37% in medium containing 25 mM NaCl [23]. *Chlamydomonas reinhardtii* displayed a high lipid content of 35.8% in medium containing 50 mM NaCl and under light stress conditions [15]. Previous studies have evaluated optimum salt stress conditions for enhancing lipid accumulation, using only NaCl.

On the other hand, other salts/ions such as magnesium (Mg^{2+}) and calcium (Ca^{2+}) reportedly influence microalgal lipid accumulation [10, 24–26]. In particular, 25 mM $CaCl_2$ reportedly increased the lipid content up to 40% in *Chlorella sorokiniana* CG12 [26]. Therefore, we hypothesized that a combination of two or more salts, e.g., NaCl and $CaCl_2$, exerts a synergistic effect in inducing lipid accumulation in microalgal cells. If this hypothesis is verified, the combination of salts stresses would potentially provide a valid method to further increase the lipid productivity of microalgae. To our knowledge, no studies have clearly reported the potential of a combination of salt stresses to induce microalgal lipid accumulation.

A freshwater microalga *C. reinhardtii* was used herein because it is a widely studied model system in cellular and molecular analyses and it has been used for biofuel production [27]. Furthermore, major salts, NaCl and $CaCl_2$, were used herein as prototypes. Therefore, the primary objectives of this study were (i) to understand the effects of combination of two different salt stresses, NaCl and $CaCl_2$, on the induction of lipid accumulation in *C. reinhardtii* and (ii) to determine the optimal NaCl/$CaCl_2$ stress conditions for lipid production by *C. reinhardtii*. *C. reinhardtii* was cultured in a two-stage culture system, comprising a growth stage and a subsequent salt stress stage with various NaCl/$CaCl_2$ concentrations. Biomass production, lipid content, and starch content were monitored during the two-stage cultures. For better understanding of lipid productivity of *C. reinhardtii* under salt stress, expression levels of target genes involved in lipid metabolism, principally triacylglycerol (TAG) metabolism (Fig. 1), were examined via reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. The expression of glycerol-3-phosphate dehydrogenase (*GPDH*), lysophosphatidic acid acyltransferase (*LPAAT*), and diacylglycerol acyltransferase (*DAGAT*), which are involved in TAG metabolism, was assessed herein (Fig. 1) [28, 29]. Furthermore, genes coding photosystem proteins, *psbA* for photosystem II (PSII) D1 and *psaA* for photosystem I (PSI) A1 [30], were selected to understand the inhibitory effects of salt stress on *C. reinhardtii* growth. Finally, the most effective NaCl/$CaCl_2$ combination for enhancing high lipid productivity of *C. reinhardtii* was determined.

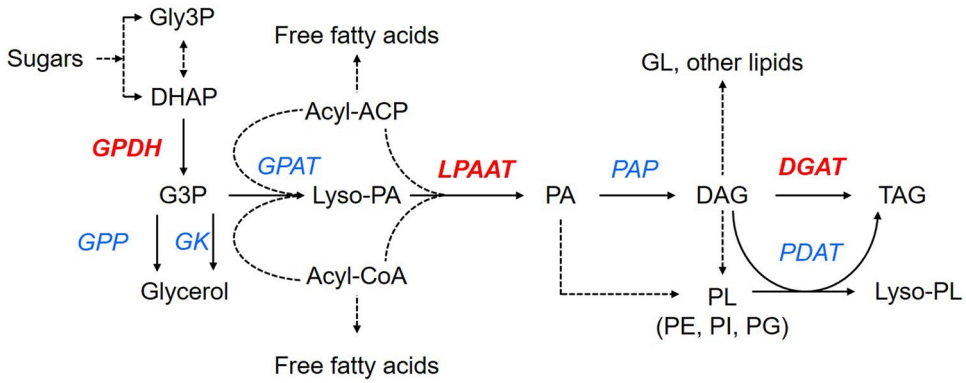

Fig. 1 Simplified pathway of lipid metabolism and key metabolic genes involved in lipid accumulation in *Chlamydomonas reinhardtii*. Key metabolites are indicated in black: *Acyl-CoA* acyl-coenzyme A, *Acyl-ACP* acyl-acyl carrier protein, *DAG* diacylglycerol, *DHAP* dihydroxyacetone phosphate, *G3P* glycerol-3-phosphate, *GL* galactoglycerolipids, *Gly3P* glyceraldehyde-3-phosphate, *Lyso-PA* lysophosphatidic acid, *Lyso-PL* lysophospholipids, *PA* phosphatidic acid, *PE* phosphatidylethanolamine, *PG* phosphatidylglycerol, *PI* phosphatidylinositol, *PL* phospholipids, *TAG* triacylglycerol. Key metabolic genes are indicated in red or blue in italics: *DAGAT* DAG acyltransferase, *GK* glycerol kinase, *GPAT* G3P acyltransferase, *GPDH* G3P dehydrogenase, *GPP* G3P phosphatase, *LPAAT* lyso-PA acyltransferase, *PAP* PA phosphatase, *PDAT* phospholipid:DAG acyltransferase. Three metabolic genes indicated in red, *DAGAT*, *GPDH*, and *LPAAT*, were used for gene expression analysis herein

Materials and methods

Chlamydomonas reinhardtii and its axenic culture

Axenic *C. reinhardtii* (NIES-2235) was obtained from the Microbial Culture Collection, National Institute for Environmental Studies, Tsukuba, Japan, and cultured in C medium containing the following: 150 mg/L $Ca(NO_3)_2{\cdot}4H_2O$, 100 mg/L KNO_3, 50 mg/L β-Na_2glycerophosphate·$5H_2O$, 40 mg/L $MgSO_4{\cdot}7H_2O$, 500 mg/L Tris(hydroxymethyl)aminomethane, 0.1 µg/L vitamin B_{12}, 0.1 µg/L biotin, 10 µg/L thiamine HCl, 3 mL/L PIV metals (1000 mg/L $Na_2EDTA{\cdot}H_2O$, 196 mg/L $FeCl_3{\cdot}6H_2O$, 36 mg/L $MnCl_2{\cdot}4H_2O$, 10.4 mg/L $ZnCl_2$, 4 mg/L $CoCl_2{\cdot}6H_2O$, 2.5 mg/L $Na_2MoO_4{\cdot}H_2O$), pH 7.5. The axenic *C. reinhardtii* culture was incubated in a flask containing C medium in a growth chamber at 28 ± 1 °C with fluorescent lamps at a photosynthetic photon flux density of 80 µmol/m^2s and a 16:8-h light–dark cycle for 1 week. Every week thereafter, cells were sub-cultured via routine transfer into fresh C medium. For subsequent experiments, *C. reinhardtii* was harvested through centrifugation (6000×*g*, room temperature, 5 min), washed with sterile C medium, and resuspended in sterile C medium. The *C. reinhardtii* cell suspension was used as the inoculum for subsequent experiments.

Enhanced lipid production of *C. reinhardtii* with a combination of salts stresses: two-stage culture system consisting of growth and salt stress stages

The potential for enhanced lipid production of *C. reinhardtii* with a combination of salt stresses was evaluated using a two-stage culture system comprising growth and salt stress stages. The growth stage was a culture of *C. reinhardtii* in C medium without any salt stress to produce a large biomass of *C. reinhardtii*. The *C. reinhardtii* cells produced in the growth stage were used for the salt stress stage, wherein two salts, NaCl and $CaCl_2$, were added to C medium at various concentrations to induce lipid accumulation in *C. reinhardtii*.

The growth stage was carried out as follows. *C. reinhardtii* cells (50 mL) were inoculated into 500 mL of C medium in a 1000-mL flask at 0.017-times the initial cell density and incubated in the growth chamber for 9 days. The growth stage *C. reinhardtii* cultures (550 mL) was carried out in 24 replicates. After 9 days of culturing, all flasks were used for the salt stress stage.

In the salt stress stage, eight different NaCl + $CaCl_2$ doses (non-salt control, 50 mM NaCl, 100 mM NaCl, 200 mM NaCl, 100 mM $CaCl_2$, 50 mM NaCl + 100 mM $CaCl_2$, 100 mM NaCl + 100 mM $CaCl_2$, 200 mM NaCl + 100 mM $CaCl_2$, Table S1) were separately added into three flasks ($n = 3$) to cultures obtained from the growth stage. All flasks were incubated in the growth chamber for 3 days.

During the growth stage, cell growth, chlorophyll $a + b$, and the dry cell weight of *C. reinhardtii* were monitored daily in three selected flasks ($n = 3$). In the salt stress stage, cell growth, chlorophyll $a + b$, the dry cell weight, starch content, and total lipid content were monitored daily in all three flasks ($n = 3$) for all eight salt doses.

Analyses of chlorophyll, dry biomass, and lipids in *C. reinhardtii*

Chlorophyll concentrations were measured spectrophotometrically after extraction in 100% methanol for 30 min [31]. The absorbance of the extract was measured at 665 nm (A_{665}) and 650 nm (A_{650}) with a spectrophotometer (UVmini-1240). The total chlorophyll (chlorophyll a + chlorophyll b: Chl $a + b$) concentration (µg/mL) was calculated as follows:

$$\text{Chl } a + b\ (\mu\text{g/mL}) = \left(4 \times A_{665}\right) + \left(25.5 \times A_{650}\right). \quad (1)$$

The dry weight of *C. reinhardtii* was measured as follows. The *C. reinhardtii* culture flask was agitated well to homogenize cultures. Fifty-milliliter cultures were harvested from each flask into a centrifuge tube, and the cells were then harvested via centrifugation (6000×*g*, 5 min, and room temperature). The cell pellet was harvested and dried at 70 °C for 1 day, the dry cell weight was weighed, and the dry cell weight (mg/L) was determined.

These dried cells were lyophilized using a BioMasher (Takara Bio, Kusatsu, Shiga, Japan) and used for starch and lipid quantification. The starch content of *C. reinhardtii* cells was determined using a total starch assay kit (Megazyme International, Wicklow, Ireland) in accordance with the manufacturer's instructions. The lipid content of *C. reinhardtii* cells was quantified in terms of the percentage of the dry biomass accounted for by lipids. *n*-Hexane/isopropanol is an extraction solvent with a relatively higher lipid yield from microalgal cells and low toxicity [32]. Based on our previous method, lipids were extracted from *C. reinhardtii* cells, using *n*-hexane/isopropanol as follows. The *C. reinhardtii* powder (20 mg) was re-crushed with 1 mL of *n*-hexane in a BioMasher. The *C. reinhardtii* sample was transferred into a 50-mL glass tube, and 9 mL of *n*-hexane and 6 mL of isopropanol were added to the tube. The tube was agitated at 225 rpm for 24 h. Thereafter, 33 mL of distilled water was added to the tube. The tube was agitated for 1 min and then centrifuged (10,000×*g*, 5 min). The *n*-hexane layer containing the lipids was harvested on a pre-weighed aluminum tray, dried at room temperature overnight,

and then dried at 90 °C for 3 h. The harvested lipids were then weighed. Finally, the lipid content (%) was determined from the dry weight of cells.

In this study, biomass productivity (g/L/days) and lipid productivity (g/L/days) throughout the two-stage culture period (12 days), including the growth (9 days) and stress (3 days) stages, were calculated as follows:

$$\text{Biomass productivity (g/L/days)} = \left[\text{final biomass (g/L)} - \text{initial biomass (g/L)}\right]/12 \text{ (days)}, \quad (2)$$

$$\text{Lipid productivity (g/L/days)} = \left[\text{final biomass (g/L)} \times \text{final lipid content (\%)} - \text{initial biomass (g/L)} \times \text{initial lipid content (\%)}\right]/12 \text{ (days)}. \quad (3)$$

RNA extraction and gene expression analysis via RT-qPCR

Chlamydomonas reinhardtii cells of salt-free control, 100 mM NaCl, 100 mM $CaCl_2$, or 100 mM NaCl + 100 mM $CaCl_2$ treatments after 1 and 3 days of salt stress were used for gene expression analysis ($n = 3$ for each salt stress condition). *C. reinhardtii* cells were harvested via centrifugation (6000×*g*, 5 min, 4 °C) from each flask and sampling day and immediately frozen in liquid nitrogen and stored at −80 °C. Total RNA was extracted from each *C. reinhardtii* frozen sample using NucleoSpin RNA Plant (Takara Bio). Thereafter, cDNA was synthesized from 1 µg of total RNA using Prime Script 1st strand cDNA Synthesis Kit (Takara Bio) in accordance with the manufacturer's instructions.

Upon gene expression analysis, five target genes were used, and 18S rRNA gene was used as a reference gene (Table S2). q-PCR was performed using TB Green Premix Ex Taq II (Takara Bio) in a Thermal Cycler Dice Real-Time System II (TaKaRa Bio). Each qPCR assay was conducted in a 25 µL reaction mixture including 12.5 µL TB Green Premix Ex Taq II, 0.5 µM of each forward and reverse primers (Table S2), 2 µL template cDNA, and 9.5 µL deionized H_2O. The reaction conditions were as follows: initial denaturation by pre-heating at 95 °C for 30 s, 40 cycles of 98 °C for 5 s, annealing at the specified temperatures (which varied with primer, Table S2) for 50 s, and an extension at 72 °C for 1 min. Quantification was based on the cycle threshold (Ct) value. Relative gene expression levels of *C. reinhardtii* under stress conditions for salt-free control conditions was determined on the basis of the $2^{-\Delta\Delta Ct}$ method [33] as follows:

$$\Delta Ct_{\text{control test}} = Ct_{\text{target gene–control test}} - Ct_{\text{reference gene–control test}}, \quad (4)$$

$$\Delta Ct_{\text{salt stress test}} = Ct_{\text{target gene–salt stress test}} - Ct_{\text{reference gene–salt stress test}}, \quad (5)$$

$$\Delta\Delta Ct = \Delta Ct_{\text{salt stress test}} - \Delta Ct_{\text{control test}}, \quad (6)$$

$$\text{Relative gene expression (fold change)} = 2^{-\Delta\Delta Ct}. \quad (7)$$

Statistical analysis

Each value represents the results of three samples ($n = 3$ replicates) per experiment. All results are expressed as mean ± SD values. Statistical significance ($p < 0.05$) was analyzed using the paired samples *t* test, with SPSS Statistics v. 22.0 (IBM, Armonk, NY, USA).

Results and discussion

Salt (NaCl) stress treatment in microalgal cultures has been recognized as a useful strategy to enhance lipid productivity [15–23]. Herein, we investigated whether a combination of two salts (e.g., NaCl and $CaCl_2$) has a synergistic effect to further enhance microalgal lipid accumulation. We cultured *C. reinhardtii* in a two-stage culture system consisting of 9-day growth stage and a 3-day salt stress stage to enhance lipid productivity, and confirmed the effect of a combination of salt stresses on lipid productivity.

Growth of *C. reinhardtii* in C medium without salt stress (growth stage)

Initially, *C. reinhardtii* was cultured in C medium without salt stress for 9 days. During this 9-day growth stage, *C. reinhardtii* grew well; chlorophyll *a* + *b* concentration and dry cell weight of *C. reinhardtii* increased from 0.48 to 142 µg/mL and 8 to 207 mg/L, respectively (Figs. 2 and 3). *C. reinhardtii* growth approached a near-stationary phase but was not saturated at the end of this 9-day growth stage.

Growth of *C. reinhardtii* in the salt stress stage

After the growth stage, *C. reinhardtii* was cultured in C medium containing various NaCl and $CaCl_2$ mixtures for the salt stress stage. In the control C medium without salt stress, chlorophyll *a* + *b* concentration and dry cell concentration of *C. reinhardtii* further increased (Figs. 2 and 3). For the *C. reinhardtii* in C medium with 50 mM NaCl or 100 mM NaCl condition, chlorophyll *a* + *b* concentration and biomass concentration of *C. reinhardtii* also tended to increase (Figs. 2 and 3). Freshwater microalgae can be

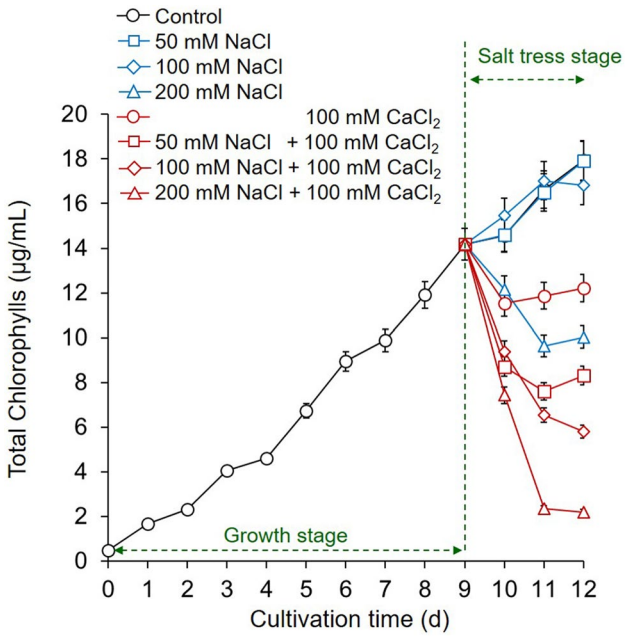

Fig. 2 Changes in chlorophyll $a+b$ (μg/mL) of *Chlamydomonas reinhardtii* cultures in growth and stress stages. Values are mean ± SD

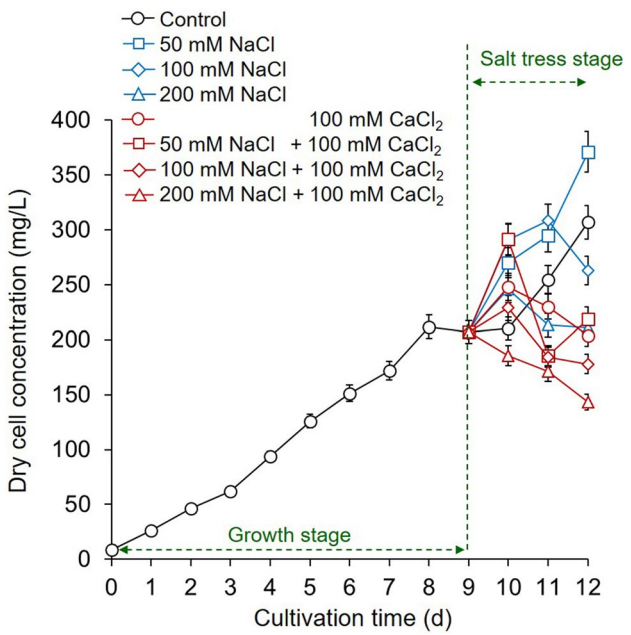

Fig. 3 Changes in dry cell concentration (mg/L) of *Chlamydomonas reinhardtii* cultures in growth and stress stages. Values are mean ± SD

adapted to moderate salinities (e.g., 18 ppt) [34]; furthermore, low NaCl levels (e.g., 25 mM) can facilitate the growth of freshwater microalgae *Chlamydomonas mexicana* and *Scenedesmus obliquus* [23]. Herein, the growth of *C. reinhardtii* in medium containing 50 mM or 100 mM NaCl was not inhibited in comparison with the control group (Figs. 2 and 3).

However, under salt stress conditions of 200 mM NaCl or 0−200 mM NaCl + 100 mM $CaCl_2$, chlorophyll $a+b$ concentration and dry cell concentration of *C. reinhardtii* markedly decreased (Figs. 2 and 3). Chlorophyll $a+b$ and biomass concentration in 200 mM NaCl or 0−200 mM NaCl + 100 mM $CaCl_2$ conditions were significantly ($p<0.05$) lower than those of the control after 3-day salt stress (Table 1).

Lipid accumulation and starch content in the salt stress stage

Changes in lipid content (%) in *C. reinhardtii* in the 3-day salt stress stage are shown in Fig. 4a. In control and 50 mM NaCl conditions, the lipid content in *C. reinhardtii* slightly increased from 13.2 to 19.2% or 13.2% to 15.0%, respectively, during the 3-day salt stress stage. High salinity (high levels of NaCl), inhibited microalgal growth and induced the three major stresses: osmotic stress, ion (salt) stress, and oxidative stress [17]. Oxidative stress was accompanied by an increase in microalgal lipid accumulation [35]. In this study, 100 mM NaCl, 200 mM NaCl, and 0−200 mM NaCl + 100 mM $CaCl_2$ treatments inhibited the growth and significantly increased the lipid content of *C. reinhardtii* in comparison with the control group (Figs. 2, 3, 4a, and Table 1). Microalgal lipid content upon culturing in medium containing 100 mM NaCl (27.2% lipid content at 3 day), 200 mM NaCl (41.1%), and 0−200 mM NaCl + 100 mM $CaCl_2$ (36.6–73.4%) after 3-day salt stress was significantly ($p<0.05$) higher than that of the control group (Table 1).

NaCl stress is a well-recognized abiotic stress inducing lipid accumulation in various microalgae. Along with NaCl stress treatment, 100 mM $CaCl_2$ was effective in inducing lipid accumulation in *C. reinhardtii*. The lipid content was significantly ($p<0.05$) higher (36.6%) in *C. reinhardtii* upon culturing in medium containing 100 mM $CaCl_2$ than in salt-free medium. Ca^{2+} contributes to various signaling pathways and influences various metabolic pathways in microalgae [24]. These functions of Ca^{2+} seem to enhance lipid synthesis in microalgal cells [26]. Furthermore, the present results show lipid accumulation in *C. reinhardtii* upon the introduction of a 100 mM $CaCl_2$ stress.

On comparing the results of 100 mM NaCl + 100 mM NaCl (i.e., 200 mM NaCl) with those of 100 mM NaCl + 100 mM $CaCl_2$, lipid accumulation of *C. reinhardtii* under the 100 mM NaCl + 100 mM $CaCl_2$ dose was significantly ($p<0.05$) higher (Fig. 4a and Table 1). Lipid content (73.4%) of *C. reinhardtii* under 100 mM NaCl + 100 mM $CaCl_2$ was 1.8-fold that of cells cultured with 200 mM NaCl (41.1%). These results clearly indicate the synergistic effect of a combination of salts, NaCl

Table 1 Chlorophyll, dry cell weight, starch content, and lipid content on day 3 of the salt stress stage, and biomass/lipid productivity of *Chlamydomonas reinhardtii* in overall 9-day growth and 3-day stress stages

Treatment in stress stage	Chlorophyll $a+b$ (μg/mL)	Dry cell weight (mg/L)	Starch content (%)	Lipid content (%)	Biomass productivity (mg/L/d)	Lipid productivity (mg/L/d)
Salt free (control)	12.0 ± 0.3	307 ± 0	39.0 ± 1.8	20.8 ± 1.7	24.9	5.3
50 mM NaCl	12.1 ± 0.6	371* ± 0	30.5 ± 9.7	15.0* ± 0.8	30.2	4.6
100 mM NaCl	11.3* ± 0.5	263* ± 0	28.1 ± 6.9	27.2* ± 1.1	21.2	6.0
200 mM NaCl	6.7* ± 0.1	211* ± 0	17.6* ± 6.9	41.1* ± 3.5	16.9	7.2
0 mM NaCl	8.1* ± 0.2	204* ± 1	14.6* ± 2.4	36.6* ± 0.9	16.3	6.2
100 mM $CaCl_2$	5.4* ± 0.2	219* ± 0	20.7* ± 0.2	48.6* ± 4.7	17.6	8.9
100 mM NaCl + 100 mM $CaCl_2$	3.9* ± 0.1	178* ± 0	16.5* ± 7.2	73.4* ± 6.2	14.2	10.9
200 mM NaCl + 100 mM $CaCl_2$	1.4* ± 0.0	143* ± 1	15.7* ± 4.2	38.0* ± 3.2	11.2	4.5

*Significant differences relative to the control ($p < 0.05$); Values are mean ± SD ($n = 3$)

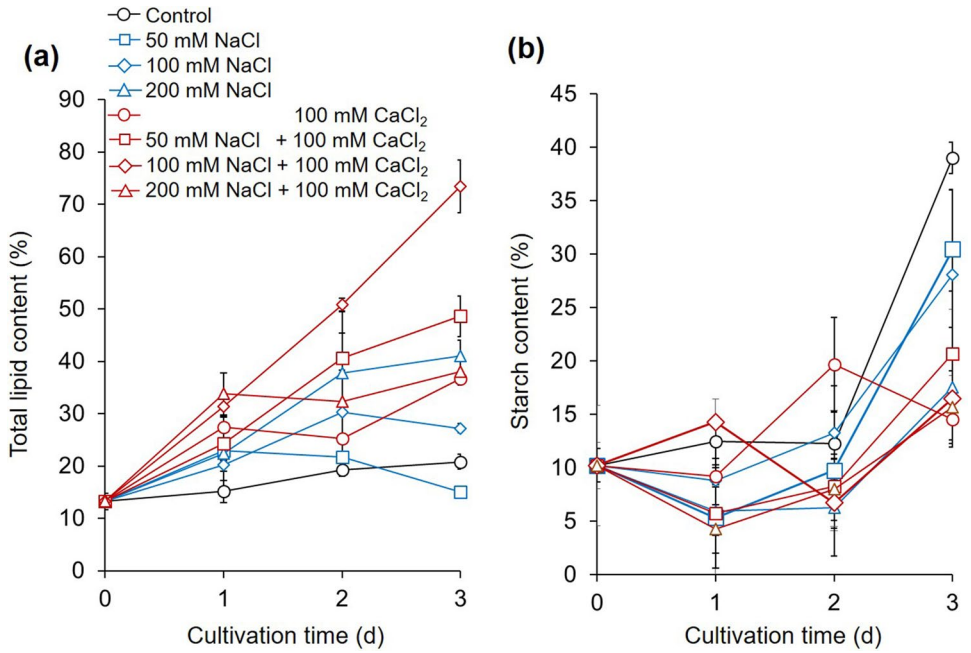

Fig. 4 Changes in **a** total lipid content (%) and **b** starch content (%) in *Chlamydomonas reinhardtii* cells in the 3-day stress stage. Values are mean ± SD

and $CaCl_2$, on lipid accumulation in *C. reinhardtii*. In experiments using a combination of salts dose conditions, lipid content in *C. reinhardtii* increased with an increase in NaCl concentrations (from 0 to 100 mM) in combination with 100 mM $CaCl_2$. However, lipid content in *C. reinhardtii* upon culturing with 200 mM NaCl + 100 mM $CaCl_2$ was significantly ($p < 0.05$) lower than with 100 mM NaCl + 100 mM $CaCl_2$ (Fig. 4a and Table 1), probably owing to higher stress damage.

In all experiments herein, 100 mM NaCl + 100 mM $CaCl_2$ stress treatment led to the highest lipid accumulation in *C. reinhardtii*; the lipid content in *C. reinhardtii* increased from 13.2 to 73.4% within 3 days. The lipid content (73.4%) in the 100 mM NaCl + 100 mM $CaCl_2$ stress condition was approximately 3.5-, 2.7-, and 2.0-fold that of *C. reinhardtii* in the salt-free control condition without any stress (20.8%), in 100 mM NaCl (27.2%) and 100 mM $CaCl_2$ (36.6%), respectively (Table 1).

Changes in starch content (%) in cultures of *C. reinhardtii* in the salt stress stage are shown in Fig. 4b. In the salt-free control, 50 mM NaCl and 100 mM NaCl conditions, starch content in *C. reinhardtii* markedly increased and approached 39.0%, 30.5%, or 28.1%, respectively, within 3 days (Fig. 4b and Table 1). However, upon culturing with 200 mM NaCl and 0−200 mM NaCl + 100 mM $CaCl_2$, starch content (%) in *C. reinhardtii* slightly increased or did not increase over 3 days; the starch content under these salts stresses after 3 days were significantly ($p < 0.05$) lower than those in the control conditions (Fig. 4b and Table 1). Previous studies have reported that the lipid content of microalgae can be increased by a metabolic switch from starch-to-lipid synthesis, wherein intracellular energy is expended in the storage

of lipids produced from starch upon NaCl treatment [16]. Herein, a similar result as the starch-to-lipid switch, i.e., a reduction in the starch content and an increase in the lipid content in *C. reinhardtii* were observed with 200 mM NaCl and 0−200 mM NaCl + 100 mM $CaCl_2$ (Fig. 4).

Expression of genes involved in TAG metabolism and photosystems in *C. reinhardtii* in the salt stress stage

Metabolism of lipids, principally TAG, in *C. reinhardtii* has been extensively analyzed (Fig. 1). Within the metabolic pathway, three genes, *GPDH*, *LPAAT*, and *DAGAT*, were monitored under salt stress conditions herein since they play key roles in lipid accumulation in *C. reinhardtii* [28]. Furthermore, *psbA* and *psaA* expression [30] was monitored to understand the inhibitory effects of salt stress on *C. reinhardtii* growth. Relative transcript levels of *GPDH*, *LPAAT* , *DAGAT*, *psbA*, and *pasA* are shown in Fig. 5. The relative gene expression levels (fold change compared to salt-free control condition) of *GPDH*, *LPAAT*, and *DAGAT* in *C. reinhardtii* upon culturing with 100 mM NaCl, 100 mM $CaCl_2$, and 100 mM NaCl + 100 mM $CaCl_2$ stress conditions were significantly higher than 1, fold change 1 was the same levels as the salt-free condition. The gene expression levels of the three genes on day 1 were greater than those on day 3. Furthermore, the expression levels of these genes upon culturing with 100 mM NaCl + 100 mM $CaCl_2$ were higher than those with 100 mM NaCl and 100 mM $CaCl_2$. Our results clearly indicate that salt stress treatments (100 mM NaCl, 100 mM $CaCl_2$, and 100 mM NaCl + 100 mM $CaCl_2$) rapidly and significantly upregulated *GPDH*, *LPAAT*, and *DAGAT* genes in *C. reinhardtii*. The upregulation of the three genes upon salt stress resulted in lipid accumulation in *C. reinhardtii*. Thus, along with NaCl, $CaCl_2$ plays a key role in lipid accumulation in *C. reinhardtii*. Moreover, compared to 100 mM NaCl and 100 mM $CaCl_2$, 100 mM NaCl + 100 mM $CaCl_2$ stress markedly upregulated these three genes in *C. reinhardtii*. Gene upregulation upon 100 mM NaCl + 100 mM $CaCl_2$ stress resulted in the highest lipid accumulation of *C. reinhardtii* in the present salt stress stage (Fig. 4a and Table 1).

However, *psbA* and *psaA* were downregulated under salt stress conditions (100 mM NaCl, 100 mM $CaCl_2$, and 100 mM NaCl + 100 mM $CaCl_2$) on days 1 and 3. Our

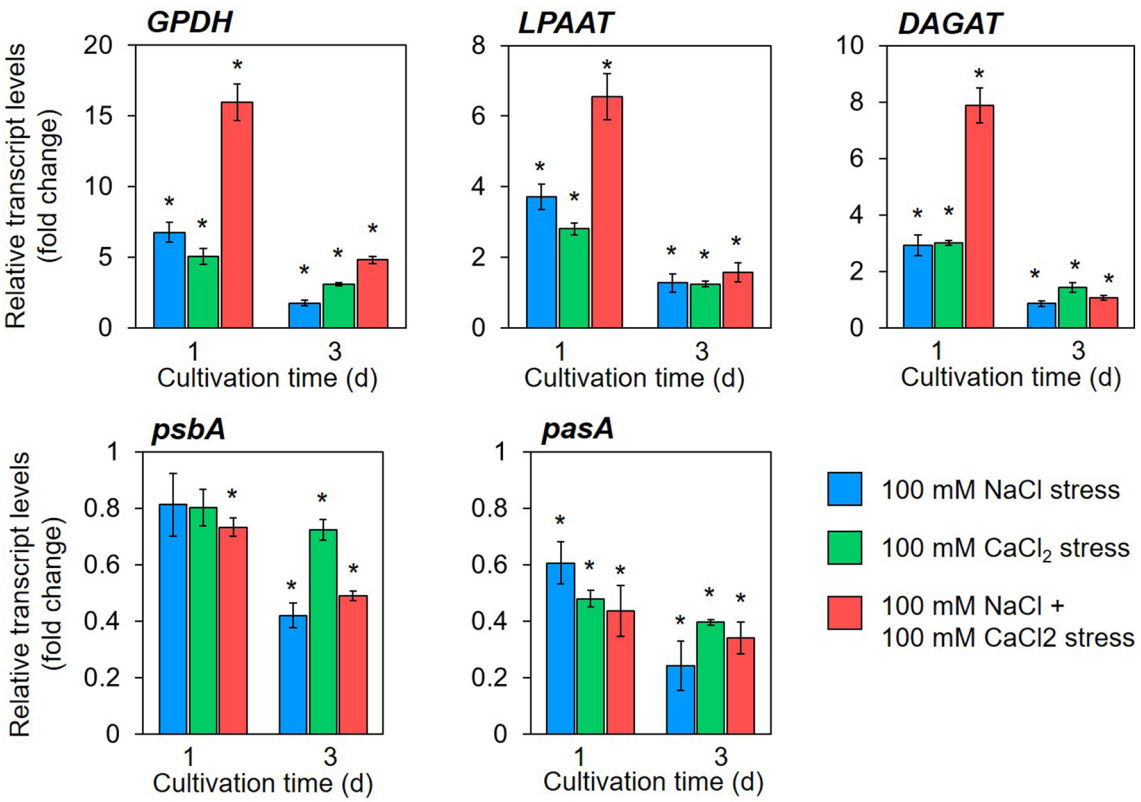

Fig. 5 Gene expression profiles of *GPDH*, *LPAAT*, *DAGAT*, *psbA*, and *psaA* genes in *Chlamydomonas reinhardtii* in the 3-day stress stage of 100 mM NaCl, 100 mM $CaCl_2$, or 100 mM NaCl + 100 mM $CaCl_2$. Data are expressed as relative transcript levels. The values are fold changes relative to transcript levels in the salt-free control condition. The relative transcript levels were determined via the $2^{-\Delta\Delta Ct}$ method. Values are mean ± SD. Asterisks indicate significant differences with respect to the salt-free control condition ($p < 0.05$)

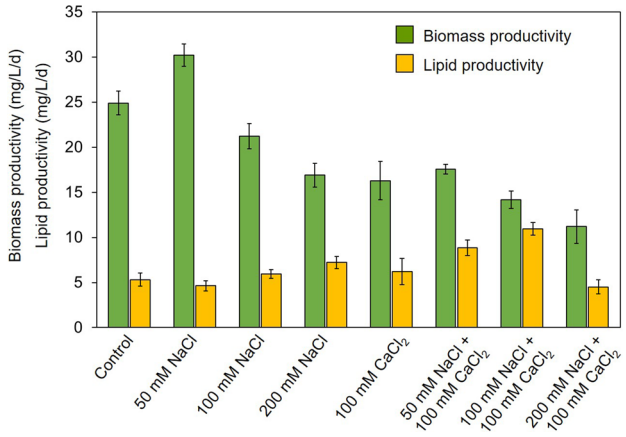

Fig. 6 Biomass and lipid production by *Chlamydomonas reinhardtii* in the two-stage culture comprising a 9-day growth and a 3-day stress stage. Values are means ± SD

results indicate that salt stress inhibited the photosystem of *C. reinhardtii*, concurrent with a previous study reporting the downregulation of genes involved in photosystem I and II in *C. reinhardtii* upon treatment with 200 mM NaCl [36]. These results indicate the inhibition of growth and reduction in chlorophyll $a+b$ levels in *C. reinhardtii* in the salt stress stage (Figs. 2 and 3).

Other salts including KCl and $MgCl_2$, and different combinations of salts including NaCl + KCl, NaCl + $MgCl_2$, and NaCl + $CaCl_2$ + KCl might have similar effects on *C. reinhardtii*. Further studies are required to determine the effects of other salts and/or their combinations on lipid accumulation and gene expression in *C. reinhardtii*.

Biomass and lipid productivity during the two-stage culture system

Dry biomass and lipid productivity (mg/L/days) in *C. reinhardtii* over the entire two-stage culture (12 days) are shown in Fig. 6. Biomass productivity of *C. reinhardtii* in the control and with 50 mM NaCl + 0 mM $CaCl_2$ stress was relatively higher than that under other salt stress conditions. The highest biomass productivity (30.2 ± 2 mg/L/days) was observed with 50 mM NaCl. However, lipid productivity of *C. reinhardtii* with 50 mM NaCl + 100 mM $CaCl_2$ and 100 mM NaCl + 100 mM $CaCl_2$ stresses was relatively higher. The highest lipid productivity (10.9 mg/L/days) was observed in the 100 mM NaCl + 100 mM $CaCl_2$ stress condition. Lipid productivity of *C. reinhardtii* with 100 mM NaCl + 100 mM $CaCl_2$ stress was approximately 2.1-, 1.8-, and 1.8-folds that of *C. reinhardtii* in the control condition without any salt stress, in 100 mM NaCl, and 100 mM $CaCl_2$, respectively (Fig. 6 and Table 1).

Our study reveals the potential of salt combinations including NaCl and $CaCl_2$ to enhance lipid productivity in *C. reinhardtii* under photo-autotrophic conditions. The effects of combinations of salts stresses on lipid content and lipid productivity in *C. reinhardtii* were compared to the effects of NaCl on various microalgae under photo-autotrophic and heterotrophic conditions (Table 2). Lipid content (73.4%) induced by 100 mM NaCl + 100 mM $CaCl_2$ in *C. reinhardtii* was the highest among those in various microalgal species upon NaCl stress. Treatment with salt combinations including NaCl and $CaCl_2$ is a potentially new, promising method to induce lipid accumulation in microalgae. However, lipid productivity reported herein (10.9 mg/L/days) was not the highest, but rather comparable to that of previously reported studies (except for those reported in [7] and [19]). Lipid productivity depends on biomass productivity and the intracellular lipid content. Therefore, in future studies, we intend to optimize the growth medium, conditions, culture periods, and NaCl/$CaCl_2$ concentrations to further increase lipid productivity in *C. reinhardtii* upon NaCl/$CaCl_2$ stress. Further studies are needed to examine the mechanisms underlying the high accumulation of lipids in *C. reinhardtii* upon NaCl/$CaCl_2$ stress.

Conclusion

This study investigated the effects of NaCl and $CaCl_2$ stress on the growth and lipid content in *Chlamydomonas reinhardtii*, using a two-stage culture system comprising growth and salt stress stages. Salt combinations including NaCl (50–200 mM) and $CaCl_2$ (100 mM), markedly inhibited the growth and increased the lipid content in *C. reinhardtii*. In particular, 100 mM NaCl/100 mM $CaCl_2$ treatment resulted in the highest lipid content (73.4%) and productivity (10.9 mg/L/days) in *C. reinhardtii*, which were 3.5-fold and 2.1-fold those in salt-free conditions, respectively. Furthermore, 100 mM NaCl/100 mM $CaCl_2$ treatment markedly upregulated *GPDH*, *LPAAT*, and *DAGAT*, which are involved in lipid accumulation in *C. reinhardtii*. The upregulation of these genes with 100 mM NaCl/100 mM $CaCl_2$ resulted in the highest lipid content and productivity in *C. reinhardtii*. Therefore, stress treatment with two salt stresses, 100 mM NaCl/100 mM $CaCl_2$, is a potentially promising method to enhance lipid production by *C. reinhardtii*.

Table 2 The effect of a combination of two salts ($NaCl/CaCl_2$) stress on lipid accumulation in *Chlamydomonas reinhardtii* and comparison with previous studies

Species	Medium	Culture condition	Incubation time (days)	Lipid content (%)	Lipid productivity (mg/L/days)	References
Chlamydomonas mexicana	BBM (photo-autotrophic)	25 mM NaCl	20 (20-day stress single culture)	37	13.6	[23]
Chlamydomonas sp. JSC4 (Marine microalga)	MB 6N (photo-autotrophic)	2% sea salt	7 (7-day stress single culture)	56.9	306	[7]
Chlamydomonas reinhardtii	TAP (heterotrophic)	50 mM NaCl + High light stress	10 (7-day growth and 3-day stress two-stage culture)	35.8	28.6	[15]
Chlorella sorokiniana	BG11 (photo-autotrophic)	20 g/L (342 mM) NaCl	12 (12-day stress single culture)	53.9	19.7	[22]
Chlorella vulgaris *Acutodesmus obliquus*	BG11 (photo-autotrophic)	400 mM NaCl	15 (15-day stress single culture)	49 43	8.6 11.7	[18]
Chlorella sorokiniana H1S	BG11 (photo-autotrophic)	60 g/L(1000 mM) NaCl	9 (7-day growth and 2-day stress two-stage culture)	38	106	[19]
Scenedesmus sp. CCNM 1077	BG11 (photo-autotrophic)	400 mM NaCl	15 (12-day growth and 3-day stress two-stage culture)	33.1	–	[20]
Scenedesmus obliquus XJ002	BG11 (photo-autotrophic)	200 mM NaCl	14 (14-day stress single culture)	32.3	–	[21]
Chlamydomonas reinhardtii	C (photo-autotrophic)	100 mM NaCl 100 mM $CaCl_2$	12 (9-day growth and 3-day stress two-stage culture	73.4	10.9	This study

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Compliance with ethical standards

Conflict of interest All the authors reviewed and agreed to submit this manuscript. The authors declare that they have no conflict of interest.

Research involving human participants and/or animals The study does not contain experiments using animals and human studies.

References

1. Chen CY, Zhao XQ, Yen HW, Ho SH, Cheng CL, Lee DJ, Bai FW, Chang JS (2013) Microalgae-based carbohydrates for biofuel production. Biochem Eng J 78:1–10
2. Peng K, Li J, Jiao K, Zeng X, Lin L, Pan S, Danquah MK (2018) The bioeconomy of microalgal biofuels. In: Jacob LE, Queiroz ZL, Queiroz M (eds) Energy from microalgae. Springer, Cham
3. Salih FM, Haase RA (2012) Potentials of microalgal biofuel production. J Pet Technol Altern Fuels 3:1–4
4. Singh A, Nigam PS, Murphy JD (2011) Mechanism and challenges in commercialisation of algal biofuels. Bioresour Technol 102:26–34
5. Su Y, Song K, Zhang P, Su Y, Cheng J, Chen X (2017) Progress of microalgae biofuel's commercialization. Renew Sustain Energy Rev 74:402–411
6. Yen HW, Hu IC, Chen CY, Ho SH, Lee DJ, Chang JS (2013) Microalgae-based biorefinery—from biofuels to natural products. Bioresour Technol 135:166–174
7. Ho SH, Nakanishi A, Ye X, Chang JS, Hara K, Hasunuma T, Kondo A (2014) Optimizing biodiesel production in marine *Chlamydomonas* sp. JSC4 through metabolic profiling and an innovative salinity-gradient strategy. Biotechnol Biofuels 7:97
8. Lin Q, Zhuo WH, Wang XW, Chen CP, Gao YH, Liang JR (2018) Effects of fundamental nutrient stresses on the lipid accumulation profiles in two diatom species *Thlassiosira weissflogii* and *Chaetoceros muelleri*. Bioprocess Biosyst Eng 41:1213–1224
9. Rios LF, Klein BC, Luz LF Jr, Maciel Filho R, Wolf Maciel MR (2015) Nitrogen starvation for lipid accumulation in the microalga species *Desmodesmus* sp. Appl Biochem Biotechnol 175:469–476
10. Gorain PC, Bagchi SK, Mallick N (2013) Effects of calcium, magnesium and sodium chloride in enhancing lipid accumulation in two green microalgae. Environ Technol 34:1887–1894
11. Hamed SM, Selim S, Klöck G, AbdElgawad H (2017) Sensitivity of two green microalgae to copper stress: growth, oxidative and antioxidants analyses. Ecotoxicol Environ Saf 144:19–25
12. Vince O, Wendy AS, Péter B, Adeyemi OA, Ambrose O, Csaba L, Zoltán M, Johannes VS (2016) Effect of temperature and nitrogen concentration on lipid productivity and fatty acid composition in three Chlorella strains. Algal Res 16:141–149
13. Abinandan S, Subashchandrabose SR, Cole N, Dharmarajan R, Venkateswarlu K, Megharaj M (2019) Sustainable production of

biomass and biodiesel by acclimation of non-acidophilic microalgae to acidic conditions. Bioresour Technol 271:316–324
14. Bartley ML, Boeing WJ, Dungan BN, Holguin FO, Schaub T (2014) pH effects on growth and lipid accumulation of the biofuel microalgae *Nannochloropsis salina* and invading organisms. J Appl Phycol 26:1431–1437
15. Fan J, Zheng L (2017) Acclimation to NaCl and light stress of heterotrophic *Chlamydomonas reinhardtii* for lipid accumulation. J Biosci Bioeng 124:302–308
16. Kato Y, Ho SH, Vavricka CJ, Chang JS, Hasunuma T, Kondo A (2017) Evolutionary engineering of salt-resistant *Chlamydomonas* sp. strains reveals salinity stress-activated starch-to-lipid biosynthesis switching. Bioresour Technol 245:1484–1490
17. Khona DK, Shirolikar SM, Gawde KK, Hom E, Deodhar MA, D'Souza JS (2016) Characterization of salt stress-induced palmelloids in the green alga, *Chlamydomonas reinhardtii*. Algal Res 16:434–448
18. Pandit PR, Fulekar MH, Karuna MSL (2017) Effect of salinity stress on growth, lipid productivity, fatty acid composition, and biodiesel properties in *Acutodesmus obliquus* and *Chlorella vulgaris*. Environ Sci Pollut Res Int 24:13437–13451
19. Kakarla R, Choi JW, Yun JH, Kim BH, Heo J, Lee S, Cho DH, Ramanan R, Kim HS (2018) Application of high-salinity stress for enhancing the lipid productivity of *Chlorella sorokiniana* HS1 in a two-phase process. J Microbiol 56:56–64
20. Pancha I, Chokshi K, Maurya R, Trivedi K, Patidar SK, Ghosh A, Mishra S (2015) Salinity induced oxidative stress enhanced biofuel production potential of microalgae *Scenedesmus* sp. CCNM 1077. Bioresour Technol 189:341–348
21. Ji X, Cheng J, Gong D, Zhao X, Qi Y, Su Y, Ma W (2018) The effect of NaCl stress on photosynthetic efficiency and lipid production in freshwater microalga—*Scenedesmus obliquus* XJ002. Sci Total Environ 633:593–599
22. Zhang L, Pei H, Chen S, Jiang L, Hou Q, Yang Z, Yu Z (2018) Salinity-induced cellular cross-talk in carbon partitioning reveals starch-to-lipid biosynthesis switching in low-starch freshwater algae. Bioresour Technol 250:449–456
23. Salama ES, Kim HC, Abou-Shanab RA, Ji MK, Oh YK, Kim SH, Jeon BH (2013) Biomass, lipid content, and fatty acid composition of freshwater *Chlamydomonas mexicana* and *Scenedesmus obliquus* grown under salt stress. Bioprocess Biosyst Eng 36:827–833
24. Chen H, Zhang Y, He C, Wang Q (2014) Ca^{2+} signal transduction related to neutral lipid synthesis in an oil-producing green alga *Chlorella* sp. C2. Plant Cell Physiol 55:634–644
25. Ren HY, Liu BF, Kong F, Zhao L, Xie GJ, Ren NQ (2014) Enhanced lipid accumulation of green microalga *Scenedesmus* sp. by metal ions and EDTA addition. Bioresour Technol 169:763–767
26. Srivastava G, Nishchal GVV (2017) Salinity induced lipid production in microalgae and cluster analysis (ICCB 16-BR_047). Bioresour Technol 242:244–252
27. Scranton MA, Ostrand JT, Fields FJ, Mayfield SP (2015) *Chlamydomonas* as a model for biofuels and bio-products production. Plant J 82:523–531
28. Lv H, Qu G, Qi X, Lu L, Tian C, Ma Y (2013) Transcriptome analysis of *Chlamydomonas reinhardtii* during the process of lipid accumulation. Genomics 101:229–237
29. Driver T, Trivedi DK, McIntosh OA, Dean AP, Goodacre R, Pittman JK (2017) Two glycerol-3-phosphate dehydrogenases from *Chlamydomonas* have distinct roles in lipid metabolism. Plant Physiol 174:2083–2097
30. Luis P, Behnke K, Toepel J, Wilhelm C (2006) Parallel analysis of transcript levels and physiological key parameters allows the identification of stress phase gene markers in *Chlamydomonas reinhardtii* under copper excess. Plant Cell Environ 29:2043–2054
31. Hipkins MF, Baker NR (1986) Photosynthesis energy transduction: a practical approach spectroscopy. IRL Press, Oxford
32. Ansari FA, Gupta SK, Shriwastav A, Guldhe A, Rawat I, Bux F (2017) Evaluation of various solvent systems for lipid extraction from wet microalgal biomass and its effects on primary metabolites of lipid-extracted biomass. Environ Sci Pollut Res 24:15299–15307
33. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. Methods 25:402–408
34. Alvensleben NV, Magnusson M, Heimann K (2016) Salinity tolerance of four freshwater microalgal species and the effects of salinity and nutrient limitation on biochemical profiles. J Appl Phycol 28:861–876
35. Kwak M, Park WK, Shin SE, Koh HG, Lee B, Bryool J, Chang YK (2017) Improvement of biomass and lipid yield under stress conditions by using diploid strains of *Chlamydomonas reinhardtii*. Algal Res 26:180–189
36. Wang N, Qian Z, Luo M, Fan S, Zhang X, Zhang L (2018) Identification of salt stress responding genes using transcriptome analysis in green alga *Chlamydomonas reinhardtii*. Int J Mol Sci 19:3359