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Increasing DNA content for cost-effective oil production in *Parachlorella kessleri*

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ABSTRACT

The aim of this work was to study salt stress effects on DNA content and oil production processes integrating harvesting, lipid accumulation and oil extraction. Salt-induced enlargement of *Parachlorella kessleri* cells, with increasing content of DNA and neutral lipid were found. The 34.77% neutral lipid content and biomass concentration of 0.83 g L^{-1} were obtained after 7 days of salt treatment, compared with that of 13.57% and 0.89 g L^{-1} cultivated under normal condition. Sedimentation efficiency increased markedly from 15% to 90% due to the cell enlargement. Disruption fraction and the recovery rate of total lipids of wet cells under salt stress were significantly higher than that of normal conditions (100% and 82.4% for salt stress vs.76.8% and 51.1% for normal conditions). This work demonstrated that salt-induced increase in cell size and DNA content was an effective strategy for the enhancement of oil production, microalgae harvesting and oil extraction.

1. Introduction

In recent years, microalgae oil is of interest for animal feed and human food industries, as well as for sustainable biofuel production (Rivera et al., 2018). However, the high cost associated with large scale production of microalgae oil infers a long road ahead for their commercialization. Microalgae culture, harvesting, the low productivity of lipid and oil extraction contribute to the high cost of microalgae oil. Various efforts are being made for cost effective production of algal oil, including enhancing lipid concentrations by using environmental stress factors (Sibi et al., 2016) and developing methods of microalgae harvesting (Milledge and Heaven, 2013) or a combination of these.

Stressful environmental conditions such as unfavorable light intensity, temperature, high salinity and nutrient limitation generally lead to lipid accumulation in microalgae (Minhas et al., 2016; Sibi et al., 2016). Among those, salt stress is of central attention. Besides enhancing lipid accumulation, it can also reduce the contamination risk and the dependence on fresh water reserves (Arora et al., 2017). In addition, salt stress is proven to be easier to achieve in mass cultivation (Kim et al., 2016). On the one hand, marine microalgal strains that could grow in brackish water or seawater have been considered as potential bioenergy producers due to their characteristics of high salt tolerance and high lipid content (Ho et al., 2014). *Chlamydomonas* sp. JSC4, *Chlorella sorokiniana*, and *Dunaliella tertiolecta* ATCC30929 have been reported to produce lipid content of 59.4%, 57.7%, and 67.0% w/w of dry weight, respectively (Chen et al., 2013; Ho et al., 2014). On the other hand, fresh water microalgae that can grow in the absence as well in the presence of salts are also used as alternative feedstock for making bioenergy. For example, *Scenedesmus* sp. IITRIND2 cultivated under saline conditions accumulated lipids in quantities in the ranges 38.9–51.8% w/w of dry weight (Arora et al., 2017).

The high cost of harvesting is another key factor limiting the commercial use of microalgae. It has been suggested that harvesting can account for 50% of the total cost (Muradov et al., 2015). Microalgae can be harvested by many methods like sedimentation, flocculation, flotation, centrifugation and filtration (Milledge and Heaven, 2013). Self-flocculation is one of the most convenient strategy for low-cost harvesting microalgae because it requires no extra investment in cultivation of microalgae and purification of bioflocculants (Milledge and Heaven, 2013). Energy consumption of sedimentation harvesting is also generally low (Milledge and Heaven, 2013). However, sedimentation has not been widely used for separation of microalgae due to their density and small size (Milledge and Heaven, 2013). Kato et al. (2017) and Arora et al. (2017) reported that salt stress induced an increase in

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cell size. It is possible that the larger cells could contribute to settlement harvesting of microalgae.

To further lower the unit cost of microalgae oil, high lipid production in association with industrial waste as sources of nutrients (Bahadar and Khan, 2013) or developing high value co-products like extracellular polymeric substance (EPS) seem very promising (Bielsa et al., 2016). *Navicula cincta* could produce both neutral lipid triacylglycerol (TAG) for biodiesel, and EPS, as co-products, under the same conditions (Bielsa et al., 2016). Besides high content of lipids, *Heynigia riparia* SX01 also produced EPS, which resulted in microalgae flocculation (Liu and Miao, 2017). In this process, cost-effective harvesting of microalgae was achieved at the same time, which thus made the production of microalgae bioenergy more conducive and appropriate. Note that, in the context of wastewater treatment, EPS can cause severe membrane biofouling in membrane bioreactors (Sepehri and Sarrafzadeh, 2018).

Parachlorella kessleri is known to accumulate starch and lipid. *P. kessleri* also has characteristics of interest for a semi-industrial scale in outdoor photobioreactors and cellular pre-treatment for lipid extraction. This microalga is therefore considered as one of the most potential feedstocks for biofuel production (Ota et al., 2016; Rivera et al., 2018). In this study, the effect of salt stress on oil production of a newly identified microalga, *P. kessleri*, was explored. An increase in DNA content and cell size were observed in salt-exposed microalgal *P. kessleri*. Neutral lipid content, settlement harvesting and the recovery rate of total lipids of wet microalgae were also investigated. From the results obtained, a new strategy of microalgae oil production was proposed.

2. Materials and methods

2.1. Microalgal cultivation

The algal species used in this study was provided by Ghopur Mijit (Xinjiang University). The Tris-acetate-phosphate (TAP) medium was utilized for cultivation of the strain A2BG1. In the experiments, the algae were cultivated in 250 mL Erlenmeyer flask containing 150 mL TAP medium under 25 ± 1 °C with shaking at 100 rpm. The light intensity was 100 μmol photons m^{-2} s^{-1}. For salt stress conditions, the cells were initially cultivated in TAP medium. After the cells entered exponential phase of growth (optical density (OD) between 0.7 and 1.0 at λ = 640 nm), they were rotated at 5000 rpm for 5 min under 25 °C, then resuspended in TAP medium without salt or with different sodium chloride concentrations (0 M, 0.1 M, 0.2 M, 0.3 M, 0.35 M and 0.55 M) (OD_{640} ~ 0.1). Because 0.35 M NaCl treatment resulted in significant changes in cell morphology, this salt concentration was chosen to use in the following studies.

2.2. Species identification by phylogeny and genetic sequencing

The sample used in this study was first identified based on the available morphological characters. Cell shape, size and arrangement of cells were investigated under the light microscopy.

For molecular identification, the multi-barcoding gene sequences of 18S rRNA, internal transcribed spacer 2 (ITS-2) and rbcL were used. Genomic DNA of microalgae was extracted using the method of CTAB (hexadecyltrimethylammonium bromide) (Porebski et al., 1997). A fragment of the nuclear rDNA including 18S, internal transcribed spacer (ITS) and rbcL was amplified by PCR. The designed primers were as follow: ITS-F: TTCTTAGTTGGTGGGTTGCCT; ITS-R: TTTCATCTTTCCC TCACGGTA; rbcL-F: CGTGACAAACTAAACAAATATGG; rbcL-R: AAGA TTTCAACTAAAGCTGGCA; 18S-F: GGGTGACGGAGRATTAGGGT; 18S-R: TGATGAMTYGSGCTTACTRG. Sequencing was conducted by Shanghai Genewiz. The sequence of 18S rRNA, rbcL, and ITS was compared with known sequences in GenBank (Clark et al, 2016). Phylogenetic tree was constructed using the method of neighbor-joining (NJ) by software MEGA5.2.2 (Tamura et al. 2011) and the genetic distance between species was calculated.

2.3. Determination of biomass production, neutral lipid content and recovery rate of total lipids

Microalgae were harvested through a mixed cellulose esters membrane filter (0.45 μm pore size, 50 mm diameter) (WX Millipore, shanghaibandao, China) after 1, 3, 7, and 12 days cultivation. Then the cells were lyophilized in a freeze drier (FD-1-50, Boyikang, China) and measured for dried biomass production.

For neutral lipid extraction, the modified method of Zonouzi was used (Zonouzi et al., 2016). Grinding and shaking replaced boiling lipid extraction. Additionally, isopropanol and hexane replaced hexane/isopropanol 3:2. After cultivation for 1, 3, 7, and 12 days, microalgae were harvested and then freeze-dried. 0.1 g freeze-dried algae power was grinded in 4 mL isopropanol. After shaking for 15 min, cell debris was removed by centrifugation (5804R, Eppendorf, Germany) (4 °C, 10 min, 12,000 rpm) and supernatant was collected. The sample was transferred to 60 °C water bath and evaporate isopropanol. 1.5 mL Hexane was added to the evaporated sample to extract neutral lipids. The solvent phase was transferred by pipette and evaporated in a water bath at 60 °C. Then the total lipids were weighed with an analytical balance (BS 124S, Sartorius, Germany).

Dry and wet microalgae with equal number of cells were used to determine recovery rate of total lipids. The total lipids were extracted from dry microalgae using a method described by Zhang et al. (2018). For wet microalgae, cells were suspended in 10 mL solvent mixture of chloroform: methanol (2:1, v/v). After shaking, the samples were centrifuged (5804R, Eppendorf, Germany) at 10,000 rpm for 10 min. The procedure was repeated three times. The solvent phase was transferred by pipette and evaporated in a water bath at 55 °C. Then the total lipids were weighed with an analytical balance (BS 124S, Sartorius, Germany). The recovery rate of total lipids was obtained by the following Eq. (1) (Chen et al., 2012):

$$\text{Recovery rate of total lipids} = \frac{W2}{W1 \times \eta} \times 100\% \quad (1)$$

where W_1 (g) was the dry weight of wet microalgae samples, η (% dry weight) was the total lipid content of dry samples, and W_2 (g) was the extracted total lipids of wet samples.

2.4. Microscopic and flow cytometry analysis

Microscopic analysis of cells was done to assess the morphological changes using an Olympus CX41 microscope (Japan). To investigate TAG distribution, samples were first harvested by centrifugation (5804R, Eppendorf, Germany) at 8000 rpm for 10 min and then stained using Nile Red. The operation process was carried out according to the protocols (GenMed Scientifics INC. U.S.A, GMS80050.1). Samples were imaged with a confocal microscope (TCS SP8 STED 3X) using an oil-immersion objective (Leica). The Nile Red signal was captured using a laser excitation line at 488 nm, and emission was captured between 539 and 591 nm. For analysis of DNA content, cells were first harvested (about 10^6 cells mL^{-1}) by centrifugation and then stained with DAPI (4′,6-diamidino-2-phenylindole) according to the manufacturers' protocol (Sangon Biotech, Shanghai, China). After incubated at room temperature for 10 min in darkness, the stained cells were detected using flow cytometer (BD FACSAria II, USA) equipped with a 375 nm laser. To investigate nuclear size, DAPI-stained cells were visualized using fluorescence microscope (ZEISS Axio Imager M2, Germany). Cells (about 10^6 cells mL^{-1}) were detected using a Cytoflex (Cytoflex, Beckman Coulter, USA) and their size were analyzed by FlowJo 10.

2.5. Measurement of sedimentation efficiency

The microalgal suspension of 10 mL was placed into 15 mL

centrifugal tube. After placing for 20 min, 40 min and 60 min, respectively, 5 mL culture was pipetted from supernatant for the evaluation of the sedimentation efficiency by ultraviolet spectrophotometer. The sedimentation efficiency was evaluated according to the Eq. (2) developed by Salim et al. (2011).

$$\text{Sedimentation efficiency}\,(\%) = (\text{A} - \text{B})/\text{A} * 100 \quad (2)$$

where A and B are the absorbance (OD) at 640 nm of the cultures before and after standing, respectively.

2.6. Investigation of cell disruption efficiency

Cell disruption efficiency was evaluated using a modified method of Taleb et al. (2016). Briefly, 30 mL of culture suspension was flowed through a high-pressure disrupter TS0.75 (China) at pressure of 1 K, 3 K, 5 K psi respectively. Cell concentrations N (in cells/ml) were determined under Olympus CX41 microscope using blood cell counting chamber before and after the culture disruption. The percentage of disrupted cells was calculated according to the following Eq. (3).

$$\text{Fraction of disrupted cells}\,(\%) = \frac{1 - \text{N after disruption}}{\text{N before disruption}} \times 100 \quad (3)$$

2.7. RNA extraction, sequencing, data analysis and validation

Algal cells from three independent cultures were harvested by centrifugation. Total RNA extraction was performed with Trizol reagent (Sangon Biotech, Shanghai, China) and purified with Plant RNA Purification Reagent (Invitrogen). After RNA quality testing by Agilent 2100 BioAnalyzer, they were sent to Majorbio company (Shanghai Majorbio Pharmaceutical Technology Co., Ltd.) for RNA-Seq and data analysis. To confirm RNA-seq results, the expression levels of several genes under normal conditions and salt stress were measured using quantitative PCR (qPCR) (Appendix). CBLP (G protein beta subunit-like polypeptide) was used as an internal reference to normalize the expression data. Primers used are listed in Appendix. qPCR was performed with the CFX96 Touch Real-Time PCR Detection System (Bio-Rad, CA, USA) using SYBR Green (Tiangen Biotech Co., Ltd. Beijing, China). The real-time PCR cycle was 95 °C for 15 min followed by 40 cycles at 95 °C for 10 s and 60 °C for 30 s. Triplicate qPCRs were performed for each sample. The ΔΔCT method was used to measure the target gene expression, in which log2 Fold change (log2 $2^{-\Delta\Delta CT}$) replaced traditional Fold change ($2^{-\Delta\Delta CT}$) (Perrineau et al., 2014).

3. Results and discussion

3.1. Morphology and phylogenetic analysis

Light and transmission electron microscopy (TEM) studies showed that the strain A2BG1 displayed the typical and distinctive characters of *Chlorella*. The cells were spherical of 4.3–8.5 μm diameter, lying dispersedly. The cell walls were thin and the pigment body was close to the cell walls. Transmission electron microscopy studies revealed the presence of ellipsoidal pyrenoid surrounded by many small starch grains. Typically, this strain A2BG1 showed asexual reproduction by autosporulation. The mother cell produced large autosporangia, often containing up to 16 autospores. The morphology corresponded best to the genus description of *Parachlorella* (Juárez et al., 2011).

The genome 18S rDNA of A2BG1 was amplified by PCR using specific primers and the amplification product was 1388 bp. Phylogenetic analyses of strain A2BG1 18S rDNA also exhibited close relationships between A2BG1 and *P. kessleri*. As can been seen from Fig. 1, the bootstrap value of A2BG1 and *P. kessleri* was 96 and the genetic distance between them is 0.000, indicating that A2BG1 had close affinity to *P. kessleri*. Herein we used a multimethod approach including microscopic and phylogenetic analyses to identify the strain A2BG1 as *P. kessleri*.

3.2. Alterations of DNA content and genes involved in DNA synthesis in Parachlorella kessleri under salt stress

In this work, a sharp increase in cell size was found in *P. kessleri* cultivated under 350 mM NaCl conditions, which may be involved in salt tolerance. Similar behavior has been observed in *Scenedesmus* sp. IITRIND (Arora et al., 2017). It is claimed that the increase in cell size play an important role for the accumulation of sodium (Dassanayake and Larkin, 2017). It is also reported that cell enlargement allows the cells to tolerate salt stress by increasing the capacity for sodium sequestration and these enlarged-cells have high level of ploidy (Barkla et al., 2018). Using fluorescence microscopy and DAPI, we found salt-treated *P. kessleri* cells possessed larger nuclei and their fluorescence intensity were higher than that of untreated cells. Nuclear DNA content is positively correlated with nuclear volume in angiosperms (Jovtchev et al., 2006). Variations of DNA content were further investigated in cells at days 1, 3, 7, and 12 using flow cytometry. As shown in Fig. 2, there was no difference in fluorescence intensity between salt-treated and untreated cells at day 1. After 1 day, however, the fluorescence value of salt-treated cells was significantly higher than that of the respective controls (Fig. 2), in line with the fluorescence microscopy observation. Our findings suggested that salt stress resulted in an increase in cell size with higher nuclear DNA content. Similarly, Barkla et al. (2018) observed that salt treatment led to an increase both in ploidy levels and cell size in the halophyte *Mesembryanthemum crystallinum*, implying that salt-induced increase in cell size and DNA content is common in plants. Cell size and nuclear DNA content are positively correlated (Jovtchev et al., 2006). Thus, one effect of enhanced DNA content is likely increased cell size.

Cells with heightened ploidy need to regulate expression of genes involved in DNA replication (De Veylder et al., 2011). In *P. kessleri* cells under salt stress, a focal set of genes associated with DNA replication was selected for specific analysis. The fold changes of this focal set of genes are presented in Table 1. DNA replication is initiated by origin recognition complex (ORC). ORC serves as the foundation for assembly of the pre-replicative complex, which includes ORC, homohexamer of the mini chromosome maintenance (MCM) protein and etc. MCM (helicase) separates the two strands of DNA, and Pol α forms a complex with primase. Once primase has created the RNA primer, Pol α starts replication elongating the primer with ~20 nucleotides. Due to its low processivity, Pol δ and Pol ε take over the strand synthesis from Pol α. In this process, single-strand binding proteins bind to the single-stranded DNA to prevent it from folding back. Clamp loader recognizes the junction between template and RNA primers and loads the clamp (PCNA). PCNA forms a sliding clamp around DNA, helping the DNA polymerase maintain contact with its template, thereby assisting with processivity. When replication is complete, the primer RNA fragments are removed by RNase and then replaced with DNA. All the complexes mentioned above were maintained at a relatively higher level in salt-treated *P. kessleri* cells than that in control ones, except for Pol δ-coding transcripts (Table 1).

Pol δ and Pol ε are high-fidelity replicative polymerases and responsible for eukaryotic genome duplication (Manhart and Alani, 2017). Pol δ was up-regulated and Pol ε down-regulated in response to salinity-induced increase of DNA content in *M. crystallinum* (Barkla et al., 2018). In our study, however, upregulation of Pol ε and down-regulation of Pol δ were observed. Rinku et al. reported that Pol ε is perhaps more sensitive than other DNA polymerases in response to oxidative stress (Jain et al., 2014). So, upregulation of Pol ε may have special significance in DNA replication in salt-treated *P. kessleri* cells. In terms of replicative helicase, PCNA loading complex and replication protein A (RPA) (single-stranded DNA binding), they showed significantly differential overexpression in salt-exposed *P. kessleri* cells, in line with the observations in salinity-induced endopolyploidy in *M.*

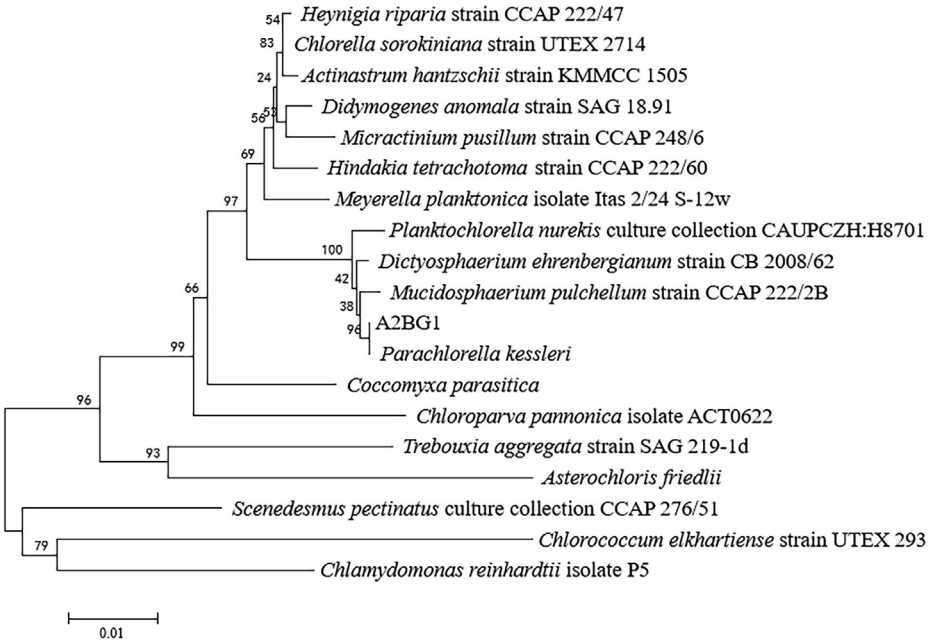

Fig. 1. The molecular phylogenetic tree of A2BG1 based on 18S rDNA sequences, inferred by neighbor-joining using MEGA5.2.2.

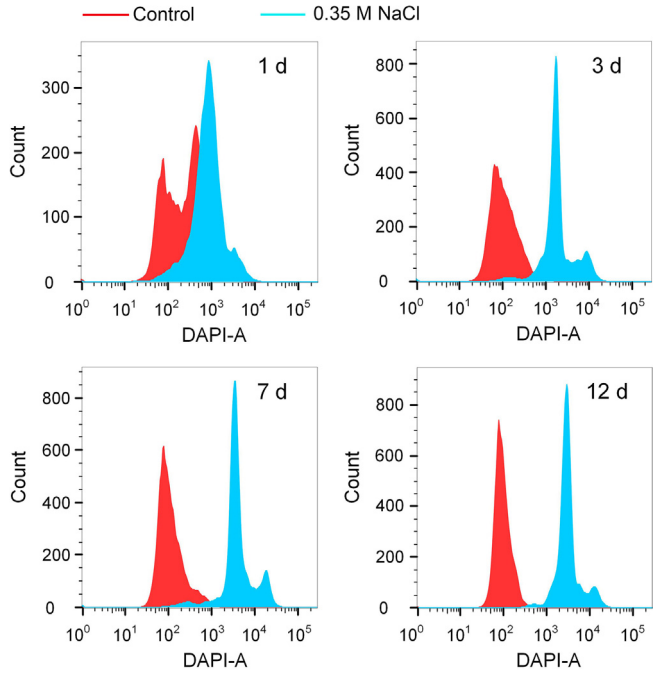

Fig. 2. Flow cytometry analysis of DNA in *Parachlorella kessleri* cells at days 1, 3, 7, and 12 respectively.

crystallinum (Barkla et al., 2018). The result suggested that upregulation of genes associated with DNA replication played an important role in DNA replication and thus contributed to total elevated DNA content.

DNA replication requires the presence of cellular deoxynucleotide (dNTP) pools and higher dNTP concentrations can facilitate DNA repair and replication (Williams et al., 2015). Inosine monophosphate (IMP) and uridine monophosphate (UMP) are nucleoside monophosphate and serve as precursor for dNTP synthesis. As shown in Fig. 3, many genes involved biosynthesis of IMP and UMP were up-regulated. Moreover,

Table 1
Differential expression genes involved in DNA replication in *Parachlorella kessleri* during salt stress. All presented fold changes are statistically significant, q value < 0.05.

DNA replication related denes	Seq_id	LogFC
ORC (Origin Recognition Complex)		
Orc3	TRINITY_DN5956_c3_g4	1.21
Orc4	TRINITY_DN5161_c0_g2	1.10
DNA polymerase α-primase complex		
α1	TRINITY_DN5878_c0_g2	1.50
Pri1	TRINITY_DN4731_c3_g10	1.31
Pri2	TRINITY_DN5475_c3_g1	1.35
DNA polymerase δ complex		
δ1	TRINITY_DN5481_c0_g3	−1.02
DNA polymerase ε complex		
ε1	TRINITY_DN4277_c0_g1	1.87
MCM complex (helicase)		
Mcm2	TRINITY_DN5045_c4_g3	1.75
Mcm3	TRINITY_DN4718_c0_g2	1.27
Mcm4	TRINITY_DN4718_c0_g1	1.16
Mcm6	TRINITY_DN5579_c2_g5	1.87
Mcm7	TRINITY_DN5579_c2_g1	2.13
RPA		
RFA1	TRINITY_DN5490_c3_g1	1.30
Clamp		
PCNA	TRINITY_DN5734_c1_g2	1.35
Clamp loader		
RFC2/4	TRINITY_DN3290_c0_g1	1.21
RFC3/5	TRINITY_DN4075_c0_g1	1.11
RNaseH		
RNaseH2B	TRINITY_DN5084_c2_g1	1.50

the gene encoding ribonucleotide reductase (RR), a rate-limiting enzyme that reduces NDP to dNDP in the dNTP biosynthetic pathway, was significantly up-regulated. The gene encoding diphosphate kinases (UDPK), which converts dNDP to dNTP, was also significantly up-regulated (Fig. 3). Almost all genes involved in dNTP biosynthesis show

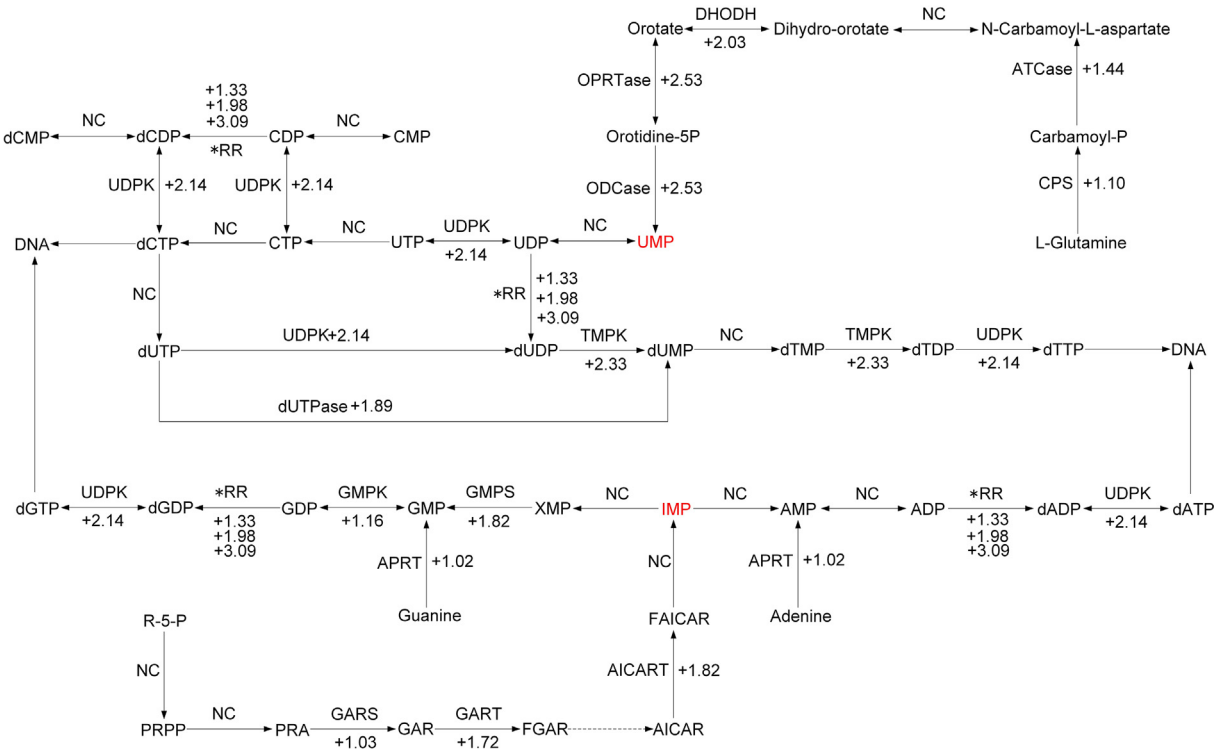

Fig. 3. Differential expression of genes involved in dNTP biosynthesis. Enzymes include: CPS, Carbamoyl phosphate synthetase (EC: 6.3.5.5); ATCase, Aspartate transcarbamoylase (EC: 2.1.3.2); DHODH, Dihydroorotate dehydrogenase (EC: 1.3.5.2); OPRTase, Orotate phosphoribosyltransferase (EC: 2.4.2.10); ODCase, Orotidine-5′-phosphate decarboxylase (EC: 4.1.1.23); UDPK, Nucleoside diphosphate kinase (EC: 2.7.4.6); TMPK, dTMP kinase (EC: 2.7.4.9); dUTPase, dUTP diphosphatase (EC: 3.6.1.23); GARS, Glycinamide ribonucleotide synthetase (EC: 6.3.4.13); GAR TFase, Glycinamide ribonucleotide transformylase (EC: 2.1.2.2); AICART, AICAR transformylase (EC: 2.1.2.3); APRT, Adenine phosphoribosyltransferase (EC: 2.4.2.7); GMPS, GMP synthase (EC: 6.3.5.2); GMPK, GMP kinase (EC: 2.7.4.8); RR, Ribonucleotide reductase (EC: 1.17.4.1). Abbreviations: ribose-5-phosphate (R-5-P); 5-phosphoribosyl-1-pyrophosphate (PRPP); β-5-phosphoribasylamine (PRA); glycinamide ribonucleotide (GAR); formyl glycinamide ribonucleotide (FGAR); 5-aminoimidazole-4-carboxamide ribotide (AICAR); and 5-formaminoimidazole-4-carboxyamideribotide (FAICAR). NC represents no change. Key enzymes are shown with an asterisk (*) next to the enzyme abbreviations. Dashed arrows denote reaction(s) for which the enzymes are not shown. All presented fold changes are statistically significant, q value < 0.05.

significant increases in transcript levels in salt-exposed *P. kessleri* cells, suggesting that ensuring dNTP pools is important for progression of DNA replication. Taken together, these results suggested that, under salt stress, the upregulation of genes associated with DNA replication and dNTP biosynthesis causes an increase in DNA content, thereby increasing cell size.

3.3. Accumulation of neutral lipids in salt-exposed P. kessleri

It was reported that the increased copies of the nuclear genome may provide the increased content of compounds and number of particular cellular organelles to combat the stress (Scholes and Paige, 2015). As can be seen from Table 2, the neutral lipid production in *P. kessleri* was greatly improved under 0.35 M salt concentration, as compared with that under untreated culture. The maximum neutral lipid content (36.13% biomass) was obtained after 12 days cultivation, which was 1.31-fold higher than that of untreated culture (15.63% biomass) (q value < 0.05) (Table 2). Neutral lipids are main component that can contribute to bio-oil production. Microalgae store neutral lipids in specialized organelles known as lipid droplets (LDs). Nile red staining of algal cells cultivated in 0.35 M salt concentration evidenced the increase of bright-yellow LDs inside the cells, as depicted in Appendix. Salt-exposed cells showed the increased neutral lipid production during the cultivation, and those cells cultivated for 7 days contained the most NR fluorescent LDs. On the contrary, LDs were almost not detected in *P. kessleri* under untreated culture (see Appendix). In addition, besides upregulation of genes involved in DNA synthesis, gene coding for lysophosphatidic acidacyltransferase (LPAAT) was also up-regulated 5.08-fold. LPAAT contributes to TAG accumulation (Kim et al., 2018). All these results suggested that enhanced DNA content in salt-exposed cells may cause an increase in neutral lipid content, thereby contributing to oil production.

Table 2
Dried biomass concentration and neutral lipid production of *Parachlorella kessleri* under the salt stress. Data are mean value of three repetitions (means ± SD). Neutral lipids changes are statistically significant, q value $<$ 0.05. There was no statistically significant change in dried biomass.

Culture Conditions	Neutral lipids (% dried biomass)		Dried biomass (g L^{-1})	
	Control	0.35 M NaCl	Control	0.35 M NaCl
1 day	8.47 ± 0.37	25.73 ± 0.55	0.30 ± 0.02	0.18 ± 0.01
3 day	10.03 ± 0.42	31.20 ± 1.68	0.89 ± 0.01	0.49 ± 0.07
7 day	13.57 ± 0.64	34.77 ± 1.01	0.89 ± 0.01	0.83 ± 0.06
12 day	15.63 ± 0.38	36.13 ± 0.57	0.92 ± 0.02	0.84 ± 0.04

Stress condition generally results in reduced growth and decreased biomass. However, the growth of *P. kessleri* was little affected by the salt stress in the present study. As shown in Table 2, the biomass production of *P. kessleri* under 0.35 M NaCl was relatively lower during the initial three days as compared with that under control condition. This phenomenon was in line with the result of *Chlamydomonas* sp. ICE-L (An et al., 2013). Surprisingly, the biomass concentration of *P. kessleri* under 0.35 M salt stress reached as high as 0.83 g L^{-1} after 7 days cultivation (Table 2), which was basically the same as that under normal condition (0.89 g L^{-1}). This increase in biomass production might be due to the increase in cell volume under salt stress.

Stress condition is generally considered as the most common and

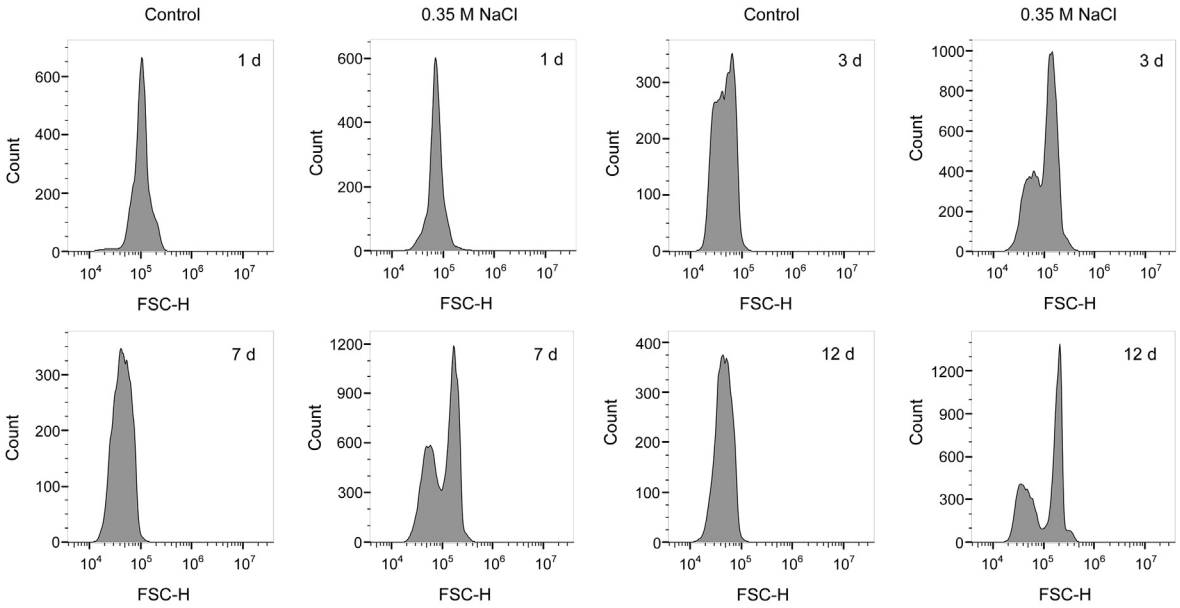

Fig. 4. Cell size comparison between 0 and 0.35 M NaCl concentrations using flow cytometry.

effective way to trigger lipid accumulation for biodiesel purpose (Sharma et al., 2012). However, slowing down cell division and photosynthesis are as secondary results of this pattern (Msanne et al., 2012). Therefore, researchers have been focusing on the tradeoff between biomass and lipid accumulation in microalgae under stress condition. In this research, we found that the neutral lipid production of *P. kessleri* were accumulated significantly after cultivated in 0.35 M NaCl for 7 days without sacrificing production efficiency. This constitutes a desired trait for economical oil production.

3.4. Enhancement of harvesting and lipids extraction under salt stress

Given that the enlarged cells could contribute to sedimentation harvesting, the more detailed cell size at days 1, 3, 7, and 12 was measured by flow cytometry. As shown in Fig. 4, cells cultivated in normal conditions showed a uniform value in FSC (forward scatter), whereas cells under 0.35 M NaCl for 3, 7, and 12 days showed a distinctive shift away from lower FSC value, with an enrichment in a high value. FSC is positively associated with cell size. The result indicated that a group of cells increased their size in response to salt stress compared to that of untreated cells, consistent with the microscope observations. Using the mean FSC as a measure of cell size, salt-treated cells for 3, 7, and 12 days respectively increased their size by 1.3-, 1.8-, and 1.8-fold, as compared to the respective controls (Fig. 5a).

Harvesting is one of the most expensive steps of production of microalgae oil, and sedimentation are one of the most convenient strategies for low-cost harvesting microalgae (Milledge and Heaven, 2013). Increased cell volume in salt stress condition could influence the efficiency of cell sedimentation, and thus could lead to a reduction in the cost of harvesting. Therefore, salt-induced sedimentation of *P. kessleri* was investigated. The cell size (Fig. 5a), biomass, and neutral lipid content (Table 2) of *P. kessleri* under salt stress at day 7 were similar to that of day12, thus cells at day 7 were selected for following studies.

The efficiency of gravity sedimentation as a function of settling was manifested in Fig. 5b. Cell size significantly influenced the sedimentation efficiency of *P. kessleri*. The sedimentation efficiency of *P. kessleri* cultivated under normal conditions was less than 15%, while the sedimentation efficiency of microalgae with 0.35 M NaCl was greatly increased, which was as high as 90% (Fig. 5b). Church et al. (2017) similarly observed the increased cell size of *Chlorella vulgaris* under salt stress and reported that algae settling was improved by 33–83%. Calculations performed by Stokes' Law show that sedimentation efficiency is proportional to the square of the radius of the microalgae cells (Milledge and Heaven, 2013). Our results indicated that salt-induced increase of cell size can contribute to the improvement of sedimentation efficiency. Both sedimentation and flocculation can simplify the dewatering process. Flocculants are usually added to induce flocculation. Self-flocculation of algal cells without flocculants addition is economically viable. However, self -flocculation does not occur in all microalgae species and can be slow and unreliable (Schenk et al., 2008). In this study, high content of lipids and cost-effective harvesting of microalgae (sedimentation) were achieved at the same time, which thus made algae-based bioenergy more economically attractive.

Oil extraction from dry microalgae is a high energy consumption process due to the drying of biomass. The disruption of microalgae cells is one method to facilitate lipid extraction in wet environment. Larger cells will experience more disruptive eddies than smaller cells and result in greater disruption (Geciova et al., 2002). We used a high pressure disrupter to evaluate disruption efficiency of salt-treated *P. kessleri* cells. As shown in Fig. 5c, salt-treated *P. kessleri* had significantly higher cell disruption fraction compared with that of untreated cell. Disruption fraction under 0.35 M NaCl at 5 K psi was almost 100%, compared with 76.8% cultivated under normal condition. High cell fragility also contributes to disruption efficiency (Taleb et al., 2016). Yao et al. (2016) reported that *Dunaliella salina* grown in 3% NaCl showed a bigger size and these cells were easy to be broken, implying an increase in cell fragility. Similarly, pressing the covering slide with the finger resulted in broken of the salt-treated *P. kessleri* cells (see Appendix). These results suggested that both cell enlargement and the resulted higher cell fragility contributed to the observed increase in disruption efficiency.

We further compared lipid extraction rate of treated and untreated *P. kessleri* cells in wet environment. The recovery rate of total lipids of wet cells under 0.35 M NaCl was 82.4%, which was 1.61 times than that of wet cells under normal conditions, indicating that salt-exposed *P. kessleri* cells presented promising results for lipid extraction in a wet environment. These results suggested that salt-induced cell size enlargement and DNA content enhancement could contribute to neutral lipid accumulation without sacrificing biomass, cell harvesting and wet oil extraction, thus leading to suitable microalgae oil production.

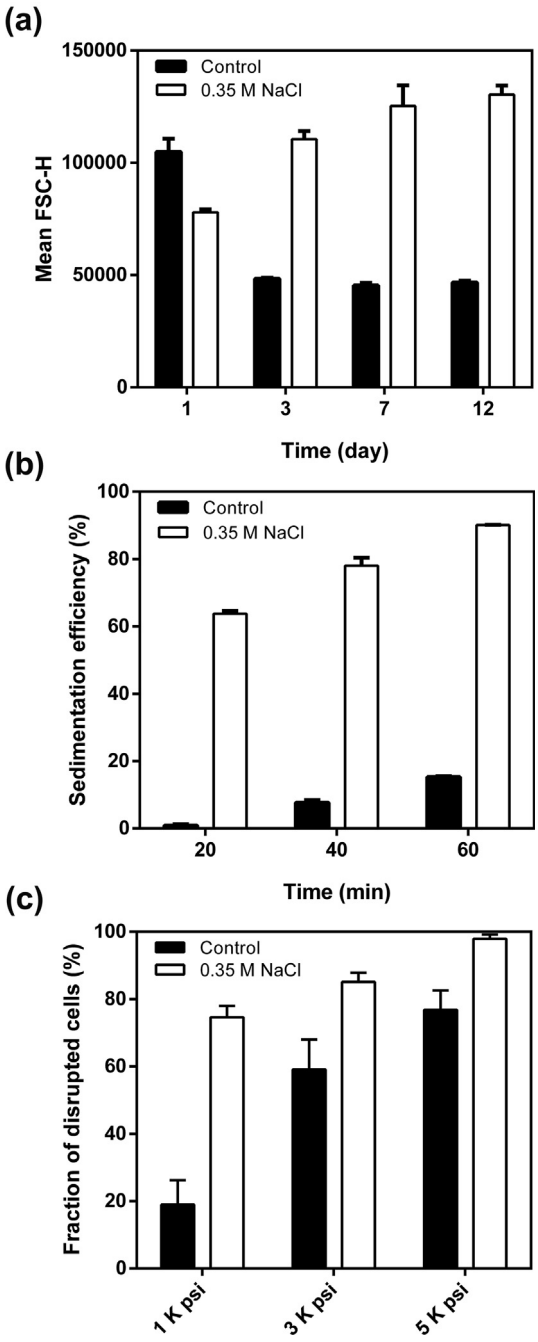

Fig. 5. Quantitative analysis of cell size using FlowJo 10 (a), sedimentation efficiency (b), fraction of disrupted cells (c) of *Parachlorella kessleri* under salt stress.

4. Conclusions

In present study, salt stress induced an increase in cell size and DNA content in *Parachlorella kessleri*. Upregulation of genes associated with DNA replication and dNTP biosynthesis were responsible for this increase, thereby likely causing 2.3-fold enhancement in neutral lipid content in salt-treated cells. The increased cell size contributed to settlement harvesting and oil extraction from wet microalgae. The sedimentation efficiency and recovery rate of total lipids in the case of 0.35 M NaCl treatments increased by 8-fold and 1.61-fold, respectively, as compared to that of controls. Based on the results, a new strategy of microalgae oil production was proposed.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2019.121332.

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