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

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HIGHLIGHTS

- Starch and lipid accumulation occurred in 4 Chlorellaceae spp. under sulfur deficient conditions.
- Lipids accumulated subsequent to a reduction in stored starch.
- A change in illumination cycle is sufficient for *Chlorella lobophora* to induce lipid accumulation.
- Fatty acid compositions were altered in *Chlorella lobophora* and *Parachlorella kessleri* by sulfur depletion.

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ABSTRACT

The influence of sulfur deficiency on biomass production was analyzed in the four Chlorellaceae species, *Chlorella vulgaris*, *Chlorella sorokiniana*, *Chlorella 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.

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; Hsieh and Wu, 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 CO_2 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

Abbreviations: TAP, tris-acetate phosphate; DMSO, dimethyl sulfoxide; FAMEs, fatty acid methyl esters; FA, fatty acid; TEM, transmission electron microscopy.

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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.: *Chlorella vulgaris*, *Chlorella sorokiniana*, *Chlorella 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 211–11 h) 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 211–11 h). 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 μmol photons·m^{-2} 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 5000*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 prewarmed 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% H_2SO_4). 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 5000*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} = 0.4–0.5) 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 2000*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 processer (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