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

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HIGHLIGHTS

- High-light-intensity is effective in improving the productivity of *Chlorella*.
- *Chlorella* shows more stable growth in the 12:12-h light–dark cycle conditions.
- The starch and lipids yields more increase in the continuous light conditions.
- Long-chain fatty acids accumulate in *Chlorella* grown in sulfur-deficient medium.
- Accumulation of starch and lipids vary in eight strains of six *Chlorella* species.

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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-μmol photons m^{-2} s^{-1}). The 12:12-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 *Chlorella viscosa* and *Chlorella 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.

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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 (g L^{-1} 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., 2012). 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-μmol photons m^{-2} s^{-1}, but growth potential is not inhibited by a light intensity of 400-μmol photons m^{-2} s^{-1} in *Chlorella sorokiniana* (Ugwu et al., 2007). Whereas cultivation at a high light intensity is not suitable for the growth of *Euglena gracillis*

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(Ogbonna and 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-μmol photons m^{-2} s^{-1}) conditions. Such high light intensity is equivalent to sunlight, which ranges from ~700-μmol photons m^{-2} s^{-1} to a maximum of ~2000-μmol photons m^{-2} s^{-1} in direct sunlight (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 (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-μmol photons m^{-2} 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. 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), *Parachlorella 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 12:12-h LD cycle at 50-μmol photons m^{-2} 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-μmol photons m^{-2} 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% CO_2 concentration, and 2-rpm using rotary culture equipment. A detailed description of the cultivation device is given in Supplementary Fig. 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 μL/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 5000×*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 Na_2HPO_4, 2 mM NaH_2PO_4, 140 mM NaCl, pH 7.4) and the cells were disrupted using an ultrasonic homogenizer for 60 s, 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 H_2O 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 H_2O 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 (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 5000×*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-μm PTFE membrane. Residual solvent in the sample filtrate was eliminated by evaporation under N_2 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 H_2O and 1 mL of n-hexane were added, and the contents were mixed well by hand.

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