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Use of conditioned medium for efficient transformation and cost-effective cultivation of *Nannochloropsis salina*

Nam Kyu Kang[a,1], Bongsoo Lee[a,1], Sung-Eun Shin[a], Seungjib Jeon[a], Min S. Park[a,b], Ji-Won Yang[a,b,*]

[a]*Department of Chemical and Biomolecular Engineering, KAIST, 291 Daehak-ro, Yuseong-gu, Daejeon 305-701, Republic of Korea*

[b]*Advanced Biomass R&D Center, KAIST, 291 Daehak-ro, Yuseong-gu, Daejeon 305-701, Republic of Korea*

* Corresponding author at: Department of Chemical and Biomolecular Engineering, KAIST, 291 Daehak-ro, Yuseong-gu, Daejeon 305-701, Republic of Korea.

Tel.: +82-42-350-3924; Fax: +82-42-350-3910.

E-mail address: jwyang@kaist.ac.kr

[1] Nam Kyu Kang and Bongsoo Lee contributed equally to this work.

A B S T R A C T

The oleaginous microalga *Nannochloropsis* sp. has been spotlighted as a promising candidate in genetic engineering research for biodiesel production. However, one of the major bottlenecks in the genetic manipulation against *Nannochloropsis* sp. is low transformation efficiency. Based on the idea that they grow rapidly in broth culture, the effect of conditioned medium on colonization and transformation efficiency of *Nannochloropsis salina* was investigated. Cells grown on agar plates with 20% to 40% conditioned medium produced colonies that were approximately 2.3-fold larger than cells grown without conditioned medium. More importantly, the transformation efficiency was about 2-fold greater on plates with 30% conditioned medium relative to those without conditioned medium. In addition, FAME productivity in liquid cultures with 100% conditioned medium increased up to 20% compared with cultures of control medium. These results suggest that conditioned medium can be applied for efficient transformation and cost-effective cultivation of *N. salina* for biodiesel production.

Keywords:

Microalgae

Nannochloropsis salina

Conditioned medium

Colonization

Transformation efficiency

Fatty acid methyl ester (FAME)

Abbreviations: *N. salina, Nannochloropsis salina*; DCW, dry cell weight; FAME, fatty acid methyl ester; CM, conditioned medium

1. Introduction

The increasing use of petroleum-based fuels has led to significant environmental problems, such as global warming and climate change. In addition, limited oil deposits and increasing oil prices have increased the demand for renewable and alternative energy sources (Chen et al., 2011). Among the various energy sources, microalgae have been considered to be a promising alternative source of biodiesel due to their high photosynthetic efficiency, rapid growth, and accumulation of abundant neutral lipids in the form of triacylglyceride (TAG) (Anandarajah et al., 2012).

Recently, *Nannochlropsis* sp. have been spotlighted as new model strain for biodiesel production in microalgal research field (Simionato et al., 2013). These species are unicellular marine microalgae that can accumulate abundant biomass, with approximately 47.5% of this biomass as lipids (Radakovits et al., 2012).

Recently, genome and transcriptome analysis results of various *Nannochloropsis* sp. have been reported (Wang et al., 2014), and this research provides a foundation for modification of many characteristics of this species through genetic engineering (Kilian et al., 2011; Radakovits et al., 2012; Vieler et al., 2012). However, despite the necessity and advantageous conditions for genetic engineering, several technical barriers affecting transformation efficiency must be overcome before researchers use *Nannochloropsis* sp. as candidate for genetic manipulation. In particular, their extremely slow growth rate on

agar plates is a major obstacle for genetic transformation; for example, the colonization of *Nannochloropsis salina* after transformation takes 6-8 weeks (Radakovits et al., 2012). It has long been known that fast and stable colonization is essential for efficient selection and maintenance of transformants. In other words, therefore, this clearly indicates that slow colonization of *N. salina* may have negative effect on transformation efficiency as well as on the recovery of transformants. In previous researches, a few methods have been used in order to obtain colonies after transformation of *Nannochloropsis* sp. (Kilian et al., 2011; Radakovits et al., 2012; Vieler et al., 2012). However, there are no available methods to induce rapid colonization of *Nannochloropsis* cells which may result in high transformation efficiency. Notably, *N. salina* grows well in broth culture but not in plate culture, indicating that certain factors present in conditioned medium might affect their growth.

In bacteria, cell-to-cell communication mechanism (quorum sensing) is well known. Bacteria utilize quorum sensing molecules affecting their behaviors including growth and virulence in a cell- density dependent manner (Waters & Bassler, 2005). It has been previously reported that chemical communication influences growth in high-cell-density cultivations of photosynthetic bacterium, *Rhodospirillum rubrum* (Carius et al., 2013). Another study indicated that *cer*I null mutant of *Rhodobacter sphaeroides*, photosynthetic bacteria which are unable to produce 7,8-cis-N-(tetradecenoyl) homoserine lactone (quorum sensing molecule) resulted in morphological changes of colonies on agar plates (Puskas et al., 1997).

Although quorum sensing molecules have not yet been identified in microalgae, it was found that microalgae shows high growth rate in appropriate concentrations of conditioned medium (Grabski et al., 2010; Grabski & Tukaj, 2008). On the other hand,

conditioned media have long been known to negatively affect growth of some microalgae (Fogg, 1971). In particular, excreted organic compounds and accumulated ions in conditioned media can stress cells and reduce growth (Alyabyev et al., 2007; Rodolfi et al., 2003). These all previous researches suggest that signaling molecules that remain in conditioned media can regulate the growth of microalgae. Recently, large-scale cultivation of microalgae by using open ponds or raceways has been attempted. Therefore, recycling of culture media may be means to reduce cultivation costs, because large amounts of water and nutrients are needed for large-scale cultivation (Yang et al., 2011). Moreover, it has been currently reported that the reused medium not only supported the growth, but also affected lipid productivity of microalgae (Farooq et al., 2015).

In the present study, conditioned medium was applied to enhance transformation efficiency by induction of fast colonization of *N. salina* on agar plate. Moreover, cell growth and fatty acid methyl ester (FAME) content were analyzed during broth cultivation by using conditioned medium in order to validate its potential use for cost-effective biodiesel production.

2. Methods

2.1. Microalgal strain and pre-culture conditions

N. salina CCMP 1776 (National Center for Marine Algae and Microbiota) was photoautotrophically maintained in sterile filtered modified F2N medium (Kilian et al., 2011). The modified F2N medium was prepared as follows: 15 g/L sea salt (Sigma-

Aldrich, USA), 427.5 mg/L $NaNO_3$, 30 mg/L $NaH_2PO_4 \cdot 2H_2O$, 5 mL/L trace metal mixture (4.36 g/L Na_2 EDTA$\cdot 2H_2O$, 3.15 g/L $FeCl_3 \cdot 6H_2O$, 10 mg/L $CoCl_2 \cdot 6H_2O$, 22 mg/L $ZnSO_4 \cdot 7H_2O$, 180 mg/L $MnCl_2 \cdot 4H_2O$, 9.8 mg/L $CuSO_4 \cdot 5H_2O$, 6.3 mg/L $Na_2MoO_4 \cdot 2H_2O$) and 2.5 mL/L vitamin stock (1 mg/L vitamin B_{12}, 1 mg/L Biotin, 200 mg/L thiamine·HCl) (Guillard & Ryther, 1962). To maintain pH, 10 mM Tris-HCl (pH 7.6) was added. *N. salina* was grown in 250 mL Erlenmeyer baffled flasks that contained 200 mL of culture medium. The cells were cultivated under 25°C, 120 rpm, under 120 µmol photons/m^2/s of fluorescent light. Air containing 2% CO_2 was directly supplied to the culture at 0.5 vvm.

2.2. Cultivation of N. salina on the agar plates containing conditioned medium

To obtain conditioned medium for agar plate cultures, cells were photoautotrophically cultivated in modified Guillard f/2 medium (Guillard & Ryther, 1962). This medium consisted of 30 g/L sea salt, 375 mg/L $NaNO_3$, 6 mg/L $NaH_2PO_4 \cdot 2H_2O$, 1 mL/L trace metal mixture and 0.5 mL/L vitamin stock. Cells were inoculated at low concentration (OD_{680nm} = 0.2; UV-1800, SHIMADZU, Japan). The supernatant was obtained at mid-exponential phase, 5 days after inoculation ($OD_{680\,nm}$ = 2.6). Then, cells were centrifuged at 4000 rpm for 15 min, and the supernatant was filtered by a 0.22 µm bottle top filter (RF 500 filter system. Sartorius Stedim Biotech, Germany). The resulting conditioned medium was diluted for preparation of agar medium, and the following nutrients were added: 375 mg/L $NaNO_3$, 6 mg/L $NaH_2PO_4 \cdot 2H_2O$, 1 mL/L trace metal mixture, 0.5 mL/L vitamin stock. *N. salina* does not grow well at low concentrations of sea salt, so sea salt was added to maintain the

concentration at 12 g/L. Bacto agar (BD Bioscience, USA) was autoclaved at 121°C for 10 min, and then mixed with the filtered conditioned medium at dilutions of 0% to 80%. Agar concentration was fixed at 15 g/L. After cultivation in the broth medium for one week, cells were counted by hemocytometer. Then, the cell culture was adjusted to 5000 cells/mL by dilution. Approximately 50 cells were spread plated on each agar medium and incubated at 25°C under 120 µmol photons/m^2/s of fluorescent.

2.3. Cultivation of N. salina in the broth culture with conditioned medium

To obtain conditioned medium for broth cultures, cells were cultivated in a 5 L cylindrical photobioreactor (PBR) that contained 3 L of modified f/2 medium. Modified f/2 medium contained 15 g/L sea salt, 375 mg/L $NaNO_3$, 6 mg/L $NaH_2PO_4 \cdot 2H_2O$, 1 mL/L trace metal mixture and 0.5 mL/L vitamin stock. Air containing 2% CO_2 was provided at 0.2 vvm, and the temperature was maintained 25°C. When the cell density was low ($OD_{680nm} < 1$), fluorescent light was maintained at 150 µmol photons/m^2/s; at higher cell density, fluorescent light was increased to 250 µmol photons/m^2/s. Cells were harvested at the mid-exponential phase ($OD_{680nm} = 3$), and the supernatant was collected. Cells and debris in the supernatant were removed by filtration through Whatman GF/C filter paper, and this solution was filtered again through a 0.22 µm bottle top filter for sterilization. The conditioned medium was diluted from 100% to 0% in 200 mL of broth culture. The following nutrients were added: 375 mg/L $NaNO_3$, 6 mg/L $NaH_2PO_4 \cdot 2H_2O$, 1 mL/L trace metal mixture, 0.5 mL/L vitamin stock. Sea salt was added to maintain a concentration of 15 g/L, except for growth in 100% conditioned medium. The cells were cultivated in 200 mL of culture medium under

25°C, 120 rpm, and 120 µmol photons/m^2/s of fluorescent light. Air containing 2% CO_2 was directly supplied to the culture at 0.5 vvm.

2.4.Bombardment transformation of N. salina

The pNsSh*ble* plasmid harboring endogenous TUB promoter, TUB terminator, and Sh*ble* gene (which encodes resistance to zeocin), was constructed (Fig. S1). Then, linearized pNsSh*ble* by XbaI was purified by ethanol precipitation method. The linearized pNsSh*ble* was coated onto microcarrier gold particles (Bio-Rad, USA) using 2.5 M $CaCl_2$ and 0.1 M spermidine. These coated gold particles were washed by 70% ethanol and resuspended in 100% ethanol. *N. salina* cells were cultivated in modified F2N medium (Kilian et al., 2011). 10^8 cells on cellulose acetate membrane filters (Sartorius Stedim Biotech, Germany) were placed on F2N agar medium. Particle bombardment was carried out using a GDS-80, low-pressure gene delivery system (Wealtec, USA), under the following conditions: 625 µg gold particles/shot coated with 1 µg linearized plasmid, 700 psi helium and 3 cm target distance. After bombardment transformation, cells on the 5 cellulose acetate membrane filters were incubated in modified F2N liquid medium at 25°C under 5 µmol photons/m^2/s of fluorescent light for 1 day (Talebi et al., 2013). All cells were harvested and plated on selective F2N agar medium containing 2.5 µg/mL zeocin and 30% conditioned medium.

2.5. Analytical procedures

2.5.1. Analysis of colony growth

Colony growth was evaluated by diameter, based on images from an inverted microscope (Eclipse TS 100, Nikon, USA). Each colony size was measured by ImageJ tool (http://rsb.info.nih.gov/ij/).

2.5.2. PCR analysis of transformants

Colonies grown on the selective medium were harvested and washed with distilled water, and 200 µL of Instagene matrix (Bio-Rad, USA) was added to suspended cells. Then, cells were incubated at 56°C for 20 min, and then at 100°C for 8 min. After centrifugation, the supernatant was used as a template for PCR. To detect the Sh*ble* gene, the S1 forward primer (5'AAGTTGACCAGTGCCGTTCCGGTG3') and S2 reverse primer (5'CTCGGCCACGAAGTGCACGCAGTT3') were used. To detect 18s rDNA, the SR6 forward primer (5'GTCAGAGGTGAAATTCTTGG3') and SR9 reverse primer (5' AACTAAGAACGGCATGCAC3') were used (Nakayama et al., 1996). PCR amplification of Sh*ble* gene and 18s rDNA was conducted in a Takara Thermal Cycler Dice gradient (Takara, Japan) with Ex-taq polymerase (Takara, Japan) through 30 cycles: 95°C (1 min), 60°C (1 min), 72°C (1 min). The expected sizes of the PCR products were 357 bp and 380 bp.

2.5.3. Growth analysis of broth culture

Growth of *N. salina* cells in broth was analyzed by measurement of dry cell weight (DCW) and cell density. For determination of DCW, cells were passed through circular glass filter paper (GF/C, 47 mm, Whatman), which was washed with deionized water, and dried in a 105°C oven overnight. After drying at 105°C oven overnight, the weight of samples was measured. Cell concentration was determined by hemocytometer.

2.5.4. Analysis of nutrient concentration of conditioned medium

For analysis of nitrate (NO_3^-), phosphate (PO_4^{3-}), and chloride (Cl^-), ion chromatography (881 compact IC pro, Metrohm, Swiss) with a Metrosep A Supp5 150 column for anions was used. Concentration of sodium (Na^+) and trace elements were determined with an inductively coupled plasma atomic emission spectrometer (Optima 7300DV, Perkin-Elmer, USA).

2.5.5. Fatty acid methyl ester (FAME) analysis

Extracted lipids from *N. salina* were converted to FAMEs by a transesterification protocol (Ryu et al., 2013). The cells were centrifuged at 4000 rpm at 25°C for 15 min (Supra-22k, Hanil Science Industrial, Republic of Korea), washed twice with deionized water, and then lyophilized (FD5508, ilShin BioBase, Republic of Korea). For lipid extraction, a chloroform and methanol mixture (2:1, v/v) was added to 10 mg of dried cells. After vigorous mixing for 10 min, 0.5 mg of heptadecanoic acid (C17:0) was added as an internal standard. Then 1 mL of methanol and 300 μL of sulfuric acid were added to samples, and the samples were held at 100°C for 20 min for transesterification.

After this reaction, 1 mL of deionized water was added, and the samples were centrifuged at 4,000 rpm for 10 min to separate the organic and aqueous phases. The lower layers, organic phase, were obtained using a syringe and filtered through a 0.20 μm RC-membrane syringe filter (Sartorius Stedim Biotech, Germany). FAME content and composition were determined by a gas chromatograph (HP 6890, Agilent, USA) that was equipped with a flame ionized detector (FID) and a HP-INNOWax polyethylene glycol column (HP 19091N-213, Agilent, USA). The temperature of the GC column increased from 50°C to 250°C at 15°C per min. FAME peaks were identified and quantified based on a 37-component FAME standard mix (F.A.M.E. MIX C8-C24, Supelco, USA).

2.5.6. Total carbohydrate (TC) analysis

Total carbohydrate in *N. salina* cells was determined according to colorimetric method (Bellou & Aggelis, 2012). 5 mg of dry biomass was suspended in 10 mL of DW, and then 1 ml of samples was mixed with 1 mL of 5% (w/v) phenol solution. Subsequently, 5 mL of sulphuric acid (95~98%) was added. After incubation at room temperature for 30 min, optical density (OD) was determined at 470 nm using UV/Vis spectrophotometer (DU 730, Beckman Coulter, USA). The amount of total carbohydrate was determined by a standard curve based on glucose concentration.

3. Results and discussion

3.1. Effect of conditioned medium on colony growth

To evaluate the effect of conditioned medium on colony growth, *N. salina* cells were spread on modified f/2 agar plates with different concentration of conditioned medium (Fig. 1). The results show that the average size of colonies grown on modified f/2 agar plates containing 20% to 40% of conditioned medium significantly increased up to approximately 1.5 mm in diameter, which is 2.3 fold larger than that of colonies grown on normal condition. Size of colonies increased in between 10% and 40% of conditioned medium supplemented plates, and then gradually decreased from plates containing 50% to 80% of conditioned medium (Fig. 1a). These results strongly suggest that conditioned medium has a positive effect on growth of *N. salina* cells on agar plates, with an optimal concentration of 20% to 40%. These results are also similar to previous research which showed that conditioned medium containing exudates from *Desmodesmus subspicatus* enhanced cell proliferation in a concentration-dependent manner, and that 2-fold diluted conditioned medium was most effective (Grabski et al., 2010).

Moreover, it was found that the size of *N. salina* colonies formed on agar plates containing conditioned medium increased continuously during plate cultivation, whereas the size of colonies formed on normal condition remained unchanged, even after 30 days (Fig. 1b). As a result, much bigger colonies were produced from conditioned medium supplemented agar plates (Fig. S2a and b) indicating that

conditioned medium induces the fast colonization of *N. salina* cells on the plates. It has been previously reported that several microalgae attained high cell density when cultivated in reused media. For example, Burkiewicz & Burkiewicz (1996) demonstrated that medium obtained after cultvation of the green algae *Dictyosphaerium pulchellum* caused a siginificant increase in cell division of *Scenedesmus armatus*. This suggests that *D. pulchellum* produces and releases biologically active substance(s) that promote the growth of *S. armatus* cells (Burkiewicz & Synak, 1996). In addition, enhancement of cell growth was observed during the cultivation of *Scenedesmus subspicatus*, when their conditioned media were supplemented to normal media (Grabski & Tukaj, 2008).

Although these positive effects of conditioned medium on growth have been characterized during the broth cultivation of microalgae, the effect of conditioned medium on the rate of colonization has not yet been reported. It should be noted that tiny colonies formed on normal growth medium have many disadvantages due to the fact that it takes longer time to maintain the plates for further study. Moreover, colonies formed on agar plates become unstable if they are not regularly transferred to fresh plates. Therefore, the method of fast colonization by use of conditioned medium as shown here can be used for effective maintenance of *N. salina* cells on agar plates.

3.2. Effect of conditioned medium on transformation

The finding that conditioned medium promotes the growth of *N. salina* on agar plates (Fig. 1) led to the hypothesis that fast colonization may improve transformation efficiency, which is crucial for efficient and successful genetic engineering. To assess

whether or not conditioned medium enhances the transformation efficiency of *N. salina*, modified f/2 agar plates containing 30% conditioned medium was used as selection medium after transformation (Fig. 2). The results show that 50.5 colonies in average grew in the presence of 30% conditioned medium, about 2-fold more than grew in the normal medium (Fig. 2a). Moreover, the size of colonies grown on plates with 30% conditioned medium was significantly larger than that of colonies produced from control plates at 21 days after plating (Fig. 2b). Especially, approximately 2.7-fold more colonies ranging between 0.4 mm and 0.6 mm in diameter were produced on conditioned medium supplemented plates. More importantly, these results further suggest that conditioned medium improves the stable recovery of colonies after transformation, and the fast colonization due to use of conditioned medium likely resulted in increased transformation efficiency.

Consequently, zeocin resistant transformants were confirmed by PCR analysis using S1 and S2 primers which could amplify the Sh*ble* gene (Fig. 3a). As a negative control, genomic DNA from *N. salina* wild type was used as template for PCR analysis. The PCR analysis revealed that Sh*ble* gene was amplified in all selected transformants, whereas PCR product of Sh*ble* gene was not observed in wild-type. This result suggests that transformants grown on plates with conditioned medium supplemented plates are also genetically stable.

Recently, many genetic transformation researches have been attempted in *Nannochloropsis* sp. (Kilian et al., 2011; Radakovits et al., 2012; Vieler et al., 2012), and these all studies gave better insights about the genetic manipulation of *Nannochloropsis* sp. However, it seems that slow colonization has still remained a barrier to overcome. Particularly, it has been reported that zeocin resistant colonies of *N.*

gaditana after transformation were detected after 5-6 weeks and picked after 7-8 weeks post-transformation (Radakovits et al., 2012). In addition, Kilian et al. (2011) also reported that transformed colonies of *Nannochloropsis* sp. appeared after 2 weeks and could be further processed after 3 weeks (Kilian et al., 2011). These previous researches together indicate that colonization of transformed *Nannochloropsis* cells takes a long time, and this likely has a negative effect on stable maintenance of colonies and transformation efficiency. It is widely known that the transformation process damages cells, due to the physical shock from electroporation, bombardment, or other procedures. Thus, stable recovery of colonies which can affect transformation efficiency is very important for successful genetic manipulation. To sum up, conditioned medium which induce early and stable colonization of *N. salina* cells can be used for efficient transformation and fast manipulation of *N. salina* transformants.

3.3. Effect of conditioned medium on broth culture

3.3.1. Growth of N. salina in broth culture with conditioned medium.

In an effort to achieve cost-effective cultivation of microalgae, previous research attempted to grow *Scenedesmus obliquus* and *Nannochloropsis* sp. with recycled media. However, these experimental researches indicated that organic metabolites accumulated in culture media lead to a decrease in biomass productivity, when culture media are recycled (Lívansky´ et al., 1996; Rodolfi et al., 2003). Moreover, it has been reported that unassimilated ions such as Na^+ and Cl^- were accumulated in culture and led to a change in media salinity which may negatively affect the biomass growth, when culture media supernatant is recycled (Alyabyev et al., 2007; Pal et al., 2011). To monitor

possible toxicity of conditioned medium used in this report, basic chemical propertises were analyzed (Table 1). The results show that more than half of the nitrate and all of the phosphate were consumed after 5 days of cultivation (Table 1). However, this would not affect the growth of microalgae if these consumed nutrients are appropriately supplemented in the broth culture. On the other hand, counter ions (eg., Na^+ and Cl^-) were slighlty accumulated in conditioned medium, but it was negligible (Table 1).

In this study, it was clearly found that use of conditioned medium is very effective to gain and maintain transformants of *N. salina* (Fig. 2); however, use of this medium will be limited if it has negative effects on growth in broth culture. To examine the effects of conditioned medium on growth of *N. salina* in broth culture, consumed nutrients were supplemented, and *N. salina* cells were cultivated in modified f/2 media containing various concentrations of conditioned medium ranging from 0% to 100% with 20% gradient (Fig. 4). The results show that *N. salina* cells grew about equally well with or without recycled medium, although there were slight variations (Fig. 4a). Moreover, total biomass based on dry cell weight (DCW) slightly increased in conditioned medium supplemented cultures after 10 days (Fig. 4b). These results suggest that conditioned medium had no negative effects on the growth of *N. salina* in broth culture.

Recently, open ponds or raceways are widely used for large-scale continuous culture of microalgae. Due to the fact that culture volume of extensive culture system is often up to several thousand cubic meters, recycling culture media become an important item to reduce the huge amounts of water and nutrient consumption for cost-effective microalgae cultivation (Hadj-Romdhane et al., 2013; Yang et al., 2011). One study reported that approximately 63% of water and 16% of nutrients can be saved by recyling the culture medium of *Scenedesmus obliquus* (Lívansky´ et al., 1996).

Moreover, Hadj-Romdhane et al. (2012) estimated that optimization of the culture medium for *Chlorella vulgaris* allowed saving approximately 77% of the water and 50% of the nutrients (Hadj-Romdhane et al., 2012). Therefore, it can be concluded that conditioned medium of *N. salina* seems very useful for the development of large-scale cultures to minimize water consumption.

3.3.2. FAME content, yield, and productivity

Lastly, FAME content and yield were analyzed in order to understand how conditioned medium affects final FAME (fatty acid methyl esters) productivity, an important factor for biodiesel production (Fig. 5). The results show that the highest FAME content was observed in broth culture containing 40% conditioned medium after 10 days, and that FAME contents under other growth conditions were at about the same level as the controls (Fig. 5a). Moreover, when considering cell biomass, FAME yield and productivity were generally greater with supplemental conditioned medium (Fig. 5b and c). The highest FAME yield and productivity were obtained in the culture medium, containing 100% conditioned medium after 10 days. In this condition, FAME yield and productivity were 452.3 mg/L and 45.2 mg/L/d, respectively, and these levels were approximately 20% greater than those under normal condition (Fig. 5b and c).

All of these results indicate that use of conditioned medium for broth cultivation did not significantly disrupt lipid production or physiological growth of *N. salina*. In general, the growth rate of microalgae is inversely proportional to their lipid content (Hu et al., 2008). Moreover, microalgae generally focus on cell division under favorable conditions, but accumulate triacylglycerols (TAGs), neutral lipids, as a protective mechanism under stressful conditions such as in the presence of nitrogen starvation or

high salinity (Sharma et al., 2012). However, the results presented here indicate that the growth rate and FAME content of *N. salina* cells grown even in 100% conditioned medium were similar to those of cells grown under normal conditions. These results therefore clearly suggest that there are no significant toxic materials in the conditioned medium of *N. salina*, which can induce dramatic fluctuations in lipid accumulation or physiological growth of *N. salina*. These results could be also supported by total carbohydrate (TC) contents, because it has been reported that synthesis of lipid and sugars is highly competitive (Bellou & Aggelis, 2012; Bellou et al., 2014). As shown in Fig. S3, there were no significant changes in total carbohydrate production even under 100% conditioned medium when comparing with normal condition. These results indicate that conditioned medium has no negative effects on production of storage compounds such as lipid and carbohydrate in *N. salina*.

4. Conclusion

The present study demonstrated that supplemental conditioned medium improved the colonization, transformation efficiency, and lipid production in *N. salina*. It was clearly found that 20% to 40% conditioned medium induced much faster colonization, and 30% conditioned medium increased transformation efficiency by about 2-fold. Moreover, *N. salina* cells grown in 100% conditioned medium showed approximately 20% increased FAME productivity. Although future work is needed to maximize the lipid contents, the present findings suggest that conditioned medium can be used for fast colonization, efficient transformation, and cost-effective cultivation of *N. salina*.

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Figure Legends

Fig. 1. Colony size analysis of *Nannochloropsis salina.* (a) Effect of different concentrations of conditioned medium on colony size at 30 days after plating. (b) Growth of colony size over 30 days. The conditioned medium was obtained at the exponential phase and diluted from 80% to 0%. About 50 cells were plated on each agar plate, and all grown cells were analyzed. *N. salina* cells was cultivated at 25°C and 120 µmol photons/m^2/s of fluorescent.

Fig. 2. Number of resistant colonies on the zeocin-containing selection medium with and without 30% conditioned medium at 21 days after transformation. (a) Total number of antibiotics-resistant colonies. (b) Total number of antibiotics-resistant colonies of different sizes. Five hundred million cells at the exponential phase were subjected to bombardment transformation. Then, cells were plated on selective medium and cultivated at 25°C under 120 µmol photons/m^2/s fluorescent light. All resistant colonies were counted and classified according to size.

Fig. 3. Confirmation of transgene analysis by PCR. (a) Schematic diagram of the pNsSh*ble* construct. S1 is forward primer (5'AAGTTGACCAGTGCCGTTCCGGTG3') and S2 is reverse primer (5'CTCGGCCACGAAGTGCACGCAGTT3') for amplifying Sh*ble* gene. Agarose gel electrophoresis for verification of the Sh*ble* PCR product (357 bp) on (b) normal selective medium and (c) selective medium with 30% conditioned medium. In (b) and (c): lane M has size markers, WT is the negative control (*N. salina* wild type), and 1 to 8 are Sh*ble* and 18s rDNA PCR bands from transformants randomly

selected from each medium.

Fig. 4. Growth analysis of *N. salina* in broth culture with conditioned medium. (a) Change of cell density over time and (b) dry cell weight at 7 days and 10 days after inoculation of cells with different concentrations of conditioned medium. *N. salina* cells were cultivated at 25°C, 120 rpm, 120 μmol photons/m^2/s of fluorescent light, and 0.5 vvm of 2% CO_2.

Fig. 5. FAME analysis of *N. salina* in broth culture with conditioned medium. (a) FAME content, (b) FAME yield, (c) FAME productivity of *N. salina* cells at 7 and 10 days after inoculation with different concentrations of conditioned medium. *N. salina* cells were cultivated at 25°C, 120 rpm, 120 μmol photons/m^2/s of fluorescent light, and 0.5 vvm of 2% CO_2.

Fig. 1.

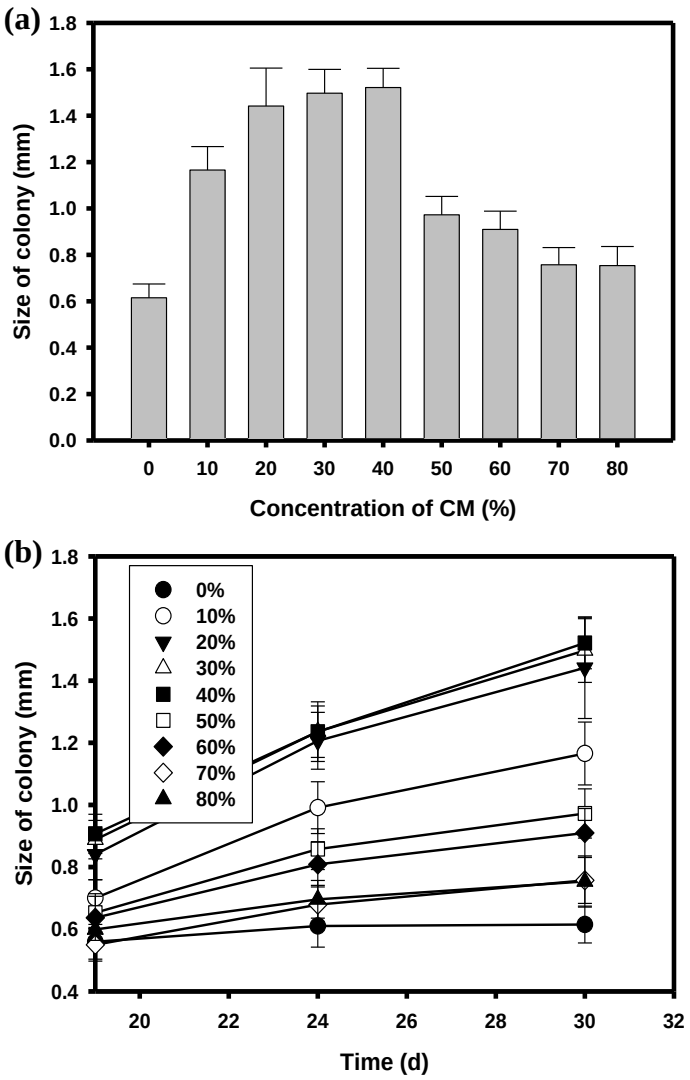

Fig. 2.

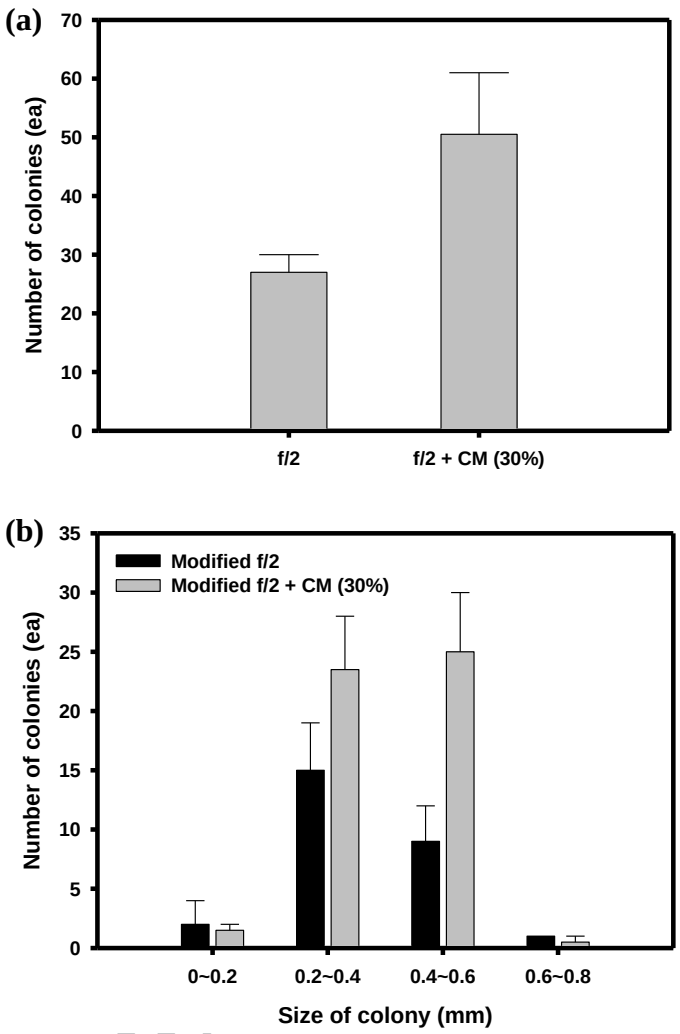

Fig. 3.

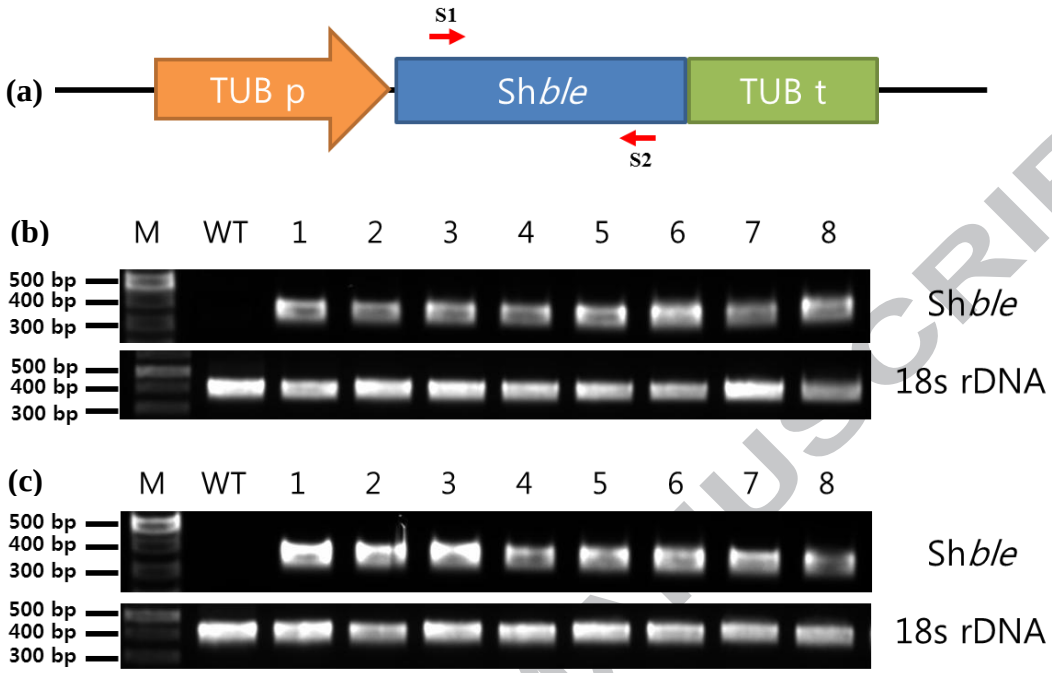

Fig. 4.

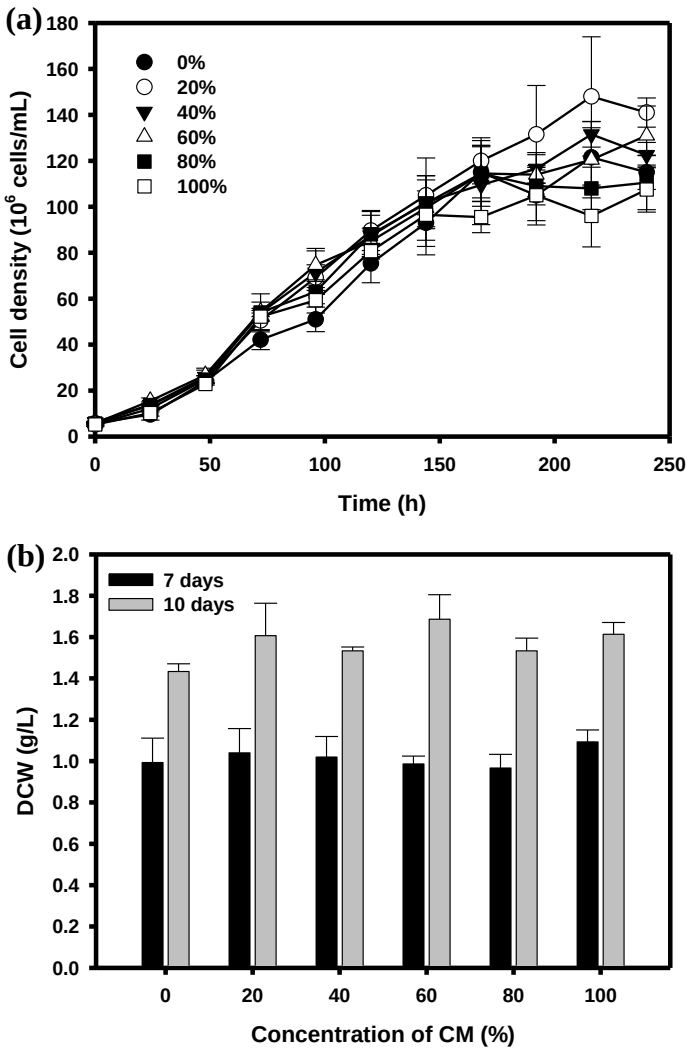

Fig. 5.

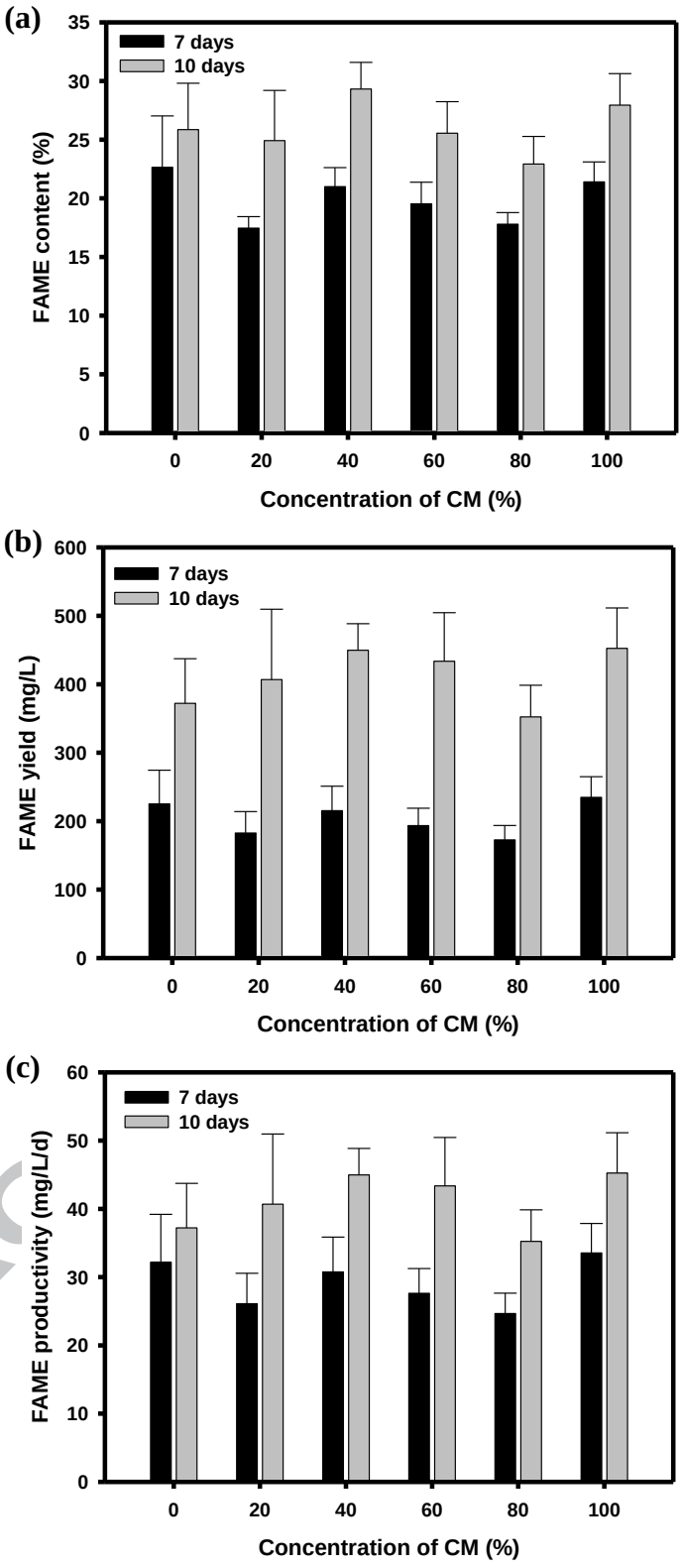

Table 1. Chemical properties of modified f/2 medium and conditioned medium.

	Modified f/2 Medium (ppm)	Conditioned Medium (ppm)
NO_3^-	268.3	106.7
PO_4^{3-}	1.7	0
Na^+	3819.6	3844.0
Cl^-	5521.6	5531.6
Fe^{2+}	0.1	0.3
Cu^{2+}	< 0.1	< 0.1
Zn^{2+}	< 0.1	< 0.1
Co^{2+}	< 0.1	< 0.1
Mn^{2+}	< 0.1	< 0.1
Mo^{7+}	< 0.1	< 0.1

[a] Conditioned medium was obtained in mid-exponential phase ($OD_{680\ nm} = 3$) after 5 days of cultivation of *N. salina* at 25°C, 120 rpm, 120 µmol photons/m^2/s fluorescent light, and 0.5 vvm of 2% CO_2. The properties of the conditioned medium were determined before addition of supplementary nutrients.

HIGHLIGHTS

- Conditioned medium was first employed for efficient transformation of *N. salina*.
- Conditioned medium induced fast colonization of *N. salina*.
- Transformation efficiency of *N. salina* was doubled by use of conditioned medium.
- FAME productivity increased up to 20% under culture containing conditioned medium.