Revised title: Sequential accumulation of starch and lipid induced by sulfur deficiency
in *Chlorella* and *Parachlorella* species

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Abbreviations: TAP, Tris-Acetate Phosphate; DMSO, dimethyl sulfoxide; FAMES, fatty acid methyl esters; FA, fatty acid. TEM, transmission electron microscopy.

ABSTRACT

The influence of sulfur deficiency on biomass production was analyzed in the four Chlorellaceae species, *Chlorella vulgaris*, *C. sorokiniana*, *C. lobophora*, and *Parachlorella kessleri*. Culturing under sulfur-deficient conditions promoted transient accumulation of starch followed by a steady increase in lipid storage. Transmission electron microscopy indicated an increase and decrease in starch granules and subsequent enlargement of lipid droplets under sulfur-deficient conditions. Chlorellaceae spp. accumulated 1.5–2.7-fold higher amounts of starch and 1.5–2.4-fold higher amounts of lipid under sulfur-deficient conditions than under sulfur-sufficient conditions. More than 75% of the fatty acids that accumulated in Chlorellaceae spp. under the sulfur-sufficient condition were unsaturated and culturing under sulfur-deficient conditions increased the saturated fatty acid content from 24.3% to 59.7% only in *P. kessleri*. These results indicate that the sequential accumulation of starch and lipid is a response to the sulfur depletion that commonly occurs in Chlorellaceae spp.

Keywords:

Sulfur deficiency

Chlorella spp.

Parachlorella kessleri

Starch

Lipids

1. Introduction

Due to high growth rates and photosynthetic efficiency, microalgae have recently received much attention as a potential renewable energy resource. Lipid production can be increased under heterotrophic and mixotrophic culture conditions (Miao and Wu, 2004; Heredia-Arroyo *et al.*, 2010; Shen *et al.*, 2010), nitrogen depletion (Liang *et al.*, 2009; Hsie *et al.*, 2009), phosphorus depletion (Rodolfi *et al.*, 2009), high salt concentrations (Takagi *et al.*, 2006), and high iron concentrations (Liu *et al.*, 2008) in some microalgae. Similarly, starch accumulation can be induced by treatments such as nitrogen depletion (Dragone *et al.*, 2011), sulfur depletion (Brányiková *et al.*, 2011), high light intensity (Brányiková *et al.*, 2011), or high CO₂ concentrations (Izumo *et al.*, 2007). However the mechanisms regulating starch and lipid accumulation in response to altered growth environments and the interrelationship between stored starch and lipid remain unclear.

Supplementation of a culture with additional carbon or nitrogen increases total biomass, starch and lipid, but the increase in cost is unacceptable to maintain competition between biofuels and fossil fuels. However, depletion of macroelements is a simple and an effective way to increase the relative starch and lipid contents per unit cell dry weight. However, this approach reduces biomass yield and, consequently, the total content of starch and lipid in comparison to relative content per unit cell dry weight. A moderate reduction in biomass and starch productivity is caused by depleting

sulfur in *Chlorella vulgaris* (Brányiková *et al.*, 2011), suggesting that sulfur depletion could be an effective and economical procedure to produce starch and lipid in microalgae. Microalgal neutral lipids are rich in polyunsaturated fatty acids (Chisti, 2007), and the process of hydrogenation of oils, which reduces double bonds and converts unsaturated fatty acids into saturated fatty acids, is required to obtain biodiesel-quality oil. Thus, some microalgae alter their fatty acid composition in response to various culture conditions (Cha *et al.*, 2011; Davidi *et al.*, 2012; Wang *et al.*, 2011). The cost of biodiesel could be reduced by screening and searching for species and culture conditions that meet biodiesel requirements.

We employed four Chlorellaceae spp.: *C. vulgaris*, *C. sorokiniana*, *C. lobophora*, and *Parachlorella kessleri* to better understand the effect of sulfur deficiency on the generation of starch and lipid in microalgae, and characterized the total biomass yield and changes in starch content and lipid content in these species under sulfur-deficient conditions. We also demonstrated the temporal relationship between starch and lipid accumulation under these conditions.

2. Methods

2.1. Strains and growth conditions

Cultures of *C. vulgaris* (NIES-2173), *C. sorokiniana* (NIES-2169), *C. lobophora* (SAG 37.88), and *P. kessleri* (PRATT/CCAP 211-11h) were obtained from the Microbial Culture Collection, National Institute for Environmental Studies, Tsukuba, Japan (NIES-2169 and NIES-2173), Die Abteilung Experimentelle Phykologie und Sammlung von Algenkulture, University of Göttingen, Göttingen, Germany (SAG 37.88), and the Culture Collection of Autotrophic Organisms, Institute of Botany, Academy of Sciences

of the Czech Republic, Třeboň, Czech Republic (PRATT/CCAP 211-11h). Cells were precultured in TAP medium (<http://mcc.nies.go.jp/02medium-e.html>) under continuous light or under a 12-h: 12-h light: dark cycle at $70 \mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ and 20°C . Cultures were transferred to TAP medium or sulfur-deficient TAP (TAP-S) 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) to a concentration of 4×10^6 cells/mL, and the cells were grown further in static culture under the same light conditions and temperature.

2.2. Biomass determination

Cell numbers were determined using a particle counter (CDA-1000, SYSMEX, Kobe, Japan). For dry weight determination, an aliquot of cell culture was withdrawn and the supernatant was removed after centrifugation at $5,000 \times g$ for 10 min. Cells were dried and weighed using an analytical balance (NewClassic MS, Mettler Toledo, Columbia, MD, USA). The experiments were repeated three times.

2.3. Starch analysis

Starch content was quantified according to Brányiková *et al.* (2011). Briefly, 1 mL of cell suspension was withdrawn and cells were recovered by centrifugation. The cell pellet was mixed with an equal volume of glass beads (0.1-mm diameter) and 0.25 mL of distilled water (DW) and the cells were disrupted by vortexing. 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 DW after centrifugation. For hydrolysis of starch, the cell suspension was kept in a water bath at 100°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 DW 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. Calibration was carried out simultaneously using glucose as the standard. The experiments were repeated three times.

2.4. Lipid extraction

Microalgal cells were extracted with *n*-hexane for rapid quantification of accumulated lipid. An aliquot of cell culture medium (1 mL) was withdrawn, and the cells were harvested by centrifugation at $5,000 \times g$ for 10 min. The cell pellet was frozen in liquid nitrogen, and 0.7-mL *n*-hexane was immediately added to the cell pellet. Cells in *n*-hexane were disrupted using an ultrasonic homogenizer (XL-2000, Misonix, Farmingdale, NY, USA), and the cell debris was centrifuged briefly. The solvent phase was transferred to a new tube and evaporated to dryness. The weight of remaining lipid was measured using an analytical balance.

Lipid was extracted from microalgal cells at the logarithmic growth phase for fatty acid analysis. Cells were harvested from 50 mL cell culture medium ($OD_{730} = 0.4\text{--}0.5$) by centrifugation and were mixed vigorously with 10-mL methanol. The suspension was mixed well with 10-mL chloroform and 5-mL DW. Chloroform was added separately to the suspension, which was shaken vigorously using a vortex mixer each time a solvent was added. The lower phase was transferred to a new tube after centrifugation at $2,000 \times g$ for 15 min at 4°C, and was concentrated by evaporation. The residue was resolved in a small volume of chloroform: methanol (2:1, v:v) solution and

stored at -24°C.

2.5. Lipid analysis

Extracted lipids were subjected to thin-layer chromatography. Spots of lipids were transferred to a vial containing 50-μg eicosanoic acid and mixed with 1.5 mL of 5% (w/v) HCl-methanol solution. After vigorously mixing the contents of the vial, it was heated at 95°C for 3 h to produce fatty acid methyl esters (FAMES). After cooling, the FAMES were re-dissolved in 3 mL *n*-hexane and concentrated by evaporation. The extraction and concentration steps were repeated twice.

FAMES were analyzed using a gas chromatograph (GC-14B, Shimadzu, Kyoto, Japan) equipped with a data processor (C-R7A plus, Shimadzu). The amount of each FAME was determined from the area ratio between peaks of each FAME and that of eicosanoic acid.

3. Results and discussion

3.1. Effect of sulfur deficiency on growth of *Chlorellaceae* species

We first examined the effects of a sulfur-deficient medium (TAP-S) and of differences in the light period on cell growth, by quantifying cells and measuring the dry biomass weight during the 3 weeks after transfer of the culture to fresh medium (Fig 1a-d). Growth rates within the first 3 days were not significantly different between cells of *C. vulgaris* and *C. sorokiniana* under continuous light (LL) cultured in TAP and TAP-S media, but cells cultured in TAP-S medium reached the stationary phase 1 day earlier than those in TAP medium (Fig. 1a). This difference likely resulted in a greater number

of *C. vulgaris* and *C. sorokiniana* cells in TAP medium than in TAP-S medium. Under the light/dark cycle (LD), the growth rates of *C. vulgaris* and *C. sorokiniana* in TAP-S medium were half of those in TAP medium until cells reached the stationary phase. Thus there was a difference in cell numbers between the *C. vulgaris* and *C. sorokiniana* culture media. At 3 weeks after transfer to TAP-S medium, the numbers of *C. vulgaris* and *C. sorokiniana* were 4.3–5.6 and 3.1–3.3 times lower than in TAP medium, respectively (Fig. 1a and b).

Sulfur deficiency had little influence on *C. lobophora* cell numbers (Fig. 1c). *P. kessleri* cells entered the stationary phase 7 days after transfer to TAP-S medium, whereas cells in TAP medium continued to propagate slowly but steadily (Fig. 1d). Sulfur deficiency had less effect on cell proliferation in *P. kessleri* than in *C. vulgaris* and *C. sorokiniana* (2.3–2.8 times fewer cells in TAP-S than that in TAP). Differences in light did not affect cell growth in either *C. lobophora* or *P. kessleri* (Fig. 1c, d).

In all species, with the exception of *C. lobophora*, the increase in dry weight comprised fast growth and slow growth phases (Fig. 1e–h). The dry weight of the cultures increased rapidly within the first 7 days after transfer but subsequently slowed. Biomass productivity from days 1–7 varied depending on culture conditions, and ranged from 52.6 to 117.0 ($\text{mg} \cdot \text{L}^{-1} \cdot \text{day}^{-1}$) in *C. vulgaris*, from 87.6 to 127.2 ($\text{mg} \cdot \text{L}^{-1} \cdot \text{day}^{-1}$) in *C. sorokiniana* and from 73.6 to 115.2 ($\text{mg} \cdot \text{L}^{-1} \cdot \text{day}^{-1}$) in *P. kessleri*. After the first 7 days, biomass productivity was lower in the three species, and a decrease in biomass was observed in their sulfur-deficient medium (Fig. 1f and h). Culturing under sulfur-deficient conditions resulted in lower dry weights of all three species (0.5–0.7-fold those under sulfur-sufficient conditions).

Cell numbers of *C. vulgaris* and *C. sorokiniana* were markedly lower in TAP-S

medium than in TAP medium. These results indicate that the cells of each species had a 3.9-fold greater biomass under sulfur-deficient conditions than under sulfur-sufficient conditions. The suppression of cell proliferation under the sulfur-deficient condition apparently caused the increase in cell dry weight. *C. lobophora* showed a linear increase in dry weight, and its biomass yield was little affected by sulfur deficiency (Fig. 1g). No significant difference in *C. lobophora* dry weight was observed under LL and LD conditions or under sulfur-deficient and -sufficient conditions. Strikingly, biomass yield and cell numbers of *C. lobophora* and *P. kessleri* were relatively lower than those of the other Chlorellaceae spp., even under sulfur-sufficient conditions. The original slow growth of *C. lobophora* and *P. kessleri* may have masked the effect of sulfur-deficiency on biomass accumulation and cell number.

3.2. Increased starch production by Chlorellaceae spp. in sulfur-deficient medium

We analyzed changes in starch content from days 1–21 after transfer to TAP-S medium to determine the cause of the increase in starch in Chlorellaceae spp. under sulfur-deficient conditions (Fig. 2). No species maintained maximum starch content, and starch storage decreased after the peak.

The time required for *C. vulgaris* to accumulate the maximum amount of starch varied according to the culture conditions (Fig. 2a). Starch content peaked 11.7% under LL at 3 days after transfer to TAP medium and 10.4% under LD at 5 days after transfer. It peaked at 13.7% under LL at 5 days after transfer to TAP-S and 22.2% under LD at 7 days after transfer. In contrast, *C. lobophora* (Fig. 2c) showed the highest starch content at 1 day after transfer (13.3% and 13.5% under LL and LD in TAP; 14% and 23% under LL and LD in TAP-S).

At 6 days after transfer, *C. sorokiniana* (Fig. 2b) and *P. kessleri* (Fig. 2d) showed the largest differences in accumulated starch after culture in TAP and TAP-S media; however, under all growth conditions, the maximum amount of starch accumulated by 7 days after transfer (Fig. 2b and d). *C. sorokiniana* showed 9.0% and 8.9% under LL and LD, respectively in TAP; 21.3% and 24.0% under LL and LD in TAP-S, whereas *P. kessleri* showed 13.8% and 20.9% under LL and LD in TAP; 32.2% and 50.5% under LL and LD in TAP-S.

3.3. Decrease in starch granule number followed by lipid drop accumulation

Starch contents decreased and never recovered after peaking in all four Chlorellaceae species. We examined the ultrastructure of these species by TEM to understand the fate of accumulated cellular starch. Since both the timing and decrease of starch accumulation differed in the four Chlorellaceae species, we used *C. sorokiniana* as a representative organism. Several starch granules were observed in cells cultured under LD in TAP for 1 week as a control (Fig. S1a and d). These sections of starch sheath surrounded the pyrenoid. Culturing *C. sorokiniana* under LL in TAP-S for 1 week resulted in cells with giant chloroplasts containing several starch granules (Fig. S1b and e). Cells with lipid droplets emerged after further culture in TAP-S (Fig. S1c). The lipid droplets coexisted with starch granules in the same cell (Fig. S1f). The chloroplasts became smaller as the number of starch granules decreased. This caused expansion of the cytoplasm, in which lipid droplets and autophagosome-like structures were present (Fig. S1g and h). These electron-transparent structures became smaller with increasing numbers and sizes of lipid drops. Lipid drops were not observed in chloroplasts, even if very small. Culture for 3 weeks in TAP-S resulted in enlarged lipid droplets under

sulfur-deficient conditions (Fig. S1i). Smaller lipid droplets were also observed beneath the cell membrane (Fig. S1f and g). These data suggest that lipids accumulate subsequent to a reduction in stored starch.

3.4. Accumulation of neutral lipids and changes in *Chlorellaceae* spp fatty acid composition

The changes in lipid content in *Chlorellaceae* spp. cultured under four different conditions were analyzed. The lipid contents of all four species gradually increased with culture in TAP-S and TAP media (Fig. 3). Lipid contents at 3 weeks after transfer ranged from 18.1% (*C. vulgaris* cultured under LL in TAP) to 49.7% (*C. lobophora* cultured under LL in TAP). *C. lobophora* exhibited a 1.78-times higher lipid content when cultured under LL in TAP than when cultured under LD in the same medium. However, differences in the light cycle did not affect the lipid content of the other three *Chlorellaceae* spp. These data suggest that *C. lobophora* is highly light sensitive, in response to which it accumulates lipids. Sulfur deficiency increased lipid content in all species tested. At 3 weeks after transfer, the lipid contents of *C. vulgaris*, *C. sorokiniana*, and *P. kessleri* were 1.8- to 2.4-fold higher when cultured in TAP-S medium than when cultured in TAP medium. Because lipid accumulation in *C. lobophora* was induced by light stress, the effect of sulfur deficiency on the lipid content was modest (1.2- and 1.5-fold increases under LL and LD in TAP-S, respectively).

Culture of *Chlorellaceae* spp. under different conditions results in altered fatty acid compositions (Patterson, 1970; Tsuzuki *et al.*, 1990; Yeh and Chang, 2011). To clarify the influence of sulfur deficiency on accumulated acyl lipids in the four *Chlorellaceae* spp., fatty acids were extracted from the cultures at 3 weeks after transfer, and fatty acid

composition was analyzed by GC (Fig. 4). Palmitic (16:0), palmitoleic (16:1), hexadecadienoic (16:2), hexadecatrienoic (16:3), stearic (18:0), oleic (18:1 (9), n-9), vaccenic (18:1 (11), n-7), linoleic (18:2), and α -linolenic (18:3) acids were identified in all four species. Saturated fatty acids comprised no more than 24.3% of the fatty acids accumulated in *Chlorella* and *Parachlorella* cells cultured in TAP, and >50% of the accumulated fatty acids were linoleic and α -linolenic acids. Transfer of cells to TAP-S resulted in a slightly altered fatty acid composition; however, the ratio of saturated to polyunsaturated fatty acids was almost unaffected in *C. vulgaris* and *C. sorokiniana*. Transfer of *C. lobophora* to TAP-S decreased the combined contents of linoleic and α -linolenic acids, from 54.2% to 44.8% under LL and from 59.1% to 48.4% under LD, respectively, but increased the combined contents of palmitic and stearic acids only under the LL condition (from 21.0% to 29.5%). Transfer of *P. kessleri* to TAP-S decreased the combined contents of linoleic and α -linolenic acids from 67.6% to 18.2% under LL and from 63.8% to 41.5% under LD, respectively, and increased the combined contents of palmitic and stearic acids from 24.3% to 59.7% under LL and from 24.2% to 43.2% under LD, respectively. These results indicate that sulfur deficiency alters the fatty acid compositions of *C. lobophora* and *P. kessleri*.

The glycolytic pathway plays a fundamental role in lipid biosynthesis and seed-oil accumulation in *Arabidopsis thaliana* (Focks and Benning, 1998). In *Chlamydomonas reinhardtii*, transcription of genes encoding starch synthase and starch phosphorylase, which function in starch synthesis and degradation respectively, are induced by sulfur depletion (Zhang *et al.*, 2004). These studies, together with our TEM observations of *Chlorella* cells (Fig. S1), suggest that accumulated starch is converted to lipid. The starch content of the Chlorellaceae spp. decreased at 2 weeks after transfer and never

recovered. If fatty acid synthesis was dependent only on acetyl-CoA, a fatty acid precursor, which is produced by glycolysis of accumulated starch, lipid content would not have increased beyond 2 weeks after transfer. Acetyl-CoA can also be supplied from the Benson–Calvin cycle through pyruvate decarboxylation by the plastid pyruvate dehydrogenase complex (Joyard *et al.*, 2010). This observation suggests that the carbon flow from starch supplies only a portion of the precursors required for lipid synthesis.

In this study, we carried out cultivation under weak light conditions ($70 \mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) and a low CO_2 concentration without aeration for 3 weeks to facilitate measurement of the effect of sequential accumulation. It is known that improving starch and lipid productivity requires high-intensity light and a high CO_2 concentration with aeration. We shortened the cultivation period by using high-intensity light and a high CO_2 concentration for about 5 days. Wileman *et al.* (2012) reported that green algae slurries display a shear thinning non-Newtonian behavior with a varying degree of sensitivity to shear rate at biomass concentrations $>60 \text{ kg/m}^3$. We plan to evaluate the rheological properties of microalgae slurries as a function of biomass concentration from 0.5 to 80 kg/m^3 during sequential accumulation of energy-rich materials such as starch and lipid induced by sulfur deficiency in *Chlorella* and *Parachlorella* species to achieve energy efficient harvesting and processing of the algal biomass.

4. Conclusion

Accumulation and loss of starch preceding lipid accumulation were induced by sulfur depletion in the Chlorellaceae spp. used in this study. TEM confirmed the increase and reduction in starch granules and subsequent enlargement of lipid droplets under sulfur-deficient conditions. The starch and lipid contents were higher in cells cultured

under sulfur-deficient conditions than in cells cultured under sulfur-sufficient conditions. Sulfur depletion altered the fatty acid composition of only *C. lobophora* and *P. kessleri*. Our results indicate that sequential accumulation of starch and lipid occurs in *Chlorella* and *Parachlorella* species under sulfur-deficient conditions.

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References

- 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.
- Cha, T.S., Chen, J.W., Goh, E.G., Aziz, A., Loh, S.H., 2011. Differential regulation of fatty acid biosynthesis in two *Chlorella* species in response to nitrate treatments and the potential of binary blending microalgae oils for biodiesel application. *Bioresour. Technol.* 102, 10633-10640.
- Chisti, Y., 2007. Biodiesel from microalgae. *Biotechnol. Adv.* 25, 294-306.
- Davidi, L., Katz, A., Pick, U., 2012. Characterization of major lipid droplet proteins from *Dunaliella*. *Planta* DOI 10.1007/s00425-011-1585-7.

Dragone, G., Fernandes, B.D., Abreu, A.P., Vicente, A.A., Teixeira, J.A., 2011. Nutrient limitation as a strategy for increasing starch accumulation in microalgae. *Appl. Energy* 88, 3331-3335.

Focks, N., and Benning, C., 1998. *wrinkled1*: a novel, low-seed-oil mutant of *Arabidopsis* with a deficiency in the seed-specific regulation of carbohydrate metabolism. *Plant Physiol.* 118, 91-101.

Heredia-Arroyo, T., Wei, W., Hu, B., 2010. Oil accumulation via heterotrophic/mixotrophic *Chlorella protothecoides*. *Appl. Biochem. Biotechnol.* 162, 1978-1995.

Hsieh, C-H., Wu, W-T., 2009. Cultivation of microalgae for oil production with a cultivation strategy of urea limitation. *Bioresour. Technol.* 100, 3921-3926.

Izumo, A., Fujiwara, S., Oyama, Y., Satoh, A., Fujita, N., Nakamura, Y., Tsuzuki, M., 2007. Physicochemical properties of starch in *Chlorella* change depending on the CO₂ concentration during growth: comparison of structure and properties of pyrenoid and stroma starch. *Plant Sci.* 172, 1138-1147.

Joyard, J., Ferro, M., Masselon, C., Seigneurin-Berny, D., Salvi, D., Garin, J. Rolland, N., 2010. Chloroplast proteomics highlights the subcellular compartmentalization of lipid metabolism. *Prog. Lipid Res.* 49, 128-158.

Liang, Y., Sarkany, N., Cui, Y., 2009. Biomass and lipid productivities of *Chlorella vulgaris* under autotrophic, heterotrophic and mixotrophic growth conditions. Biotechnol. Lett. 31, 1043-1049.

Liu, Z-Y., Wang, G-C., Zhou, B-C., 2008. Effect of iron on growth and lipid accumulation in *Chlorella vulgaris*. Bioresour. Technol. 99, 4717-4722.

Miao, X., Wu, Q., 2004. High-yield bio-oil production from fast pyrolysis by metabolic controlling of *Chlorella protothecoides*. J. Biotechnol. 110, 85-93.

Patterson, G.W., 1970. Effect of culture temperature on fatty acid composition of *Chlorella sorokiniana*. Lipids 5, 597-600.

Rodolfi, L., Zittelli, G.C., 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.

Shen, Y., Yuan, W., Pei, Z., Mao, E., 2010. Heterotrophic culture of *Chlorella protothecoides* in various nitrogen sources for lipid production. Appl. Biochem. Biotechnol. 160, 1674-1684.

Takagi, M., Karseno, Yoshida, T., 2006. Effect of salt concentration on intracellular accumulation of lipids and triacylglyceride in marine microalgae *Dunaliella* cells. J. Biosci. Bioeng. 101, 223-226.

Tsuzuki, M., Ohnuma, E., Sato, N., Takaku, T., Kawaguchi, A., 1990. Effects of CO₂ concentration during growth on fatty acid composition in microalgae. *Plant Physiol.* 93, 851-856.

Yeh, K-L., Chang, J-S., 2011. Nitrogen starvation strategies and photobioreactor design for enhancing lipid production of a newly isolated microalga *Chlorella vulgaris* ESP-31: implications for biofuels. *Biotechnol. J.* 6, 1358-1366.

Wang, S-T., Pan, Y-Y., Liu, C-C., Chuang, L-T., Chen, M-J.N., 2011. Characterization of a green microalga UTEX 2219-4: effects of photosynthesis and osmotic stress on oil body formation. *Bot. Stud.* 52, 305-312.

Wileman, A., Ozkan, A., Berberoglu, H., 2012. Rheological properties of algae slurries for minimizing harvesting energy requirements in biofuel production. *Bioresource Technology* 104, 432-439.

Zhang, Z., Sharagaer, J., Jain, M., Chang, C-W., Vallon, O., Grossman, A.R., 2004. Insights into the survival of *Chlamydomonas reinhardtii* during sulfur starvation based on microarray analysis of gene expression. *Eukaryot. Cell* 3, 1331-1348.

Figure legends

Fig. 1. Effect of sulfur starvation on cell growth under different light conditions.

Chlorellaceae spp. were cultured in TAP medium under continuous light (LL) (diamonds) or a 12-h: 12-h light: dark cycle (LD) (squares), or in sulfur-deficient TAP (TAP-S) medium under LL (triangles) or LD (circles). Aliquots of the cultures were taken at the indicated time points to count cell numbers (a-d) and determine dry weights (e-h). (a, e) *Chlorella vulgaris*, (b, f) *C. sorokiniana*, (c, g) *C. lobophora*, and (d, h) *Parachlorella kessleri*. Vertical bars represent standard deviations.

Fig. 2. Starch production in Chlorellaceae spp. under various culture conditions.

Chlorellaceae spp. were cultured in TAP medium under continuous light (LL) or a 12-h: 12-h light: dark cycle (LD), or in sulfur-deficient TAP medium under continuous light (SLL) or a 12-h:12-h light: dark cycle (SLD) and starch production levels were determined. The ratios of starch content to cell dry weight (%) were plotted.

Fig. 3. Lipid production in Chlorellaceae spp. under various culture conditions.

Chlorellaceae spp. were cultured in TAP medium under continuous light (LL) or a 12-h: 12-h light: dark cycle (LD); or in sulfur-deficient TAP medium under continuous light (SLL) or a 12-h: 12-h light: dark cycle (SLD), and lipid accumulation at the indicated time points was analyzed. Lipid content per cell dry weight at the indicated time points.

Fig. 4. Influence of sulfur starvation on fatty acid synthesis.

Chlorellaceae spp. were cultured in TAP medium under continuous light (LL) or a 12-h:12-h light/dark cycle (LD); or in TAP-S medium under continuous light (SLL) or a

12-h:12-h light/dark cycle (SLD). 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 and the positions of the double bonds in each fatty acid are indicated in the legend (number of carbon atoms: number of double bonds-positions of the double bonds).

Fig1

Fig2

Fig3

Fig4