Journal of Microbiology (2018) Vol. 56, No. 1, pp. 56–64
DOI 10.1007/s12275-018-7488-6
eISSN 1976-3794
pISSN 1225-8873

Application of high-salinity stress for enhancing the lipid productivity of *Chlorella sorokiniana* HS1 in a two-phase process[§]

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(Received Nov 8, 2017 / Revised Nov 19, 2017 / Accepted Nov 20, 2017)

Increased lipid accumulation of algal cells as a response to environmental stress factors attracted much attention of researchers to incorporate this stress response into industrial algal cultivation process with the aim of enhancing algal lipid productivity. This study applies high-salinity stress condition to a two-phase process in which microalgal cells are initially grown in freshwater medium until late exponential phase and subsequently subjected to high-salinity condition that induces excessive lipid accumulation. Our initial experiment revealed that the concentrated culture of *Chlorella sorokiniana* HS1 exhibited the intense fluorescence of Nile red at the NaCl concentration of 60 g/L along with 1 g/L of supplemental bicarbonate after 48 h of induction period without significantly compromising cultural integrity. These conditions were further verified with the algal culture grown for 7 days in a 1 L bottle reactor that reached late exponential phase; a 12% increment in the lipid content of harvested biomass was observed upon inducing high lipid accumulation in the concentrated algal culture at the density of 5.0 g DW/L. Although an increase in the sum of carbohydrate and lipid contents of harvested biomass indicated that the external carbon source supplemented during the induction period increased overall carbon assimilation, a decrease in carbohydrate content suggested the potential reallocation of cellular carbon that promoted lipid droplet formation under high-salinity stress. These results thus emphasize that the two-phase process can be successfully implemented to enhance algal lipid productivity by incorporating high-salinity stress conditions into the pre-concentrated sedimentation ponds of industrial algal production system.

Keywords: *Chlorella sorokiniana* HS1, high-salinity stress, lipid induction, microalgae, two-phase process

Introduction

The capacity of algae to grow under different environmental conditions with high lipid accumulation potential and the possibility of cultivating algal biomass in arid and/or marginal areas with higher aerial biomass productivities than conventional energy crops support algae as a feasible candidate for the production of biofuels (Hu *et al*., 2008; Smith *et al*., 2010; Davis *et al*., 2011). However, the current techno-economic status of algae-based biofuels is not promising enough mainly because high costs associated with the cultivation and harvesting of algal biomass at the commercial- scale (Davis *et al*., 2011). In particular, achieving the bulk production of lipid-rich algal biomass is an imperative precondition to make the price of algae-based biofuels competitive enough to replace at least a portion of current market share of conventional fossil fuels. Nonetheless, there is an obvious trade-off between optimizing the yields of total algal biomass vs. lipids in algal biomass production facilities designed to harvest algal biomass for downstream processes (Shurin *et al*., 2013). It has therefore attracted the keen interest of researchers to develop cost-effective strategies to achieve high lipid accumulation in algal cells without significantly compromising biomass productivity by carefully optimizing culture operation conditions in both photobioreactors and open pond systems (Narala *et al*., 2016; Sung *et al*., 2017).

In particular, it is well established that the production of algal lipids can be enhanced with environmental stress factors that generally induce hyper-accumulation of lipids as a response to the stress conditions (Mata *et al*., 2010; Mulders *et al*., 2014; González *et al*., 2015; Minhas *et al*., 2016). Up to date, an array of stress factors have been explored to induce hyper-accumulation of algal lipids, including nutrient starvation (i.e., nitrogen, phosphorus), metal ions (i.e., iron, cadmium, copper and zinc), high irradiance, and temperature (Pal *et al*., 2011; Yeesang and Cheirsilp, 2011; El-Kassas, 2013; Ho *et al*., 2014). Nonetheless, combining these stress factors into algal cultivation process is likely to incur additional costs associated with the fabrication and operation of superfluous facilities for high lipid induction. It is therefore preferable to invent a lipid induction strategy that utilizes readily available platforms at industrial algal biomass production. For

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[§]Supplemental material for this article may be found at http://www.springerlink.com/content/120956.

example, Narala *et al.* (2016) suggested a hybrid system that combines exponential biomass production in bioreactors with a separate synchronized high-lipid induction phase in open ponds, and achieved high algal lipid productivity (Narala *et al.*, 2016). Furthermore, Sung *et al.* (2017) demonstrated a serially-connected photobioreactor system that exhibited significantly increased overall lipid productivity because of nitrogen limiting condition in the photobioreactor connected at the end of the serial system (Sung *et al.*, 2017).

Incorporation of lipid inducing stress condition into sedimentation ponds may also provide a promising strategy that eliminates the necessity of assembling additional facilities for high lipid induction. Generally, an industrial algal cultivation system requires a two-step harvesting process of bulk harvesting and thickening, and the sedimentation ponds are utilized during the bulk harvesting process to separate algal biomass from the bulk suspension using flocculation, flotation, or gravity sedimentation (Chen *et al.*, 2011). Applying stress factors to these sedimentation ponds is likely to substantially reduce capital and operating costs for lipid induction process (Brennan and Owende, 2010; Uduman *et al.*, 2010; Chen *et al.*, 2011).

In previous study, we isolated a novel *Chlorella* strain that hyper-accumulates lipids under high-salinity stress condition; however, it was found that the high-salinity condition significantly limited the production of algal biomass (Kim *et al.*, 2016b). Separating biomass growth and high lipid induction in a two-phase process may provide a promising strategy for enhancing algal lipid productivity by successfully applying high-salinity stress to fully-grown algal culture during the later stage of two-phase process. In addition, high-salinity stress can be relatively easily incorporated into industrial algal cultivation platforms as schematically described in Fig. 1 by supplying saline water sources or inorganic salts into the sedimentation ponds.

The purpose of this study was thus applying high-salinity stress condition to later lipid induction phase of two-phase process with the aim of enhancing algal lipid productivity. While NaCl was supplemented into concentrated algal cultures to induce high lipid accumulation, we initially tested the influences of salinity level, the density of concentrated algal culture, and supplemental bicarbonate on algal lipid accumulation during high lipid induction phase. The identified lipid induction conditions were subsequently validated at lab scale to assess the lipid productivity-enhancing potential of two-phase process by comparing the lipid yields of (1) the culture amended with two-phase process and (2) the culture operated in conventional one-step cultivation mode in either freshwater or saline growth media under conditions identical to those of the first growth-stage of two-phase process.

Materials and Methods

Strain selection

We have previously isolated *Chlorella sorokiniana* HS1 from a local swine wastewater source (Kim *et al.*, 2016b). Notably, *C. sorokiniana* HS1 exhibited its growth in a wide range of sodium chloride concentrations (0–60 g/L) (Kim *et al.*, 2016b). While the biomass accumulation of *C. sorokiniana* HS1 was significantly reduced above the salt concentration of 40 g/L, this strain exhibited a 59% increase in lipid content at high salt concentration (Kim *et al.*, 2016b). The halotolerance and lipid accumulation potential of this freshwater microalga, therefore, supported its use in the demonstration of the two-phase process in which algal growth and high lipid induction phases are separated.

Cultivation of *Chlorella sorokiniana* HS1

C. sorokiniana HS1 was initially cultivated in a 1 L bottle reactor with a working volume of 600 ml using freshwater BG-11 medium with the following composition per L: $NaNO_3$ (1.5 g), $MgSO_4{\cdot}7H_2O$ (75 mg), $CaCl_2{\cdot}2H_2O$ (36 mg), citric acid (6 mg), H_3BO_3 (2.86 mg), $MnCl_2{\cdot}4H_2O$ (1.81 mg), $ZnSO_4{\cdot}7H_2O$ (222 μg), $Na_2MoO_4{\cdot}2H_2O$ (390 μg), $CuSO_4{\cdot}5H_2O$ (79 μg), $Co(No_3)_2{\cdot}6H_2O$ (49.4 μg), ferric ammonium citrate (6 mg), Na_2CO_3 (20 mg), and KH_2PO_4 (30.5 mg) (Rippka *et al.*, 1979). Algal reactor was inoculated at the cell concentration of 0.02 g DW/L with bacteria-free seed culture that was obtained by treating the xenic culture of *Chlorella sorokiniana* HS1 in BG-11 medium with ultrasonication, fluorescence activated cell sorter (FACS), and micropicking (Cho *et al.*, 2013). Prior to the inoculation of algal reactor, samples withdrawn from bacteria-removed seed cultures were streaked onto five different types of agar plates that contained organic carbon sources (i.e., YM, R2A, LB, TSA, and BG-11 + 100 ppm of glucose) to assure the absence of culturable bacteria (Cho *et al.*, 2013, 2015). Continuous illumination was provided with white fluorescent lamp at the intensity of 400 μmol/m^2/sec following inoculation, and the reactor was operated at 25°C

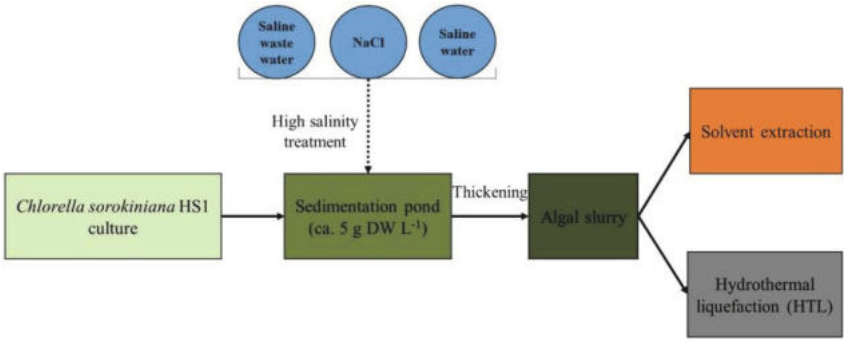

Fig. 1. A schematic diagram of applying high-salinity stress to the sedimentation pond of industrial algal cultivation system for high lipid induction.

with the supplementation of 2% CO_2 at 0.4 vvm throughout the cultivation period. The culture reached late-exponential phase at the culture density of 2.5 g DW/L after a 7-day-long growth period, and it was subsequently harvested to screen salinity stress conditions for high lipid induction.

Screening of salinity stress conditions for high lipid induction

Because the industrial implementation of two-phase process will necessitate the careful optimization of operational conditions, we initially screened favorable salinity level and algal culture concentration for high lipid induction based on the fluorescence intensity of algal cells after staining neutral lipids with Nile Red (9-diethylamino-5 H-benzo[α]phenoxazine-5-one; HPLC grade, Sigma Aldrich). Each experimental group was tested in test tubes (10 × 50 mm) in triplicate under the identical light and temperature conditions of initial biomass accumulation phase without supplemental CO_2 and the results were presented as mean values.

Screening of salinity level

The influence of different NaCl concentrations on the Nile red fluorescence intensity of the harvested *C. sorokiniana* HS1 culture was initially explored throughout the course of a 48 h induction period. Each test tube was subjected to different salinity levels with the supplemental NaCl at the concentrations of 0, 30, 60, and 90 g/L.

Screening of culture density condition

While sedimentation ponds can serve as a suitable platform to induce high lipid accumulation by incorporating stress conditions into the culture that reached late-exponential to stationary phase, it should be noted that total solid matter in sedimentation ponds generally reaches around 2–7% using flocculation, flotation, or gravity sedimentation (Brennan and Owende, 2010; Chen *et al.*, 2011). Therefore, it is necessary to explore the influence of concentrating algal culture within the range of culture density in sedimentation ponds to achieve industrial operation of high salinity-based lipid induction.

After harvesting the culture of *C. sorokiniana* HS1 that reached late-stationary phase at the density of 2.5 g DW/L, we further concentrated the harvested culture with centrifugation at 4,500 × *g* for 5 min to reach the culture densities of 5, 7.5, and 10 g DW/L, assuming the case of allowing more time and/or flocculants in the operation of sedimentation ponds at industrial algal biomass production. Each culture density condition, including the density of unconcentrated culture following the first growth phase, was tested under initially screened salinity level during a 48 h long lipid induction period to explore suitable culture density for high lipid accumulation.

Testing potential influence of supplemental bicarbonate on lipid induction

Because this study presumes the application of high salinity stress into the sedimentation ponds of algal cultivation facilities, supplying external carbon source as gaseous CO_2 may be limited due to a possible mechanical disturbance to the sedimentation of algal cells and the costs associated with supplying gaseous CO_2 in sedimentation ponds. We thus further evaluated the influence of supplying powdered sodium bicarbonate on high lipid induction under the identified salinity and culture concentration conditions in triplicated 250 ml flasks. Sodium bicarbonate was supplemented at the concentration of 1 g/L, and its influence on high lipid induction during a 48 h long induction period was explored by comparing the lipid content of harvested biomass with and without additive sodium bicarbonate.

Laboratory-scale demonstration of two-phase process

1 L bottle reactors were operated under identical conditions as the reactor used in initial screening for high lipid induction conditions to demonstrate the two-phase process with separate biomass growth and lipid induction phases. Upon reaching late exponential phase at the cell concentration of 2.5 g DW/L on Day 7, the culture was harvested by centrifugation at 4,500 × *g* for 5 min, and a portion of supernatant was properly removed to ensure that the resuspended culture reached the desired concentration. Following the resuspension of algal culture in triplicated 250 ml flasks, NaCl was added to expose concentrated algal culture to high-salinity stress under the identified lipid induction conditions. Subsequently, algal biomass was harvested by centrifugation and was freeze-dried for further analysis.

To compare the extent of lipid enhancing capacity of the proposed two-phase process, we also conducted the cultivation of *Chlorella sorokiniana* HS1 in 1 L bottle reactors using (i) freshwater BG-11 medium and (ii) BG-11 medium supplemented with 60 g/L of NaCl under the above-mentioned cultivation conditions in conventional single-step cultivation mode for 9 days. The harvested culture at the end of a 9-day-long cultivation period was lyophilized for further biochemical analysis, and the lipid productivity of each reactor was calculated to compare lipid productivity between the culture amended with two-phase process and the culture operated in conventional single-step cultivation mode.

Analytical methods

Culture growth measurement : The microalgal cell growth was monitored by measuring optical density (OD) at 660 nm with spectrophotometer (UV-2450, Shimadzu). The dry weight (DW) was also determined by filtration onto preweighed GF/C filters (Whatman). After rinsing with distilled water, the filters were dried at 105°C overnight. The filters were reweighed after drying and the DW was calculated from the difference between the filter weight with and without algal biomass.

Nile red staining and analysis : Algal cultures were sampled throughout the course of lipid induction phase, and 1 ml of each sample was stained with Nile red stain to reach the final concentration of 0.1 μg/ml. The samples were kept in dark for 5 min, followed by fluorescence imaging using Olympus BX51 fluorescence microscope set with Olympus model UMF2 filter with excitation at 451–490 nm and emission at 491–540 nm (Olympus). Afterwards, the fluorescence intensity of Nile red was measured in triplicated samples using a microplate fluorescence reader (BioTek) (Ramanan *et al.*,

2013).

Biochemical analyses : Total lipid, protein, and carbohydrate contents were measured as mentioned in previous studies (Ramanan *et al*., 2013; Habiby *et al*., 2014; Kim *et al*., 2016b). Briefly, total lipids were extracted by mixing chloroform-methanol (2:1 [v/v]) with the samples in a proportion of 1:1 using a slightly modified version of Bligh and Dyer's method (Bligh and Dyer, 1959). The lipid fraction was subsequently obtained by evaporating the solvent using a rotary evaporator, and the weight of crude lipid was measured for each sample (Kang *et al*., 2015). Total protein was indirectly quantified from nitrogen measurement by micro-Kjeldahl digestion method (Miller and Houghton, 1945; Wang *et al*., 2016), and carbohydrate content was determined based on the phenol-sulfuric acid methods by using Total Carbohydrate Assay Kit (Sigma-Aldrich).

Fatty acid methyl ester composition analysis

Fatty acid methyl ester (FAME) composition analysis was performed using a gas chromatograph (Shimadzu GC-2010). 50 mg of each sample was first placed into capped test tubes, saponified with 1 ml of a saturated KOH-CH_3OH solution at 75°C for 10 min, and then submitted to methanolysis with 5% HCl in methanol at 75°C for another 10 min (Kang *et al*., 2015). Fatty acids containing phase was subsequently separated by adding 2 ml of distilled water and recovered (Kang *et al*., 2015). The components were identified by comparing their retention times and fragmentation patterns with those for standards (Xu *et al*., 2001; Kang *et al*., 2015). Six fatty acids ($C_{16:1}$, $C_{17:0}$, $C_{18:0}$, $C_{18:1}$, $C_{18:2}$, and $C_{18:3}$) were used as the standard materials (Lee *et al*., 2010; Kang *et al*., 2015).

Transmission electron microscopy (TEM) imaging analysis

The concentrated cultures of *C. sorokiniana* HS1 with and without high-salinity stress treatment were centrifuged (1,000 × *g*, 10 min) and each pellet was fixed in 25 g/L paraformaldehyde-glutaraldehyde mixture buffered with 0.1 mol/m^3 phosphate (pH 7.2) for 2 h, post-fixed in 10 g/L osmium tetroxide in the same buffer for 1 h, dehydrated in graded ethanol and propylene oxide, and embedded in Epon-812 (Kim *et al*., 2016b). Ultra-thin sections were prepared by using a Leica ULTRACUT E ultramicrotome (Leica), and they were subsequently examined under a CM-20 electron microscope (Philips Electron Optics) after uranyl acetate and lead citrate staining.

Results and Discussion

Screening of salinity and culture density conditions for lipid induction under high-salinity stress

The initial screening of NaCl concentration for high lipid induction revealed that the Nile red fluorescence intensity was greatest with the addition of NaCl at the concentration of 60 g/L, whereas increasing NaCl concentration to 90 and 120 g/L reduced the fluorescence intensity throughout the course of a 48 h long lipid induction period (Fig. 2A). Subsequently, the potential influence of concentrating algal culture above 2.5 g DW/L was tested at the NaCl concentration of 60 g/L. The results indicated that the Nile red fluorescence intensity was slightly increased in the concentrated culture at the density of 5.0 DW/L after a 48 h lipid induction period compared to the culture at the density of 2.5 g DW/L, whereas there was a significant decrease in the fluorescence intensity of Nile Red as the algal culture was further concentrated above 5 g DW/L (Fig. 2B). Given a substantial decrease in the Nile red fluorescence intensity at the culture density above 5.0 g/DW and more time and flocculants necessary for achieving higher culture density in sedimentation ponds, 5 g DW/L of culture density is considered to be suitable for the application of high-salinity stress in the sedimentation ponds of algal biomass production system (Brennan and Owende, 2010; Chen *et al*., 2011).

Although our previous study reported that just 6 h of salt stress was enough to reach the maximum lipid content of *C. sorokiniana* HS1, the results of initial screening suggested at least 48 h of high lipid induction period for the concentrated algal culture, indicating potential influence of culture density on the amount of time required for high lipid induc-

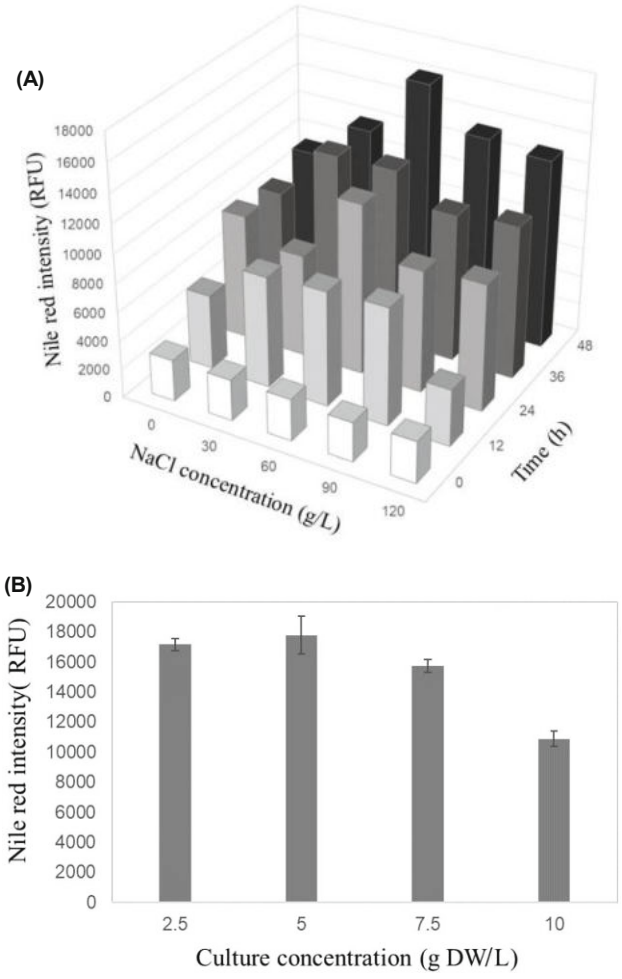

Fig. 2. Nile red intensity of the harvested culture of *Chlorella sorokiniana* HS1 at the density of 2.5 g DW/L throughout the 48 h course of high-salinity treatment at different NaCl concentrations (A), and Nile red intensity of algal culture at four different densities following the treatment of 60 g/L of NaCl for 48 h with 95% confidence interval for the mean (B).

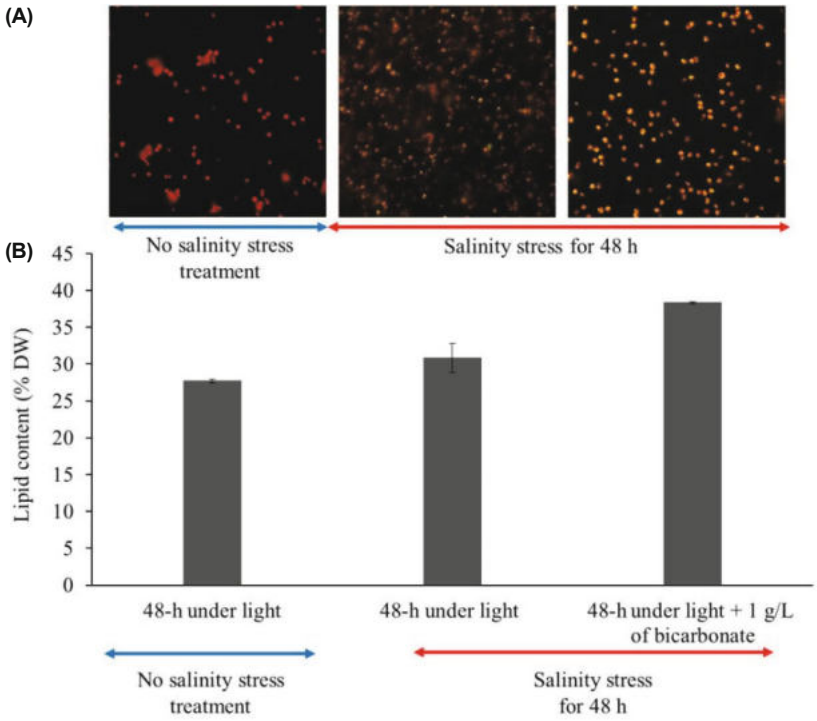

Fig. 3. Fluorescence microscopic images of the concentrated culture of *Chlorella sorokiniana* HS1 at the density of 5 g DW/L after 48 h under identical light and temperature conditions as the initial biomass growth phase with and without supplemental sodium bicarbonate (A), and the lipid content of harvested algal biomass with 95% confidence interval for the mean (B). 60 g/L of NaCl was applied to all salinity stress treatments for 48 h.

tion under high-salinity stress (Kim *et al.*, 2016b). The cell density-dependent impact of osmotic stress has been already reported in the study of *Candida glabrata* in which cells are protected and survived at least for 4 days at a higher cell density against osmotic stress by releasing substances that support growth of this strain (Almshawit *et al.*, 2014). While the lysis of algal cells was not observed under the high-salinity conditions tested in this study, we chose a 48 h lipid induction period at the cell density of 5 g DW/L for the subsequent demonstration of two-phase process.

Influence of supplemental bicarbonate on lipid induction under high-salinity stress

It is evident that high lipid induction in algal cells requires the assimilation of external carbon sources that exist within culture medium. While the addition of inorganic carbon sources has been known to enhance both growth and lipid accumulation in cultures of *Chlorella*, our results clearly indicated that the supplementation of bicarbonate under continuous illumination induced a significant increase in the lipid content of harvested algal biomass, suggesting the use of bicarbonate as an external carbon source during the lipid induction phase (Fig. 3).

Compared with the lipid content of the culture received continuous illumination for 48 h without supplemental NaCl and CO_2, high salinity stress increased the lipid content of harvested biomass by 5% and 11% under the continuous illumination and the treatment that received both continuous illumination and bicarbonate, respectively (Fig. 3). The results thus clearly indicated that the salinity stress played an important role in inducing high lipid accumulation of *C. sorokiniana* HS1, and further supported the addition of sodium bicarbonate into the concentrated algal culture under continuous illumination to enhance lipid accumulation during the second phase of two-phase process.

It should be, however, noted that other additive carbon sources can also serve as a suitable external carbon source for high lipid induction of selected algal species. For example, several microalgae are known to use organic carbon sources under heterotrophic conditions for the production of lipids (Lohman *et al.*, 2015; Minhas *et al.*, 2016). Nonetheless, the effect of organic carbon sources on metabolism of microalgae varies significantly, and the risks of biological contamination associated with supplying organic carbon sources encourage the application of inorganic carbon sources in sedimentation ponds (Minhas *et al.*, 2016). Although supplying inorganic carbon source as gaseous CO_2 is likely to be limited due to a possible mechanical disturbance to the sedimentation of algal cells, bicarbonate can be a suitable external carbon source to enhance lipid accumulation at a greater extent in sedimentation ponds, especially given that there is a readily available method to capture sodium bicarbonate without energy intensive compression (Kim *et al.*, 2017).

Influence of high-salinity stress on the composition of harvested algal cells

Based on the initial screening of salinity stress conditions, we tested the potential influence of high-salinity stress on the biochemical composition of *C. sorokiniana* HS1 at the flask scale. Upon reaching the late exponential phase in a 1 L reactor, the culture density was 2.5 g DW/L and it was further concentrated by centrifugation to 5.0 g DW/L and subsequently exposed to the identified high-salinity stress conditions. The analysis of cellular biochemical composition confirmed an increase in the lipid content of dried biomass from 25% to 38% compared to the culture grown in fresh-

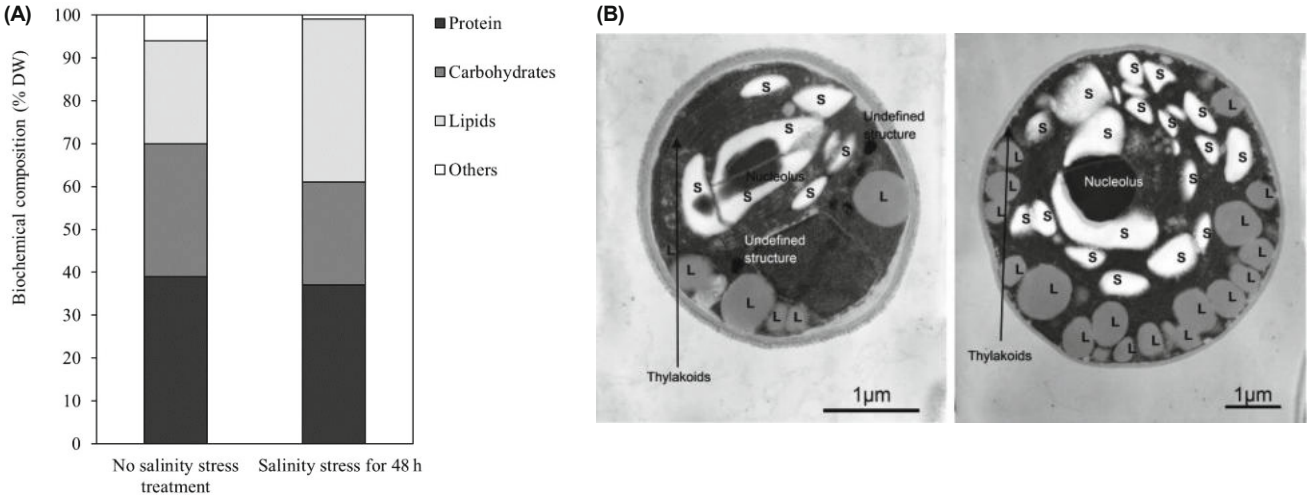

Fig. 4. Biochemical composition of the concentrated culture of *Chlorella sorokiniana* HS1 at the density of 5 g DW/L with salinity stress treatment for 48 h with 60 g/L of NaCl and 1 g/L of sodium bicarbonate (right) and the 9-day-old culture of *C. sorokiniana* HS1 without salinity stress treatment under continuous external CO_2 supplementation (left) (A), and their corresponding transmission electron micrographs (B). L denotes lipid droplet and S denotes starch granule.

water BG-11 medium for 9 days with supplemental CO_2, supporting high lipid accumulation under high-salinity stress (Fig. 4A). Contrary to the increment in the lipid content, the carbohydrate and protein contents of the harvested biomass were declined: under high-salinity stress, the carbohydrate content was lowered from 31% to 24%, and the protein content exhibited a slight decrease from 39% to 37% compared to the culture grown in freshwater BG-11 medium (Fig. 4A).

It is however noteworthy that the sum of lipid and carbohydrate contents was increased by 6% under high-salinity stress. Such increment may correspond to the supplemental bicarbonate during the lipid induction period: increased carbon availability is likely to enhance the overall carbon assimilation in algal cells. Nonetheless, the decrease in the carbohydrate content indicated that lipid assimilation pathway was more active under high-salinity stress. Indeed, an increase in osmotic pressure under salinity stress is known to trigger the synthesis of glycerol, which is a major precursor for the formation of lipid droplets in algal cells (Azachi *et al.*, 2002; Kim *et al.*, 2016b). Previous studies further reported that the salinity stress can induce high triacylglycerol accumulation in several microalgal species including *Chlorella pyrenoidosa*, *Chlorella vulgaris*, and *Acutodesmus obliquus* as a response to the oxidative stress under high-salinity conditions (Fan *et al.*, 2014; Kim *et al.*, 2016b; Pandit *et al.*, 2017). It has to be also noted that the increase in lipid content is also likely a result of the reallocation of carbon flux from carbohydrate for synthesizing lipids (Minhas *et al.*, 2016). Li *et al.* (2012), for instance, showed that accumulation of lipids occurs through the conversion of either starch or carbon to lipids, while the conversion depends on microalgal strains (Li *et al.*, 2012; Minhas *et al.*, 2016).

The results of TEM analysis further confirmed a significant increase in the number of lipid droplets within algal cells under high-salinity stress (Fig. 4B). In particular, the diameter of *C. sorokiniana* HS1 was at least doubled under high-salinity condition, indicating greater osmotic pressure on algal cells under high-salinity stress. The enlarged cell size is also a likely result of the arrest in cell division, which is associated with cellular response to the salinity stress (Kim *et al.*, 2016a, 2016b). Church *et al.* (2017) similarly observed the increased cell size of *C. vulgaris* under high-salinity stress, and reported the increase of average cell diameter from initial 2.7 μm (with no NaCl) to 4.0 μm for algae grown in 30 g/L of NaCl (Church *et al.*, 2017). While the response to increasing salinity is likely to be strain specific, osmosensing and osmoregulation were likely to induce metabolic changes, which subsequently influenced cell volume and biochemical composition (Ahmad and Hellebust, 1984; Church *et al.*, 2017).

Although there was no significant change in the FAME composition of *C. sorokiniana* HS1, the proportion of linoleate ($C_{18:2}$) was decreased from 36.2% to 32.3% under high-sali-

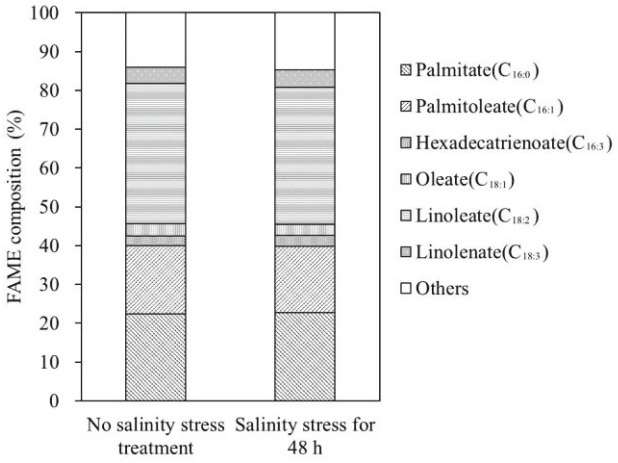

Fig. 5. FAME composition of *Chlorella sorokiniana* HS1 with salinity stress treatment for 48 h with 60 g/L of NaCl and 1 g/L of sodium bicarbonate and the 9-day-old culture of *C. sorokiniana* HS1 without salinity stress treatment under continuous external CO_2 supplementation.

nity stress (Fig. 5). This linoleate is a major fatty acid in triacylglycerol in most of *Chlorella* strains and the decrease of this unsaturated fatty acid under high-salinity stress was previously reported (Kobayashi *et al*., 2013). Azachi *et al*. (2002), for example, noted the decrease of unsaturated C_{18} fatty acids in *Dunaliella salina* under high-salinity stress, and it was further reported that high-salinity stress resulted in the increase of the proportion of saturated fatty acids (Xu and Beardall, 1997; Azachi *et al*., 2002). While Church *et al*. (2017) similarly observed significantly increased portions of saturated fatty acids in the harvested biomass of *Chlorella vulgaris* under the salinity stress with 45 g/L of NaCl, no significant increase in the proportion of saturated fatty acids was acknowledged in our results (Church *et al*., 2017).

Applying high-salinity stress condition in industrial algal cultivation

While most of the lipid-inducing stress factors are known to impose negative influence on the growth of algal biomass, the results of our two-phase process indicated that the lipid productivity of *C. sorokiniana* HS1 was 0.106 g DW/L/day following a 2-day lipid induction period, whereas it was 0.067 g DW/L/day for the culture grown in conventional single-step cultivation mode for 9 days. Such increment corresponded to a 58% increase in the lipid productivity, supporting that the biphasic strategy of dividing the cultivation process into biomass production and high lipid induction phases has a great industrial potential to enhance the production of algal lipids (Minhas *et al*., 2016).

Furthermore, the growth of *C. sorokiniana* HS1 under one-step cultivation in BG-11 medium with 60 g/L of additive NaCl indicated significantly low accumulation of algal biomass and a potential time lag in growth that results in a longer cultivation time to reach stationary phase (Fig. 6). Comparison of algal lipid productivity between two-phase process and single-step cultivation in saline BG-11, therefore, indicated significant lower lipid productivity of 0.046 g DW/L/day in single-step cultivation with saline BG-11, although 6% higher lipid content was observed (Table 1). The results thus suggest a significant compromise in the growth of biomass when the culture of *C. sorokiniana* HS1 was cultivated in saline BG-11, and reiterate the necessity of implementing high-salinity stress treatment to the cultures that reached the maximum possible growth in freshwater medium to enhance the lipid productivity of harvested biomass.

High-salinity stress, in particular, has been acclaimed for its great potential to increase the lipid productivity with a promising economic outlook because of the potential application of hypersaline wastewater sources into the lipid induction process (Church *et al*., 2017). Saline wastewater is generally produced by several industrial processes including food processing, wine production, tanneries, textiles, aquaculture, and oil production (Lefebvre and Moletta, 2006; Church *et al*., 2017), and combined operation of algal cultivation and the treatment of saline wastewater sources has been proposed (Church *et al*., 2017). Furthermore, some municipal wastewater may provide a saline water source for high lipid induction because sewage systems often exhibit significantly high-salinity levels due to landfill leachate and seawater infiltration (Linarić *et al*., 2013; Church *et al*., 2017). Nonetheless, utilizing NaCl during the lipid induction phase as demonstrated in this study is also plausible option since it is considered as an inexpensive and readily available source of nutrients (Minhas *et al*., 2016).

It is, however, noted that the addition of mineral salts may interfere with the downstream extraction processes and may incur additional costs in the operation and maintenance of overall facilities designed for the production of algal biofuels (Schlagermann *et al*., 2012). Nonetheless, osmotic shock was demonstrated to increase lipid recovery approximately 2 times when applied directly to wet *Chlamydomonas reinhardtii* biomass (Yoo *et al*., 2012). Roberts *et al*. (2013) also proposed that the presence of alkali species in the harvested algal biomass catalyze the HTL reactions and thus could be advantageous for producing a biocrude with lower oxygen and larger HHV (Roberts *et al*., 2013). Church *et al*. (2017), furthermore,

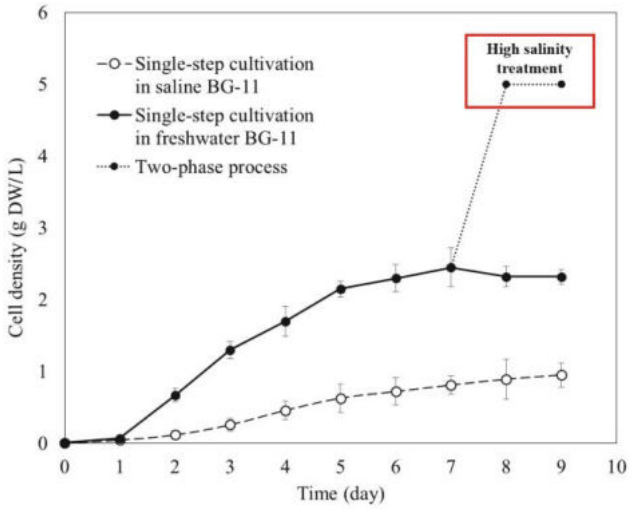

Fig. 6. Growth curves of *C. sorokiniana* HS1 grown in (1) freshwater BG-11 and (2) saline BG-11 with 60 g/L of NaCl. Triplicated 1 L bottle reactors reached stationary phase on Day 7 with freshwater BG-11, whereas the growth curve suggested a potential time lag in growth with saline BG-11 that resulted in a longer cultivation time to reach the stationary phase. The culture grown in freshwater BG-11 for 7 days was harvested and subsequently exposed to the identified high-salinity stress conditions for 48 h. Error bars represent standard error of the mean.

Table 1. Comparisons of the lipid content, biomass productivity, and lipid productivity of the cultures operated in (1) two-phase process and (2) conventional single-step mode in freshwater and saline BG-11 media

Operation mode		Total operation period (day)	Lipid content (% DW)	Biomass productivity (g DW/L/day)	Lipid productivity (g DW/L/day)
Single-step cultivation	BG-11	9	25	0.268	0.067
	BG-11 + 60 g/L of NaCl	9	44	0.104	0.046
Two-phase process		9	38	0.279	0.106

noted that the enlarged cell size as a response to high salinity facilitates more efficient settling (i.e., 33–83% increased efficiency) of *Chlorella vulgaris* (Church *et al*., 2017). While the lipid induction demonstrated in this study was performed after concentrating a dense laboratory culture only by a factor of two, our independent cultivation trial of *Chlorella sorokiniana* HS1 in an outdoor setting with the culture volume of 200 L indicated that the culture density did not exceed 0.5 g DW/L at the stationary phase (Supplementary data Fig. S1). Applying high-salinity stress to the sedimentation ponds is therefore likely to significantly reduce capital and operating costs associated with directly applying salinity stress to bulk algal cultures, and it may also provide potential advantages in subsequent harvesting and downstream extraction processes.

Although this study was focused on the demonstration of two-phase process with previously isolated *C. sorokiniana* HS1, it should be also noted that any osmotolerant algal strains that hyper-accumulate lipids or other desirable biomolecules under varying salinity levels may be selected for the implementation of two-phase process. Kobayashi *et al*. (1997) for example, demonstrated a two-stage batch process for light-independent astaxanthin production from *Haematococcus pluvialis*, a freshwater green alga, and reported substantially high accumulation of astaxanthin under high salinity stress (Kobayashi *et al*., 1997). Ra *et al*. (2015) further demonstrated a two-stage culture strategy to allow maximum biomass production during the first growth stage and subsequently exposed marine microalgal species under low salinity stress to induce high oil production (Ra *et al*., 2015). Kim *et al*. (2016b), however, indicated that not all freshwater *Chlorella* strains exhibit high lipid accumulation under high salinity stress (Kim *et al*., 2016b); selecting reliable algal species will thus be imperative to successfully incorporate different salinity stress conditions into industrial algal cultivation.

While the techno-economic assessment on incorporating salinity stress into the production of algal biomass is yet to be performed, carefully adjusting the concentration of NaCl and/or combining other stress factors (e.g., nitrogen limitation and high irradiance) with salinity stress could contribute to further reducing the costs associated with lipid induction under high salinity stress and enhance the lipid productivity and biofuels properties of selected algal strain. We therefore urge more extensive study on optimizing the proposed two-phase process with stress-tolerant commercial strains by carefully incorporating different saline water sources, including wastewater, and other stress factors into high lipid induction phase to economically achieve high lipid productivity in industrial algal cultivation platforms.

Acknowledgements

This work was supported by the Advanced Biomass R&D Center (ABC) of Global Frontier Project funded by the Ministry of Science and ICT of the Republic of Korea (ABC-2016922286), a grant of the Korea Health technology R&D Project through the Korea Health Industry Development Institute (KHIDI), funded by the Ministry of Health & Welfare of the Republic of Korea (HN13C0080), and a grant from the KRIBB (Korea Research Institute of Bioscience and Biotechnology) Research Initiative Program (www.kribb.re.kr).

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