Starch and lipid accumulation in eight strains of six *Chlorella* species under comparatively high light intensity and aeration culture conditions

Authors:

Tsuyoshi Takeshita^a; Shuhei Ota^{a,c}; Tomokazu Yamazaki^{a,c}; Aiko Hirata^a; Vilem Zachleder^b; Shigeyuki Kawano^{a,c,*}

Affiliations:

^a *Department of Integrated Biosciences, Graduate School of Frontier Sciences,
University of Tokyo, 5-1-5 Kashiwanoha, Kashiwa, Chiba 277-8562, Japan*

^b *Laboratory of the Cell Cycles of Algae, Institute of Microbiology, Academy of Sciences
of the Czech Republic, Opatovický mlýn, Třeboň 379 81, Czech Republic*

^c *Japan Science and Technology Agency, CREST, 5-1-5 Kashiwanoha, Kashiwa, Chiba
277-8562, Japan*

**For post-publication correspondence: (fax +81-471363679; kawano@k.u-tokyo.ac.jp)*

Keywords

Accumulation shift; Lipid; *Chlorella*; Starch; Strong light.

Abstract

The microalgae family *Chlorella* species are known to accumulate starch and lipids. Although nitrogen or phosphorous deficiencies promote starch and lipids formation in many microalgae, these deficiencies also limit their growth and productivity. Therefore, the Chlorellaceae strains were attempted to increase starch and lipids productivity under high-light-intensity conditions ($600\text{-}\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$). The 12-h:12-h light-dark (LD) cycle conditions elicited more stable growth than the continuous light (LL) conditions, whereas the starch and lipids yields increased in LL conditions. The amount of starch and lipids per cell increased in *C. viscosa* and *C. vulgaris* in sulfur-deficient medium, and long-chain fatty acids with 20 or more carbon atoms accumulated in cells grown in sulfur-deficient medium. Accumulation of starch and lipids was investigated in eight strains. The accumulation was strain-dependent, and varied according to the medium and light conditions. Five of the eight *Chlorella* strains exhibited similar accumulation patterns.

1. Introduction

Rapid progress in the field of biofuel production from microalgae is being made on all levels, from identifying high-yield strains of microalgae to improving harvest and extraction techniques (Greenwell et al., 2010). However, the cost reduction potential for the industrial use of microalgae in biofuel production may depend on maximizing lipid

content and on maximizing growth yield (Davis et al., 2011).

The production efficiency in microalgae is estimated by the productivity ($\text{g}\cdot\text{L}^{-1}\cdot\text{day}^{-1}$) of the generated material, including biomass, lipids and carbohydrates (Ho et al., 2010; Fu et al., 2012; Converti et al., 2009; Rodolfi et al., 2009; Ho et al., 2013). Because these productivities are indicated per unit time, the duration of culture is important. Efficiency is increased as the time required for culture is reduced and the growth rate increases. Therefore, it is necessary for increasing productivity to produce much material to decrease period of cultivation time.

Most reports describe microalgae cultured under light intensity conditions of $300\text{-}\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, but growth potential is not inhibited by a light intensity of $400\text{-}\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ in *C. sorokiniana* (Ugwu et al., 2007). Whereas cultivation at a high light intensity is not suitable for the growth of *Euglena gracilis* (Ogbonna & Tanaka 2000; Kitaya et al., 2005), *Chlorella vulgaris* (Yeh et al. 2010, Lv et al. 2010) or *Parachlorella kessleri* (Li et al. 2012), higher productivity than low light intensity conditions has been reported in *Anabaena variabilis* and *Scenedesmus almeriensis* (Yoon et al. 2008, Sánchez et al. 2008) grown under very high light intensity ($1600\text{-}\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) conditions. Such high light intensity is equivalent to sunlight, which ranges from $\sim 700\text{-}\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ to a maximum of $\sim 2000\text{-}\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ in direct sunlight (Masojídek et al., 2010; Přibyl et al., 2012). Therefore, it is necessary to investigate productivity and growth under high-light-intensity conditions and to identify strains that show productivity to industrialize microalgae biofuel production under sunlight. A previous study showed an increase in the starch

content in cells grown under sulfur-deficient conditions (Brányiková et al., 2011). Furthermore, it has been shown that lipids are produced when sulfo-lipids are decomposed under sulfur-deficient conditions (Sugimoto et al., 2007). Sulfo-lipids exist in the chloroplast membrane system, and sulfate is supplied by decomposition of sulfo-lipids. Therefore, while sulfur deficiency decreased the growth yield per culture, it enhanced intracellular starch and lipid accumulations.

Microalgae are known to produce proteins, oils, and carbohydrates as major intracellular components (P. John et al., 2011; Perez-Garcia et al., 2011; Choix et al., 2012). *Chlorella* accumulates starch and that of lipids as carbon storage materials, and a trade-off between the accumulation of starch and lipids has been observed in four *Chlorella* species: *C. vulgaris*, *C. sorokiniana*, *C. lobophora* and *P. kessleri* (Mizuno et al., 2013). This trade-off has also been reported under high-light-intensity ($1200\text{-}\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) conditions in *P. kessleri* (Fernandes et al. 2013).

In this study, the relationships between accumulation of starch and lipids were investigated in eight strains of *Chlorella* under high light intensity. Productivity was examined using sulfur-deficient and complete media, light and dark (LD) cycles and continuous light (LL) conditions. The results show that the accumulation of starch and lipids depends on the strains or the culture conditions. A similar relationship under different conditions was observed in five strains of *Chlorella*. These results suggest that *Chlorella* produces constant yields of materials regardless of the growth conditions.

2. Materials and methods

2.1 Strains and culture conditions

Cultures of *C. vulgaris* (NIES-2170), *C. sorokiniana* (NIES-2169), *C. emersonii* (NIES-2151), *P. kessleri* (NIES-2152, NIES-2159), *C. viscosa* (SAG 2338), *P. beijerinckii* (SAG 2046) and *P. kessleri* (CCALA 255) were obtained from the Microbial Culture Collection, National Institute for Environmental Studies, Tsukuba, Japan (NIES-2170, NIES-2169, NIES-2151, NIES-2152 and NIES-2179), Die Abteilung Experimentelle Phykologie und Sammlung von Algenkulture, University of Göttingen, Göttingen, Germany (SAG 2338 and SAG 2046) and the Culture Collection of Autotrophic Organisms, Institute of Botany, Academy of Sciences of the Czech Republic, Třeboň, Czech Republic (CCALA 255).

Cells were precultured for 20 days in Tris-acetate-phosphate (TAP) medium (<http://mcc.nies.go.jp/02medium-e.html>) under a 12-h:12-h LD cycle at 50- $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ and 23°C. Cultures were transferred to TAP medium or sulfur-deficient TAP (STAP) medium (MgSO_4 , ZnSO_4 , FeSO_4 , and CuSO_4 in TAP medium were replaced with MgCl_2 , ZnCl_2 , FeCl_3 and CuCl_2 , respectively). The continuous light (LL) and LD cycle irradiance of 600- $\mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ incidence on the tube surface was provided by fluorescent lights, cold-cathode fluorescent lamp (CCFL, Nippon Medical & Chemical Instruments Co., Ltd, Osaka, Japan) units and light-emitting diode (LED) lamps (LUS-BF, Beaubelle, Ehime, Japan). Four culture conditions were created for the study from combinations of culture media (TAP and STAP media) and light conditions (LL and LD conditions): TAP medium under LD condition (LD), TAP medium under

LL condition (LL), STAP medium under LD condition (SLD), and STAP medium under LL conditions (SLL). Cells were cultivated at 23°C, with 20 mL/min air content, 2–3% CO₂ concentration, and 2-rpm using rotary culture equipment. A detailed description of the cultivation device is given in Supplementary Figure 1. The temperatures of the culture media were fixed using an incubator CLE-303 (TOMY SEIKO CO., LTD., Tokyo, Japan). There were 8.0×10^8 cells inoculated into 93-mL test tubes containing 80-mL culture media. Since the test tubes were 30-mm thick and 200-mm long, changes by aeration in the volume of culture media, if any, was always approximately 500 μ L/day during the survey.

2.2 Cell counts and dry weight measurement

Cell numbers were counted using a particle counter (CDA-1000, Sysmex, Kobe, Japan). For dry weight measurement, an aliquot of cell culture was centrifuged at $5,000 \times g$ for 10 min, the supernatant was removed and the cell pellet was suspended in 1-mL ethanol. Cells were dried at 105°C and weighed using an analytical balance (NewClassic MS, Mettler Toledo, Columbia, MD, USA).

2.3 Starch measurement

Starch content was quantified as described previously (Brányiková et al., 2011). Briefly, 1 mL of cell suspension was withdrawn and the cells were recovered by centrifugation. The cell pellet was suspended in 0.25 mL of PBS (8 mM Na₂HPO₄, 2 mM NaH₂PO₄, 140 mM NaCl, pH 7.4) and the cells were disrupted using an ultrasonic homogenizer

for 60 sec, interrupted every 20 s, on ice (XL-2000, Misonix, Farmingdale, NY, USA). Pigments in the cells were extracted using 80% ethanol pre-warmed to 50°C, and the starch-containing cell pellet was suspended in 0.15-mL H₂O after centrifugation. For starch hydrolysis, the cell suspension was incubated in a heat block at 90°C for 15 min and then mixed with 0.25-mL 60% perchloric acid after cooling. After stirring for 15 min, the suspension was mixed with 0.6 mL H₂O and centrifuged. Subsequently, 0.4 mL of the supernatant was mixed with 2-mL anthrone solution (0.2-g anthrone in 100-mL 75% H₂SO₄). The mixture was kept in a water bath at 100°C for 8 min. It was then cooled to room temperature, and the absorbance at 625 nm was measured using a spectrophotometer (Viento nano, BioTek Japan, Tokyo, Japan). Glucose was used simultaneously as the standard.

2.4 Lipid extraction

Total extractable lipids were assessed according to the method of Matyash et al. (2008) with the following modifications: An aliquot of cell culture medium (~7–8 mL) was withdrawn, and the cells were harvested by centrifugation at $5,000 \times g$ for 10 min. Subsequently, 1.5 mL of methanol was added and the tube was vortexed. Then, 5 mL of methyl tertiary-butyl ether (MTBE) were added and the tube was vortexed. The mixture was disrupted using an ultrasonic homogenizer for 20 s on ice.

Tubes were affixed to a shaker and subjected to continuous shaking at 150 rpm for 2 h. After the extraction step, the solvent-biomass mixture was filtered through a 0.45- μ m PTFE membrane. Residual solvent in the sample filtrate was eliminated by evaporation

under N₂ at an evaporation temperature below 55°C overnight. Next, 1 mL of 3-N methanolic HCl (Supelco, St. Louis, MO) was added to the dried lipid in a test tube and heated in a water bath at 85°C for 2.5 h. After cooling the mixture to room temperature, 0.5 mL of H₂O and 1 mL of n-hexane were added, and the contents were mixed well by hand. The hexane layer containing the trans-esterification fatty-acid methyl ester (FAME) was collected and the remaining methanol/water was extracted two more times with 1-mL hexane. The sample was stored at –20°C. The nonadecanoic acid solution of the carbon number 19 was used as an internal standard.

FAMEs were analyzed using a gas chromatograph (GCMS-QP2010 Plus, Shimadzu, Kyoto, Japan) equipped with a Supelco SP-2380 capillary column (30 m × 0.25 mm × 0.20 µm) and flame ionization detector (FID). Operating conditions were as follows: split ratio 1:40.5; injection volume 1 µL; helium carrier gas with constant linear velocity 24.2 cm/s; make up gas (helium) 20 mL/min; injector and detector temperature 250°C; and oven temperature beginning at 140°C for 1 min and increasing at a rate of 4°C/min to 220°C. The Supelco 37-component FAME mix standard (Supelco, St. Louis, MO) provided the calibration curve for each FAME compound. The peaks from a sample were identified by comparing retention times with those of standard compounds; additionally, the identities were confirmed by gas chromatography–mass spectrometry GC–MS. Compound concentrations in samples were quantified based on the area under the chromatogram peak in comparison with the mix standards.

3. Results and discussion

3.1 Stable growth of eight strains of six *Chlorella* species under high light intensity

A large number of diverse microalgae species accumulate high levels of lipids in the form of triacylglycerol (TAG) (Day et al., 2012; Hu et al., 2008). Although many algal strains have been isolated and assessed in terms of their production of lipids for biofuels, no ‘silver-bullet’ strain has yet been identified.

Eight strains (*C. viscosa*, *C. vulgaris*, *C. sorokiniana*, *C. emersonii*, *P. beijerinckii*, *P. kessleri* CCALA255, NIES-2159, NIES-2152 strains) of six *Chlorella* species were used in this study. Cultivation was performed under high light intensity in LL and LD conditions, and the cultures reached stationary phase by day 5 post-inoculation (Fig. 1a–h). The cells grew better and faster under the LD condition than under the LL condition. Thus, although only half the light energy of the LL condition was available under LD condition, the growth and efficiency was more than that under the LL condition. Electron microscopy of cells cultured under LD conditions confirmed the existence of starch and oil droplets in the cells (Fig. S2). Of the eight strains of six species, *C. vulgaris* showed the greatest growth with an increase of about 70-fold the initial input. However, even *P. beijerinckii*, which showed the worst growth of the eight strains, showed growth of about 25-fold the initial input.

The effects of sulfur deficiency on growth appeared at day 2 post-inoculation. Eight strains of six species showed a lag phase at day 1 post-inoculation in both the sulfur-deficient and TAP media. All strains entered the exponential growth phase from day 2 post-inoculation. All eight strains showed weak growth during the exponential

growth phase in STAP medium, which was markedly different compared to growth in TAP medium. The number of cells on day 5 post-inoculation increased by 1.4–3.2-fold the initial input in all eight strains in STAP medium (Fig1 a–h).

It is necessary to use species and strains adapted to outdoor light intensity in the cultivation of algae using outdoor photobioreactors. Outdoor sunlight ranges from several 100 $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ in cloudy weather to more than 1000 $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ in moderate weather to a maximum of over 2000 $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ in the direct sunlight of midsummer (Quentin et al., 2013; Masojídek et al., 2011). Therefore, it is necessary to maintain stable cultivation under high light intensities. Although few studies have cultivated *Chlorella* under high light intensity ($>300\text{-}\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) one study used $500\text{-}\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ (Pribyl et al., 2012). A high light intensity ($600\text{-}\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) and aeration conditions were adopted in this study. Eight strains of six species of *Chlorella* completed the exponential growth phase by day 4 post-inoculation, and the strains reached the stationary phase on day 5 post-inoculation (Fig. 1). *P. kessleri* NIES-2152 required ~ 14 days to reach the stationary phase under a light intensity of $\sim 100\text{ }\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ (data not shown). The results showed that cultivation at a high light intensity decreased the time required, and that growth inhibition did not occur in all eight strains examined in this study. In this research, sulfur deficiency reduced cell growth in all the strains, even under high light intensity conditions (Fig. 1). This was presumably because the sulfur-deficient medium diminished the availability of the sulfur-containing amino acids cysteine and methionine, which reduced growth. In contrast, it is important to note that the number of

cells was either maintained or increased slightly under sulfur-deficient conditions, showing that limited growth is possible under such conditions.

3.2 Changes in starch and lipids levels

Starch was assayed by the modified method of Brányiková et al. (2011). To determine the influence of sulfur on cell growth and the accumulation of starch and lipids, cells cultured in TAP and STAP media were compared. Lipid accumulation in cells and reduced cellular growth potential have been reported using various nutrient sources, including nitrogen and phosphorus, but not sulfur (Brányiková et al., 2011; Rodolfi et al., 2009; Breuer et al., 2012). Accumulation of starch peaked on day 4 or 5 post-inoculation under LD or LL conditions (Fig. 2a), with the greatest accumulation observed in *P. kessleri* NIES-2152 (875 mg/L), *C. emersonii* (699 mg/L) and *P. kessleri* NIES-2159 (662 mg/L). Starch storage tended to decrease after peaking in all strains. Maximum accumulation of starch was seen on day 4 post-inoculation under LL conditions. *C. viscosa* (170 mg/L) had the lowest amounts of starch under LL conditions. Compared with under LD conditions, all strains accumulated more starch under LL conditions than LD conditions; *P. kessleri* NIES-2152 accumulated about 1.69-fold more, *C. emersonii* accumulated 1.59-fold more, *P. kessleri* NIES-2159 accumulated 1.69-fold more and *C. viscosa* accumulated 1.53-fold more starch.

In seven strains, the maximum starch accumulation occurred earlier in the STAP medium than in the TAP medium, with the maximum accumulation at days 2 to 5

post-inoculation (Fig. 2a, c). Amount of starch showed trends to decrease after reaching the maximum in STAP medium as well as TAP medium. Starch accumulation under SLL conditions was observed in *P. kessleri* NIES-2159 (393 mg/L) and *P. kessleri* NIES-2152 (292 mg/L). *P. kessleri* NIES-2159 showed starch accumulation (237 mg/L) in SLD conditions. In all strains, greater accumulation of starch occurred under SLL conditions than under SLD conditions. *P. kessleri* NIES-2159 showed about 1.66-fold the amount of starch under SLL conditions compared to SLD conditions, and *P. kessleri* NIES-2152 showed 1.75-fold the amount of starch.

Lipids accumulation increased rapidly after day 3 post-inoculation, peaking on day 5 post-inoculation (Fig. 2b). The greatest lipids accumulation occurred in *P. kessleri* NIES-2152 (1.72 g/L), *C. vulgaris* (1.48 g/L) and *C. emersonii* (1.26 g/L) under LL conditions. Compared to under LD conditions, *P. kessleri* NIES-2152 accumulated 2.15-fold more lipids under LL conditions, *C. vulgaris* 1.78-fold more, and *C. emersonii* 1.75-fold more lipids under LL conditions. In fact, greater accumulation of both lipids and starch was observed under LL conditions than under LD conditions.

No significant difference was observed between the accumulation of lipids under SLL conditions compared with SLD conditions (Fig. 2d). Similar to the time course of starch accumulation, lipids accumulation peaked earlier in STAP medium. Maximum lipids accumulation was accelerated in *C. sorokinana*, *C. emersonii*, *P. beijerinckii*, *P. kessleri* CCALA255, NIES-2159 and NIES-2152 under SLL conditions, and in *P. kessleri* CCALA255 under SLD conditions. Although the growth rate under LL conditions was somewhat slower than under LD conditions, the amount of biomass,

starch and lipids accumulation was greater under LL conditions than LD conditions at all time points (Fig. 2, Table. 1). Lipid accumulation in *P. kessleri* NIES-2152 more than doubled under the LD condition (Fig. 2b). Therefore, the duration of the light period was important for the production of materials. Previous studies have reported greater biomass production under LL than under LD conditions (Han et al., 2013; Mizuno et al., 2013). Han *et al.* (2013) found that biomass production under LL conditions in *C. pyrenoidosa* was twofold that under LD conditions, and interpreted that this was due to the fact that biomass quantity decreased during the dark period under LD conditions. It has been shown that biomass decreases because of the reduction of carbohydrate at night (James et al., 1996). Since cells cannot photosynthesize at night, the production of starch not only stops but the cells also decompose starch. It appears that the reduction of biomass also occurred in the dark period in this research. It is likely that this same phenomenon occurred in all strains.

3.3 Effects of sulfur deficiency under strong light conditions on yields

Lipid levels peaked on day 5 post-inoculation (Fig. 2b, d). The cells were stained with Nile Red to identify intracellular oil droplets and observed by using fluorescence microscopy (Fig. S3). Red staining indicated the autofluorescence of chloroplasts and yellow staining showed the localization of oil droplets. Such staining was observed in *C. emersonii*, *P. beijerinckii*, *C. sorokiniana*, *P. kessleri* NIES-2152, CCALA255, and NIES-2159 strains under LL conditions (Fig. S3c–h); however, this staining was not observed in *C. viscosa* and *C. vulgaris* (Fig. S3a, b).

The yellow fluorescence indicating oil localization was seen in all strains under SLL conditions (Fig. S3i–p). Although strong yellow fluorescence derived wholly from the cell was observed in *C. vulgaris* and *C. viscosa* under SLL conditions (Fig. S3a, b), no oil droplets were observed in these strains under LL conditions (Fig. S3i, j). The amount of oil per cell increased under sulfur-deprivation conditions compared to TAP medium.

3.4 Fatty acid composition of accumulated lipids

In this study, lipids were extracted from the cells, converted to FAME by methyl esterification, and the fatty-acid composition determined by GC-MS (Fig. 3). The lipids comprised mainly 16-carbon-atom fatty acids and 18-carbon-atom fatty acids with 0–3 degrees of unsaturation. Palmitic acid (C16:0), oleic acid (C18:1), linoleic acid (C18:2) and α -linolenic acid (C18:3) comprised more than 70% of the total fatty acids. In addition, fatty acids with more number than 20 carbon atoms comprised 3% of the total *C. viscosa* under all culture conditions, and in *C. sorokiniana*, *P. kessleri* CCALA255, and NIES-2152 strains under the SLD and SLL conditions.

The fatty acids composition of *C. viscosa* and *C. vulgaris* was compared under all culture conditions. The proportion of C18:3 was highest under the LD condition and that of C18:1 under the LL, SLD, and SLL conditions in *C. viscosa* and *C. vulgaris*. The proportion of C18:1 was also highest in *P. kessleri* NIES-2152 and *C. emersonii* under all culture conditions. Different fatty acids in every culture condition accounted for the maximum rate in *C. sorokiniana*, *P. beijerinckii*, *P. kessleri* CCALA255 and NIES-2159.

These findings suggest that the types of fatty acid accumulated differed according to culture duration, species and strain. Previous studies have shown changes in the composition of fatty acids by defective nutrition stress. C16:0 and C18:1 increased with nitrogen deprivation in *Dunaliella salina* (Lamers et al., 2012). In this research, fatty acid composition changed in various ways because of sulfur deprivation. The increase in C16:0 or C18:1 was similar to that in a previous study of nutrient starvation, and the characteristic increase in fatty acids with chain length more than C20 was seen.

3.5 Accumulation shift from starch to lipids under high light intensity

Accumulation shifts from starch to lipids differed under the LL and SLL conditions. Maximum starch and lipids accumulation in *C. viscosa* occurred simultaneously under LL conditions. In contrast, the maximum starch accumulation decreased as the lipids accumulation peaked under SLL conditions. This relationship between the maximum amounts of starch and lipids was evident in *C. vulgaris*, *C. emersonii*, *P. beijeirinkii*, and *P. kessleri* CCALA255, NIES-2159 and NIES-2152 strains under both LL and SLL conditions (Fig. 2a, b). Maximum accumulation of starch and lipids occurred simultaneously in *C. sorokiniana* under SLL conditions. Thus, starch accumulation occurred first, followed by lipids accumulation; alternatively, both might have occurred simultaneously. The shift from starch to lipids accumulation was evident under LL conditions (Fig. 2a, b) but not under LD or sulfur-deficient conditions (Fig. 2c, d).

The ratio of starch to lipids accumulation was measured on a dry weight basis (Fig. 4). Although little starch accumulation occurred in cells grown in sulfur-deficient

medium, the proportion of lipids increased in *C. viscosa* and *C. vulgaris*, comprising 61% under SLL conditions in *C. viscosa*, and ~65% under SLD and SLL conditions in *C. vulgaris*. Lipids accumulation decreased under sulfur-deficient conditions in *C. sorokiniana*; however, starch accumulation was induced, comprising ~40% under SLL conditions.

The rate of starch accumulation peaked (~12–36%) at days 1 to 2 post-inoculation, while the rate of lipids accumulation during the same period was lowest (~10–24%) in five strains (*C. emersonii*, *P. beijerinckii*, and *P. kessleri* CCALA255, NIES-2159 and NIES-2152 strains). Thereafter, lipid content increased with decreasing starch content under both LD and LL conditions. This relationship was also seen under sulfur-deficient conditions. This has been reported previously under low light intensity (Mizuno et al., 2013; Fernandes et al., 2013). In this study, the shift between starch and lipids accumulation was confirmed by dry weight measurements in *C. emersonii*, *P. beijerinckii*, *P. kessleri* CCALA255, NIES-2159 and NIES-2152 (Fig. 4). In addition, the shift from starch to lipids accumulation was indicated by the total amounts of both materials in cultures under both LL and SLL conditions (Fig. 2). The above results showed that the shift to lipids accumulation from starch accumulation occurred even when cultivation was accelerated by high light intensity. The duration of the culture period before reaching the stationary phase was decreased under high light intensity conditions. Therefore, the entire incubation system was accelerated and the shift to lipids from starch was induced by high light intensity. Previous studies have shown that intracellular starch and cellulose are disassembled to monosaccharides such

as glucose, and lipid accumulation is induced under high light intensity (Ho et al., 2013).

3.6 Comparison of productivity in eight strains of six *Chlorella* species

The dry weights of lipids and starch in eight strains of six *Chlorella* species cultured under LL conditions are shown in Figure 5 and Table 1. The maximum yield of starch occurred before the final day of cultivation. However, while many studies have reported lipid productivity, few have investigated starch productivity, presumably because starch is in the process of shifting to lipids. Because productivity is dependent on the number of days in culture ($\text{g}\cdot\text{L}^{-1}\cdot\text{day}^{-1}$), the culture period exerts a direct effect on yield (Ho et al., 2010).

In this study, high productivity was achieved by reducing the culture time and using a high light intensity. *P. kessleri* NIES-2159 achieved under LL conditions the greatest biomass productivity ($1.04 \text{ g}\cdot\text{L}^{-1}\cdot\text{day}^{-1}$), which was about twofold that in *C. vulgaris* ($0.55 \text{ g}\cdot\text{L}^{-1}\cdot\text{day}^{-1}$). Lipids ($0.3 \text{ g}\cdot\text{L}^{-1}\cdot\text{day}^{-1}$) and starch ($0.22 \text{ g}\cdot\text{L}^{-1}\cdot\text{day}^{-1}$) productivity was greatest under LL conditions in *P. kessleri* NIES-2152. Biomass productivity ($0.80 \text{ g}\cdot\text{L}^{-1}\cdot\text{day}^{-1}$) and lipid productivity ($0.60 \text{ g}\cdot\text{L}^{-1}\cdot\text{day}^{-1}$) in *Parachlorella* have been reported (Li et al., 2013). Another group also reported high biomass ($1.291 \text{ g}\cdot\text{L}^{-1}\cdot\text{day}^{-1}$) and lipid ($0.526 \text{ g}\cdot\text{L}^{-1}\cdot\text{day}^{-1}$) productivity (Přibyl et al. 2012). While the biomass productivity in this study approached that reported previously, these results suggest that lipid productivity could be increased. Future studies of different light conditions and media are required to determine the optimum conditions for maintenance

of high biomass productivity to induce lipid production.

Biomass productivity has been reported for *C. vulgaris* ($1.05 \text{ g} \cdot \text{L}^{-1} \cdot \text{day}^{-1}$; Přibyl et al., 2012) and *C. pyrenoidosa* ($1.10 \text{ g} \cdot \text{L}^{-1} \cdot \text{day}^{-1}$; Li et al., 2011). Lipid productivity in *C. vulgaris* has been reported as $0.64 \text{ g} \cdot \text{L}^{-1} \cdot \text{day}^{-1}$ (Li et al., 2011) and $0.604 \text{ g} \cdot \text{L}^{-1} \cdot \text{day}^{-1}$ (Přibyl et al., 2012). The biomass productivity of *C. emersonii*, which was the highest of the strains tested in this study, was equal to that in a previous report ($1.03 \text{ g} \cdot \text{L}^{-1} \cdot \text{day}^{-1}$). *C. vulgaris* showed the maximum lipid productivity ($0.23 \text{ g} \cdot \text{L}^{-1} \cdot \text{day}^{-1}$), which was less than the previously result. Examination of a culture condition is a future subject like *Parachlorella*. The cost of attaining LL condition industrially may be problematic. LL condition can be cheaply attained by using sunlight in the daytime and artificial illumination in bad weather or at night. Direct rays have 2–3 times the light intensity of LED lights, so they are perfect as a substitute for lighting. However, too strong light intensity may inhibit the growth of *Chlorella*. Nevertheless, further basic data are required on the use of sunlight.

4. Conclusions

Eight strains of six *Chlorella* species showed stable growth under LD conditions under high light intensity, and showed high productivity under LL conditions. The amount of lipids per *C. viscosa* and *C. vulgaris* cell increased under sulfur-deprivation conditions. The rate of accumulation of lipids containing fatty acids with carbon numbers of 20 or more increased under sulfur-deprivation conditions. The shift from starch accumulation to lipids accumulation was evident under LL conditions. This accumulation pattern was

shared by five of the eight strains of six species, regardless of the culture conditions.

Acknowledgments

We thank the members of the Plant Life System Laboratory for their technical assistance with this research. This study was supported by JST, CREST.

References

1. Béchet, Q., Muñoz, R., Shilton, A., Guieysse, B., 2013. Outdoor cultivation of temperature-tolerant *Chlorella sorokiniana* in a column photobioreactor under low power-input. *Biotechnol. Bioeng.* 110, 118–126.
2. Brányiková, I., Maršáľková, B., Doucha, J., Brányik, T., Bišová, K., Zachleder, V., Vítová, M., 2011. Microalgae—novel highly efficient starch producers. *Biotechnol. Bioeng.* 108, 766–776.
3. Breuer, G., Lamers, P.P., Martens, D.E., Draaisma, R.B., Wijffels, R.H., 2012. The impact of nitrogen starvation on the dynamics of triacylglycerol accumulation in nine microalgae strains. *Bioresour. Technol.* 124, 217–226.
4. Choix, F., De-Bashan, L., Bashan, Y., 2012. Enhanced accumulation of starch and total carbohydrates in alginate-immobilized *Chlorella* spp. induced by *Azospirillum brasilense*: II. Heterotrophic. *Enzyme Microb. Technol.* 51, 294–299.

5. Converti, A., Casazza, A.A., Ortiz, E.Y., Perego, P., Del Borghi, M., 2009. Effect of temperature and nitrogen concentration on the growth and lipid content of *Nannochloropsis oculata* and *Chlorella vulgaris* for biodiesel production. Chem. Eng. Process. 48, 1146–1151.
6. Davis, R., Aden, A., Pienkos, P., 2011. Techno-economic analysis of autotrophic microalgae for fuel production. Appl. Energy 88, 3254–3531.
7. Day, J., Slocombe, S., Stanley, M., 2012. Overcoming biological constraints to enable the exploitation of microalgae for biofuels. Bioresour. Technol. 109, 245–251.
8. Fernandes, B., Teixeira, J., Dragone, G., Vicente, A.A., Kawano, S., Bišová, K., Příbýl, P., Zachleder, V., Vítová, M., 2013. Relationship between starch and lipid accumulation induced by nutrient depletion and replenishment in the microalga *Parachlorella kessleri*. Bioresour. Technol. 144, 268–274.
9. Fu, W., Gudmundsson, O., Feist, A.M., Herjolfsson, G., Brynjolfsson, S., Palsson, B.Ø., 2012. Maximizing biomass productivity and cell density of *Chlorella vulgaris* by using light-emitting diode-based photobioreactor. J. Biotechnol. 161, 242–249.

10. Greenwell, H.C., Laurens, L.M.L., Shields, R.J., Lovitt, R.W., Flynn, K.J., 2010. Placing microalgae on the biofuels priority list: a review of the technological challenges. *J. R. Soc. Interface* 7, 703–726.
11. Han, F., Wang, W., Li, Y., Shen, G., Wan, M., Wang, J., 2013. Changes of biomass, lipid content and fatty acids composition under a light-dark cyclic culture of *Chlorella pyrenoidosa* in response to different temperature. *Bioresour. Technol.* 132, 182–189.
12. Ho, S., Chen, C., Chang, J., 2012. Effect of light intensity and nitrogen starvation on CO₂ fixation and lipid/carbohydrate production of an indigenous microalga *Scenedesmus obliquus*. *Bioresour. Technol.* 113, 244–252.
13. Ho, S.-H., Chen, W.-M., Chang, J.-S., 2010. *Scenedesmus obliquus* CNW-N as a potential candidate for CO₂ mitigation and biodiesel production. *Bioresour. Technol.* 101, 8725–8730.
14. Hu, Q., Sommerfeld, M., Jarvis, E., Ghirardi, M., Posewitz, M., Seibert, M., Darzins, A., 2008. Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances. *Plant J.* 54, 621–639.
15. James C., and Tanaka H., 1996. Night biomass loss and changes in biochemical

composition of cells during light/dark cyclic culture of *Chlorella pyrenoidosa*.

Journal Of Fermentation And Bioengineering. 82, 6, 558–564.

16. John, R., Anisha, G., Nampoothiri, K., Pandey, A., 2011. Micro and macroalgal biomass: a renewable source for bioethanol. Bioresour. Technol. 102, 186–193.

17. Kitaya, Y., Azuma, H., Kiyota, M., 2005. Effects of temperature, CO₂/O₂ concentrations and light intensity on cellular multiplication of microalgae, *Euglena gracilis*. Adv. Space Res. 35, 1584–1588.

18. Lamers P., Janssen M., Vos R., Bino R., Wijffels R., 2012. Carotenoid and fatty acid metabolism in nitrogen-starved *Dunaliella salina*, a unicellular green microalga. J. Biotechnol. 2012, 21–27.

19. Li, P., Miao, X., Li, R., Zhong, J., 2011. In situ biodiesel production from fast-growing and high oil content *Chlorella pyrenoidosa* in rice straw hydrolysate. J. Biomed. Biotechnol. 2011, 141–207.

20. Li, X., Přibyl, P., Bišová, K., Kawano, S., Cepák, V., Zachleder, V., Čížková, M., Brányiková, I., Vítová, M., 2013. The microalga *Parachlorella kessleri*—a novel highly efficient lipid producer. Biotechnol. Bioeng. 110, 97–107.

21. Li, Y., Zhou, W., Hu, B., Min, M., Chen, P., Ruan, R.R., 2012. Effect of light intensity on algal biomass accumulation and biodiesel production for mixotrophic strains *Chlorella kessleri* and *Chlorella protothecoides* cultivated in highly concentrated municipal wastewater. *Biotechnol. Bioeng.* 109, 2222–2229.
22. Lv, J., Cheng, L., Xu, X., Zhang, L., Chen, H., 2010. Enhanced lipid production of *Chlorella vulgaris* by adjustment of cultivation conditions. *Bioresour. Technol.* 101, 6797–6804.
23. Masojídek, J., Kopecký, J., Giannelli, L., Torzillo, G., 2011. Productivity correlated to photobiochemical performance of *Chlorella* mass cultures grown outdoors in thin-layer cascades. *J. Ind. Microbiol. Biotechnol.* 38, 307–317.
24. Masojídek, J., Prášil, O., 2010. The development of microalgal biotechnology in the Czech Republic. *J. Ind. Microbiol. Biotechnol.* 37, 1307–1317.
25. Matyash, V., Liebisch, G., Kurzchalia, T. V., Shevchenko, A., Schwudke, D., 2008. Lipid extraction by methyl-tert-butyl ether for high-throughput lipidomics. *J. Lipid Res.* 49, 1137–1146.
26. Mizuno, Y., Sato, A., Watanabe, K., 2012. Sequential accumulation of starch and lipid induced by sulfur deficiency in *Chlorella* and *Parachlorella* species. *Bioresour.*

Technol. 129, 150–155.

27. Ogbonna, J., Tanaka, H., 2000. Light requirement and photosynthetic cell cultivation—Development of processes for efficient light utilization in photobioreactors. *J. Appl. Phycol.* 12, 207–218.
28. Perez-Garcia, O., Escalante, F.M.E., de-Bashan, L.E., Bashan, Y., 2011. Heterotrophic cultures of microalgae: metabolism and potential products. *Water Res.* 45, 11–36.
29. Přibyl, P., Cepák, V., Zachleder, V., 2012. Production of lipids in 10 strains of *Chlorella* and *Parachlorella*, and enhanced lipid productivity in *Chlorella vulgaris*. *Appl. Microbiol. Biotechnol.* 94, 549–561.
30. Rodolfi, L., Chini Zittelli, G., Bassi, N., Padovani, G., Biondi, N., Bonini, G., Tredici, M.R., 2009. Microalgae for oil: strain selection, induction of lipid synthesis and outdoor mass cultivation in a low-cost photobioreactor. *Biotechnol. Bioeng.* 102, 100–112.
31. Sánchez, J.F., Fernández-Sevilla, J.M., Ación, F.G., Cerón, M.C., Pérez-Parra, J., Molina-Grima, E., 2008. Biomass and lutein productivity of *Scenedesmus almeriensis*: influence of irradiance, dilution rate and temperature. *Appl. Microbiol.*

Biotechnol. 79, 719–729.

32. Sugimoto, K., Sato, N., Tsuzuki, M., 2007. Utilization of a chloroplast membrane sulfolipid as a major internal sulfur source for protein synthesis in the early phase of sulfur starvation in *Chlamydomonas reinhardtii*. FEBS Lett. 581, 4519–4522.
33. Ugwu, C., Aoyagi, H., Uchiyama, H., 2007. Influence of irradiance, dissolved oxygen concentration, and temperature on the growth of *Chlorella sorokiniana*. Photosynthetica 45, 309–311.
34. Yeh, K.-L., Chang, J.-S., chen, W., 2010. Effect of light supply and carbon source on cell growth and cellular composition of a newly isolated microalga *Chlorella vulgaris* ESP-31. Eng. Life Sci. 10, 201–208.
35. Yoon, J., Shin, J., Ahn, E., Park, T., 2008. High cell density culture of *Anabaena variabilis* with controlled light intensity and nutrient supply. J. Microbiol. Biotechnol. 18, 918–925.

Figure captions

Figure 1. Time-course of growth of eight strains of six *Chlorella* species (cells/mL). Effect of sulfur deprivation and the light-dark (LD) cycle under various culture

conditions. (a) *Chlorella viscosa*, (b) *C. vulgaris*, (c) *C. sorokiniana*, (d) *C. emersonii*, (e) *Parachlorella beijerinckii*, (f) *P. kessleri* CCALA255, (g) *P. kessleri* NIES-2159, (h) *P. kessleri* NIES-2152.

Figure 2. Time-course of changes in starch and lipid contents in eight strains of six *Chlorella* species. Effect of sulfur deprivation and the LD cycle under various culture conditions. (a) Starch concentration (mg/L) in Tris acetate phosphate (TAP) medium, (b) lipid concentration (g/L) in TAP medium, (c) starch concentration (mg/L) in sulfur-deficient TAP (STAP) medium, (d) lipid concentration (g/L) in STAP medium.

The solid lines represent the TAP medium, and the dashed lines represent the STAP medium. Black lines represent LD condition, and red lines represent LL condition. The strains and their symbols are as follows: “*Chlorella viscosa* (○), *C. vulgaris* (Δ), *C. sorokiniana* (□), *C. emersonii* (◇), *Parachlorella beijerinckii* (●), *P. kessleri* CCALA255 (▲) *P. kessleri* NIES-2159 (■), and *P. kessleri* NIES-2152 (◆).

Figure 3. Fatty acid profiles (percentage of total fatty acids) in eight strains of six *Chlorella* species under four different culture conditions. Accumulated lipids were extracted and their fatty acid compositions were analyzed by gas chromatography. The ratios of accumulated fatty acids are shown. The numbers of carbon atoms and double bonds in each fatty acid are indicated in the legend (number of carbon atoms: number of double bonds).

Figure 4. Time-course of changes in starch and lipid contents (percentage of dry weight biomass = % Dry Weight) in eight strains of six *Chlorella* species under various culture conditions. The cultivation conditions are indicated in the left column. Red lines show the amount of starch, and blue lines show the amount of lipids.

Figure 5. Biomass, lipid and starch productivity of eight strains of six *Chlorella* species cultured in TAP medium under continuous light (LL) conditions. Biomass and lipid productivity were measured on day 5 post-inoculation. The day of maximum starch productivity is indicated.

Table 1. Biomass, lipid and starch productivity data in eight strains of six *Chlorella* species. Biomass, lipids and starch weights in eight strains of six *Chlorella* species cultured in TAP medium under LL conditions. Biomass and lipid productivity were measured on day 5 post-inoculation. The day of maximum starch productivity is indicated.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Table 1. Biomass, lipid and starch productivity data in eight strains of six *Chlorella* species.

Strain	Biomass		Lipid		Starch	
	Final g/L	g/L/Day	Final g/L	g/L/Day	Max g/L	g/L/Day
<i>C. viscosa</i>	2.9	0.57	0.42	0.081	0.170 (5)	0.034
<i>C. vulgaris</i>	2.8	0.55	1.50	0.290	0.277 (4)	0.069
<i>C. sorokiniana</i>	2.9	0.57	0.21	0.039	0.471 (5)	0.094
<i>C. emersonii</i>	5.4	1.00	1.30	0.230	0.699 (4)	0.180
<i>P. beijerinckii</i>	4.6	0.87	1.00	0.190	0.326 (5)	0.065
<i>P. kessleri</i> CCALA 255	3.4	0.61	1.10	0.200	0.405 (4)	0.100
<i>P. kessleri</i> NIES-2159	5.6	1.00	1.20	0.210	0.662 (4)	0.170
<i>P. kessleri</i> NIES-2152	4.9	0.93	1.70	0.330	0.875 (4)	0.220