Journal of Applied Phycology (2018) 30:2725–2735
https://doi.org/10.1007/s10811-018-1388-3

6TH CONGRESS OF THE INTERNATIONAL SOCIETY FOR APPLIED PHYCOLOGY

Improvement of biomass and fatty acid productivity in ocean cultivation of *Tetraselmis* sp. using hypersaline medium

Hanwool Park[1,2] · Daewoo Jung[1,2] · Jongchan Lee[1,2] · Philhan Kim[1,2] · Yonghee Cho[2] · Injae Jung[1,2] · Z-Hun Kim[3] · Sang-Min Lim[1,2] · Choul-Gyun Lee[1,2]

Received: 2 August 2017 / Revised and accepted: 2 January 2018 / Published online: 20 January 2018

Abstract
In this study, hypersaline media were used for ocean cultivation of the marine microalga *Tetraselmis* sp. KCTC12432BP for enhanced biomass and fatty acid (FA) productivity. Hypersaline media (55, 80, and 105 PSU) were prepared without sterilization by addition of NaCl to seawater obtained from Incheon, Korea. The highest biomass productivity was obtained at 55 PSU (0.16 g L^{-1} day^{-1}) followed by 80 PSU (0.15 g L^{-1} day^{-1}). Although the specific growth rate of *Tetraselmis* decreased at salinities higher than 55 PSU, prevention of contamination led to higher biomass productivity at 80 PSU than at 30 PSU (0.03 g L^{-1} day^{-1}). FA content of algal biomass increased as salinity increased to 80 PSU, above which it declined, and FA productivity was highest at 80 PSU. Ocean cultivation of *Tetraselmis* was performed using 50-L tubular module photobioreactors and 2.5-kL square basic ponds, closed- and open-type ocean culture systems, respectively. Culturing microalgae in hypersaline medium (80 PSU) improved biomass productivities by 89 and 152% in closed and open cultures, respectively, compared with cultures with regular salinity. FA productivity was greatly improved by 369% in the closed cultures. The efficacy of salinity shift and N-deficiency to enhance FA productivity was also investigated. Lowering salinity to 30 PSU with N-starvation following cultivation at 80 PSU improved FA productivity by 19% in comparison with single-stage culture without N-deficiency at 30 PSU. The results show that salinity manipulation could be an effective strategy to improve biomass and FA productivity in ocean cultivation of *Tetraselmis* sp.

Keywords Hyposalinity shock · Marine photobioreactor · Ocean culture systems · Salinity shift · Two-stage cultivation · Contamination

Introduction

Despite the many advantages of microalgae over terrestrial energy crops as feedstocks for biofuels, there are many challenges that need to be overcome for commercialization of algal biofuels. Every step in the algal biofuel production process needs to be improved. Especially, cultivation plays a significant role in the economic feasibility and sustainability of algal biofuels, and improvements in biomass productivity and fatty acid (FA) content with reduced energy consumption and production costs are of great interests (Rodolfi et al. 2009; Norsker et al. 2011; Richardson et al. 2014; Park and Lee 2016). Conventionally, microalgae have been cultivated in open ponds and photobioreactors (PBRs) based on land. In recent years, studies on floating culture systems in the ocean for growing microalgae have increased due to their various advantages, including availability of large areas, utilization of waves for culture mixing, thermoregulation by seawater, easy access to seawater, and use of dissolved nutrients in seawater (Wiley et al. 2013; Kim et al. 2015, 2016b, 2016c; Novoveská et al. 2016; Park and Lee 2016).

In large-scale cultivation, contamination by other microalgae, bacteria, protozoa, ciliates, viruses, and fungal parasites can severely reduce biomass and FA productivity via various mechanisms (Borowitzka 1999; Hoffman et al. 2008; Das et al. 2011; Bartley et al. 2013; Fon-Sing et al. 2014;

Hanwool Park and Daewoo Jung contributed equally to this work.

✉ Choul-Gyun Lee
leecg@inha.ac.kr

[1] Department of Marine Science & Biological Engineering, Inha University, Incheon 22212, South Korea

[2] National Marine Bioenergy R&D Center, Inha University, Incheon 22212, South Korea

[3] Culture Techniques Division, Freshwater Bioresources Culture Research Division, Nakdonggang National Institute of Biological Resources, Sangju 37242, South Korea

Karpov et al. 2014). Being mostly aquatic species, microalgae require a lot of water for their cultivation. In order to avoid conflicts with agriculture for freshwater resources, use of seawater or wastewater is desired, although it increases chances of contamination (Carney et al. 2014; Novoveská et al. 2016; Park and Lee 2016). Sterilization and disinfection by heat, filtration, sonication, and chemicals are often used in small-scale operations or for high-value products, but these methods are difficult to be economically feasible in large-scale algal cultures for biofuel production. Furthermore, application of such methods in algal cultures floating on the ocean would be difficult. Commercialized algal cultures often exploit the ability of some microalgae to grow under extreme, highly selective culture conditions, such as cultivation of *Dunaliella salina* at hypersalinity, *Arthrospira* at high pH, and *Chlorella* at high nutrient concentrations (Borowitzka 1999).

Manipulation of culture conditions are also commonly used to promote accumulation of biochemicals in microalgae. For example, production of astaxanthin from *Haematoccocus* at high light intensity with nitrogen deficiency is a well-known process (Suh et al. 2006; Kim et al. 2016d). Limiting nitrogen sources is widely used to induce lipid accumulation. Lately, improvement of FA productivity by manipulation of salinity in various algae, such as *Dunaliella*, *Nannochloris*, *Nannochloropsis*, *Navicula*, and *Tetraselmis*, has gained popularity for its potential efficacy in biofuel production (Takagi & Karseno 2006; Das et al. 2011; Gu et al. 2012; Bartley et al. 2013; Kim et al. 2016a; Saadaoui et al. 2016). Changes in culture conditions often lead to reduced biomass productivity in exchange for accumulation of desired biochemicals; a two-stage culture strategy has been suggested for algal cultivation (Das et al. 2011; Su et al. 2011; Gleason et al. 2014). In *Haematoccocus* cultures, two-stage cultivation has long been used since large accumulation of astaxanthin occurs in cyst cells induced by high light intensity and N-starvation (Suh et al. 2006; Kim et al. 2016d). Combining N-deficient condition with a salinity shift to higher salinity could also be used to increase FA productivity, and such a strategy has been successfully applied to *Tetraselmis* and *Scenedesmus* (Xia et al. 2013; Ho et al. 2014; Kim et al. 2016a).

For the floating algal cultures in the ocean, preventing contamination by addition of chemicals, heating, filtration, or sonication is extremely difficult, but exploiting extreme conditions could be feasible due to simplicity of application. Other studies have shown that *Tetraselmis* sp. could be cultivated in outdoor with minimal contamination using hypersaline media or frequent harvesting (Moheimani 2013; Fon-Sing et al. 2014; Fon-Sing and Borowitzka 2016). In this study, application of hypersalinity in ocean cultivation to prevent contamination and increase biomass and FA productivity was investigated. A suitable salinity for a locally isolated strain of *Tetraselmis* sp. KCTC12429BP, which genus includes many halotolerant species, was selected to improve biomass productivity by preventing contamination and increase FA accumulation (Fon-Sing et al. 2014; Fon-Sing and Borowitzka 2016; Kim et al. 2016a). The selected salinity was applied to open- and closed-type ocean culture systems without any sterilization or disinfection measures. The possibility of two-stage cultivation using salinity shift along with nitrogen deficiency was then assessed to improve FA content and productivity of the microalgae.

Materials and methods

Microalgae and seed culture maintenance The green microalga *Tetraselmis* sp. KCTC12432BP in this study was isolated from natural seawater (NSW) from the ocean test-bed of Marine Bioenergy R&D Consortium (MBE) at Inha University in Youngheung Island, Incheon, Korea. The culture medium used for the maintenance of seed culture was NSW from Incheon supplemented with modified f/2-Si medium consisting of 450 mg L^{-1} of $NaNO_3$, 30 mg L^{-1} of NaH_2PO_4, 3.15 g L^{-1} of $FeCl_3{\cdot}6H_2O$, 4.36 g L^{-1} of $Na_2EDTA{\cdot}2H_2O$, 180 mg L^{-1} of $MnCl_2{\cdot}4H_2O$, 22 mg L^{-1} of $ZnSO_4{\cdot}7H_2O$, 10 mg L^{-1} of $CoCl_2{\cdot}6H_2O$, 9.8 mg L^{-1} of $CuSO_4{\cdot}5H_2O$, and 6.3 mg L^{-1} of $Na_2MoO_4{\cdot}2H_2O$ without vitamins. The medium was filter-sterilized (Millipore, pore size 0.22 μm) before use. The seed cultures were maintained in 400-mL glass bubble column photobioreactors (BC-PBRs) in a room with temperature set at 20 ± 1 °C under continuous illumination at a light intensity of 100 ± 5 μmol photons m^{-2} s^{-1} by two 55-W fluorescent light bulbs (FL 18D, OSRAM Korea Co., Korea). The culture pH was maintained at 8 with aeration of 2% CO_2 at 0.1 vvm.

Effect of salinity on fatty acid productivity of *Tetraselmis* sp. Effects of four different salinities, 30 (NSW), 55, 80, and 105 PSU, on the growth and FA accumulation of the microalgae were investigated. Hypersaline media were prepared by addition of 25, 50, or 75 g L^{-1} of NaCl to NSW from Incheon without sterilization. The microalgae were cultivated in 400-mL BC-PBRs under the same light intensity, temperature, aeration, and nutrition conditions used for the seed cultures. The experiments were performed in duplicates. Cells were harvested 3 days after depletion of nitrate by centrifugation at 3000 rpm for 10 min for analyses of FAs in the algal biomass. The specific growth rates of *Tetraselmis* sp. at four salinities were calculated using the following formula:

$$\mu = \frac{\ln\left(\frac{C_2}{C_1}\right)}{t_2 - t_1} \quad (1)$$

where μ is the specific growth rate expressed in day^{-1} and C_2 and C_1 are the biomass concentrations of samples collected at

time t_2 (day 2.5) and t_1 (day 0), respectively, in g L^{-1}. Volumetric biomass productivity was calculated for experiments in the lab with BC-PBRs, using the following formula:

$$VBP = \frac{C_2 - C_1}{t_2 - t_1} \quad (2)$$

where *VBP* is the volumetric biomass productivity expressed as g L^{-1} day^{-1} and C_2 and C_1 are the biomass concentrations of samples collected at time t_2 (harvest) and t_1 (inoculation), respectively, in g L^{-1}. FA productivity was calculated by multiplying the volumetric biomass productivity with FA content of harvested microalgae and expressed in mg L^{-1} day^{-1}.

Ocean culture systems and operation Using hypersaline medium to enhance biomass and FA productivity of *Tetraselmis* sp. in ocean cultivation was evaluated at the ocean test-bed of MBE located on the nearshore of Youngheung Island (36.23° N, 126.43° E, Incheon, Korea). The ocean test-bed consisted of pontoon cubes forming square openings for installation of culture systems (Fig. 1A). Two different culture systems were used to cultivate *Tetraselmis* sp. on the ocean: tubular module PBRs (TM-PBRs) and square basic floating ponds (SB-Ps) for closed and open culture systems, respectively.

TM-PBRs made of polyurethane (0.6-mm thick and 80% light transmittance) were 4.3 m in length and 0.33 m in width. The working volume was 50 L, and the working area was 1.075 m^2 based on a diameter of 0.25 m when inflated. The TM-PBRs were inflated with air and deployed on the ocean in 2.5 m × 5-m openings (Fig. 1B). SB-Ps were made of white, 0.5-mm thick polyvinylchloride tarpaulin sheet in a square shape with 5.4 m in sides. The SB-Ps were installed in square openings to make the working area of 16 m^2 (4 m × 4 m), and working volume was 2500 L (Fig. 1C). As the culture media, NSW was supplemented with the modified f/2-Si medium that used for seed culture maintenance. For hypersaline medium, 50 g L^{-1} of NaCl was added, and both media with normal and elevated salinity were used without sterilization or disinfection. The experiments were performed in duplicate. Areal biomass productivity was calculated for experiments in the ocean with TM-PBRs and SB-Ps, using the following formula:

$$ABP = \frac{V(C_2 - C_1)}{A(t_2 - t_1)} \quad (3)$$

where *ABP* is the areal biomass productivity expressed as g m^{-2} day^{-1}, *V* is the volume of culture system in L, *A* is the area of culture system, and C_2 and C_1 are the biomass concentrations of samples collected at t_2 (harvest) and time t_1 (inoculation), respectively, in g L^{-1}. FA productivity was calculated by multiplying the areal biomass productivity with FA content of harvested microalgae and expressed in mg m^{-2} day^{-1}.

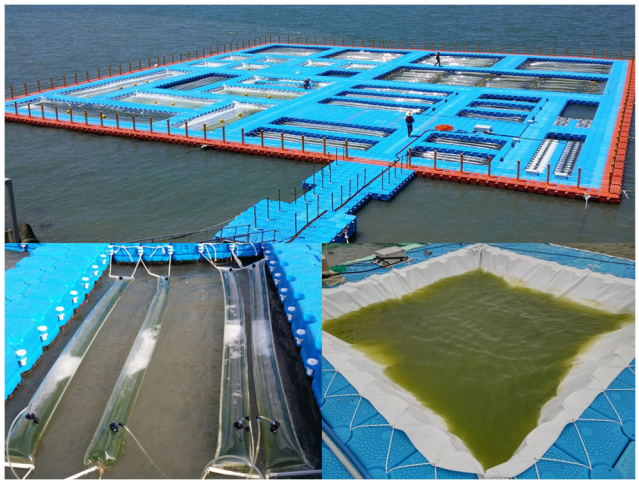

Fig. 1 Photographs of ocean test-bed of MBE at Youngheung Island (top), tubular module PBRs (bottom left), and a square basic pond (bottom right)

Effects of shifts to higher or lower salinity on FA productivity of *Tetraselmis* sp. The effects of salinity changes on FA productivity were investigated in the laboratory using BC-PBRs. The culture conditions and media were identical to those of the seed culture maintenance and salinity survey experiments. Microalgae were cultivated at normal or elevated salinity, and then cells were harvested by centrifugation at 3000 rpm for 10 min and transferred to fresh media with elevated or normal salinity, respectively, after nitrogen source were depleted. As controls, only nitrogen deficiency was applied to algal cultures at normal or elevated salinity. The algal biomass was harvested by centrifugation at 3000 rpm for 10 min 2 days after salinity shift for analysis of FAs. The volumetric biomass productivity was calculated using Formula (2), and FA was calculated by multiplying the volumetric biomass productivity and FA contents, expressed in mg L^{-1} day^{-1}.

Measurements and analyses of samples and environmental factors Cell size distributions and concentrations of algal cultures were measured using a Coulter Counter (Multisizer 3, Beckman Coulter, USA) and converted by Multisizer 3 software after diluting samples with an electrolyte solution. Then, data were exported to an Excel spreadsheet to estimate dry cell weight (DCW). The correlation between DCW and data from the Coulter Counter was occasionally confirmed by measuring DCW with 20 or 500 mL of samples for the laboratory and ocean experiments, respectively (Kim et al. 2016b). Each sample was centrifuged at 3000 rpm for 10 min and washed twice with deionized water to remove residual salts. The washed pellet was transferred to a pre-dried, pre-weighed crucible and dried at 90 °C overnight. The dried pellet was then cooled to room temperature in a desiccator cabinet and weighed. Drying was repeated until the weight of the pellet did not change.

FA contents of the microalgal biomass were analyzed by gas chromatography with a flame ionization detector (GC-FID) (Acme 6000 GC, Young Lin, Anyang, Korea) after transesterification of FA in the biomass to FA methyl esters. Detailed methods for the transesterification of FAs and GC-FID measurements are described elsewhere (Tran et al. 2009).

Salinity of the culture medium was measured using a portable conductivity meter (EC-40N, Istek, Seoul, Korea). Samples with salinities of 80 and higher were diluted with deionized water before measurements. Nitrate concentration in the culture media was measured with a UV-Vis after diluting the cell-free media with 0.1-M HCl (Kim et al. 2016b). Light intensity of photosynthetically active radiation (wavelengths 400–700 nm) was measured with a LI-COR quantum sensor (LI-190SA, LI-COR, USA) equipped with a Data Logger (LI-1400, LI-COR). Culture pH was measured with a pH meter (P25-A, Istek, Korea). Microscopic pictures of algal cultures were taken with a phase-contrast microscope (Eclipse Ci, Nikon Corp., Japan) equipped with a digital camera (HK5U3, K-Optic, Korea).

Results

Effect of salinity on fatty acid productivity of *Tetraselmis* sp. Elevation of salinity by addition of NaCl to NSW increased maximum biomass concentrations (Fig. 2). The specific growth rates of algae from inoculation to day 2.5 decreased at 80 and 105 PSU (1.71, 1.71, 1.39, and 0.87 day^{-1} at 30, 55, 80, and 105 PSU, respectively). In the cultures with 30 PSU, biomass concentrations decreased after reaching 0.43 g L^{-1} on day 2.5, whereas maximum biomass concentrations were 1.14 ± 0.09 g L^{-1} in hypersaline media. Nitrate was depleted on day 4.5 in cultures with 30, 55, and 80 PSU and was exhausted 5 days later in culture with 105 PSU (data not shown). Lysed microalgae with motile particles, decolorized microalgae, and bacteria were observed in cultures with 30 and 55 PSU (Fig. 3A and B), while cultures consisted of mostly intact microalgae at 80 and 105 PSU (Fig. 3C and D).

The overall volumetric biomass productivities were 0.03, 0.16, 0.15, and 0.08 g L^{-1} day^{-1} at 30, 55, 80, and 105 PSU, respectively (for 7.5 days at 30, 55, and 80 PSU and for 12.5 days at 105 PSU) (Fig. 4). In 30 PSU cultures (control), biomass concentration initially increased and then sharply decreased, leading to lower biomass productivity compared to the cultures with other salinities. FA contents of algal biomass were in the order of 80, 55, 105, and 30 PSU, and their values were 11.2, 8.9, 8.4, and 5.6%, respectively (Fig. 4). The resulting FA productivity was highest (16.9 mg L^{-1} day^{-1}) at 80 PSU, followed by 14.1 mg L^{-1} day^{-1} at 55 PSU, 6.4 mg L^{-1} day^{-1} at 105 PSU, and 1.5 mg L^{-1} day^{-1} at 30 PSU. Based on its minimal contamination and highest FA content, 80 PSU was selected for the ocean cultivation of *Tetraselmis* sp.

Use of hypersaline medium for ocean cultivation Use of hypersaline medium for ocean cultivation improved biomass productivities in both TM-PBRs and SB-Ps. Time profiles of

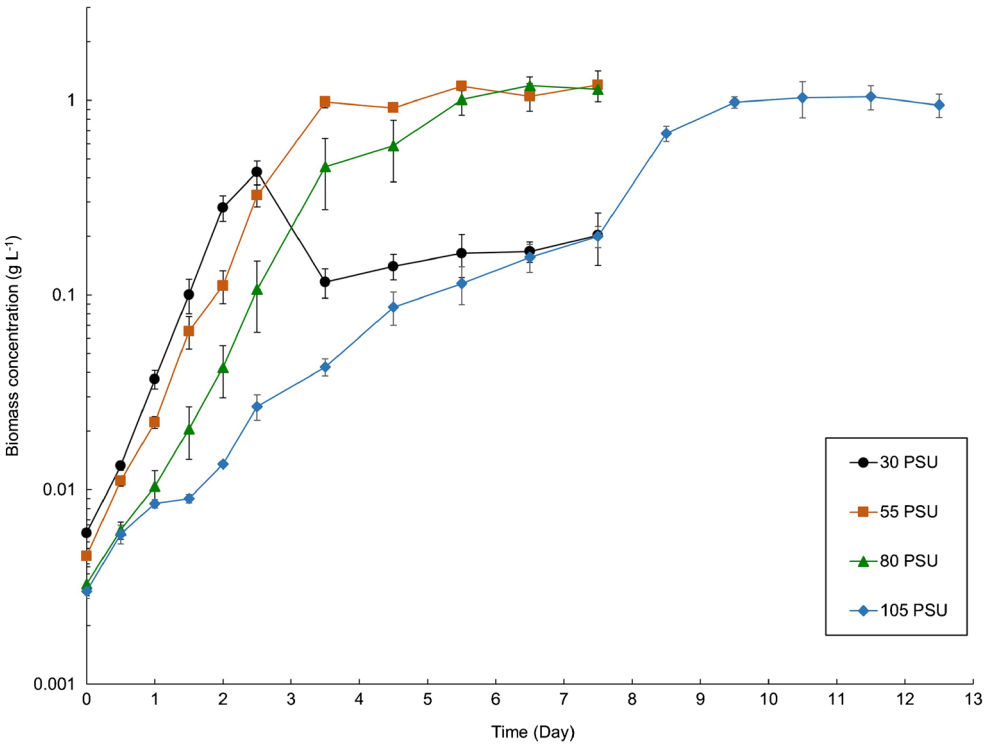

Fig. 2 Time profiles of biomass concentrations in different salinities (error bars indicate range of duplicates)

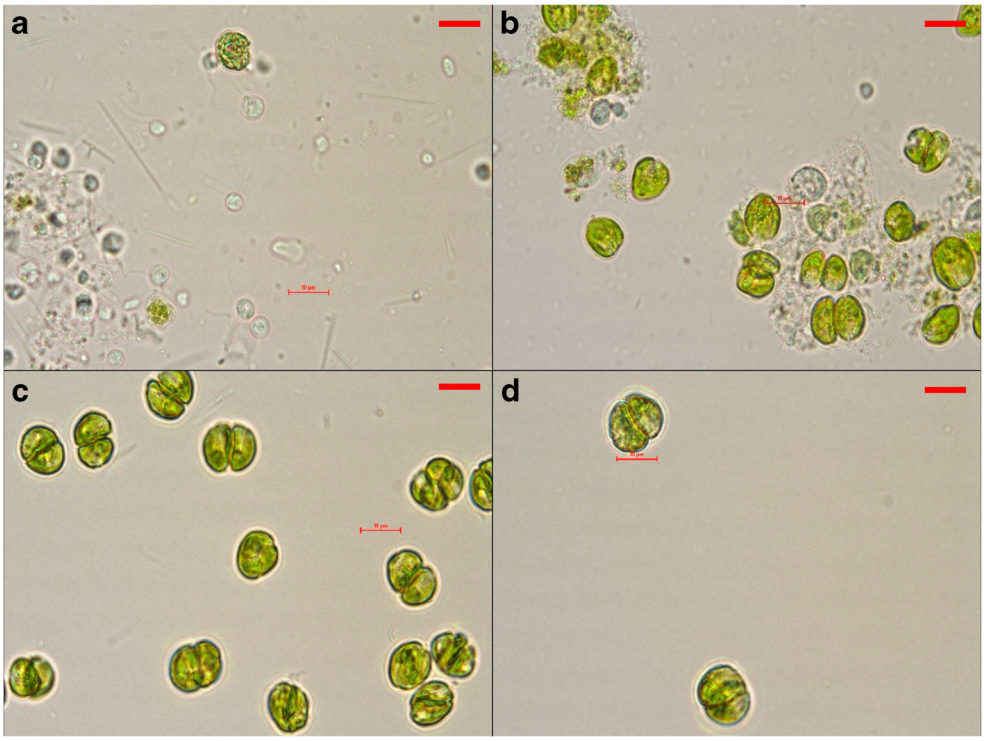

Fig. 3 Micrographs of cultures with 30 PSU (**a**), 55 PSU (**b**), 80 PSU (**c**), and 105 PSU (**d**) (scale bars = 10 μm)

biomass concentrations in the cultures with regular and hypersaline media are presented in Fig. 5A. In both culture systems, maximum biomass concentrations were higher at 80 PSU than at 30 PSU. In the TM-PBRs with hypersaline medium, maximum biomass concentration was 0.77 g L^{-1} whereas that in TM-PBRs with NSW control was 0.23 g L^{-1}. The maximum biomass concentrations in SB-Ps were 0.38 and 0.11 g L^{-1} at 80 and 30 PSU, respectively. Use of hypersaline media improved areal biomass productivities by 89 and 152% in TM-PBRs and SB-Ps, respectively, during the 17-day cultivation. Competing microalgae (mainly diatoms) and lysed *Tetraselmis* cells with motile particles were detected in the

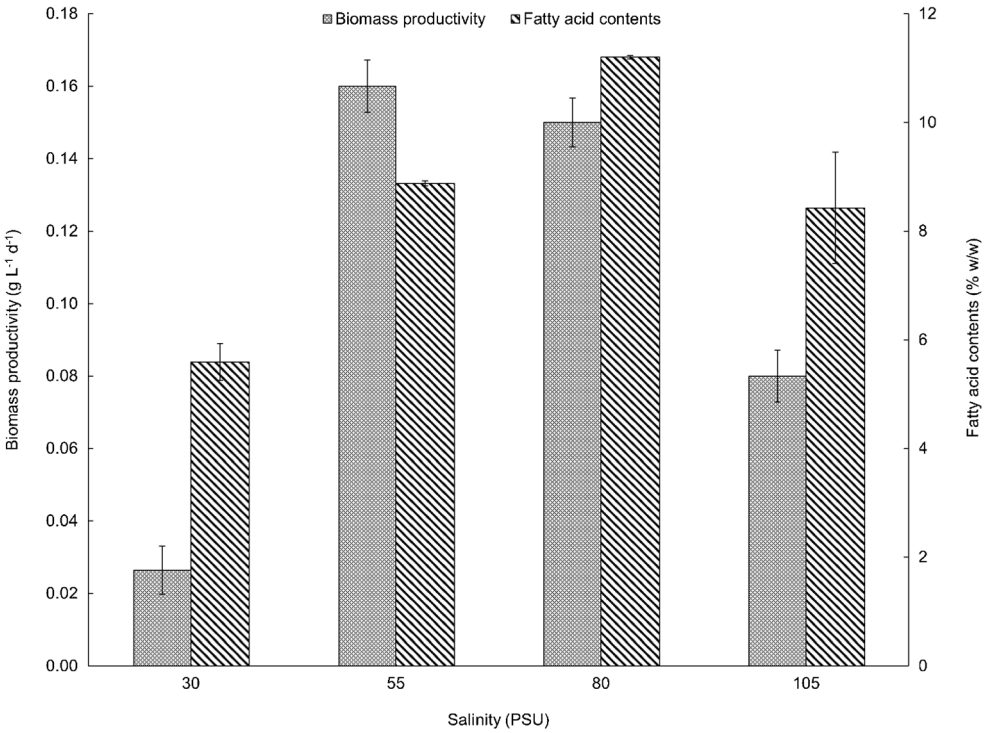

Fig. 4 Biomass productivities and fatty acid contents by salinities (error bars indicate range of duplicates)

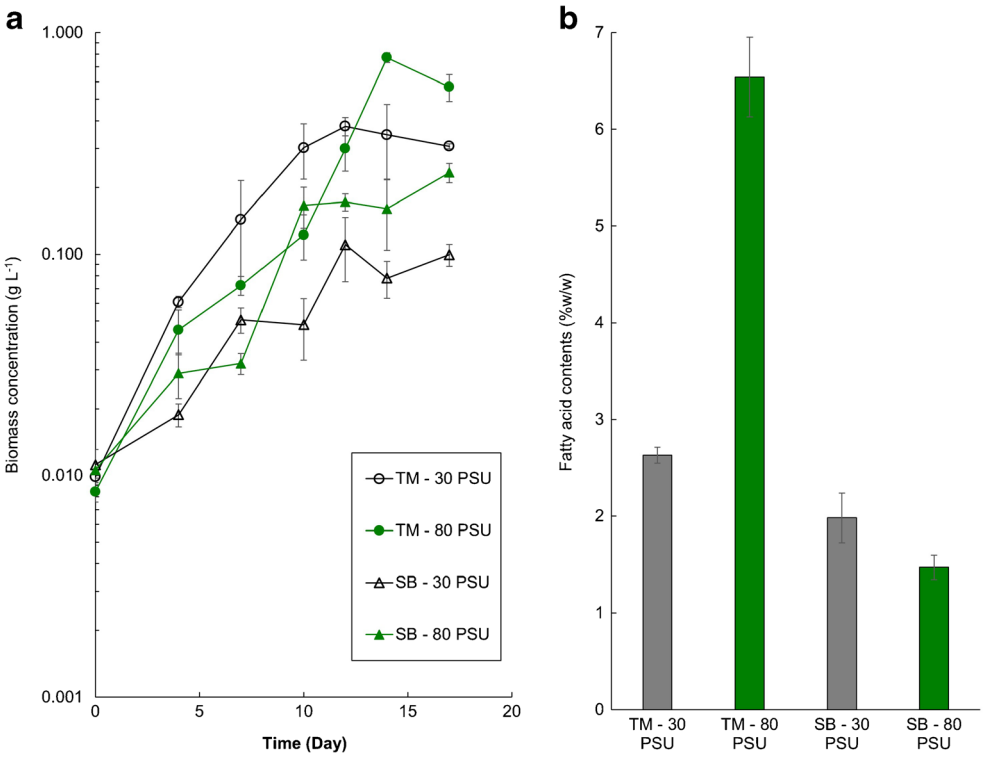

Fig. 5 Changes of biomass concentration in TM-PBRs and SB-Ps with 30 (empty symbols) and 80 PSU (filled symbols) (**a**) and fatty acid contents after cultivation (**b**) (error bars indicate range of duplicates)

cultures at 30 PSU (Fig. 6A and C), whereas mainly *Tetraselmis* cells were found in the cultures at 80 PSU (Fig. 6B and D). Nitrate was not depleted in the TM-PBRs and SB-Ps during cultivation (data not shown).

FA content of the algal biomass was increased by 149% in the TM-PBRs by using hypersaline medium, compared with when NSW was used (Fig. 5B). On the other hand, FA content decreased by 35% when hypersaline medium was used in SB-

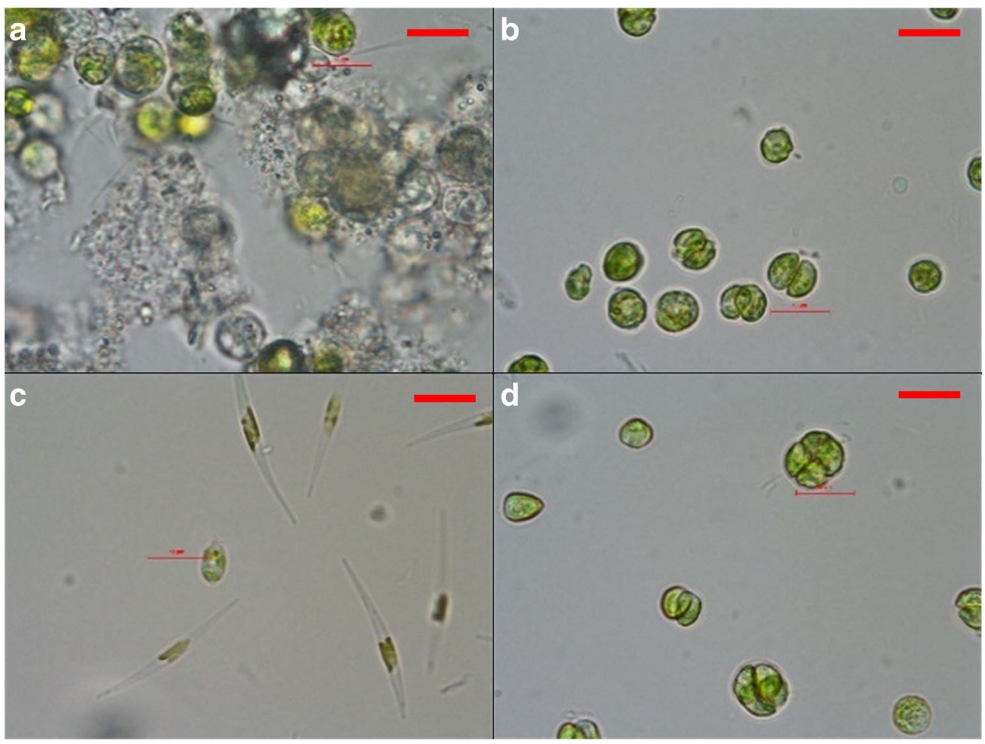

Fig. 6 Micrographs of cultures with 30 PSU in TM-PBR (**a**), 80 PSU in TM-PBR (**b**), 30 PSU in SB-P (**c**), and 80 PSU in SB-P (**d**) (red bars on the top-right corner indicate 10 μm)

Ps. Nevertheless, due to substantial improvements in biomass productivities, FA productivities were enhanced by 369 and 87% in the TM-PBRs and SB-Ps, respectively, with hypersaline medium. In the TM-PBRs, FA productivities were 100.1 and 21.3 mg m^{-2} day^{-1} at 80 and 30 PSU, respectively. Those were 30.1 and 16.1 mg m^{-2} day^{-1} at 80 and 30 PSU, respectively, in the SB-Ps.

Effects of increasing or decreasing salinity on FA productivity of *Tetraselmis* sp. The effects of hyposalinity or hypersalinity shock on FA productivity were investigated to assess the possibility of two-stage cultivation for further improvement of FA production. Changes in biomass concentrations of algal cultures during cultivation are shown in Fig. 6A. Maximum biomass concentrations were attained after 4 and 6 days in cultures at 30 and 80 PSU, respectively. Similar to the results of the salinity survey experiment, biomass concentration was reduced by 86% after reaching 1.2 g L^{-1} in cultures at 30 PSU. When the cells grown at 30 PSU were transferred to 80 PSU, biomass concentration still decreased by 42%. As the result, biomass productivity was increased by 320% in the cultures transferred to 80 PSU compared with the cultures stayed at 30 PSU. Biomass concentration increased relatively slowly in the 80 PSU cultures, but it did not decline after nitrate was exhausted. Shifting from 80 to 30 PSU did not lead to significant differences in biomass productivities. Nitrate was depleted on days 4 and 5.5 in the 30 and 80 PSU cultures.

Shifts to lower and higher salinities increased FA content of the algal biomass compared to nitrogen starvation condition alone (Fig. 7B). In NSW cultures without salinity shift, FA content decreased after exhaustion of nitrate, whereas shift to 80 PSU increased FA content of the biomass. Compared to the NSW control, FA content increased by 173% upon hypersalinity shock along with nitrogen starvation. In the cultures with 80 PSU, FA contents were increased by 39 and 109% without and with hyposalinity shock, respectively. Although biomass reduction was not completely prevented in the cultures with hypersalinity shock, FA content was 22% higher compared with the cultures that stayed at 80 PSU. FA productivities before nitrogen starvation and salinity shift were 24.0 and 17.0 mg L^{-1} day^{-1} at 30 and 80 PSU, respectively. After N-depletion, the highest FA productivity of 28.5 mg L^{-1} day^{-1} was attained in the cultures that underwent a salinity shift from 80 to 30 PSU, followed by 17.4 mg L^{-1} day^{-1} in the cultures with the 30 to 80 PSU salinity shift. Despite lower final biomass concentration in the 30 to 80 PSU cultures, higher FA contents resulted in higher FA productivity than in the cultures that stayed at 80 PSU.

Discussion

Our results suggest that use of hypersaline medium at 80 PSU could increase biomass and FA productivity in *Tetraselmis* sp. KCTC12432BP by preventing contamination and inducing

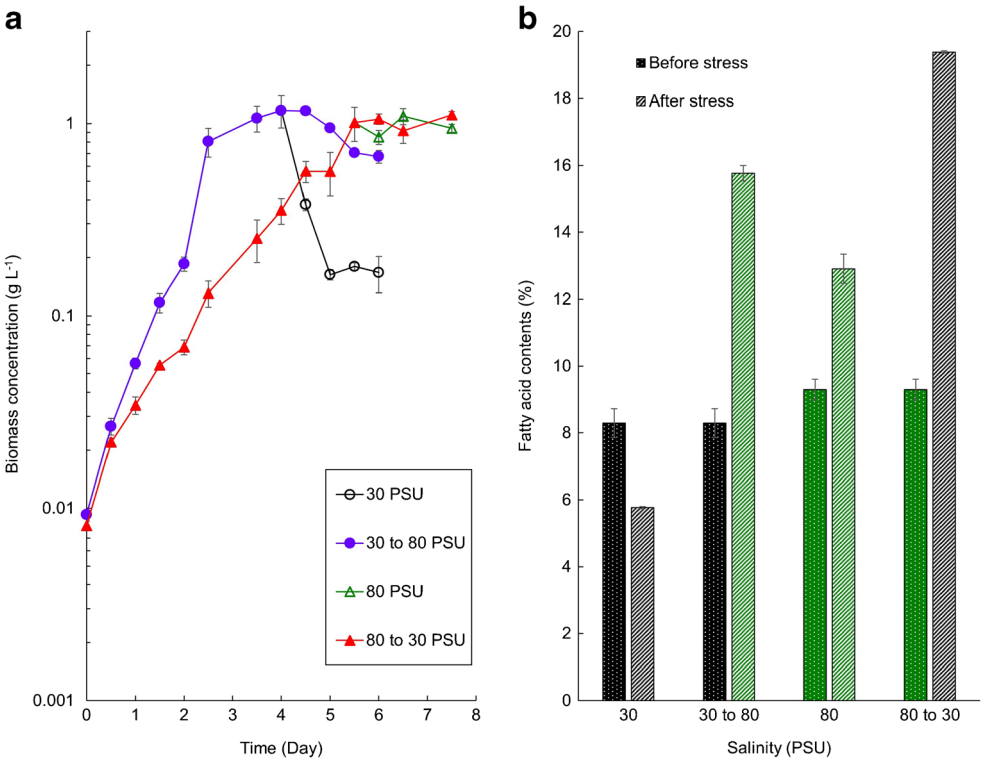

Fig. 7 Time profiles of biomass concentration with (filled symbols) or without (empty symbols) salinity shifts (**a**) and fatty acid contents before and after salinity shifts and N-deficiency (**b**) (error bars indicate range of duplicates)

FA accumulation in both open and closed ocean culture systems as well as in the well-regulated laboratory condition. Furthermore, a salinity shift from 80 to 30 PSU combined with nitrogen starvation was able to enhance FA productivity by substantially increasing FA content.

The specific growth rate of *Tetraselmis* sp. KCTC12429BP was reduced at salinities higher than 55 PSU (Fig. 2). While *Tetraselmis* generally exhibit some extent of halotolerance, their optimal salinities vary even within the same genus (Fon-Sing and Borowitzka 2016). They can tolerate high salinity because of their ability to produce and accumulate compatible solutes as well as efficient active transportation system for Na^+ (Fon-Sing and Borowitzka 2016; Hagemann 2016). Lowered growth rate also allows the algae to maintain its homeostasis better than when they are growing faster (Fanesi et al. 2014). Besides the metabolic burden for osmoregulation, dehydration and increased intracellular Na^+ levels at high salinity could also disrupt structural integrity of the photosystem and electron transport chain, thereby lowering photosynthetic efficiency (Hellebust 1985; Zakhozhii et al. 2012).

Although the specific growth rate of *Tetraselmis* sp. was reduced at higher salinities, the gain in biomass productivity from prevention of contamination was greater (Fig. 4). When the microalgae were cultivated in BC-PBRs, biomass concentration rapidly increased initially and sharply declined as nitrate became scarce in the medium (< 2 mg L^{-1}, data not shown) in the cultures at 30 PSU (Figs. 2 and 6). Microscopic observation showed that a considerable portion of microalgae were lysed, and contaminants were present in cultures at 30 PSU (Fig. 3A). Contaminants included bacteria, ciliates, and motile particles, all of which may cause cell lysis or predate on algae (Pretorius and Engelbrecht 1980; Bartley et al. 2013; Karpov et al. 2014). Some ciliates thrive at 34 PSU and can ultimately cause a total crash of the culture (Bartley et al. 2013). Even though ciliates were observed in our cultures, they were rather rarely seen. Most notable contaminants in these experiments were the motile particles that resided within the microalgae. When the microalgae were exponentially growing with sufficient NO_3^-, they were not visible nor lysed cells were observed. However, as the culture enters a stationary phase due to limitation on N sources, their presence manifested. Their presence in the algae coincided with decolorization, formation of holes, and lysis of the cells with a dark particle inside. Although the population was not completed eradicated, algal cell concentration was greatly decreased as well as FA content (Figs. 2 and 7). We could not isolate or identify these motile particles, but their behavior and phenotype are close to *Aphelidium*, a group of parasitoids of algae that were described as fungal parasites (Pretorius and Engelbrecht 1980; Gleason et al. 2014; Karpov et al. 2014). Similar contaminants and lysis of microalgae were also observed in cultures at 55 PSU but to a much lower extent (Fig. 3B). The final cell concentration did not decrease unlike the cultures at 33 PSU (Fig. 2). At higher salinities (80 and 105 PSU), contaminants were rarely observed, and most cells remained intact until harvest (Fig. 3C and D). The result implies that the parasitoids may be unable to survive at high salinity, but related information has not been revealed as interests on aphelids have been raised very recently (Karpov et al. 2014). Although biomass productivity was highest at 55 PSU, due to the presence of contaminants, a salinity higher than 55 PSU would be appropriate for the cultivation of *Tetraselmis* sp. KCTC12429BP when NSW is used without disinfection or sterilization.

Elevation of salinity led to higher FA content in *Tetraselmis* (Fig. 4). FA content increased until 80 PSU and then decreased at 105 PSU. Other microalgae also accumulate biochemicals under hyperosmotic conditions. Cultivation at a salinity higher than NSW increased the intracellular total lipid contents of *Dunaliella tertiolecta* (Takagi & Karseno 2006), *Navicula* sp. (Al-Hasan et al. 1990), *Nannochloropsis salina* (Bartley et al. 2013), and *Tetraselmis* sp. (Kim et al. 2016a). Similar to the results of the present study, lipid contents decreased at very high salinities after rapidly increasing at moderately higher salinities in *Navicula* sp. (13.4% at 99 PSU to 4.3% at 146 PSU) (Al-Hasan et al. 1990) and *N. salina* (37.5% at 34 PSU to 21.8% at 46 PSU) (Bartley et al. 2013), though the effective salinities and extent of lipid accumulation varied. As for the specific growth rates, lipid contents over a range of salinity differed among *Tetraselmis* strains (Fon-Sing and Borowitzka 2016). Many of the *Tetraselmis* strains they isolated showed increased lipid contents even at 11% NaCl compared with lower salinity (e.g., 9% NaCl). Their *Tetraselmis* strains were isolated from saline lakes at which salinities were 6.4–13.1% NaCl whereas the *Tetraselmis* sp. used in this study was isolated from seawater at 30 PSU. The difference in the salinity of their natural habitats may have led to various characteristics. High salinity suppresses photosynthesis by exerting chemical and structural stresses on the photosynthesis apparatus caused by increased intracellular Na^+ levels and changes in cell volume (Zakhozhii et al. 2012). Consequently, surplus NADPH or ATP could be used for the conversion of lipids from glyceraldehyde-3-phosphate along with elevation of glycerol content in cells in response to osmotic pressure (Hellebust 1985; Zakhozhii et al. 2012; Klok et al. 2014; Kim et al. 2016a). Furthermore, a recent study reported that the activity of Rubisco, an important enzyme in carbon fixation, increased at 58 PSU, and extracellular carbon anhydrase (CA), a key enzyme in inorganic carbon uptake, was most active at 117 PSU in *Dunaliella viridis* (Wang et al. 2016). Higher activities of these enzymes could increase the internal carbon pool to promote FA synthesis. As optimal salinity for microalgae varies by strains, their enzymes would have different optimal salinities, but if the enzymes involved in carbon fixation for *Tetraselmis* sp. KCTC12432BP are more active at 80 PSU, they could contribute to increased FA content. Extremely high

salinities could drastically increase energy-consuming cellular respiration for maintenance of homeostasis and reduction of Rubisco and CA activities (Zeng and Vonshak 1998; Gu et al. 2012; Wang et al. 2016). In the case of *Tetraselmis* sp. KCTC12432BP, increasing salinity beyond 80 PSU was undesirable as biomass productivity and FA content were both reduced.

Use of hypersaline medium improved FA productivity during ocean cultivation in both closed and open culture systems (Fig. 5A). Nitrate consumption patterns were similar at 30 and 80 PSU (data not shown), but biomass concentrations differed due to cell lysis and invasion by contaminants. In the TM-PBRs at 30 PSU, lysed cells were detected by microscopic observation, which was similar to the result of laboratory experiments (Fig. 6A). On the other hand, shifts in dominant species from *Tetraselmis* to diatoms were observed in SB-Ps (Fig. 6C). While NSW was the initial source of contaminants during inoculation in TM-PBRs, contaminants could have been introduced multiple times into open cultures in SB-Ps, a common risk in open systems (Das et al. 2011; Fon-Sing et al. 2014). Lysis of *Tetraselmis* by contaminants such as *Aphelidium* gave other microalgae opportunities to overtake the culture in SB-Ps at 30 PSU (Fig. 6C). Such contamination was prevented by high salinity, resulting in higher biomass productivity (Fig. 5). Significant contamination was not detected upon microscopic observation as in the laboratory experiments with 80 PSU media (Fig. 6B and D). For the large-scale cultures of *Tetraselmis* sp. in outdoor raceway ponds, hypersaline medium was effective to enhance biomass productivity with minimal contamination (Fon-Sing et al. 2014). Our results also suggest that using hypersaline medium is an effective cultivation strategy for ocean cultivation as well.

FA contents were considerably lower in ocean cultures than in laboratory cultures (Figs. 3 and 5B). When pH increased from 7.5 to 9.5, total lipid content of *Tetraselmis* sp. decreased (Khatoon et al. 2014). Moreover, it was shown that lipid content was 38% lower when pH was unregulated compared with when pH 7.5 was maintained with CO_2 bubbling (Moheimani 2013). Since TM-PBRs and SB-Ps were dependent on dissolution of atmospheric CO_2, there would have been insufficient carbon supply for seamless FA synthesis, and the culture pH increased to above 9.0 in the absence of pH modulation by CO_2 gas. Elevation of pH above 9 could lead to precipitation of salt, thereby increasing ash content and reducing FA content (Renaud and Parry 1994). Since these ocean culture systems were prototypes still under development, productivity cannot be directly compared with data from conventional culture systems, which have been extensively studied. Nevertheless, the results demonstrate that use of hypersaline medium could improve biomass and FA productivity in the cultivation of *Tetraselmis* in the ocean.

The lowered FA content may be increased with two-stage cultivation, wherein culture conditions are rapidly altered to induce accumulation of FA (Gleason et al. 2014). The efficacy of two-stage cultivation using a salinity shift was investigated under laboratory conditions to eliminate the effects of other parameters, such as solar irradiance, L/D cycle, and wave conditions. The results indicate that alteration of salinity to a lower or higher level both increased FA productivity (Fig. 7). When *Tetraselmis* was cultivated at 30 PSU, biomass and FA content were reduced as nitrate was depleted, which is the same result shown in Fig. 2. A hypersalinity shock, in which the algae were transferred from 30 to 80 PSU, reduced biomass loss by 50% and increased FA content by 90% compared with the cultures staying at 30 PSU (Fig. 7). However, in comparison to single-stage cultivation without nitrogen starvation (i.e., microalgae harvested on the fourth day in Fig. 7A), FA productivity was 27.4% lower in the cultures with a hypersalinity shock. Since contamination was inhibited at 80 PSU, the reduction in biomass after transferring to 80 PSU would be caused by lysis of cells that were infected with the parasitoids prior to the salinity shift. Applying nitrogen deficiency condition at 80 PSU did not increase FA content as much as salinity shifts, and FA productivity was 7.6% lower than that of the cultures with a salinity shift from 30 to 80 PSU despite higher biomass productivity. A hyposalinity shock, shifting salinity from 80 to 30 PSU, did not affect biomass concentration but largely increased FA content by 109% than before the salinity changed (Fig. 7). Overall, highest FA productivity of 28.5 mg L^{-1} day^{-1} was obtained from the two-stage cultivation with a hyposalinity shock. FA productivity of single-stage cultivation at 30 PSU followed as 24 mg L^{-1} day^{-1}. Numerous studies have reported that FA or total lipid productivity could be enhanced by shifting salinity to a higher (Takagi & Karseno 2006; Xia et al. 2013; Ho et al. 2014) or lower level (Kim et al. 2016a). However, this study is the first to report the effects of salinity shift from elevated salinity to regular levels on the FA productivity of microalgae for the first time.

In this study, improving biomass and FA productivity in *Tetraselmis* sp. KCTC12432BP using hypersaline medium was investigated. When NSW was used without sterilization, increasing salinity to 80 PSU by addition of NaCl enhanced biomass and FA productivity be limiting contamination and inducing FA accumulation in a controlled environment. Hypersaline medium was also effective in improving biomass and FA productivity in ocean cultivation using both closed- and open-type culture systems. Using hypersaline medium prepared by evaporation of NSW, or reusing the hypersaline medium showed the same effect with NaCl-added medium or using fresh medium, respectively (data not shown), which could alleviate the cost increase when using hypersaline medium. Application of two-stage cultivation, growing phase at 80 PSU with nitrate-replete condition followed by FA accumulation phase at 30 PSU without nitrogen source, could further enhance FA productivity in *Tetraselmis* culture. Further

studies in two-stage cultivation at large-scale in a long term are in progress to assess feasibility of this method in mass production of algal biomass for biofuels.

Funding information This research was supported by a grant from the Marine Biotechnology Program (Project No.: 20090267, Development of Marine Microalgal Biofuel Production Technology) funded by the Ministry of Oceans and Fisheries, Korea. Authors are also grateful to the Manpower Development Program for Marine Energy from the same ministry (Project No.: 201405502). Hanwool Park was also supported by a NRF (National Research Foundation of Korea) Grant funded by the Korean Government (NRF-2013H1A2A1032623).

References

Al-Hasan RH, Ali AM, Ka'wash HH, Radwan SS (1990) Effect of salinity on the lipid and fatty acid composition of the halophyte *Navicula* sp.: potential in mariculture. J Appl Phycol 2:215–222

Bartley ML, Boeing WJ, Corcoran AA, Holguin FO, Schaub T (2013) Effects of salinity on growth and lipid accumulation of biofuel microalga *Nannochloropsis salina* and invading organisms. Biomass Bioenergy 54:83–88

Borowitzka MA (1999) Commercial production of microalgae: ponds, tanks, tubes and fermenters. J Biotech 70:313–321

Carney LT, Reinsch SS, Lane PD, Solberg OD, Jansen LS, Williams KP, Trent JD, Lane TW (2014) Microbiome analysis of a microalgal mass culture growing in municipal wastewater in a prototype OMEGA photobioreactor. Algal Res 4:52–61

Das P, Aziz SS, Obbard JP (2011) Two phase microalgae growth in the open system for enhanced lipid productivity. Renew Energy 36: 2524–2528

Fanesi A, Raven JA, Giordano M (2014) Growth rate affects the responses of the green alga *Tetraselmis suecica* to external perturbations. Plant Cell Environ 37:512–519

Fon-Sing S, Borowitzka MA (2016) Isolation and screening of euryhaline *Tetraselmis* spp. suitable for large-scale outdoor culture in hypersaline media for biofuels. J Appl Phycol 28:1–14

Fon-Sing S, Isdepsky A, Borowitzka MA, Lewis D (2014) Pilot-scale continuous recycling of growth medium for the mass culture of a halotolerant *Tetraselmis* sp. in raceway ponds under increasing salinity: a novel protocol for commercial microalgal biomass production. Bioresour Technol 161:47–54

Gleason FH, Karpov SA, Lilje O, Macarthur DJ, Van Otgen F, Sime-Ngando T (2014) Zoosporic parasites of phytoplankton. In: Gareth Jones EB, Hyde KD, Pang K-L (eds) Freshwater fungi and fungal-like organisms, Walter de Gruyter, Berlin, pp 279-304

Gu N, Lin Q, Li G, Tan Y, Huang L, Lin J (2012) Effect of salinity on growth, biochemical composition, and lipid productivity of *Nannochloropsis oculata* CS 179. Eng Life Sci 12:631–637

Hagemann M (2016) Coping with high and variable salinity: molecular aspects of compatible solute accumulation. In: Borowitzka MA, Beardall J, Raven JA (eds) The physiology of microalgae. Springer, Cham, pp 359-372.

Hellebust JA (1985) Mechanisms of response to salinity in halotolerant microalgae. Plant Soil 89:69–81

Ho S-H, Nakanishi A, Ye X, Chang J-S, 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

Hoffman Y, Aflalo C, Zarka A, Gutman J, James TY, Boussiba S (2008) Isolation and characterization of a novel chytrid species (phylum Blastocladiomycota), parasitic on the green alga *Haematococcus*. Mycol Res 112:70–81

Karpov SA, Mamkaeva MA, Benzerara K, Moreira D, López-García P (2014) Molecular phylogeny and ultrastructure of *Aphelidium* aff. *melosirae* (Aphelida, Opisthosporidia). Protist 165:512–526

Khatoon H, Rahman NA, Banerjee S, Harun N, Suleiman SS, Zakaria NH, Lananan F, Hamid SHA, Endut A (2014) Effects of different salinities and pH on the growth and proximate composition of *Nannochloropsis* sp. and *Tetraselmis* sp. isolated from South China Sea cultured under control and natural condition. Int Biodeterior Biodegrad 95:11–18

Kim G, Lee C-H, Lee K (2016a) Enhancement of lipid production in marine microalga *Tetraselmis* sp. through salinity variation. Korean J Chem Eng 33:230–237

Kim Z-H, Park H, Hong S-J, Lim S-M, Lee C-G (2016b) Development of a floating photobioreactor with internal partitions for efficient utilization of ocean wave into improved mass transfer and algal culture mixing. Bioprocess Biosyst Eng 39:713–723

Kim Z-H, Park H, Lee C-G (2016c) Seasonal assessment of biomass and fatty acid productivity by *Tetraselmis* sp. in the ocean using semi-permeable membrane photobioreactors. J Microbiol Biotechnol 26: 1098–1102

Kim Z-H, Park H, Lee H-S, Lee C-G (2016d) Enhancing photon utilization efficiency for astaxanthin production from *Haematococcus lacustris* using a split-column photobioreactor. J Microbiol Biotechnol 26:1285–1289

Kim Z-H, Park H, Ryu Y-J, Shin D-W, Hong S-J, Tran H-L, Lim S-M, Lee C-G (2015) Algal biomass and biodiesel production by utilizing the nutrients dissolved in seawater using semi-permeable membrane photobioreactors. J Appl Phycol 27:1763–1773

Klok AJ, Lamers PP, Martens DE, Draaisma RB, Wijffels RH (2014) Edible oils from microalgae: insights in TAG accumulation. Trends Biotechnol 32:521–528

Moheimani NR (2013) Inorganic carbon and pH effect on growth and lipid productivity of *Tetraselmis suecica* and *Chlorella* sp (Chlorophyta) grown outdoors in bag photobioreactors. J Appl Phycol 25:387–398

Norsker N-H, Barbosa MJ, Vermuë MH, Wijffels RH (2011) Microalgal production—a close look at the economics. Biotechnol Adv 29:24–27

Novoveská L, Zapata AKM, Zabolotney JB, Atwood MC, Sundstrom ER (2016) Optimizing microalgae cultivation and wastewater treatment in large-scale offshore photobioreactors. Algal Res 18:86–94

Park H, Lee C-G (2016) Theoretical calculations on the feasibility of microalgal biofuels: utilization of marine resources could help realizing the potential of microalgae. Biotechnol J 11:1461–1470

Pretorius J, Engelbrecht AHP (1980) The occurrence of a fungal parasite on a *Tetraselmis* (*Prasinophyceae*) species. Water SA 6:181–185

Renaud SM, Parry DL (1994) Microalgae for use in tropical aquaculture II: effect of salinity on growth, gross chemical composition and fatty acid composition of three species of marine microalgae. J Appl Phycol 6:347–356

Richardson JW, Johnson MD, Zhang X, Zemke P, Chen W, Hu Q (2014) A financial assessment of two alternative cultivation systems and their contributions to algae biofuel economic viability. Algal Res 4:96–104

Rodolfi L, Chini Zittelli G, Bassi N, Padovani G, Biondi N, Bonini G, Tredici MR (2009) Microalgae for oil: strain selection, induction of lipid synthesis and outdoor mass cultivation in a low-cost photobioreactor. Biotechnol Bioeng 102:100–112

Saadaoui I, Al Ghazal G, Bounnit T, Al Khulaifi F, Al Jabri H, Potts M (2016) Evidence of thermo and halotolerant *Nannochloris* isolate suitable for biodiesel production in Qatar Culture Collection of Cyanobacteria and Microalgae. Algal Res 14:39–47

Su C-H, Chien L-J, Gomes J, Lin Y-S, Yu Y-K, Liou J-S, Syu R-J (2011) Factors affecting lipid accumulation by *Nannochloropsis oculata* in a two-stage cultivation process. J Appl Phycol 23:903–908

Suh IS, Joo H-N, Lee C-G (2006) A novel double-layered photobioreactor for simultaneous *Haematococcus pluvialis* cell growth and astaxanthin accumulation. J Biotech 125:540–546

Takagi M, Karseno YT (2006) Effect of salt concentration on intracellular accumulation of lipids and triacylglyceride in marine microalgae *Dunaliella* cells. J Biosci Bioeng 101:223–226

Tran H-L, Hong S-J, Lee C-G (2009) Evaluation of extraction methods for recovery of fatty acids from *Botryococcus braunii* LB 572 and *Synechocystis* sp. PCC 6803. Biotechnol Bioprocess Eng 14:187–192

Wang D, Wang W, Xu N, Sun X (2016) Changes in growth, carbon and nitrogen enzyme activity and mRNA accumulation in the halophilic microalga *Dunaliella viridis* in response to NaCl stress. J Ocean Univ China 15:1094–1100

Wiley P, Harris L, Reinsch S, Tozzi S, Embaye T, Clark K, McKuin B, Kolber Z, Adams R, Kagawa H (2013) Microalgae cultivation using offshore membrane enclosures for growing algae (OMEGA). J Sust Bioenergy Syst 3:18–32

Xia L, Ge H, Zhou X, Zhang D, Hu C (2013) Photoautotrophic outdoor two-stage cultivation for oleaginous microalgae *Scenedesmus obtusus* XJ-15. Bioresour Technol 144:261–267

Zakhozhii I, Matalin D, Popova L, Balnokin YV (2012) Responses of photosynthetic apparatus of the halotolerant microalga *Dunalliella maritima* to hyperosmotic salt shock. Russ J Plant Physiol 59:42–49

Zeng M-T, Vonshak A (1998) Adaptation of *Spirulina platensis* to salinity-stress. Comp Biochem Physiol A 120:113–118