博　士　論　文

Studies on starch and lipids accumulation by laboratory and large scale outdoor cultures in the *Chlorella* species

（クロレラのデンプンとオイル蓄積に関する
実験室と屋外大量培養による研究）

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Studies on starch and lipids accumulation by laboratory and large scale outdoor cultures in the *Chlorella* species

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CONTENTS

PREFACE

Microalgae are unicellular photosynthetic organisms that can fix carbon dioxide and synthesize carbohydrates as well as terrestrial plants. Currently, microalgae are attractive next-generation sources of biofuel and high-value fatty acids because there is no competition for crops and they exhibit high lipid productivity. The microalgae genus *Chlorella* belongs to the green algae class Chlorophyceae. Some *Chlorella* species belong to the class that is still confused in taxonomy. Moreover, little is known about *Chlorella* biomass and lipid productivity in high light (stress) conditions. To address this, biomass and lipid productivities of six species and eight strains of *Chlorella* were determined in this study. PK4, a mutant strain of *Parachlorella kessleri*, was also examined for its usefulness as a raw material for biofuel production in an outdoor cultivation system.

In the early phase of *Chlorella* culture, starch accumulation occurred faster than lipid accumulation. During the middle phase of culture, lipid levels increased as starch levels decreased and, by the end of culture, lipid levels were at a maximum (Fig. P-1). It has been reported that there is a trade-off between starch and lipid accumulation in *Chlorella* (Mizuno at al. 2013; Li et al. 2015).

Chapter I contains high-throughput and compendium methods for starch and lipid quantification. Traditional methods for starch and lipid quantification are time-consuming. For this study, two simple methods for starch and lipid quantification were used. Lugol's iodine and Nile Red were used to stain starch and lipids, respectively. Starch and lipid levels were then quantified by absorbance and fluorescence measurements, respectively. Assays were performed in a 96-well plate, which allowed for many samples to be measured simultaneously.

In Chapter II, six species and eight strains of *Chlorella* were examined to understand starch and lipid accumulation under comparatively high light intensity and aeration culture conditions. It is well known that microalgae synthesize storage substances such as lipids when nitrogen sources are removed from the culture medium. However, amino acids that are synthesized in the presence of nitrogen are necessary for protein synthesis; therefore, nitrogen deficiency influences biomass production. In this regard, amino acid and protein synthesis are directly related. In contrast to nitrogen deficiency, sulfur deficiency's influence on biomass productivity is relatively low, because only two sulfur-containing amino acids, methionine and cysteine, are required for protein synthesis. High biomass productivity must be accompanied by high lipid accumulation for biofuel production; however, this cannot be achieved using only nutrient limitation to induce lipid accumulation. Two methods have been suggested to improve biomass productivity: (1) the addition of saccharide to the culture medium under heterotrophy conditions, and (2) lipid accumulation induction by culture under conditions of high light intensity. In this study, the second method was investigated. Specifically, an experimental system for high-light *Chlorella* cultivation was developed to improve biomass productivity.

In Chapter III, lipid production by the dilution culture method in an outdoor cultivation system (150-L) was optimized using PK4, a mutant strain of *P. kessleri*. PK4 genetic variations were also investigated. Specifically, the use of SS medium as an economically viable nutrient medium for outdoor cultivation was examined. SS medium contains urea as the nitrogen source (Zachleder and Šetlík 1982), and there are few organisms that can use urea; therefore, culture with this medium minimizes contamination, which can be compose problematic in outdoor culture systems. It has been reported that the dilution culture method,

which involves dilution of the whole medium with SS medium, can induce lipid accumulation (Přibyl et al. 2012; Li et al. 2013). In the present study, PK4, a mutant strain resulting from heavy-ion-beam irradiation of wild-type *P. kessleri* (Ota et al. 2013), was used for outdoor cultivation. Heavy-ion-beam irradiation has been used to breed agricultural land plants, and was recently used to breed microalgae such as *Chlamydomonas reinhardtii, Desmodesmus* sp. and *Nannochloropsis* (Li et al. 2010; Hu et al. 2013; Ma et al. 2013). Whole-genome sequencing was used to identify point mutations in PK4 that were induced by heavy-ion-beam irradiation (Ota et al. 2016). Sequencing results showed mutations at two genes were responsible for the phenotypic differences between PK4 and wild-type.

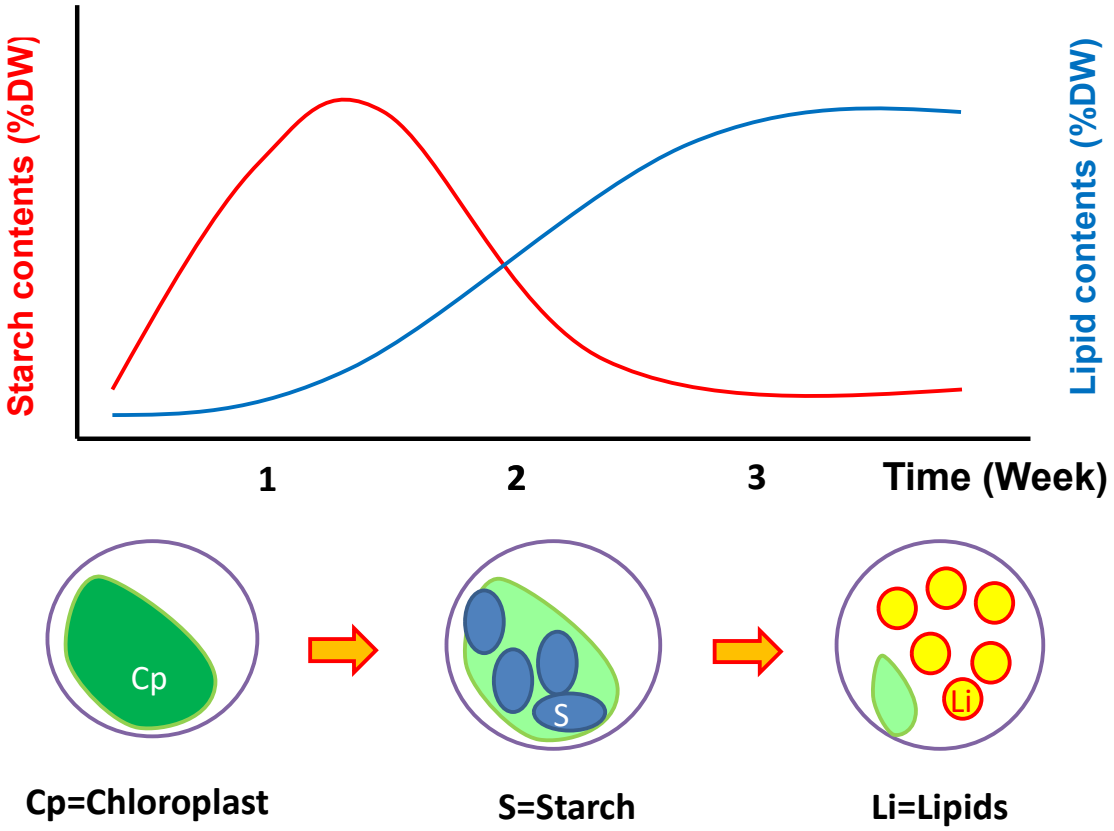

Fig. P-1 Graph showing the trade-off between starch and lipids accumulation. Starch accumulates during the early phase of culture. In the middle phase, starch levels decrease and lipid levels increase. During this period, chloroplasts shrink and oil bodies are formed in cells.

CHAPTER I

A simple method for measuring the starch and lipid contents in the cell of microalgae

SUMMARY

I used a microplate-based method to quantify microalgal lipids with Nile Red staining and a fluorescence microplate reader. However, a method to quantify starch that combines microplates with staining has not been reported. Therefore, I examined microplate-based quantification of lipids using Nile Red staining and of starch using Lugol staining. Neither starch nor lipids accumulated during the zero phase of cultured *Parachlorella kessleri*, only starch accumulated during the starch phase, and starch was subsequently lost and lipids accumulated during the oil phase. The quantities of starch and lipids were measured using a microplate-based method, which indicated linear production of starch and lipids within limited ranges (lipids, 0.071–0.380 g mL^{-1}; starch, 155–404 mg mL^{-1}) when standard curves were prepared for lipids extracted with methyl tertiary-butyl ether and for starch extracted with anthrone. The concentration of starch produced during the starch phase was 0.77–1.32 mg $mL^{-1}$and that of lipids during the oil phase was 1.96–2.20 mg mL^{-1}. The concentration of starch produced was 0.07–0.42 mg mL^{-1} during phases other than the starch phase, and lipids were not detected other than during the oil phase because lipid contents were approximated based on the quantity of triacylglycerol, which stains with Nile Red.

INTRODUCTION

Starch and lipids have different structures and accumulate in different parts of cells, but both function to store carbon. Starch and lipid accumulation are associated in *Chlorella* (Mizuno et al. 2013; Takeshita et al. 2014; Li et al. 2015). Starch is the primary product of photosynthesis and is metabolized to fatty acids through acetyl-CoA. Triacylglycerol (TAG) is a neutral lipid synthesized from fatty acids (Andre et al. 2012) and is the principal lipid component in *Chlorella*. TAG contains fatty acids with carbon numbers from 14 to 20 (Zhang et al. 2014). These fatty acids have attracted attention as biodiesel feedstock (Chisti 2007; Liu et al. 2010). The colorimetric anthrone method is commonly used to quantify starch(McCready et al. 1950), but it requires a particular sample quantity for analysis. A glucose colorimetric method has been developed to measure starch in very small samples by decomposing the starch to glucose using enzymes. The Lugol staining method and the starch-iodine reaction make the observation of intracellular starch by microscopy possible. Starch forms a helical glucose structure and the iodine becomes trapped in the glucose helix, producing a purple-black color. No method to quantify starch using this color reaction has been reported. *n*-Hexane, chloroform, and methanol extraction methods are used to quantify lipids (von Soxhlet 1879, Bligh and Dyer 1959), and the quantities of the extracted lipids are measured as residuals after the solvent is volatilized. In contrast, Nile Red, which accumulates in cellular lipids, can be used to observe lipid accumulation by fluorescence microscopy (Greenspan and Fowler 1985). A method to quantify lipids using Nile Red and a fluorescence plate reader has also been commonly used in recent years to quantify lipids (Chen et al. 2009, Rumin et al. 2015).

Chlorella (*Parachlorella kessleri*) cells were observed under a microscope after Lugol and Nile Red fluorescent staining, which allowed us to observe the trade-off between starch and lipid accumulation. Almost no starch or lipids were observed in cells just after inoculation, but starch and lipids accumulated alternately as the culture aged. Here, I quantified starch and lipids using a simple microplate method and small samples.

MATERIALS AND METHODS

Strain and culture conditions

The *Parachlorella kessleri* NIES-2152 strain was obtained from the Microbial Culture Collection, National Institute for Environmental Studies, Tsukuba, Japan (http://mcc.nies.go.jp/). Cells were precultured using a method described previously (Ota et al. 2013). The cultures were transferred to Tris–acetate–phosphate (TAP) medium under continuous light at 300–μmol photons m^{-2} s^{-1} and 23°C. The other culture conditions were the same as previously reported (Takeshita et al. 2014).

Microscopic observations

Intracellular starch granules were stained with Lugol's solution by staining 1–mL of cell suspension with 10–μL of Lugol's solution (5 g I_2 and 10 g KI dissolved in 100–mL distilled water), andsamples were observed after a 5 min incubation at 90°C. Intracellular oil droplets were stained with Nile Red (9–diethylamino–5H–benzo[a] phenoxazine–5–one; Polyscience, Inc., Warrington, PA, U.S.A.;Wayama et al. 2013).

Gravimetric determination of total lipids

Total lipids were extracted using a MTBE (methyl tertiary–butyl ether)–methanol–water system according to Matyash et al. (2008) with some modifications as described previously (Takeshita et al. 2014). The extract was evaporated under a fume hood to remove the solvents. The dried residuals were placed in a pre–weighed aluminum Petri dish and weighed.

Quantification of starch by the extraction method

Starch hydrolysis procedures and spectrophotometric measurements were as described by Brányiková et al. (2011).

Microplate–based quantitative analyses of starch and lipids

Starch and lipid quantitative results using a conventional method and a microplate–based quantitative method were plotted to prepare standard curves. Day 6 post–inoculation culture medium was used to quantify starch and day 9 post–inoculation medium was used to quantify lipids. The culture medium was serially diluted up to 1 : 25 by adding distilled water. The coefficient of variation between quantification methods and the effective standard curve range were calculated. The microplate–based method was modified from a previous report (Ota et al. 2013) A 200–μL aliquot of culture medium was transferred to each well of a 96–well microplate (1860–096; Iwaki, Holliston, MA, U.S.A.) to measure starch content. A 5–μL aliquot of Lugol's solution was added to each well, and the suspension was mixed. After a 10–min incubation at 50°C, absorbance was read at 660 nm using a microplate reader (Viento nano; DS Pharma Biomedical, Osaka, Japan). The $OD_{660\ nm}$ value of an unstained control was used for normalization, and the difference between the $OD_{660\ nm}$ value of stained distilled water and unstained DW was the blank control. Normalization was performed as follows: $(OD_{Lugol\ stained\ sample}–OD_{unstained\ sample})–(OD_{Lugol\ DW}–OD_{DW})$. A 100–$\mu$L aliquot of culture medium was transferred to each well of a black 96–well microplate (Nunc 165305; Thermo Scientific, Rockford, IL, U.S.A.) to quantify lipids. A 100–μL aliquot of Nile Red solution or 100–μL of dimethyl sulfoxide was added to each well, and the suspensions were mixed. After a 10–min incubation at 37°C, Nile Red fluorescence intensity was determined at 570 nm excitation and 530 nm emission using an ARVO SX microplate reader (Perkin–Elmer Japan,

Kanagawa, Japan). A 100–μL aliquot of distilled water was used as the blank control. Normalization was performed as follows: (fluorescence intensity $_{\text{Nile Red stained sample}}$–fluorescence intensity $_{\text{unstained sample with DMSO)}}$–(fluorescence intensity $_{\text{DW with Nile Red}}$–fluorescence intensity $_{\text{DW}}$).

RESULTS

Microscopic observations at various **P. kessleri** *culture ages*

P. kessleri was inoculated into TAP medium and precultured for four days. Exponential (day 6) and stationary phase (day 18) cells were observed by a microscope after inoculation and Lugol and Nile Red staining. Well–developed cup–shaped chloroplasts were dominant on day 0 post–inoculation as indicated by strong chlorophyll auto–fluorescence (Fig. 1–1A, B). No starch or lipids had accumulated in the cells as indicated by Nile Red staining and bright field observations of Lugol stained cells (Fig. 1–1C, D). Therefore, this period was called the zero phase.

Large mother cells divided into four endogenous spores, and small daughter cells separated from the mother cell wall on day 6 post–inoculation. Cup–shaped chloroplasts developed in both cell types and strong chlorophyll auto–fluorescence was observed (Fig. 1–1E, F). No lipids accumulated during this period based on Nile Red staining observations; however, mother cells with endogenous spores and small daughter cells accumulated starch granules as detected by Lugol staining (Fig. 1–1H). Therefore, this period was called the starch phase.

The large mother cells did not proliferate, but relatively small 1–3–μm diameter cells were observed on day 18 post–inoculation (Fig. 1–1I). Chloroplasts degenerated in many cells, and chlorophyll auto–fluorescence had diminished (Fig. 1–1J). Oil droplets were observed in cells with degenerated chloroplasts. Strong signals were observed in Nile Red–stained oil droplets, indicating that lipids were accumulating (Fig. 1–1K). In contrast, almost no cells were Lugol stained and almost no starch accumulated (Fig. 1–1L). Therefore, this period was called the oil phase.

Starch and lipid standard curves generated using the microplate method

I used small samples and a simple microplate method to quantify starch and lipids during the starch and oil phases, respectively. The starch standard curve was prepared using day 4 post–inoculation culture medium diluted 1 : 25. The diluted medium was dispensed to a 96–well microplate and Lugol's solution was added to each well. After the microplate was incubated for 10 min at 50°C, absorbance was measured with a spectrophotometric plate reader.

The absorbance values of the Lugol's solution–treated wells were measured for every 10 nm of wavelength, and the measurement wavelength was set to λ=660 nm. Because Lugol's solution is brown, sample absorbance was measured before and after Lugol staining to exclude the effect of color, and the difference was the starch signal. The results were plotted with the absorbance of Lugol's–stained cells on the vertical axis, and the results of the conventional anthrone method on the horizontal axis (Fig. 1–2A), and a high correlation (R^2= 0.972) was detected. The range indicating the highest correlation and linearity was determined using Sigma Plot 8.0 software (Systat Software Inc., San Jose, CA, U.S.A.). Linearity was described by Eq. (1) with a small range of $155 \leq x \leq 404$.

$$f = 0.00257x - 0.139 \ (R^2 = 0.998, \ 155 \leq x \leq 404) \quad (1)$$

The lipid standard was prepared from day 18 post–inoculation culture medium. The diluted culture medium was dispensed into a 96–well microplate and the results were plotted as Nile Red stained fluorescence of cells on the vertical axis and total extracted lipids on the horizontal axis (Fig. 1–2B). The results approximated a sigmoid curve with a high linear correlation (R^2= 0.961) described by Eq. (2) and a range of $0.071 \leq x \leq 0.380$.

$$f = 1.23 \times 105\, x + 26.8\ (R2 = 0.999,\ 0.071 \leq x \leq 0.380) \quad (2)$$

Microplate–based quantitative analyses of starch and lipids during the starch and oil phases

My method shows that the quantities of starch and lipid could be determined within very limited ranges, so we determined these quantities during the starch and oil phases with attention to the dilution. The quantities of starch on culture days 0, 3, 6, 9, 12, 15 and 18 post–inoculation were measured by microplate–based quantitative analyses and the anthrone method (Table. 1-1). Starch was detected on all days using the microplate–based method as same as the extraction method. The largest quantity of starch was detected during the starch phase on day 9 post–inoculation (1.32 mg mL^{-1}). No differences were detected between the values using the two methods. However, lipids were detected by the microplate–based method only on day12, 15 and 18 post–inoculation, but were detected on all days in the extraction method. The largest quantity of lipids was detected during the oil phase on day 18 post–inoculation (2.20 mg mL^{-1}).

DISCUSSION

Quantitative starch analysis using the microplate–based method within a very limited measurement range

Previously, starch quantities have been calculated using extraction methods. A starch quantitative analytical method using a microplate was reported for plant cells (Smith and Zeeman 2006), but the extracted starch was decomposed to glucose by enzymes and measured by colorimetric reaction with the Smith and Zeeman method. In this study, I used intracellular starch stained with Lugol's solution and developed a simple and easy method of quantification by measuring absorbance. A high correlation was observed using the microplate–based method within a limited range of 155–404 μg mL^{-1}, which was much smaller than that of the anthrone method (10–1000 μg mL^{-1}; McCready et al. 1950; Takeshita et al. 2014) and allowed detection of only a 2.5–fold change. I regard this to be the reason that no examples of this method have been reported until now. However, microalgae are cultured in liquid medium, so diluting and concentrating the culture medium is easy, allowing measurements in the linear range to be made.

Lipids quantified by Nile Red staining and the microplate–based method

The microplate–based quantitative analysis of lipids using Nile Red staining is a simple and easy method for various microalgal species (Chen et al. 2009; Balduyck et al. 2015; Rumin et al. 2015). The effective range for quantification of lipids by the microplate–based method was 0.071–0.380 mg mL^{-1} which allowed for measurement of a wider range than for starch. Lipids were detected by extracting with the MTBE–methanol–water system, but lipids were not detected on days 0 and 6 post–inoculation (Table 1–1). Because Nile Red only stains

neutral lipids and parts of polar lipids (Chen et al. 2009; Balduyck et al. 2015), neutral lipids and parts of the polar lipids appear to not be included in the lipids of these phases. The lipids were not detected by fluorescence microscopic observations using Nile Red on days 0 and 6 post–inoculation (Fig. 1–1C, G). Because the MTBE–methanol–water system extracts total lipids, these may be membrane lipids, which were detected by extraction on days 0 and 6 post–inoculation.

Chlorella and *Nannochloropsis* accumulate neutral lipids (65–80%), phospholipids (10–15%), and oligo lipids (5–10%) during the oil phase (Liu et al. 2010; Xiao et al. 2015). The total quantity of TAG, which is a neutral lipid, was 1.61 mg mL^{-1}, and approximately 70% of total lipids was TAG on day 18 post–inoculation (data not shown). The microplate–based method with Nile Red staining provided the best method to quantify total lipids as a fixed percentage of TAG in the culture medium used to prepare the standard curve.

This assay has limitations, such as the small measuring range and that the estimate of total lipids is based on a fixed percentage of TAG, but the method is simple and easy and small samples can be used to determine the quantities of starch and lipids. This method is suited for repeated sampling experiments when elapsed time is important during different phases. The microplate based quantitative method will be useful for studies on the production of substances with microalgae.

TABLES AND FIGURES

Table 1–1. Starch and lipid concentrations measured by different methods. The quantities of starch and lipids at day0, 3, 6, 9, 12, 15 and 18 were determined using microplate–based methods (*n*=4).

Time (Phase)	Starch concentrations [mg/mL]			Lipids concentrations [mg/mL]		
	Anthrone method	Microplate method	P value	MTBE-MeOH-Water method	Microplate method	P value
Day 0 (Zero Phase)	0.05 ± 0.001	0.07 ± 0.010	**	0.02 ± 0.01	—	—
Day 3	0.17 ± 0.004	0.19 ± 0.019	*	0.22 ± 0.04	—	—
Day 6 (Starch Phase)	0.91 ± 0.028	0.77 ± 0.300	*	0.49 ± 0.13	—	—
Day 9	1.37 ± 0.024	1.32 ± 0.081	*	1.10 ± 0.15	—	—
Day 12	0.83 ± 0.012	0.91 ± 0.359	*	1.70 ± 0.13	1.04 ± 0.04	—
Day 15	0.41 ± 0.007	0.42 ± 0.094	*	2.16 ± 0.20	1.96 ± 0.06	**
Day 18 (Oil Phase)	0.39 ± 0.001	0.42 ± 0.023	*	2.33 ± 0.23	2.20 ± 0.03	**

Values are mean ± standard deviations. (n=4)
* P<0.05, **P<0.01

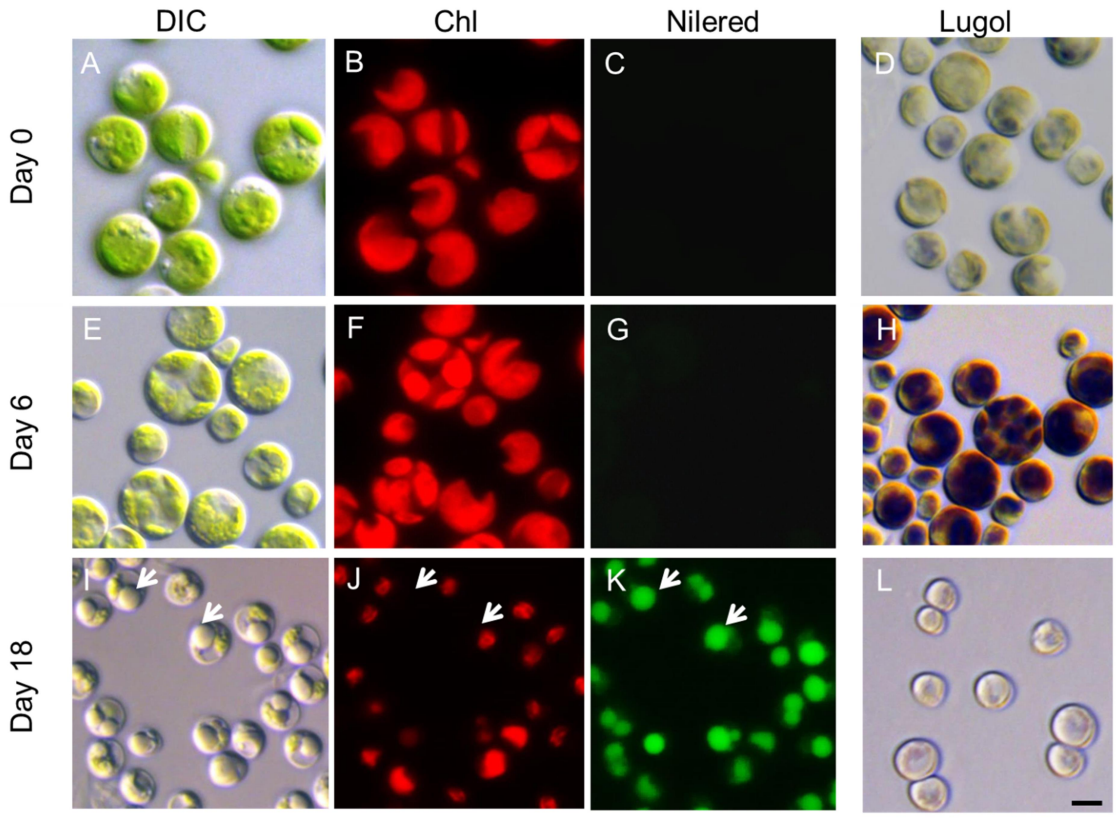

Fig. 1–1. A–D. Day 0 post–inoculation, E–H. Day 6 post–inoculation, and I–L. Day 18 post–inoculation cells shown in Nomarski (DIC), auto–fluorescence (Chl), Nile Red–stained (Nile Red), and Lugol stained (Lugol) images. The DIC, chlorophyll (Chl), and Nile Red images are of the same field of view, and the Lugol stain image is of a different field of view. Days 0, 6, and 18 post–inoculation were the zero, starch, and oil phases, respectively. Arrows indicate oil drops. Scale bar =2 μm.

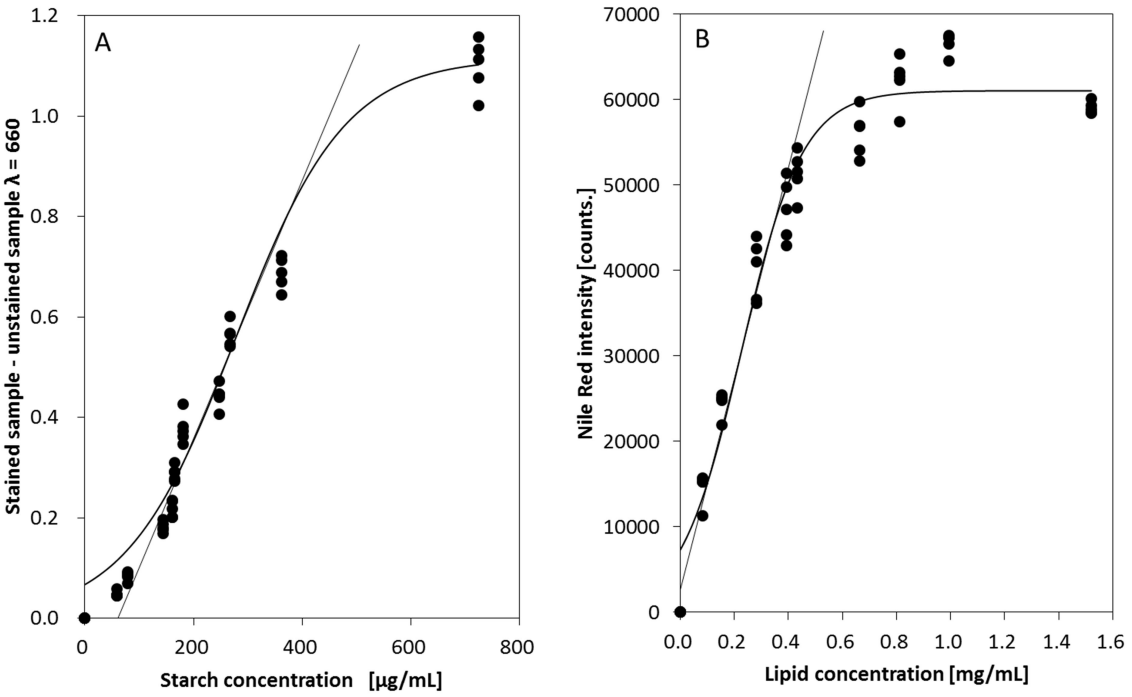

Fig. 1–2. Standard curve for the microplate–based method and the effective measurement range. A: Standard curve for the microplate–based quanttative analysis using Lugol staining of samples measured by the anthrone method, B: Standard curve for the microplate–based quantitative analysis using Nile Red staining of samples extracted from a MTBE–methanol–water system. Curves are approximate sigmoids drawn using Sigma Plot 8.0 software.

CHAPTER II

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

SUMMARY

The microalgae genus *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 Chlorella strains were attempted to increase starch and lipids productivity under high–light–intensity conditions (600–μmol photons m^{-2} 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.

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 ($g \cdot L^{-1} \cdot day^{-1}$) 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 producitvity to produce much material to decrease period of cultivation time.

Most reports describe microalgae cultured under light intensity conditions of 300–µmol photons$\cdot m^{-2}$ s^{-1}, but growth potential is not inhibited by a light intensity of 400–µmol photons$\cdot m^{-2}$ s^{-1} in *C. sorokiniana* (Ugwu et al. 2007). Whereas cultivation at a high light intensity is not suitable for the growth of *Euglena gracillis* (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–µmol photons$\cdot m^{-2}$ 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 (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 (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.

MATERIALS AND METHODS

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–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^{-1} 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 Figure 2–S1. 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^{-1} during the survey.

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 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 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.

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 × *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 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 H_2O 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^{-1}; 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^{-1} 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.

RESULTS

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

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. 2–1a–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. 2–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 (Fig. 2–1 a–h).

Changes in starch and lipids levels

Starch was assayed by the modified method of Brányiková et al. (2011) Accumulation of starch peaked on day 4 or 5 post–inoculation under LD or LL conditions (Fig. 2–2a), with the greatest accumulation observed in *P. kessleri* NIES–2152 (875 mg L^{-1}), *C. emersonii* (699 mg L^{-1}) and *P. kessleri* NIES–2159 (662 mg L^{-1}). 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^{-1}) 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. 2–2a, 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^{-1}) and *P. kessleri* NIES–2152 (292 mg L^{-1}). *P. kessleri* NIES–2159 showed starch accumulation (237 mg L^{-1}) 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. 2–2b). The greatest lipids accumulation occurred in *P. kessleri* NIES–2152 (1.72 g·L^{-1}), *C. vulgaris* (1.48 g·L^{-1}) and *C. emersonii* (1.26 g·L^{-1}) 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. 2–2d). 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. 2–2, Table. 2–1). Lipid accumulation in *P. kessleri* NIES–2152 more than doubled under the LD condition (Fig. 2–2b).

Fatty acid composition of accumulated lipids

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 (Fig. 2–3). 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.

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. beijerinckii, and P. kessleri* CCALA255, NIES–2159 and NIES–2152 strains under both LL and SLL conditions (Fig. 2–2a, 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. 2–2a, b) but not under LD or sulfur–deficient conditions (Fig. 2–2c, d).

The ratio of starch to lipids accumulation was measured on a dry weight basis (Fig. 2–4). 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.

DISCUSSION

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.

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 μmol photons m^{-2} s^{-1} in cloudy weather to more than 1000 μmol photons m^{-2} s^{-1} in moderate weather to a maximum of over 2000 μmol photons m^{-2} s^{-1} in the direct sunlight of midsummer (Béchet 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–μmol photons m^{-2} s^{-1}) one study used 500–μmol photons m^{-2} s^{-1} (Přibyl et al. 2012). A high light intensity (600–μmol photons m^{-2} 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. 2–1). *P. kessleri* NIES–2152 required ~14 days to reach the stationary phase under a light intensity of ~100 μmol photons m^{-2} 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. 2–1). 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.

Changes in starch and lipids levels

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).

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 (Ogbonna and Tanaka 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.

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. 2–3). 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.

Accumulation shift from starch to lipids under high light intensity

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. 2–4). 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. 2–2). The above results showed that the shift to lipids accmulation 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. 2010, 2012).

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 Fig. 2–5 and Table 2–1. 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 (g L^{-1} 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 g L^{-1} day^{-1}), which was about twofold that in *C. vulgaris* (0.55 g L^{-1} day^{-1}). Lipids (0.3 g L^{-1} day^{-1}) and starch (0.22 g L^{-1} day^{-1}) productivity was greatest under LL conditions in *P. kessleri* NIES–2152. Biomass productivity (0.80 g L^{-1} day^{-1}) and lipid productivity (0.60 g L^{-1} day^{-1}) in *Parachlorella* have been reported (Li et al. 2013). Another group also reported high biomass (1.291 g L^{-1} day^{-1}) and lipid (0.526 g L–1 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 g L^{-1} day^{-1}; Přibyl et al. 2012) and *C. pyrenoidosa* (1.10 g L^{-1} day^{-1}; Li et al. 2011). Lipid productivity in *C. vulgaris* has been reported as 0.64 g L^{-1} day^{-1} (Li et al. 2011) and 0.604 g L^{-1} 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 g L^{-1} day^{-1}). *C. vulgaris* showed the maximum lipid productivity (0.23 g L^{-1} day^{-1}), which was less than the preciously 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.

TABLES AND FIGURES

Table 2–1 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.

Strain	Biomass		Lipid		Starch	
	Final g/L	g/L/Day	Final g/L	g/L/Day	Max g/L	g/L/Day
C. vulgaris	2.8	0.55	1.5	0.29	0.277 (4)	0.069
C. sorokiniana	2.9	0.57	0.21	0.039	0.471 (5)	0.094
C. viscosa	2.9	0.57	0.42	0.081	0.170 (5)	0.034
C. emersonii	5.4	1.0	1.3	0.23	0.699 (4)	0.18
P. kessleri NIES-2152	4.9	0.93	1.7	0.33	0.875 (4)	0.22
P. kessleri NIES-2159	5.6	1.0	1.2	0.21	0.662 (4)	0.17
P. kessleri CCALA 255	3.4	0.61	1.1	0.20	0.405 (4)	0.10
P. beijerinckii	4.6	0.87	1.0	0.19	0.326 (5)	0.065

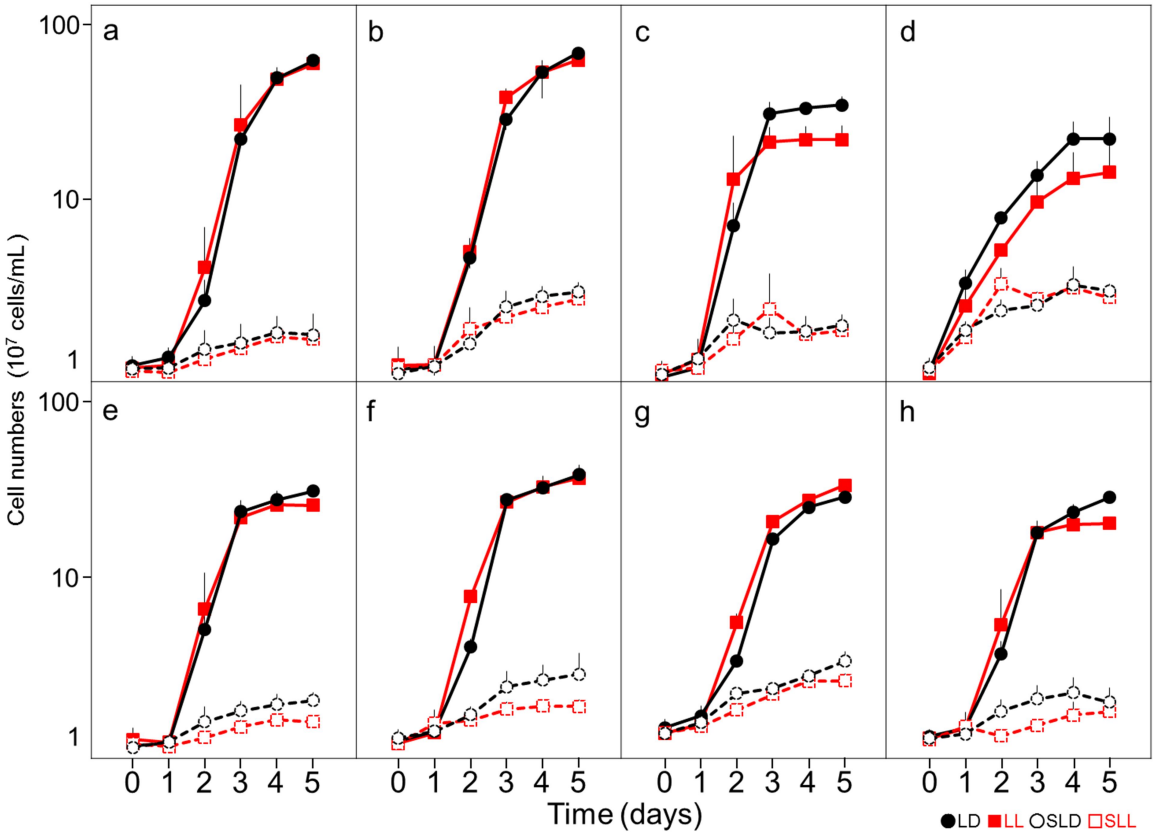

Fig. 2–1. Time–course of growth of eight strains of six *Chlorella* species (cells mL^{-1}). Effect of sulfur deprivation and the light–dark (LD) cycle under various culture conditions. (a) *Chlorella viscosa,* (b) *C. vulgaris*, (c) *C. sorokiniana*, (d) *C. emersonii,* (e) *Parachlorella beijerinckii*, (f) *P. kessleri* CCALA255, (g) *P. kessleri* NIES–2159, (h) *P. kessleri* NIES–2152.

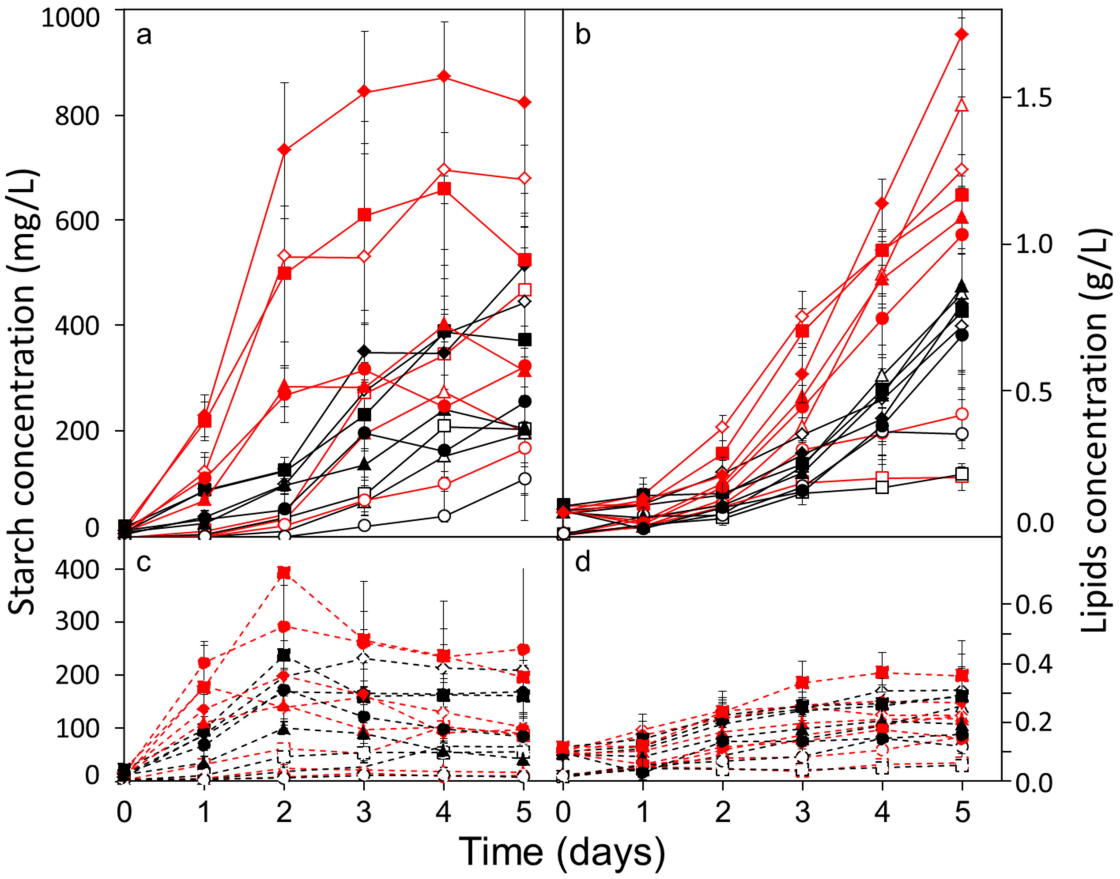

Fig. 2–2. Time–course of changes in starch and lipid contents in eight strains of six *Chlorella* species. Effect of sulfur deprivation and the LD cycle under various culture conditions. (a) Starch concentration (mg/L) in Tris acetate phosphate (TAP) medium, (b) lipid concentration (g/L) in TAP medium, (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* (△), *C. sorokiniana* (□), *C. emersonii* (◇), *Parachlorella beijerinckii* (●), *P. kessleri* CCALA255 (▲) *P. kessleri* NIES–2159 (■), and *P. kessleri* NIES–2152 (◆).

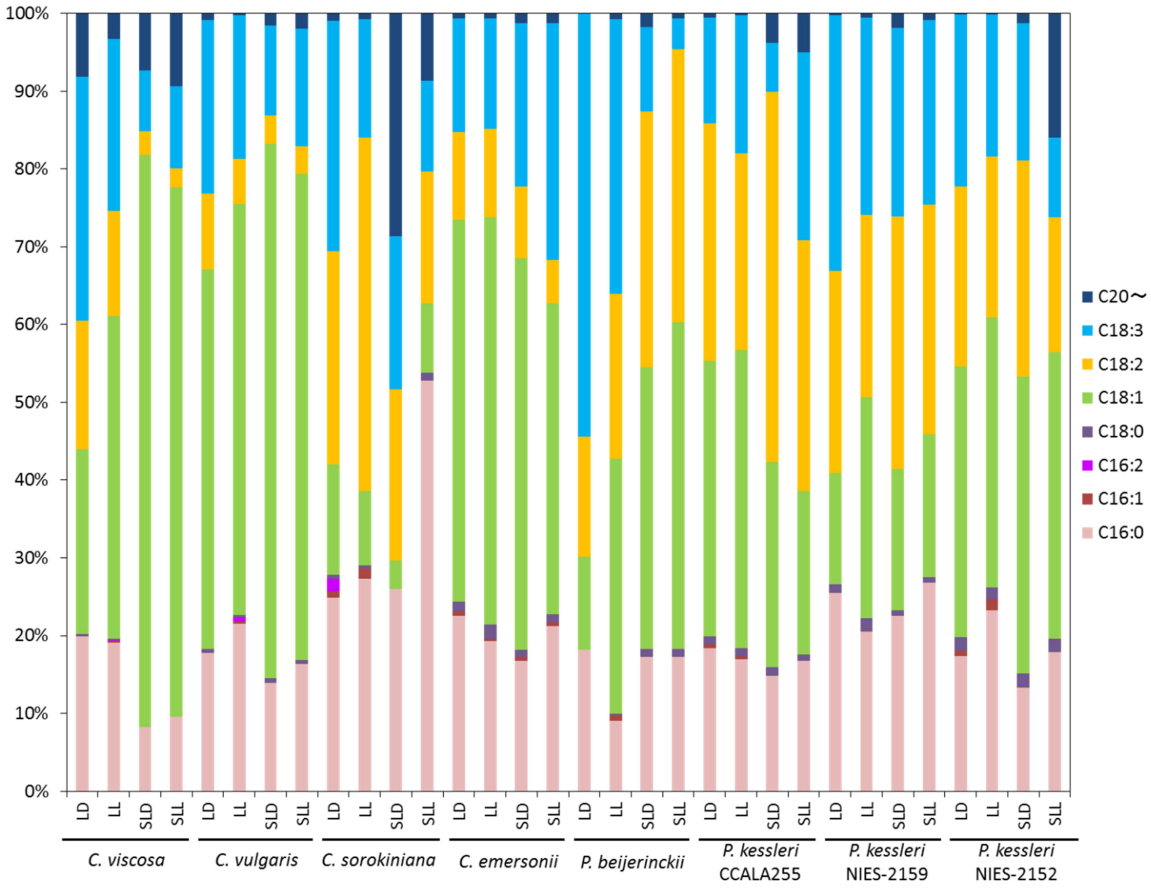

Fig. 2–3. Fatty acid profiles (percentage of total fatty acids) in eight strains of six *Chlorella* species under four different culture conditions. Accumulated lipids were extracted and their fatty acid compositions were analyzed by gas chromatography. The ratios of accumulated fatty acids are shown. 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).

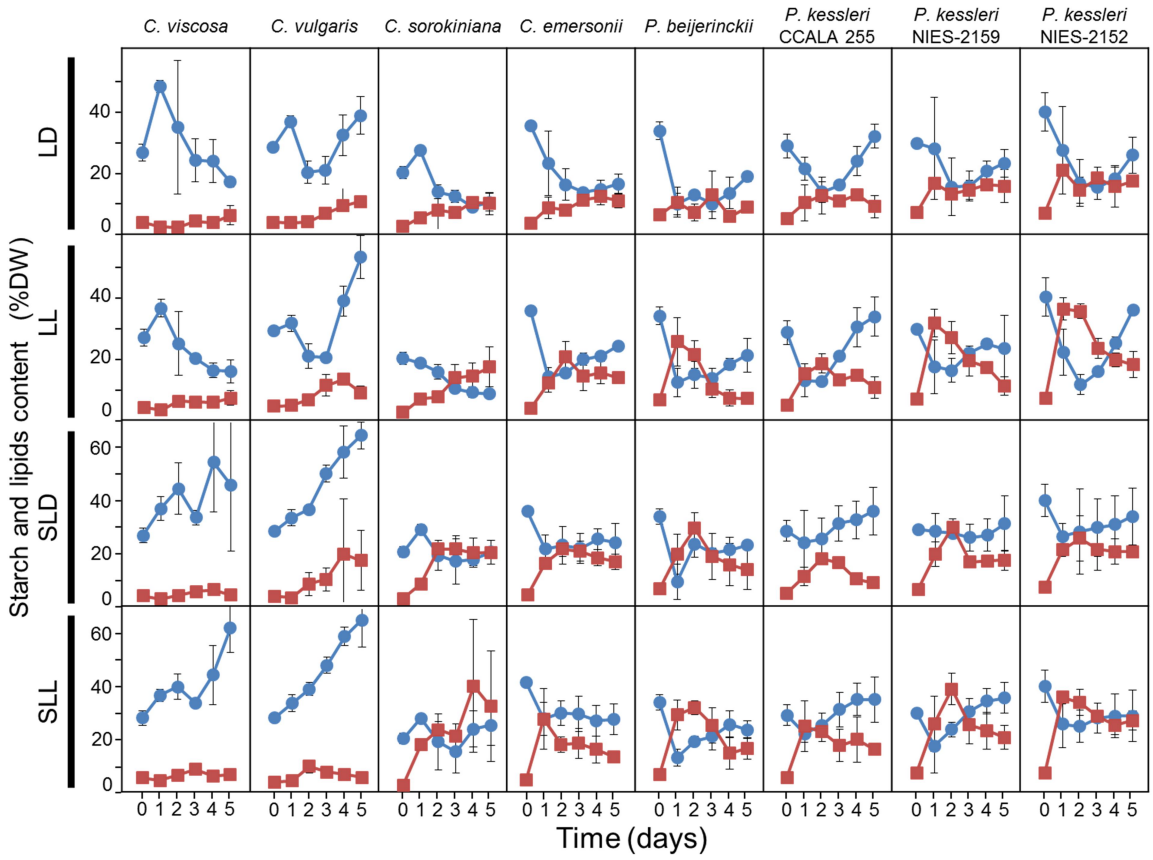

Fig. 2–4. Time–course of changes in starch and lipid contents (percentage of dry weight biomass = % Dry Weight) in eight strains of six *Chlorella* species under various culture conditions. The cultivation conditions are indicated in the left column. Red lines show the amount of starch, and blue lines show the amount of lipids.

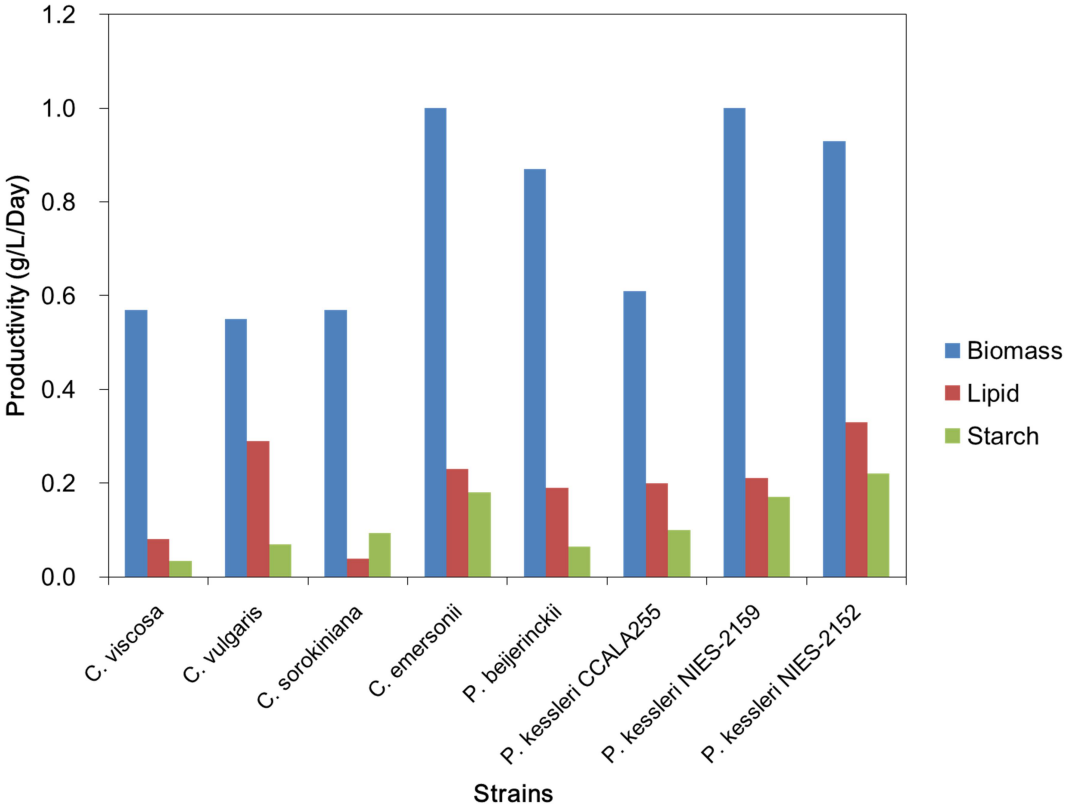

Fig. 2–5. Biomass, lipid and starch productivity of eight strains of six *Chlorella* species cultured in TAP medium under continuous light (LL) conditions. Biomass and lipid productivity were measured on day 5 post–inoculation. The day of maximum starch productivity is indicated.

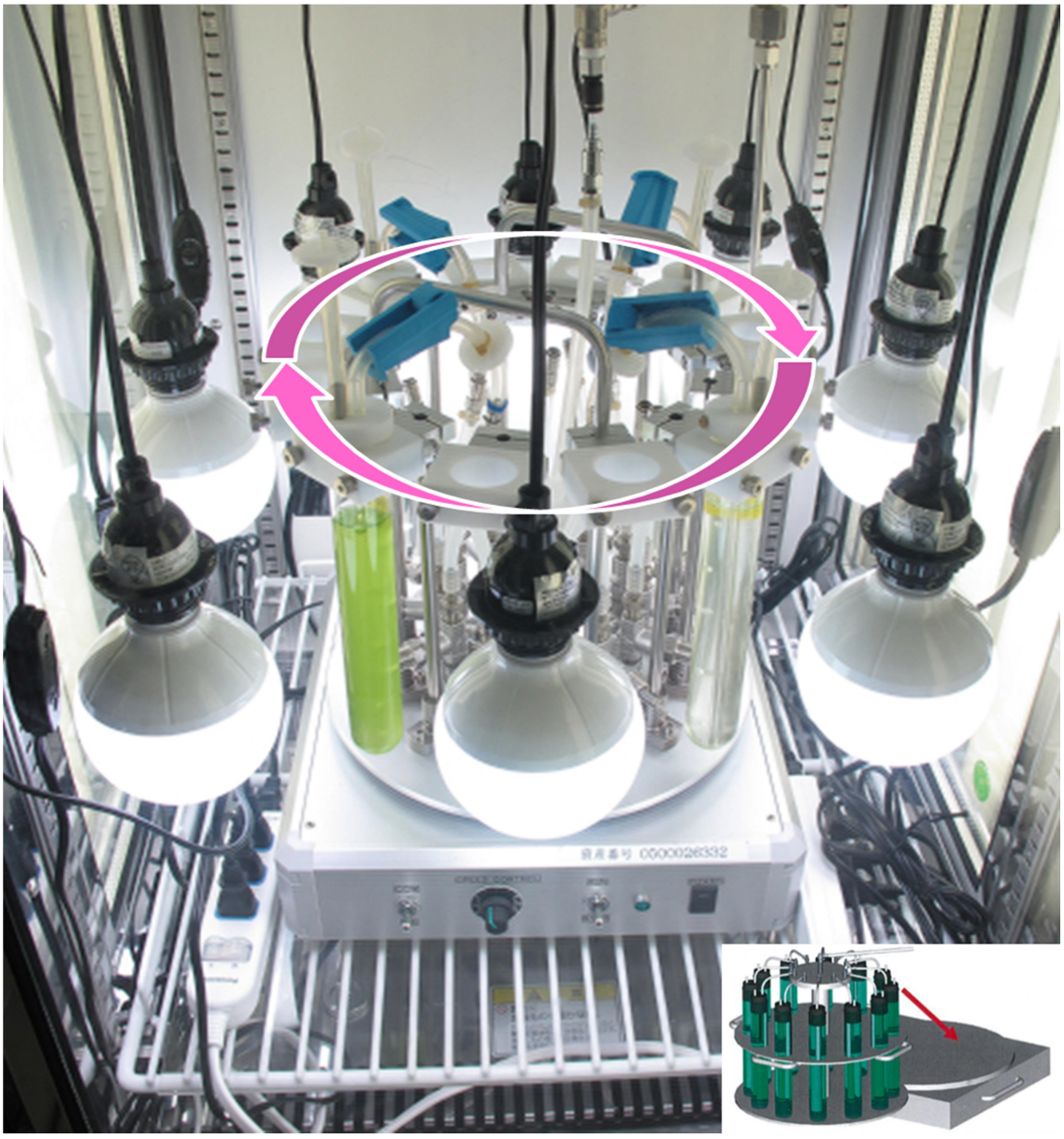

Fig. 2–S1. Rotary culture equipment. Cultivation was performed in a maximum of 12 test tubes fixed to a central platform holder, which was rotated at 2 rpm.

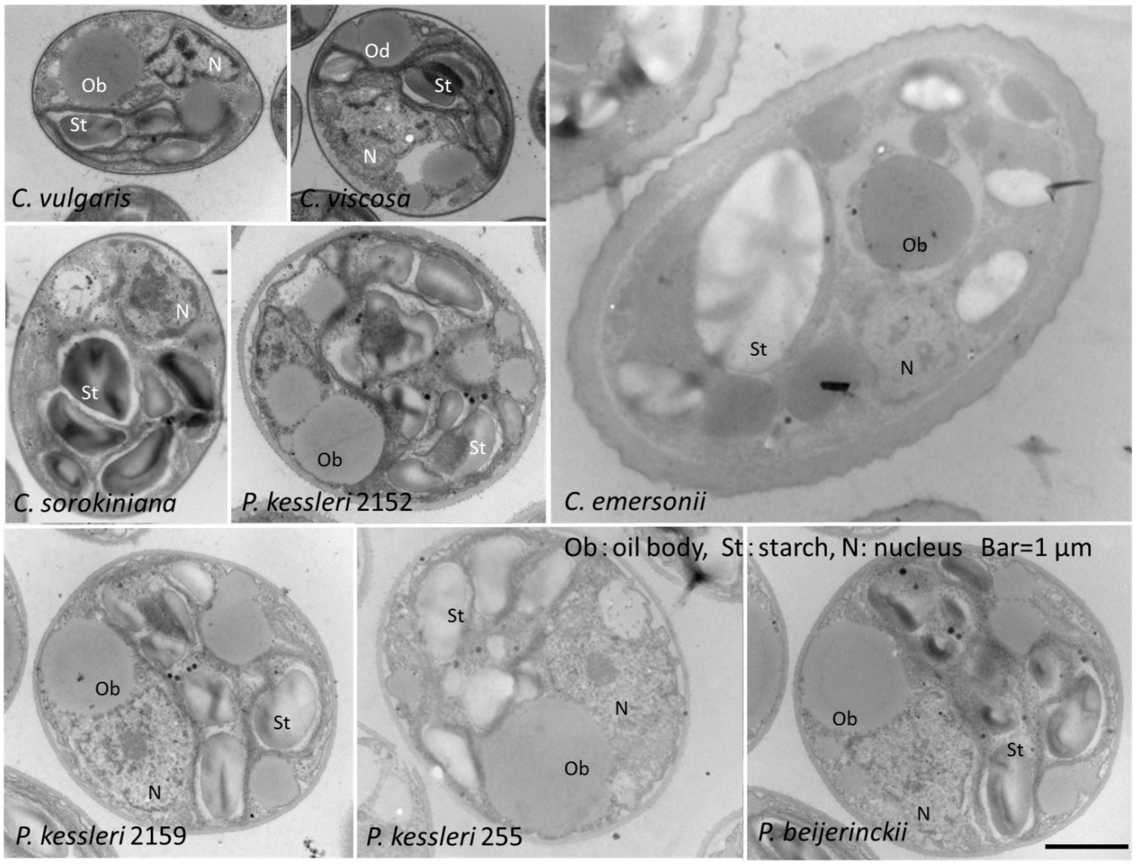

Fig. 2–S2. Cytology of the ultrastructures of eight strains of six *Chlorella* species cultured in TAP medium under LD conditions. Ob: Oil body; St: starch, N: nucleus. Scale bar: 1 µm. Cultivation time is day 5 post–inoculation.

Supplementary methods

Transmission electron microscopy

The cells were fixed with 2.5% glutaraldehyde in cacodylate buffer (pH 7.0). Fixed cells were washed several times in buffer. The fixed cells were postfixed in 1% OsO4 for 2 h at room temperature. After washing in distilled water, the cells were dehydrated by passing them through a series of increasing concentrations of ethanol and absolute acetone, and then they were embedded in Spurr's resin. Ultrathin ections were cut on a Reichert Ultracut S ultramicrotome (Leica, Vienna, Austria) using a diamond knife.

The sections were mounted on copper grids cotated with polyvinyl formvar films and stained with 3% uranyl acetate(2 h) and lead citrate(10 min). Sections were viewed with an H–7650 TEM(Hitachi High Technologies, Tokyo, Japan) at 100kV.

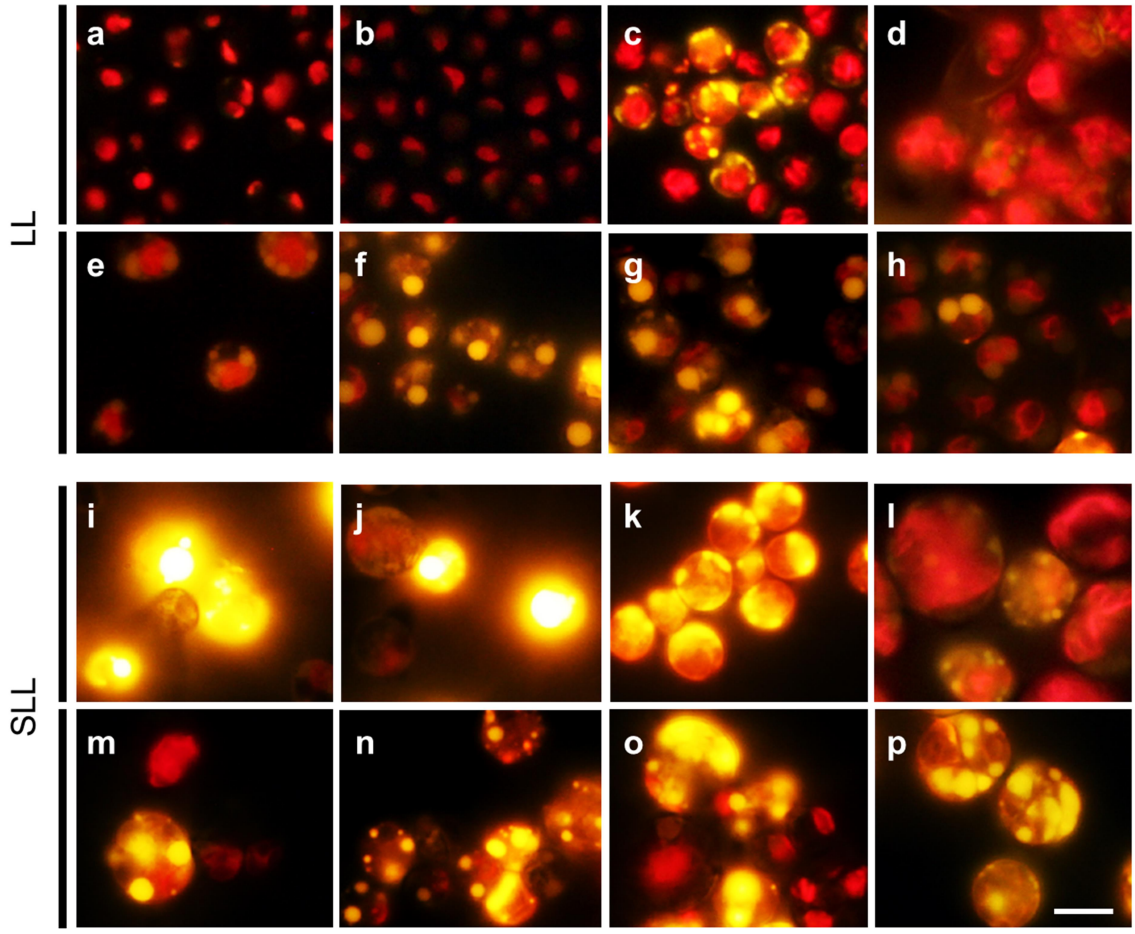

Fig. 2–S3. Fluorescence images; oil droplets were stained using Nile Red (yellow–crimson); autofluorescence of chloroplasts is shown in red; oil droplets are shown in yellow. (a, i) *C. viscosa,* (b, j) *C. vulgaris*, (c, k) *C. sorokiniana*, (d, l) *C. emersonii,* (e, m) *P. beijerinckii*, (f, n) *P. kessleri* CCALA255, (g, o) *P. kessleri* NIES–2159, (h, p) *P. kessleri* NIES–2152. Images were obtained on day 5 post–inoculation. The cultivation conditions are indicated in the left column. Bar = 5 µm.

Supplementary methods

Visualization of intracellular oil droplets

The intracellular oil droplets were observed by using Nile red (9–diethylamino–5 H–benzo [alpha] phenoxazine–5–one). The supernatant was discarded after centrifugation of 1–

mL cell suspension, and the cell pellet was vortexed for 4 min with the addition of 10 mM Nile red solution in DMSO (1 μL). Oil droplets were visualized by using an Olympus BX 52 fluorescence microscope, Olympus DP70 color CCD camera and Olympus U–NMWB3 filter.

CHAPTER III

Increase of lipid production by the dilution culture method upon outdoor cultivation of a *Parachlorella kessleri* mutant, PK4, and identification of its genetic variations

SUMMARY

Background: *Chlorella* has the ability to selectively induce starch and lipid accumulation by controlling light and nutrient salt levels. Although *Parachlorella kessleri* is a superior *Chlorella* species in terms of productivity, it is necessary to develop a selective breeding technology to further improve its productivity. Heavy-ion-beam irradiation is a breeding technology that uses mutagenesis to breed land plants. This technology has recently been used for microalgae breeding. Its validity has been proven on a laboratory-scale but not on a large-scale outdoors. Current studies show that PK4 is a potentially excellent mutant strain generated from *P. kessleri* by heavy-ion-beam irradiation. A laboratory-scale experiment showed that PK4 accumulated approximately 6X more lipids than wild-type (WT) in diluted nutrient medium with 0.2X nitrogen concentration.

Results: An inexpensive SS medium was used and induction of lipid accumulation was attempted by the dilution culture method. PK4 accumulated more lipids and at an earlier stage than WT. An experiment with an outdoor mass culture system also showed high lipid accumulation (66% of dry weight) and biomass productivity associated with lipid productivity. In addition, whole genome sequencing of PK4 by next generation sequencing revealed mutations in two genes that encode endo-1,4-beta-mannase and an ATP/ADP transporter.

Conclusions: PK4 balances high lipid accumulation with high biomass productivity and is a promising strain of biofuel production that is cultivable on a large-scale outdoors. Single-nucleotide polymorphisms were found in two genes in PK4 that are thought to influence its phenotype. However, these influences are currently unknown.

INTRODUCTION

Chlorella and *Chlamydomonas* are microalgae genera that have been studied for a long time all over the world. Green microalgae have similar morphological features, such as a spherical shape and immobile endospores. Numerous species of green microalgae had been classified as *Chlorella*.

However, molecular phylogenetic information has now resulted in definitive classifications(Huss et al. 1999). Although the majority of *Chlorella* species live in freshwater, several live in seawater. *Parachlorella kessleri*, a species of the *Chlorella* genus, has high biomass productivity and is also known for having high-level accumulation of starch and lipids (Fernandes et al. 2013).

In response to nutrient starvation under sulfur or nitrogen conditions, *P. kessleri* initially accumulates starch, followed by lipids (Mizuno et al. 2013, Ota et al. 2013).

Spontaneous mutation breeding technologies have been used to improve the productivity of crops such as corn and rice. To improve the productivity of microalgae materials such as starch or lipids, studies have applied a spontaneous mutation technology to obtain improved strains of microalgae. Some resultant mutant strains have exhibited excellent characteristics. Exposure of *Chlorella pyrenoidosa* to ultraviolet (UV) irradiation resulted in the generation of a starch-less mutant with improved lipid accumulation (Ramazanov and Ramazanov 2006). UV irradiation also generated a *Chlorella* sp. mutant that had increased dry weight and lipid accumulation (Liu et al. 2015).

A heavy ion is an atom with an electron stripped off. The heavy ion accelerated is called heavy-ion-beam. Studies on mutation breeding using heavy-ion-beams have led to the development of farm products and gardening plants. Heavy-ion-beam irradiation leads to

double-stranded DNA breaks (Yokota et al. 2007) and induces large-scale deletions resulting from extensive linear energy transfer (LET) (Hirano et al. 2012). These effects are very different from DNA damage that is induced by UV radiation or gamma rays. PK4 is a mutant derived from heavy-ion-beam-irradiated *P. kessleri* that exhibits high lipid accumulation under nitrogen-starved conditions (Ota et al. 2013). Heavy-ion-beam irradiation of microalgae such as *Chlamydomonas reinhardtii* (Li et al. 2010), *Nannochloropsis* (Ma et al. 2013) and *Desmodesmus* sp. (Hu et al. 2013) have also led to the production of mutants with practical applications. All of these mutant strains had higher lipid accumulation than wild-type (WT). However, these mutants were produced on a small-scale experimental system in the laboratory and so far, no studies have focused on large-volume cultivation under an outdoor open system. Nutrient source limitation has been shown to be effective at utilizing the characteristics of PK4 (Ota et al. 2013). On a laboratory-scale, the usual method to induce microalgae starch and lipid accumulation is to exchange the medium by centrifugal separation. However, problems associated with large-scale culture include excessive costs and the time required to exchange the medium by centrifugal separation (Li et al. 2007). An alternative for the induction of lipid accumulation is the dilution culture method that reduces the nutrient salt concentration not by exchange of the nutrient medium but by dilution of the whole culture medium with water. A previous study showed that in the cultivation of *P. kessleri,* a five- or 10-fold dilution of the medium induced lipid accumulation (Li et al. 2013). Furthermore, this led to a smaller decrease in biomass than other methods that induce lipid accumulation by limiting major elements such as nitrogen, sulfur and phosphorus (Fernandes et al. 2013).

The choice of medium is also an important factor in dilution culture techniques. Tris-acetate-phosphate (TAP) medium, containing acetic acid as the carbon source, is

commonly used for the culture of *Chlamydomonas* and can be used for the culture of *Chlorella* (Sueoka 1960). For *Scenedesmus* culturing, SS medium, which contains urea as the nitrogen source, has also been used for the culture of *Chlorella* (Zachleder and Šetlík 1982). To date, the use of SS medium by the dilution culture method has led to an increase in lipid accumulation in outdoor large-scale culture systems (Přibyl et al. 2012, Li et al. 2013). Three species, *C. sorokiniana*, *C. vulgaris* and *P. kessleri*, have been successfully cultured outdoors using a thin-layer photobioreactor (T-PBR).

The T-PBR has two sloping flat boards at a water depth of approximately 7 mm. Sunlight can be used to irradiate the medium flowing around two pieces of sloping flat boards to the bottom of the culture (Doucha et al. 2005). Oxygen produced by photosynthesis can be removed by combining small cascades during the course with ups and downs. With the exception of *Chlorella*, good results were obtained in previous studies using the same type of photobioreactor (PBR) (Doucha and Lívanský 2006; 2009).

In the current study, *P. kessleri* WT and PK4 were cultivated and analyzed in terms of starch and lipid accumulation using two experimental systems: a laboratory-based small-scale culture and an outdoor large-scale culture. From the results regarding induction of lipid accumulation by the dilution culture method in SS medium, the mutant strain PK4 exhibited higher lipid accumulation than the WT. The WT and PK4 strains were also cultivated in an outdoor mass culture (150-L) using SS medium, and the induction of lipid accumulation in PK4 was attempted by the dilution culture method. In addition, whole genome sequencing of PK4 revealed genetic variation caused by heavy-ion-beam irradiation. To the best of our knowledge, this is the first time such an approach has been used in microalgae.

MATERIALS AND METHODS

Strains and culture conditions

Strain of *Parachlorella kessleri* (NIES–2152) was obtained from the Microbial Culture Collection, National Institute for Environmental Studies, Tsukuba, Japan. The *P. kessleri* mutant PK4 by irradiation with a heavy–ion–beam was described in our previous report (Ota et al. 2013). The PK4 is amutant that was generated by heavy–ion–beam irradiation with C ions (LET: 22.6 keV μm^{-1}) at a dose of 10–Gy at the RIKEN RI–beam factory (Wako, Saitama, Japan; http://www.rarf.riken.go.jp/Eng/facilities/RIBF.html). Cells were precultured for 20 days in TAP medium (http://mcc.nies.go.jp/02medium–e.html) or SS medium (Zachleder and Šetlík 1982) under a 12:12–h light–dark cycle at 50–μmol photons m^{-2} s^{-1} and 23 °C. Continuous light irradiance of 600–μmol photons m^{-2} s^{-1} on the tube surface was provided by light–emitting diode lamps (LUS–BF; Beaubelle, Ehime, Japan). After pre–cultivation, cells were cultivated at 23 °C, with air flow of 20 mL·min^{-1}, CO_2 concentration of 2–3%, and rotation at 2 rpm using rotary culture equipment (REV–Light12; B.E. Marubishi Co., Ltd., Tokyo, Japan and CLE–303; Tomy Seiko Co., Ltd., Tokyo, Japan). A total of the pre–cultured 8.0×10^7 cells were inoculated into 93–mL test tubes containing 80–mL of culture medium. The culture solution was obtained by diluting sterilized distilled water 4 to 10 times on day 4 post–inoculation.

Outdoor cultivation

The preculture with a cell density of ~2×10^7 cells in SS medium was inoculated in 3–L photobioreactors and was cultivated for 3 days. The WT and mutant strains were grown in a thin layer photobioreactor (T–PBR, volume of 150–L) located in a temperature–uncontrolled

greenhouse in Třeboň, Czech Republic (49°0′21.546″ N, 14°46′21.538″ E), in August 2013. The T–PBR consisted of two glass lanes (each 6 m long and 1.1 m wide) with an inclination of 1.6%, and the two glass lanes were arranged with antiparallel and connected by a trough. Algal culture was delivered from a retention tank by a pump to the upper rim of the glass plate. The culture was enriched with CO_2 released directly into the cultivation medium immediately behind the pump at a flow rate of 2 L h^{-1}. The pH of the culture solution was measured every morning. The amount of NaOH to be added was adjusted to keep the pH of the solution at 7. The culture solution was diluted by water four times on day 7 post–inoculation. A precise description of the culture system and a schematic were published previously (Doucha et al. 2005). During these experiments, cell number, dry weight, starch content, and lipid content were determined at two or three times in a day. For the measurement of light intensity, a quantum/radiometer–photometer (LI–COR, Inc.) was used.

Determination of cell growth and dry weight

Optical density (OD) was measured using a spectrophotometer. Cultures were diluted to an OD of less than one, to fall within the linear range of measurement. The actual OD was determined by multiplying by the dilution factor. For dry weight determination, biomass was separated from the medium by centrifugation of 2–mL of the cell suspension in pre–weighed microtubes at 3,000 *g* for 5 min; the sediment was dried at 105 °C for 12 h and weighed on an analytical Sartorius 1601 MPB balance (as described elsewhere Brányiková et al. 2011). Cell volume and concentration were measured using a Beckman Coulter Multisizer III (Coulter Corporation, Miami, FL, USA) by diluting 10–50–mL of fixed (0.2% glutaraldehyde) cell suspension into 10–mL of 0.9% NaCl (w/v) electrolyte solution.

Microscopic observations

Intracellular oil droplets were stained with Nile Red (9–diethylamino–5H–benzo[a]phenoxazine–5–one; Polyscience, Inc., Warrington, PA, USA; Takeshita et al. 2015). A total of 1–mL of the cell suspension was fixed with 10 μL of glutaraldehyde. An Olympus BX 60 LM microscope equipped with the filter combination U–MNU2 (360–370 nm excitation and >515 nm barrier filter) was used. Photomicrographs were taken with a digital camera (Camedia C–5050 Zoom; Olympus Corp., Tokyo, Japan).

Determination of starch contents

Starch content in the laboratory cultivations was determined by the Lugol staining method. The analyses were carried out as described previously (Takeshita et al. 2015). Starch content in the outdoor cultivations was determined by a modified version of a previously reported method (McCready et al. 1950), as described previously (Brányiková et al. 2011).

Determination of lipid contents

Lipid contents in laboratory cultures were determined by Nile Red staining. The analyses were carried out as described previously (Takeshita et al. 2015). Lipid content in outdoor cultivations was determined by Nile Red staining and the gravimetric method. The algal suspension was fixed with glutaraldehyde to a final concentration of 0.25% (v/v) and loaded into the wells (100 μL per well) of a 96–well plate. Four microliters of Nile Red solution was added to the wells and the fluorescence intensity was measured using a 96–well plate luminometer (Tecan Infinite 200; Tecan, Männedorf, Switzerland) with the following filters:

excitation 485 nm (bandwidth 20 nm) and emission 595 nm (bandwidth 10 nm). The gravimetric lipid determination method was carried out as described previously (Přibyl et al. 2012).

Whole–genome sequencing and identification of unique mutations

The cell pellets of 1–L culture were used for DNA preparation. Cultures were grown under 100 μmol photons m^{-2} s^{-1} under a 12 h:12 h light:dark cycle at 23 °C. DNA was extracted in accordance with a previous report (Ota et al. 2016), followed by the DNeasy Plant Maxi Kit (Qiagen, Hilden, Germany). The genomic sequencing of *P. kessleri* PK4 was carried out on an Illumina Miseq (Illumina, San Diego, CA, USA), as shown in Supplementary Fig. 3–2 (300–bp paired end sequencing). All sequences were trimmed using the Fastq Quality Trimmer (http://hannonlab.cshl.edu/fastx_toolkit/) and mapped to *P. kessleri* NIES–2152 (WT) using the Burrows–Wheeler Aligner (Li and Durbin 2010). Format conversion was performed with SAMtools (Li et al. 2009). Variants were identified and filtered with Pindel (Ye et al. 2009) and SomaticSniper (Larson et al. 2012).

RESULTS

Comparison between TAP medium and SS medium

During *P. kessleri* cultivation, the effects of TAP and SS media on cell proliferation and starch and lipid accumulation were compared. These two media are supplemented with different nutrient salts. TAP medium contains acetic acid and SS medium contains urea. Urea, which is found in SS medium, is a cheaper nitrogen source than ammonium chloride, which is found in TAP medium. Urea is typically used as a fertilizer for crops. *P. kessleri* WT and PK4 were investigated in terms of their differences upon being cultivated in TAP medium and SS medium. Culture absorbance values were measured over 8 days of cultivation (Fig. 3-1A). The results showed that WT and PK4 stopped growing in TAP medium on day 6 post-inoculation and reached the stationary phase, but both WT and PK4 continued to proliferate in SS medium even on day 8 post-inoculation.

The amount of starch was measured by a microplate-based method using Lugol staining. In TAP medium, the amount of starch for the WT and PK4 increased until day 6 post-inoculation and started to decrease (Fig. 3-1B).In SS medium, the amount of starch for the WT and PK4 strains continued to increase until day 8 post-inoculation (Fig. 3-1 B). The amount of lipids was measured by a microplate-based method using Nile Red staining. The amount of lipids in both WT and PK4 in TAP medium continued to increase even after 8 days post-inoculation (Fig. 3-1C). In SS medium, a small amount of lipids accumulated in both WT and PK4 during cultivation.

Effect of the dilution cultivation method in SS medium

The induction of lipid accumulation was examined by a dilution cultivation method in SS medium. All dilution steps were performed using the aseptic technique. Samples of culture medium on day 4 post-inoculation were collected and diluted four-, five- and 10-fold with sterilized water, and individual cultivations were continued. The culture absorbances of both WT and PK4 decreased temporarily following the dilutions (Fig. 3-2A–C, arrow). However, the absorbances of all dilution cultivations eventually recovered to pre-dilution values (Fig. 3-2A).

The dilution cultivation method initially reduced the amount of starch produced by WT and PK4 but eventually recovered to pre-dilution levels and increased afterward (Fig. 3-2B). The amount of starch produced by WT and PK4 in the four- and five-fold dilutions increased until day 3 post-dilution (WT: 12–14-fold; PK4: 7.4–8.5-fold). The amount of starch in the culture diluted 10-fold increased until day 2 post-dilution (WT: 12-fold; PK4: 9.4-fold).Both WT and PK4 did not accumulate lipids before dilution in SS medium but started to accumulate lipids suddenly after dilution. There were no significant differences between WT and PK4 in terms of absorbance or the amount of starch. On the other hand, PK4 had significantly faster lipid accumulation than WT.

Outdoor large-scale culture using a thin-layer photobioreactor

The WT and PK4 strains were cultivated in SS medium using an outdoor large-scale culture system with large environmental changes during individual cultivation periods. This system was comprised of a T-PBR consisting of 150-L of SS medium in a semi-closed outdoor environment. WT and PK4 culture samples were removed from the T-PBR every day and its color was monitored (Fig. 3-3). The color of the WT culture was dark green on days 4–

6 post-inoculation and changed to olive green afterwards. The color of the PK4 culture was dark green on days 4–9 post-inoculation and rapidly changed to yellowish-green on days 11–15 post-inoculation.

Cellular lipids were stained by Nile Red, which is used to visualize neutral lipids, and can be observed by fluorescence microscopy. Autofluorescence of chlorophyll located in chloroplast thylakoid membranes resulted in a red signal, and neutral lipids stained with Nile Red resulted in a yellow signal. In both WT and PK4, almost no Nile Red staining was observed until day 7 post-inoculation. However, this staining was detected in almost all cells on day 10 post-inoculation, with PK4 lipid signals being higher than WT lipid signals (Fig. 3-4).

Lipid accumulation in PK4 by the dilution cultivation method in an outdoor large-scale culture system changed during cultivation

The dilution cultivation method that induced lipid accumulation in both WT and PK4 in the laboratory-scale culture system with SS medium was performed in the T-PBR system using the same medium. This experiment was performed from August 26th to September 15th, 2013. The water temperature and light intensity fluctuated every day during the cultivation period (Fig. 3-5A). The results show that cell density changed throughout the cultivation period (Fig. 3-5B). The initial cell concentration was 5.8×10^6 cells mL^{-1} and the cells proliferated 24 times and reached 1.4×10^8 cells mL^{-1} on day 7 post-inoculation. A sample of the culture was discarded on day 7 post-inoculation and the culture was diluted four-fold with water (Fig. 3-5B, C, arrow). The dry weight (DW, $g \cdot L^{-1}$) increased similarly to the cell density until day 7 post-inoculation and peaked at 5.8 $g \cdot L^{-1}$ (Fig. 3-5B). Both the number of cells and

DW decreased temporarily after medium dilution but recovered 3 days after dilution later to pre-dilution levels. The time-course data of starch and lipids levels are shown in Fig. 3-5C. Starch levels decreased upon medium dilution and recovered to pre-dilution levels after one day. Thereafter, starch levels decreased from day 12 to day 13 post-inoculation (0.35 $g \cdot L^{-1}$), and then peaked (1.06 $g \cdot L^{-1}$). Lipids hardly accumulated until the medium was diluted, but began to increase gradually from day 9 post-inoculation and showed a tendency to increase until the last day of cultivation.

The results of the outdoor large-scale culture were summarized and are shown together with the results of preceding studies using WT in Table 3–1. The maximum biomass was 5.8 g L^{-1}; the biomass productivity, represented by the increase in biomass per day, was up to 0.82 g L^{-1} day^{-1}.

In addition, for PK4, the greatest lipid productivity, similarly represented by the increase of lipids per day, was 0.59 g L^{-1} day^{-1}, and the maximum amount of lipids per unit biomass that was achieved was 66% of DW, by the dilution method. The results for PK4 by the dilution culture method were compared with the results using the same type of bioreactor as reported in preceding studies, and with the results upon culture indoors in SS medium. The maximum biomass of PK4 did not reach the result obtained for *P. kessleri* CCALA 255 in a preceding study, which was in the range of 5.7–14 g L^{-1}. However, the biomass productivity was 0.71–0.94 g L^{-1} day^{-1} in that preceding study, and in this study, the results for PK4 using the dilution culture method were the highest value among outdoor results. Lipid productivities in the preceding studies were in the range of 0.30–0.50 g L^{-1} day^{-1}, and maximum lipid levels were 25–51% of DW. The lipid results for PK4 in this study are thus superior to those in preceding studies.

Elucidation of PK4 genetic variation induced by heavy-ion-beam irradiation

The locations of genetic variations in PK4 were investigated by next generation sequencing (NGS) (Illumina MiSeq). Short read sequences by MiSeq were mapped on the *P. kessleri* WT draft genome sequence (Ota et al. 2016). After mapping, Pindel and SomaticSniper were used to identify the following types of candidate genetic variations: deletion, inversion, small or large insertion, tandem duplication, and single-nucleotide polymorphism (SNP) (Fig. 3-S2, (Ye et al. 2009; Larson et al. 2012)). No deletions, inversions, insertions, or tandem duplications were identified by Pindel, but SNP candidates were found at 148 sites, eight of which were selected as being particularly promising by SomaticSniper (QV > 40, Somatic Score > 100). Next, associations of the eight candidate SNPs with predicted genes were analyzed (Table. 3–2). Three SNPS were located in introns (No. 42, 69, 70), one was located in an intergenic region (No. 145), and four were located in exons (No. 27, 62, 74, 82). Among these four candidates in exons, at two sites, a base substitution was confirmed, but this was only a synonymous substitution (No. 27, 62). Candidate No. 82 in an exon, representing base number 21,293 in Scaffold 00082 was a non-synonymous substitution of G to A, corresponding to the amino acid replacement of Gly by Arg. The best homologous gene was a hypothetical protein identified in *C. variabilis* by a BLAST search, but high homology was also found with genes from *Coccomyxa*, *Chlamydomonas* and *Volvox* that encode an ATP and ADP transporter. Candidate No. 74, representing base number 107,506 in Scaffold 00057, exhibited a non-synonymous substitution of C to T, corresponding to Gln being replaced by a stop codon. According to the Kyoto Encyclopedia of Genes and Genomes (KEGG), this gene encodes a protein that is an

endo-1,4-beta-mannase and is mapped to a sugar metabolism pathway connected to galactose, an amino sugar and nucleotide sugar, N-glycan biosynthesis, and glycolysis metabolism.

DISCUSSION

The influence of TAP medium and SS medium on lipid accumulation in P. kessleri

When *P. kessleri* WT and PK4 were cultured, they exhibited healthy proliferation in both TAP and SS media and similarly accumulated starch (Fig. 3-1A, B), but they differed in terms of lipid accumulation (Fig. 3-1C). TAP and SS media are composed of different nitrogen and carbon sources. TAP and SS media were initially developed for the cultivation of *Chlamydomonas* and *Scenedesmus,* respectively (Sueoka 1960; Zachleder and Setlík 1982). The source of carbon in TAP medium is acetic acid, while it is urea in SS medium. When comparing molar amounts, TAP medium contains 1.89 times as much carbon as SS medium (TAP: 3.46×10^{-2} mol L^{-1}; SS: 1.83×10^{-2} mol L^{-1}). On the other hand, the source of nitrogen in TAP medium is ammonium chloride, while it is urea in SS medium. SS medium contains 4.90 times as much nitrogen as TAP medium (TAP: 7.47×10^{-3} mol·L^{-1}; SS: 3.66×10^{-2} mol·L^{-1}). The molar amounts of other elements in the media are not significantly different. In this regard, TAP medium has a relatively high amount of carbon and SS medium has a relatively high amount of nitrogen. It was previously asserted that the amount of nitrogen affected the relationship between cell proliferation and lipid accumulation (Hu et al. 2008). Therefore, it seemed that, the nitrogen source was exhausted at an early stage and then lipid accumulation occurred in TAP medium. In SS medium, however, lipid accumulation was late in comparison with TAP medium. It seemed about because of the nitrogen source of the SS medium had been still left with richness.

Lipid accumulation induced by the dilution culture method in the laboratory

The induction of lipid accumulation by the dilution culture method was reported in SS medium (Přibyl et al. 2012; Fernandes et al. 2013; Li et al. 2013). There are two methods for medium dilution: (1) the medium can be diluted at the beginning of cultivation or (2) the whole culture can be diluted later. The latter method was adopted in this study. When the medium was diluted at the beginning of cultivation, biomass productivity decreased dramatically, similar to cases with limited nitrogen, sulfur, or phosphorus (Breuer et al. 2012; Cakmak et al. 2012; Fernandes et al. 2013; Li et al. 2013). However, periods of biomass production, starch accumulation and lipid accumulation can be controlled by dilution during cultivation (Vitova et al. 2015). Lipid accumulation after dilution was observed for both WT and PK4, but PK4 accumulated lipids more rapidly than WT (Fig. 3-2C). PK4 accumulated approximately six times more lipids than WT in nitrogen-starved medium (Mizuno et al. 2013). In a previous study, TAP medium depleted of nutrients was used from the beginning of cultivation, and PK4 accumulated more lipids than WT, regardless of whether TAP or SS medium was used.

Outdoor large-scale culture by the dilution culture method and the effect of urea in SS medium

PK4 also exhibited high lipid accumulation (66% of DW) by the dilution culture method using the outdoor large-scale culture system. Previous reports described high lipid accumulation by *Chlorella* (approximately 30–60% of DW). Values were as high as 65.1% of DW in *C. protothecoides*, 64.7% of DW in *C. zofingiensis* and 63.1% of DW in *A. protothecoides* (Feng et al. 2012; Chen et al. 2015; Rismani-Yazdi et al. 2015). In these studies, glucose was added to the medium, and the results were achieved under heterotrophic

conditions. Hardly any reports describe the lipid content exceeding 60% of DW under phototrophic conditions. Lipid accumulation greater than 60% of DW is possible in the laboratory under heterotrophic conditions; however, it would be difficult to achieve this in an outdoor large-scale culture system. I have shown that PK4 can achieve both high lipid and biomass productivity; therefore it can be used to promote the commercialization of biofuel production.

A less expensive medium was required for large-scale culture.SS medium contains a relatively inexpensive nitrogen source, namely, urea (Zachleder and Setlík 1982). However, it has been reported that, for some microalgae, urea cannot be used as a nitrogen source because growth decreases compared with other nitrogen sources such as nitric acid or ammonia (Antia et al. 1977; Lourenco et al. 2002). To date, urea-containing media has been used to culture 44 species of microalgae. Fourteen of these are not able to grow in urea. Specifically, they includes six types of diatom (e.g. *Chaetoceros*), two types of *Chlorophyta* (e.g. *Chlamydomona*), *Heterocapsa circularisquama*, *Emiliania huxleyi,* and three types of *Raphidophytes* (e.g. C*hattonella*) (Carpenter et al. 1972; Yamaguchi et al. 2001, Zhang et al. 2006). It has also been reported that bacteria such as *Staphylococcus* and *Klebsiella* cannot use urea (Varalakshmi and Devi 2014). Therefore, SS medium can be used to prevent contamination by organisms that are unable to use urea.

Analysis of the location of genetic variation in PK4

Sequencing identified eight SNPs as potential candidates for the unique characteristics of PK4; among these, it was estimated that two sites have a direct influence on the phenotype, that is, they involve non-synonymous substitution. A previous study reported variants of

Chlamydomonas produced by UV irradiation: one example had mutations at three sites (two substitutions and one insertion) and another had a mutation at one site (a substitution) (Schierenbeck et al. 2015). A gamma-ray variant of the diatom *Nitzschia* was also analyzed in a different study, in which 1,701 genetic mutations were reported (Cheng et al. 2014). Thus, the types of variation can differ markedly and depend on the algae species and the source of mutagen. In this study, re-sequencing was used to perform genetic-deficiency analysis. The function of the protein encoded by the gene at one of the two sites with genetic variation was not clear (9067_t, hypothetical protein), but there was high homology with *Coccomyxa*, *Chlamydomonas* and *Volvox* genes that encode proteins related to the ATP and ADP transporter, suggesting there may be phenotypic variation in the transportation of ATP and ADP associated with this site. For the other site of variation (8741_t), the single-base substitution involved the amino acid Gln being replaced by a stop codon. The affected gene was found in the KEGG metabolic pathway and encodes endo-1,4-beta-mannase. Mannan is one of the polysaccharides present in the cell wall of plant cells; 1,4-beta-mannase hydrolyzes β-1,4-mannosidic linkages of D-mannan at random sites and cuts them (Bien-Cuong et al. 2009). Mannan is a component of the algal cell wall from many species of the genus *Chlorella*, and the composition varies greatly according to species (Takeda 1991). *P. kesssleri,* which was used in this study, has mannan in its cell wall (Takeda 1993), and the resolution of mannan may be controlled in PK4. When the level of endo-1,4-beta-mannase decreases, the level of D-mannose decreases; this affects the metabolic pathway because D-mannose is a starting material for the production of amino sugars and nucleotide sugar metabolism. The relationship between glucose metabolism and lipid accumulation is unclear. However, it is thought that there is a trade-off between the accumulation of starch and lipids (Mizuno et al.

2013; Takeshita et al. 2014). The metabolism of mannose is also involved in the synthesis of glyceraldehyde 3-phosphate, which is a starting material in starch and lipid metabolism.

TABLES AND FIGURES

Table 3–1 Comparison between preceding studies and the results of outdoor mass culture. The experimental results for the same type of bioreactor are provided.

	This study	This study	Přibyl, et al. (2012)	Li, et al. (2013)	Přibyl, et al. (2012)
Strain	*P. kessleri* PK4	*P. kessleri* NIES-2152	*C. vulgaris* CCALA 256	*P. kessleri* CCALA 255	*P. kessleri* CCALA 255
Cultute condition	150 L with dilution	150 L with dilution	150 L with dilution	150L with dilution	Lab-scale with dilution
Biomass (maximum, g/L)	5.8	5.6	5.7	14	6.6
Biomass productivity (g/L/d)	0.82	0.68	0.71	0.64	0.94
Lipid productivity (g/L/d)	0.59	0.26	0.30	0.50	0.48
Lipid contents (maximum %DW)	66	32	31	25	51

Table 3–2 The single-nucleotide polymorphisms identified by gene variation analysis for PK4. The eight single-nucleotide polymorphisms selected for detailed analysis are listed.

SNP No.	Gene id	Region	Scaffold	Position	PK4	WT	Number of reads				Pattern	Substitution	KO number	Annotation
							Supporting reference in Pk4	Supporting variant(s) in Pk4	Supporting reference in WT	Supporting variant(s) in WT				
27	3780_t	exon	scaffold 00014	624615	G	C	0	44	47	0	Synonym	-	-	S-adenosylmethionine-dependent methyltransferase domain-containing protein
42	4990_t	intron	scaffold 00021	332190	C	T	0	47	69	0	-	-	-	Hypothetical protein
62	8038_t	exon	scaffold 00048	228466	T	C	0	37	35	0	Synonym	-	K13211	GC-rich sequence DNA-binding factor
69	8229_t	intron	scaffold 00051	347538	T	A	8	34	64	0	-	-	-	Vacuolar protein sorting-associated protein 18 homolog
70	8229_t	intron	scaffold 00051	347542	G	C	7	33	64	0	-	-	-	Vacuolar protein sorting-associated protein 18 homolog
74	8741_t	exon	scaffold 00057	107506	T	C	0	55	51	0	Nonsynonym	Gln ->stop	K19355	Endo-1,4-beta-mannanase
82	9067_t	exon	scaffold 00062	21293	A	G	3	36	59	0	Nonsynonym	Gly ->Arg	-	Hypothetical protein (ATP/ADP transporter)
145	-	intergenic	scaffold 00328	1418	A	G	1	38	52	0	-	-	-	-

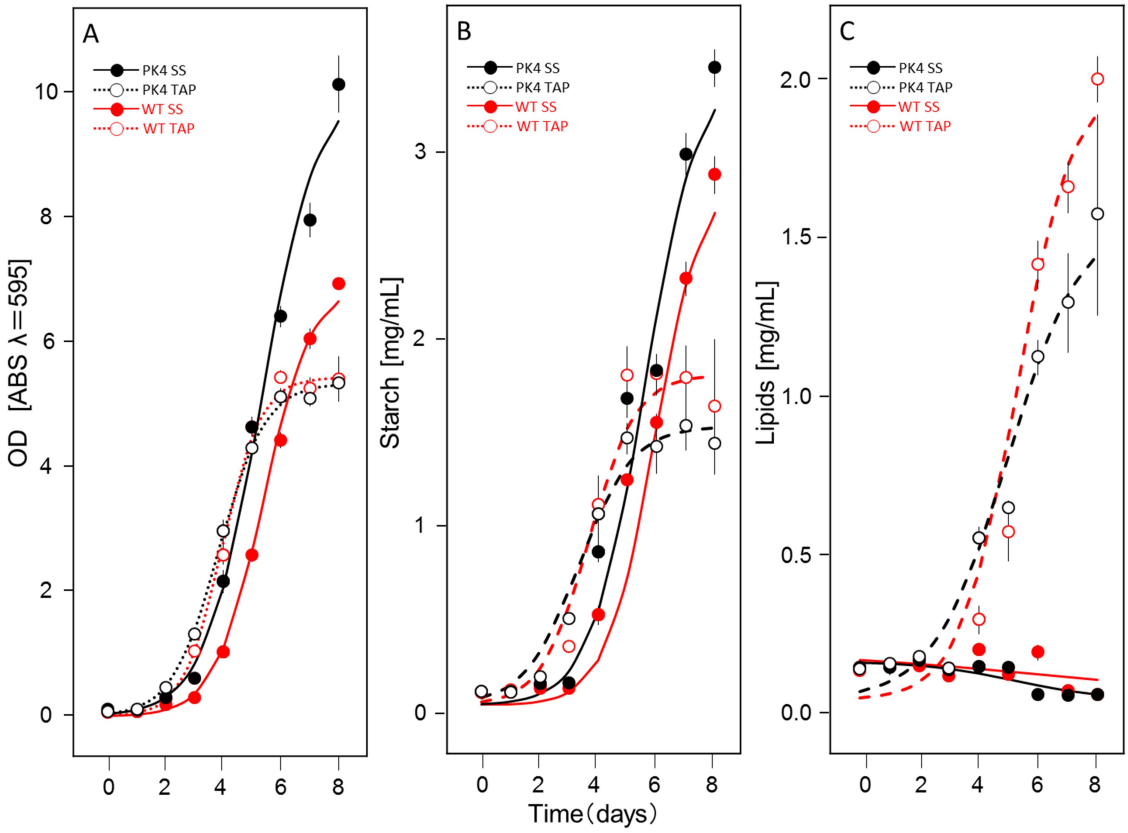

Fig. 3–1. Time-course data on optical density, starch content, and lipid content in Tris–acetate–phosphate (TAP) medium and SS medium. A: Changes in optical density over time. B: Changes in starch content over time. C: Changes in lipid content over time. The data on optical density, starch content, and lipid content were obtained during the culturing of the wild-type (WT) and PK4 in TAP medium and SS medium.

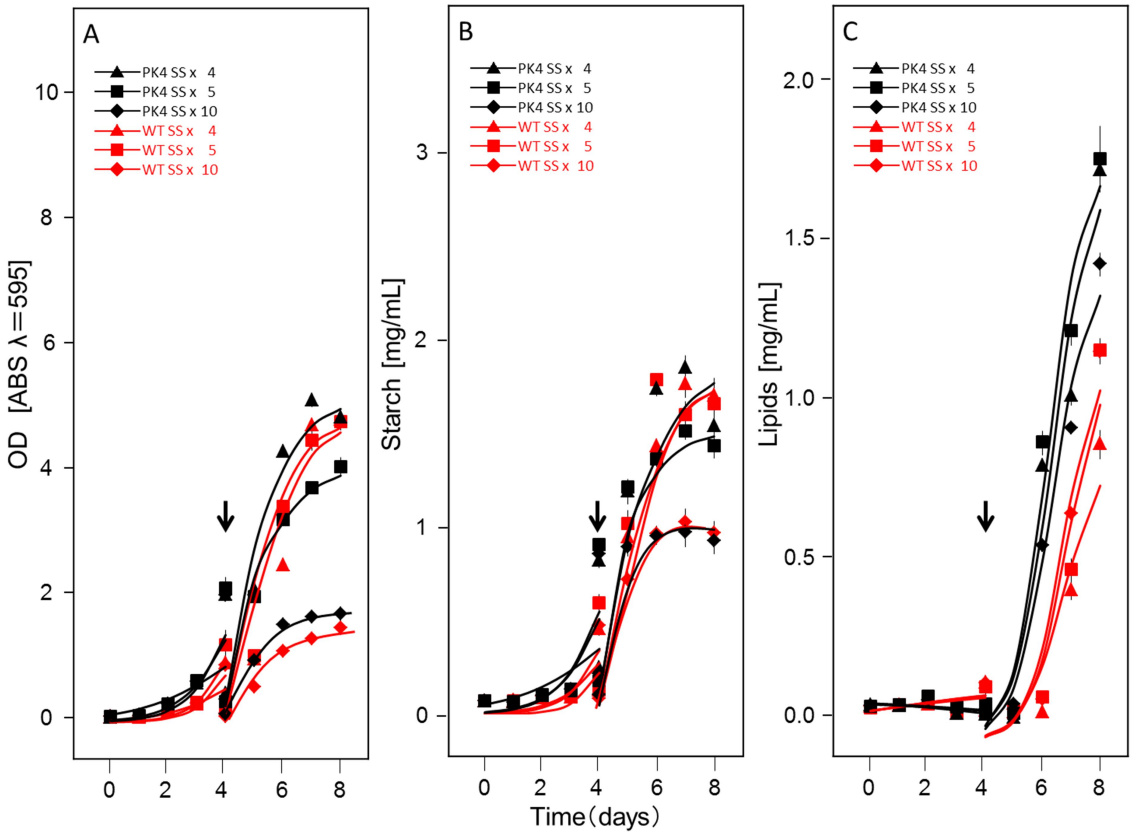

Fig. 3–2. The effect of dilution at different times in SS medium. A: Changes in optical density over time. B: Changes in starch content over time. C: Changes in lipid content over time. The data on optical density, starch content, and lipid content were obtained during the culturing of WT and PK4 strain in SS medium, when the cultures were diluted with sterilized water after a sample of the cultures had been thrown away on day 4.

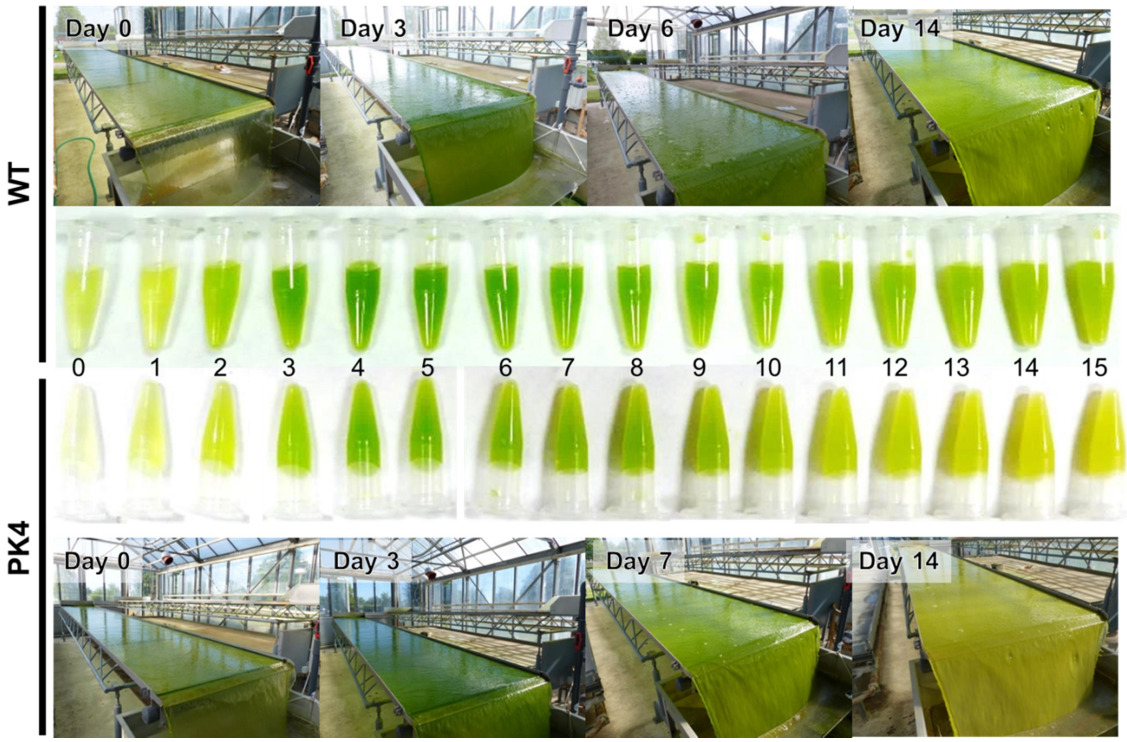

Fig. 3–3. Changes of the outdoor mass cultures of WT and PK4 over time. Upper section: WT; lower section: PK4. During the culturing in a thin-layer photobioreactor, the green thickened maximum on days 4–5 in boss cultures and a culture age advanced, the yellow color of PK4 became stronger than that of WT.

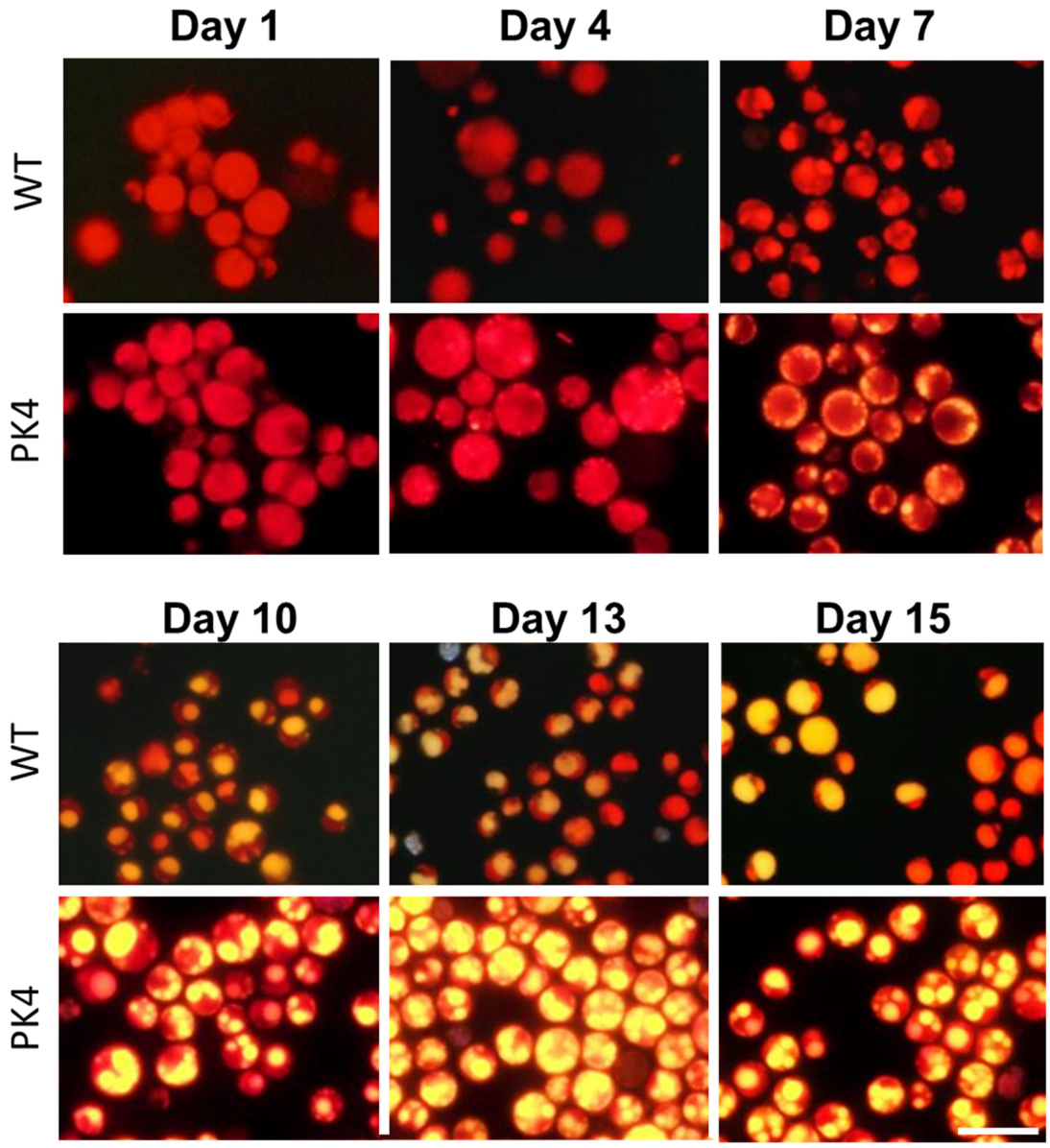

Fig. 3–4. Time course of Nile Red staining in outdoor mass cultures. The details of lipid accumulation in WT and PK4 were indicated by Nile Red staining and observation with a fluorescent microscope. Bar = 10 μm.

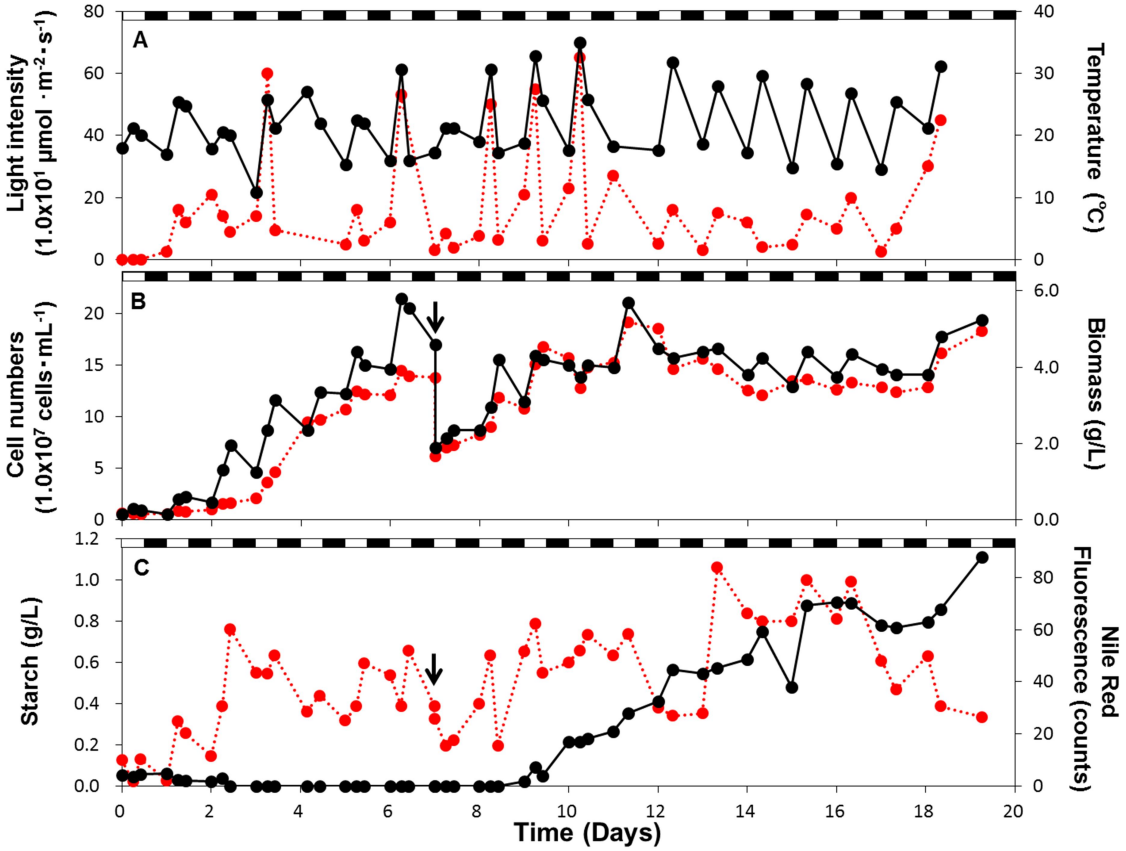

Fig. 3–5. Time-course data for the PK4 strain by the dilution culture method during outdoor mass culturing. A: Black solid line: temperature; red dashed line: light intensity. B: Black solid line: biomass concentration; red dashed line: cell number. C: Black solid line: Nile Red fluorescent intensity; red dashed line: starch content. The results were obtained during outdoor mass culturing using a thin-layer photobioreactor for PK4. The culture was diluted four-fold on day 7 (arrows).

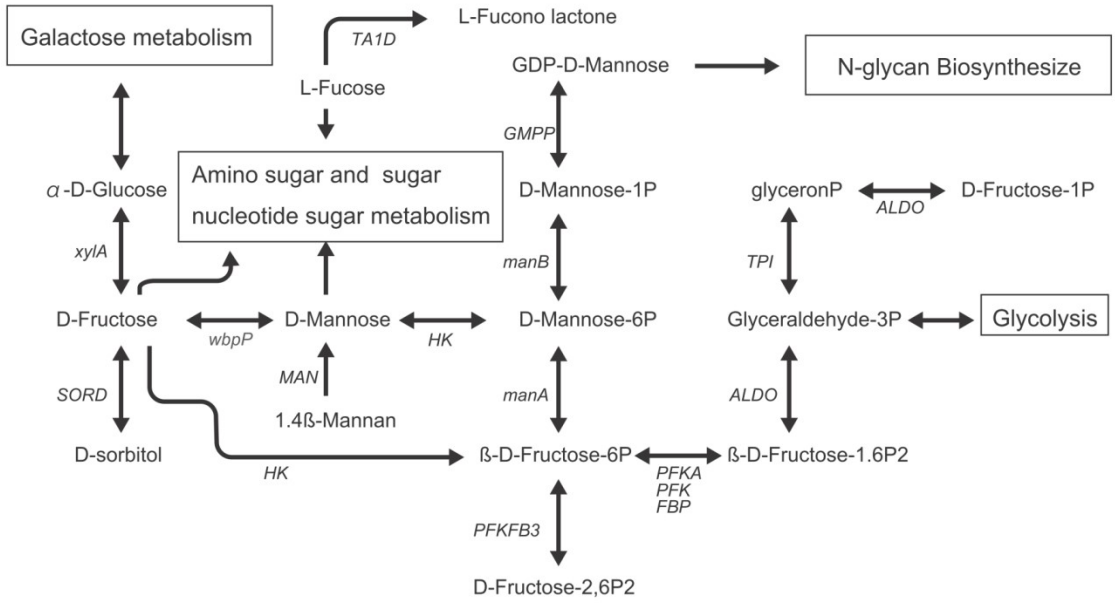

Fig. 3–S1. Metabolism of 1,4-β-mannnan. 1,4-β-mannan is broken down into D-mannose and becomes the starting material for glucose metabolism. Furthermore, it leads to the formation of lipids through glyceraldehyde 3-phosphate.

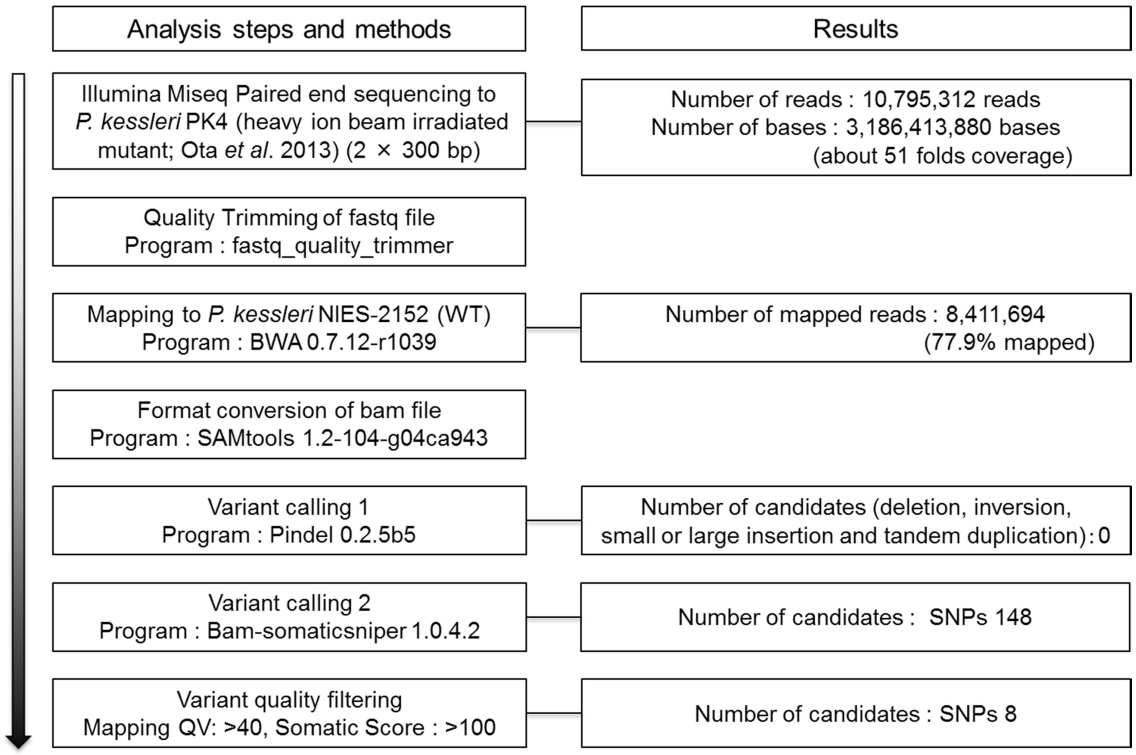

Fig. 3–S2. Flow chart of limiting selections. Analysis of the gene variations and results are shown in a flow chart.

CONCLUSIONS

In this study, I studied on the accumulation of starch and lipids by *Chlorella* under various conditions. I first reported "a simple method for measuring the starch and lipid " in Chapter I.

1. I examined microplate–based quantification of lipids using Nile Red staining and of starch using Lugol staining.
2. Neither starch nor lipids accumulated during the zero phase of cultured *Parachlorella kessleri*, only starch accumulated during the starch phase, and starch was subsequently lost and lipids accumulated during the oil phase.
3. The concentration of starch produced during the starch phase was 0.59 mg mL^{-1} and that of lipids during the oil phase was 2.49 mg mL^{-1}. The concentration of starch produced was 0.05–0.13 mg mL^{-1} during phases other than the starch phase, and lipids were not detected other than during the oil phase because lipid contents were approximated based on the quantity of triacylglycerol, which stains with Nile Red.

I reported "the culture of eight strains of six *Chlorella* species under strong light and aeration conditions" in Chapter II.

4. The eight strains of six *Chlorella* species were attempted to increase starch and lipids productivity under high–light–intensity conditions (600–μmol photons m^{-2} s^{-1}).
5. 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.
6. 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.

7. The accumulation was strain–dependent, and varied according to the medium and light conditions. Five of the eight *Chlorella* strains exhibited similar accumulation patterns.

I reported “increase of lipid production by the dilution culture method upon outdoor cultivation of *Parachlorella kessleri* PK4 and genetic variation of this strain” in Chapter III.

8. Strain PK4, a potentially excellent mutant produced from *P. kessleri* by heavy–ion–beam irradiation, analyzed in this study.
9. The inexpensive SS medium was used and the induction of lipid accumulation was attempted by the dilution culture method. PK4 could be cultured in an outdoor mass culture system and showed lipid accumulation up to 66% of DW and high lipid productivity of 0.82 g L^{-1} day^{-1} upaon culture by the dilution culture method in SS medium.
10. The whole genome of PK4 was sequenced with a next–generation sequencer to clarify the genetic variation upon heavy–ion–beam irradiation by comparison with the WT. Genetic differentiation of PK4 was found at two domains related to endo–1,4–beta–mannase and ATP/ADP transport.

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