1 Regulation of Starch and Lipid Accumulation in a Microalga *Chlorella*
2 *sorokiniana*

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Abstract

Microalgae have attracted growing attention due to their potential in biofuel feedstock production. However, current understanding of the regulatory mechanisms for lipid biosynthesis and storage in microalgae is still limited. This study revealed that the microalga *Chlorella sorokiniana* showed sequential accumulation of starch and lipids. When nitrogen was replete and/or depleted over a short period, starch was the predominant carbon storage form with basal levels of lipid accumulation. After prolonged nitrogen depletion, lipid accumulation increased considerably, which was partially due to starch degradation, as well as the turnover of primary metabolites. Lipid accumulation is also strongly dependent on the linear electron flow of photosynthesis, peaking at lower light intensities. Collectively, this study reveals a relatively clear regulation pattern of starch and lipid accumulation that is basically controlled by nitrogen levels. The mixotrophic growth of *C. sorokiniana* shows promise for biofuel production in terms of lipid accumulation in the final biomass.

Keywords: microalgae; *Chlorella sorokiniana*; starch; lipids.

1. Introduction

Microalgae offer an alternative feedstock for biofuel production due to their high growth rates, high photosynthetic efficiency, and relatively high content of energy-rich molecules. Starch and lipids are two major carbon and energy storage compounds in microalgal cells, particularly under conditions of stress or nutrient starvation. If biofuel feedstock is to be used at a refinery to make diesel, jet fuel or gasoline, lipid is a preferable option due to their higher energy density and lower downstream energy cost from reduced hydrogen requirements (Sivakumar et al., 2010). Therefore, enhancing lipid production in algae has garnered considerable attention.

Ways to increase lipid production have been explored through genetic and metabolic engineering of microalgae and plants. Most efforts have focused on stimulating fatty acid biosynthesis, increasing the availability of glycerol as a backbone of lipids, and overexpressing the genes involved in the triacylglycerol (TAG) biosynthesis pathway (Radakovits et al., 2010). However, these efforts have shown limited success, typically yielding less than a 50% increase in lipid content. Another approach to increasing cellular lipid content is to block starch synthesis pathways. Several studies have reported the overaccumulation of lipid bodies in starch-devoid mutants of *Chlamydomonas reinhardtii* (Li et al., 2010; Wang et al., 2009; Work et al., 2010). However, impairing starch synthesis does not necessarily result in higher lipid content (Siaut et al., 2011). Therefore, the mechanisms involved in rerouting carbon metabolism from carbohydrates to fatty acids are still unknown.

Many studies have demonstrated that a variety of stress conditions such as excessive light, sulfur deprivation and nitrogen deprivation can induce the storage of carbohydrates and/or oil accumulation in microalgae. For example, lipid levels of *Chlorella sorokiniana* under mixotrophic conditions are 2- to 3-fold higher than those under heterotrophic conditions, provided that the same amount of organic carbon is supplied (Li et al., 2014). Starch synthesis shares common carbon precursors with lipid synthesis. However, it is still unclear whether starch or lipids are more likely to be accumulated, and how this accumulation is regulated. More research on carbon partitioning of algal cells into lipids and/or starch would provide key insights for cultivation strategy development and for biofuel production.

This study provides a greater understanding of carbon allocation between starch and lipids and of the regulatory mechanisms controlling this distribution in *C. sorokiniana* in response to altered growth environments. In addition to analyzing the time course of starch and lipid content, quantitative real time (RT)-PCR was used to examine the expression levels of four important genes involved in starch and lipid metabolism: acetyl-CoA carboxylase (ACCase) beta subunit (accD gene), ADP-glucose pyrophosphorylase (AGPase) large subunit (AGP-L gene), β -amylase (BAM gene), and iso-amylase (ISA1 gene). ACCase catalyzes the irreversible conversion of acetyl-CoA to malonyl-CoA in fatty-acid synthesis (Cronan Jr & Waldrop, 2002); AGPase catalyzes the rate-limiting step of starch synthesis from glucose 1-phosphate and ATP to ADP-glucose and pyrophosphate (Ball & Morell, 2003); β -amylase and iso-amylase play critical roles in the hydrolysis of the starch granules that hydrolyze the 1,4- and 1,6-glycosidic bonds, respectively (Zeeman et al.,

2010). This study also included an analysis of metabolites in order to profile the major metabolite accumulation patterns.

Results reveal a nitrogen-level controlled temporal relationship between starch and lipids, with starch accumulated in the nitrogen (N)-replete condition and lipid accumulated in the N-depleted condition. Accumulation of both starch and lipids in mixotrophically-grown cells was found to be strongly dependent on the linear electron flow of photosynthesis, while lipid content peaked at lower light intensities. Understanding the interdependence of starch and lipids as well as their regulation is integral to the metabolic engineering of alga *C. sorokiniana* for improved biofuel productivity. This study also demonstrates that converting previously assimilated carbon in the form of starch and other components would provide carbon/energy for lipid biosynthesis, and suggests that mixotrophically-grown *C. sorokiniana* is a good candidate for lipid-based biofuel feedstock production.

2. Materials and Methods

2.1 Organism and culture conditions

The green microalga *Chlorella sorokiniana* (UTEX 1602) was obtained from UTEX, the Culture Collection of Algae at the University of Texas at Austin (USA). This strain was maintained at 4 °C on an agar slant of the Kuhl medium (Li et al., 2014). In routine experiments, the inocula were cultured at 25 °C in a 250-mL flask containing 150 ml Kuhl medium, agitated with filter-sterilized air enriched with CO₂ to 1% (v/v). Cool-white fluorescent lamps gave a constant light intensity of 100 μmol m⁻² s⁻¹. In experimental cultures, 2% (v/v) exponentially growing seeds were inoculated into each flask, which

101 contained 50-mL Kuhl medium supplemented with glucose and grew at 25 °C on an orbital
102 shaker at 150 rpm. For mixotrophic cultures, illumination was provided; for heterotrophic
103 cultures, flasks were covered with foil.

104 For treatment with the PSII inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU),
105 the compound (in methanol) was added directly to the growth media. Control cells were
106 treated with methanol alone. To inhibit *de novo* protein synthesis, cycloheximide (CHX)
107 was added at the beginning of the experiments. The final concentration in the mineral
108 medium was 1 mg L⁻¹. CHX and DCMU were purchased from Sigma-Aldrich.

109 **2.2 Analytical procedure**

110 To determine the biomass dry weight (DW), a 5 mL cell suspension sample was
111 centrifuged at a speed of 1000 g for 5 min. Cell pellets were washed twice with distilled
112 water and dried in a pre-weighed aluminum dish at 105 °C for 3 hrs. They were
113 subsequently cooled to room temperature in a desiccator before weighing.

114 Glucose concentrations were analyzed by a Dionex ICS-3000 ion chromatography system
115 (Dionex Corporation) equipped with a CarboPac TM PA 20 (4 × 50 mm) analytical column
116 and CarboPac TM PA 20 (3 × 30 mm) guard column. Samples were filtered with 0.2 µm
117 pore-size filter before injection and eluted with 0.01 M NaOH at a flow rate of 0.500 ml
118 min⁻¹. The analytes were detected and quantified against standard curves by
119 electrochemical detection in a pulsed amperometric detector.

Nitrate-nitrogen and lipids were analyzed as described previously (Li et al., 2013). Briefly, nitrogen was determined with the H₂SO₄-salicylic acid method, while the lipids were analyzed with gas chromatography using tridecanoic acid (C13:0) as an internal standard.

For starch analysis, samples of 1-2 mL of the algal suspension were harvested by centrifuging at 3000 g for 5 min. Algal pastes were disintegrated by vortexing with 0.5 mL of glass beads (0.5 mm diameter) in 1 mL of deionized water. The pigments were extracted three times with 5 mL of 80% ethanol for 5 min at 80 °C. After centrifuging, concentrations of starch in the pellets were measured enzymatically with the Sigma Starch Assay Kit (STA 20, Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions.

2.3 Quantitative RT-PCR conditions and analysis

Total RNA was isolated with a Trizol reagent (Ambion) from the cell pellet. One microgram of total RNA was used as a template for each RT-reaction, following manufacturer's instructions (qScript cDNA SuperMix, Quanta Biosciences). Gene-specific primers were designed to amplify fragments of approximately 100–150 bp in length. For the quantification of gene expression, qPCR was carried out on a Mastercycle Realplex 2 (Eppendorf) using the Perfecta Syber Green Fast Mix (Quanta Biosciences, Gaithersburg, MD). The 18S gene served as the internal control for quantification assays. For gene expression analysis by qPCR, the expression values were calculated according to the 2- $\Delta\Delta C_t$ method (for each gene, $\Delta C_t = C_{t, \text{Gene}} - C_{t, \text{housekeeping}}$). The $\Delta\Delta C_t$ calculation was validated using the plot of the log cDNA dilution versus ΔC_t . See Table 1 for all primer sequences used in this work.

2.4 Metabolite analysis

Relative levels of amino acids, organic acids and sugars were determined by gas chromatography- mass spectroscopy (GC-MS). The lyophilized cells were transferred to a 2 mL eppendorf tube and disrupted with a steel ball (5 mm). Then, 0.75 mL of extraction buffer methanol: chloroform: water (5:2:2) was added to each sample and vortexed for 10 min at room temperature. Cell debris was centrifuged at 16,000 g for 2 min. The supernatant was evaporated and spiked with 2 μ L of internal standard mixture solution of methyl esters and C30 linear chain-length fatty acids. Ninety minutes of incubation (5 μ L pyridine methoxyamine hydrochloride, added to each sample) was performed in a thermostatic bath at 30 °C. Samples were derivatized by adding 45 μ L N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA + 1%TMCS, Pierce, Rockford, IL, USA) for 30 min at 37 °C. Time of flight mass spectrometer (Pegasus 4D, LECO, St. Joseph, MI), equipped with an MPS-2 autosampler (Gerstel, Muehlheim, Germany), was used for analysis with an RTX[®]-5Sil MS with Integra-Guard[®] column (30 m $\times$ 0.25 mm ID $\times$ 0.25 μ m film thickness) from Restek (GmbH, Bad Homburg, Germany). The column was held isothermally at 50 °C for 1 min and ramped to at 20 °C min⁻¹ to 330 °C over 5 min. All injections were performed in splitless mode (1 μ L), with helium as a carrier gas at a constant flow of 1 mL min⁻¹. The ChromaTOF software version 4.41 equipped with the LECO/Fiehn Metabolomics database was used for primary metabolite data processing.

2.5 Statistical analysis

All experiments were conducted in triplicates and results are presented as either means of the replicates or means along with standard deviation in graphs.

3. Results and Discussion

3.1 Sequential accumulation of starch and lipids

To gain insight into the relationship between starch and lipid accumulation, the time course of changes in starch and lipids as a result of mixotrophic and heterotrophic growth was monitored. Following inoculation, starch levels in mixotrophic cells increased rapidly during exponential growth, with the maximum content of 27% after 2 d, which then steadily decreased (Fig. 1A). The accumulation of lipids lagged behind the starch, with a rapid increase observed after 3d of cultivation, accompanied by declined starch accumulation. The lipid content continued to increase to 44% by the end of the time course. Similarly, in heterotrophic growth, the maximum accumulation of starch occurred after 2 d of cultivation, and a marked loss of starch was followed by enhanced lipid production (Fig. 1B). However, the maximum starch and lipid content during the time course were 14% and 19%, respectively, which were much less than those observed in the mixotrophic culture.

Levels of the remaining glucose and nitrate in the medium were also quantified. The assimilation of nitrate occurred much faster than that of glucose, which lasted for 1 d for the mixotrophic culture and 2 d for the heterotrophic culture (Fig. 1C). However, glucose was used up in the medium after 3 d in the heterotrophic culture and 4 d in the mixotrophic culture.

The above results show that lipid content in the mixotrophic and heterotrophic cells began to increase as nitrate ran out. Coincidentally, the time in which the lipid level began to increase was exactly when the starch accumulation slowed down or began to decrease.

Therefore, it appears that nitrate level correlates with both starch and lipid accumulation in reverse directions. Li et al. (2011) have reported similar starch and lipid accumulation patterns in the microalga *Pseudochlorococcum*, suggesting that algal cells use starch as a short-term carbon and energy storage product, and that cells shift the carbon partitioning into neutral lipids as a secondary storage product after prolonged nitrogen depletion.

Previous studies demonstrate several different kinds of starch and lipid regulation patterns in microalgae. Both starch and lipid content in *C. sorokiniana* are enhanced with 2% CO₂ as opposed to air (VanderGheynst et al., 2014). A marine microalgae *Tetraselmis subcordiformis* reached 54% of starch immediately following nitrogen deprivation without lipid accumulation (Yao et al., 2012). Takeshita et al. (2014) found a strain-dependent accumulation pattern. For *C. sorokiniana*, starch accumulation was induced and accompanied by decreased lipid accumulation. For other *Chlorella* strains, lipid content increased with decreasing starch content. It has been reported that in *C. reinhardtii* mutant, which lacks cell walls, starch is the dominant sink for carbon storage, and rapid lipid synthesis occurs only when the carbon supply exceeds the capacity of starch synthesis (Fan et al., 2012). Furthermore, a microalga *Chlorella zofingiensis* possesses basal levels of starch and lipids under nitrogen-replete conditions, and large amounts of starch accumulate under nitrogen-starvation conditions. This is followed by increased lipid content with partially degraded starch (Zhu et al., 2014). It suggests that distinct factors are involved in sensing and rapidly responding to the changed environment to adjust starch and lipid accumulation in different alga cells. Future research to identify and characterize the role of these specific regulatory proteins will provide a clear understanding of regulation process,

especially how the switch to nitrogen-depleted medium is indeed sensed and then transduced to large metabolic changes, which enables this aquatic, single-celled organism to survive under large environmental stresses.

3.2 Accumulation of starch and lipids depends on nitrogen status

To further characterize the metabolic changes triggered by nitrogen levels, algal cells were transferred to the nitrogen (N)-free Kuhl medium supplemented with glucose and cultured for 2 d. In N-starved cultures supplemented with 4 g L⁻¹ glucose, there was still an increase in starch content (Fig. 2A); however, starch levels peaked much faster (12 h) and showed much lower values (14%, in the light; 6%, in the dark) than cultures supplemented with nitrogen (Fig. 1A, 1B).

One possible reason for lower starch levels in the N-free medium was probably glucose limitation, yet increasing glucose from 4 to 20 g L⁻¹ did not lead to a significant difference in the maximum level of starch in both dark and light grown cells. After excluding the factor of insufficient carbon availability, another explanation for lower starch levels is nitrogen limitation. It is possible that the limited endogenous nitrogen of algal cells is depleted quickly in N-starved culture conditions, so the protein levels involved in starch synthesis and accumulation was negatively influenced.

Results show that lipid accumulation lagged behind that of starch, and lipid content increased rapidly after a decrease in starch. Under the same light conditions, algal cells supplied with 20 g L⁻¹ glucose in the N-free medium accumulated similar levels of lipids as the N-starved cells with 4 g L⁻¹ glucose (Fig. 2). This result counters the report that lipid

accumulation in N-starved cells depends strictly on increased carbon, and that lipid content increases steadily as acetate increases (Fan et al., 2012). This suggests that algal cells in N-depleted conditions utilize carbon sources differently, and that other factors beyond carbon availability determine the upper limits of lipid accumulation in *C. sorokiniana*.

3.3 The biosynthesis of starch and lipids are both benefitted from active photosynthetic activity

The aforementioned results showed lower levels of starch and lipids in the heterotrophic culture than in the mixotrophic culture. This highlights the significant role played by photosynthesis in the production of energy storage. To determine the role of photosynthetic electron transport in storage product accumulation, the PSII inhibitor DCMU was applied, which inhibits photosynthesis by interrupting the flow of electrons from PSII to plastoquinone (Rippka, 1972).

As shown in Fig. 3, adding DCMU reduced the accumulation of both starch and lipids in the mixotrophic culture significantly after 3 and 6 d, and kept the levels at similar values with the corresponding heterotrophic culture. These results support the notion that the linear electron flow of photosynthesis is an important source of free energy and reductants for the synthesis of starch and lipids in *C. sorokiniana* under mixotrophic conditions. In addition, the refixation of CO₂ through photosynthetic activity has also been proved to be critical for biomass synthesis in mixotrophic cells, which provides higher carbon availability to produce energy storage products (Li et al., 2014). Excess carbon from photosynthesis has been found to partition into the lipid synthesis pathway (Suen et al.,

1987). Therefore, photosynthetic activity also provides a carbon source for lipid accumulation along with energy.

On the other hand, significantly higher lipid production may serve as a protective mechanism in mixotrophic cells. Under optimal growth conditions, large amounts of algal biomass are produced, partly driven by the ATP and NADPH produced from photosynthesis. When cell growth and proliferation is impaired by N limitation, the pool of NADP⁺, the major electron acceptor for photosynthesis, can become depleted as it becomes over-reduced (due to high levels of NADPH build-up). This leads to over-oxidation of the PSII reaction center, causing photo-oxidative damage, reactive oxygen species generation, and overall cellular damage. Since NADPH is consumed in fatty acid biosynthesis, increased lipid production may be a means used by the cell to protect itself from oxidative damage (Hu et al., 2008).

3.4 Lipid biosynthesis does not require high light intensity

To further understand the role of light in the formation of storage reserves, we determined the amounts of starch and lipids in cells starved for N under three light conditions; 0 (dark condition), 50, and 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$. As shown in Fig. 2, N-starved cells grown under light intensities of 50 and 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$ accumulated equal amounts of starch, which were higher than that of N-starved cells grown in the dark condition.

Generally, lipid content showed a slow increase at the beginning of cultivation. This increase was enhanced after starch content peaked under all light conditions (Fig. 2A, 2B). In addition, light exerted a considerable effect on lipid content. As observed for starch, N-

starved cells grown under light conditions showed higher lipid levels than cells grown in the dark. However, the maximum lipid level (28%) was obtained under a light intensity of $50 \mu\text{mol m}^{-2} \text{s}^{-1}$ instead of $100 \mu\text{mol m}^{-2} \text{s}^{-1}$. It should be also noted that under a light intensity of $100 \mu\text{mol m}^{-2} \text{s}^{-1}$, N-starved cells grown for 2 d underwent a drastic decrease in chlorophyll content and eventually died (data not shown). Thus, the degradation of photosynthetic machinery probably intensified cellular damage under higher light intensity and weakened the contribution of photosynthesis to fatty acid production. Therefore, although photosynthetic light reactions are beneficial for lipid accumulation, lipid biosynthesis does not require a high light intensity in the N-starved cells.

3.5 Declining starch levels are mainly attributed to starch catabolism

To determine whether the declining starch level was due to a decrease in starch anabolism or an increase in starch catabolism, the expression levels of three important genes involved in starch metabolism under the mixotrophic growth of *C. sorokiniana* were investigated (Fig. 4). For β -amylase and iso-amylase, the expression levels after 3 d were increased by 40% and 288%, respectively, compared with those after 1 d. The mRNA level of iso-amylase continued to increase after 6 d, which was 9.4-fold higher than that after 1 d. The increased mRNA levels of β -amylase and iso-amylase were consistent with decreased starch content, which suggests that starch degradation occurred. Consistently, the significantly increased levels of glucose and glucose 1-phosphate were indicators of starch degradation after 3 d (Fig. 7). However, transcript levels of AGPase increased after cultivation for 3 and 6 d, which may indicate feedback regulation by increased levels of glucose 1-phosphate and/or indicate enhanced starch synthesis. Even it is considered that

the increased mRNA levels of AGPase indicate enhanced starch synthesis, the starch degradation rate is higher than the starch synthesis rate.

3.6 Lipid synthesis is largely dependent on starch degradation

The aforementioned results demonstrate that starch degradation occurs as lipid levels increases. To determine whether starch degradation facilitates carbon partitioning into the fatty acid synthesis pathway and increases lipid content, CHX was added to the medium. CHX is an antibiotic that inhibits eukaryotic cytoplasmic protein synthesis without significantly reducing protein synthesis in the chloroplast. It is important to note that CHX inhibits the induction of β -amylase mRNAs, which requires *de novo* synthesis of proteins in the cytoplasm (Ohto et al., 1992). In this study, there was no significant difference in starch and lipid content for algal cells with or without CHX after 3 d (Fig. 5). This indicates that CHX had no influence on the synthesis of starch and lipids, which reside largely within the chloroplast. However, algal cultures without CHX showed greatly reduced starch levels after 6 d. At the same time, algal cultures with CHX showed a much smaller decrease, and contained twice the starch of cells without CHX. On the other hand, algal cultures without CHX showed a 1.8-fold increase in lipid content after 6 days, while lipids in algal cultures with CHX did not show an obvious increase.

Thus, the substantially increased lipid levels in algal cells correspond to significantly decreased starch levels. A similar phenomenon has been observed in microalga *Pseudochlorococcum* sp. (Li et al., 2011). The partial inhibition of starch degradation resulted in a decrease of lipid content, indicating the conversion of starch to lipid in *C.*

sorokiniana, which was also supported by major metabolite accumulation patterns (Fig. 7). After 6 days, levels of glucose and glucose 1-phosphate in algal cells significantly increased, reflecting drastic starch degradation (Smith et al., 2005). On the other hand, most fatty acids showed very strong positive values compared to those measured at 1 and 3 d, indicating enhanced lipid synthesis. However, the gene expression level of ACCase dramatically declined when lipid content increased. Similar results have been reported in *Chlamydomonas reinhardtii*, indicating ACCase gene expression was not upregulated during lipid accumulation. It suggests that this enzyme may not be regulated at the transcriptional level (Lv et al., 2013).

Taken together, these results indicates that lipid synthesis is largely dependent on starch degradation, and that *C. sorokiniana* cells show the strategy of rerouting the carbon skeleton from starch to lipids.

3.7 Metabolite profiling in *C. sorokiniana*

In this study, gas chromatography-time of flight mass spectrometry in conjunction with automatic mass spectral deconvolution and database annotation to profile primary metabolites was performed. From a total of 612 unique metabolic peaks positively detected at $s/n > 5$ in at least three extracts, 124 non-redundant metabolites were structurally identified. Principal component analysis (PCA) was used to determine how metabolite abundance patterns relate to the time course of *C. sorokiniana* growth under mixotrophic conditions. The loadings plot from the PCA analysis showed three distinct groups at each time point (Fig. 6A). The group including the 1 d samples was closer to the 3 d samples

than to the 6 d samples. Major metabolite accumulation patterns also reflected this relationship, which accords with differential metabolite profiling within these three time points (Fig. 6B). Levels of most of the metabolites involved in the TCA cycle (organic acids) and amino acids decreased after 6 d and remained suppressed thereafter. However, two central metabolites, malate and glutamine, showed rapid and high accumulation. Elevated levels of malate suggest suppressed respiration in these cells, resulting from a slowing of cell growth in response to N deprivation. Glutamine is known to play a critical role in C/N partitioning and N assimilation in plant cells. Considering the N-depleted conditions in the tested cells after 6 d (Fig. 1C), it appears that the cells actively maintain high levels of glutamine to enable rapid response to changing N levels. A marked decrease in nitrogen-rich compounds essential for CO₂ fixation, such as chlorophyll and enzymes Rubisco, has been observed in nitrogen-starved *Chlamydomonas* cells, and the decreased cellular components are suggested to be recycled for neutral lipid biosynthesis (Fan et al., 2014; Msanne et al., 2012). In this study, increased level of glutamine was accompanied by increased fatty acids, which further indicates the turnover of non-lipid components to lipids under N-deficient condition.

3.8 Comparison of starch and lipid accumulation

Most of studies indicate a competitive relationship between starch and lipid synthesis due to their common precursors. Blocking the starch synthesis pathway results in substantially higher lipid accumulation in *Chlamydomonas* and *Chlorella* (Fan et al., 2012; Ramazanov & Ramazanov, 2006). Therefore, this is a promising technique to enhance oil production in microalgae. However, the complexity of carbon partitioning goes beyond mere competition

between starch and lipid synthesis. Starchless mutants of *C. reinhardtii* cannot over-accumulate lipids (Siaut et al., 2011), show restricted growth rates (Darzins et al., 2010), and exhibit a decrease in overall anabolic processes (Work et al., 2010). The supply of carbon from starch is vital for normal plant growth. In *Arabidopsis*, like most vascular plants, starch plays an important role in the carbohydrate metabolism of the leaf. Glucose produced through photosynthesis during the day is retained mainly in the form of starch granules in the chloroplast. Starch is then degraded at night to provide a constant supply of carbohydrate in the absence of photosynthesis. Mutant *Arabidopsis* plants that cannot synthesize starch or degrade it at night show reduced growth rates under most conditions (Smith & Stitt, 2007). Therefore, the increase in starch content in *C. sorokiniana* during N-replete culturing probably plays an important physiological role and represents a mechanism to increase the synthesis of a primary bioenergy carrier (starch) at the expense of other cellular constituents used for cell division (proteins and nucleic acids).

Compared with lipids, starch synthesis is an efficient means of storing carbon. The synthesis of fatty acids, which are major components of lipids, represents a significant loss of carbon due to the conversion of pyruvate (C3) to acetyl-CoA (C2) by pyruvate dehydrogenase. Furthermore, if we account for the energy of starch and lipids with the active import of CO₂, the ATP and NADPH requirements for starch synthesis are 50% and 45% lower, respectively than those required for TAG (C₅₅H₉₈O₆) synthesis (Subramanian et al., 2013). In addition, glucose can be directly converted into starch without prior conversion to triose-phosphates (C3) (Fettke et al., 2010), which requires only one ATP per glucose.

377 On the other hand, although starch represents a more accessible form of carbon storage for
378 plant cells than fatty acids, the energy recovery from fatty acid oxidation is greater than that
379 of starch oxidation. When fatty acids are oxidized via the β -oxidation pathway and the
380 citric acid cycle, the energy recovery is approximately 6.7 ATP equivalents per carbon for
381 palmitic acid. For glucose oxidation via glycolysis as well as the citric acid cycle, energy
382 recovery is 5 ATP equivalents per carbon (Johnson & Alric, 2013). This higher
383 energy/mass ratio for oil may explain why oils are often a preferred storage reserve in seeds.
384 Namely, smaller and lighter seeds may confer a selective advantage for dissemination.

385 In *C. sorokiniana*, starch as the preferred carbon storage in N-replete condition guarantees
386 the highest efficiency for energy capture. Furthermore, *C. sorokiniana* cells can reroute all
387 the carbon into only one sink (lipids) instead of two sinks (starch and lipids) in the N-
388 depleted condition. The straight substitution of lipids for starch in the final biomass offers
389 an effective strategy for energy accumulation. By degrading starch in the chloroplast, algal
390 cells generate more physical space and have a higher capacity to host lipid bodies. This has
391 been shown to relate to increased lipid levels in starchless mutants of *C. reinhardtii*
392 (Goodson et al., 2011). Therefore, it is not necessary to build engineered strains of *C.*
393 *sorokiniana* with blocked starch synthesis pathway to increase lipid content, which mostly
394 accompanied by decreased growth.

395 To determine the oil-producing ability of microalgae, both lipid content and biomass
396 production should be considered simultaneously. Mixotrophic growth of *C. sorokiniana* as
397 a promising lipid production strategy only requires optimizing the cultivation conditions. In

addition, engineering this strain by disrupting lipid catabolism shows promise. Most studies of this strategy focused on higher plants and yeast, and their limited success may have been due to the requirement of cellular energy from fatty-acid oxidation for proper seedling development (Fulda et al., 2004). However, the microalga *Thalassiosira pseudonana* showed greatly increased lipid content without compromising growth through the knock-out of lipase genes involved in lipid catabolism (Trentacoste et al., 2013).

4. Conclusions

This study reveals the dynamic carbon partitioning that occurs between starch and lipids during cell growth of *C. sorokiniana*. This species uses starch as a primary carbon and energy storage compound under nitrogen-replete conditions, and lipids become major storage products accompanied by starch degradation as nitrogen is depleted. Starch degradation is necessary for partial lipid accumulation under nitrogen-depleted conditions. The distinct accumulation pattern of starch and lipids, high growth rate, low light intensity requirement for lipid accumulation, along with high lipid content, all point to the promise of mixotrophic cultivation of *C. sorokiniana* as an efficient production strategy.

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523 **Figure legends**

524 **Figure 1.** Mixotrophic and heterotrophic cultivation of *C. sorokiniana*. (A) Time course of
525 DW, lipid, and starch in mixotrophic culture; (B) Time course of DW, lipid, and starch in
526 heterotrophic culture; (C) Time course of glucose and KNO₃ in mixotrophic and
527 heterotrophic culture.

528 **Figure 2.** Time course of lipid and starch accumulation in *C. sorokiniana* in response to N
529 starvation. Cells were first grown in Kuhl medium until the exponential phase, and then
530 switched to N-free Kuhl medium supplemented with 4 g L⁻¹ (A) or 20 g L⁻¹ glucose (B) and
531 further cultured for 2 d. Lipid (closed symbols) and starch (open symbols) content was
532 monitored under light intensities (μmol m⁻² s⁻¹) of 0 (down-triangle), 50 (square), and 100
533 (circle).

534 **Figure 3.** Effects of DCMU on changes of starch and lipid content in mixotrophic growth
535 of *C. sorokiniana*.

536 **Figure 4.** RT-PCR results of critical enzymes involved in the synthesis of starch and lipid
537 and in the degradation of starch.

538 **Figure 5.** Effects of cycloheximide (CHX, 1 mg L⁻¹) on changes of starch and lipid content
539 in mixotrophic growth of *C. sorokiniana*.

540 **Figure 6.** Metabolite profiling in mixotrophic cells of *C. sorokiniana*. (A) Principal
541 component analysis illustrates differences in metabolite levels among the three time points
542 (1, 3 and 6 d). (B) Primary metabolite profiles at three time points (1, 3 and 6 d).

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

559

Figure 6

563 **Tables**

564 **Table 1.** Primers for quantitative real-time PCR detection of expression genes in *C.*

565 *sorokiniana*

Gene	Primer sequence (5'→3')	Length (bp) of production
accD	F: ACGCCACTCAAGAAGGCTTA	100
	R: GAAATTTTTTGCCATTTGCATT	
AGP-L	F: CCATGAGCAACTGCATCAAC	74
	R: GGTTGAGCGAGGTGGAGTT	
BAM	F: GTACCCGTCCTACCCAGAGG	72
	R: TGTCGTAGCACTGGAACTGG	
ISA1	F: CCACCGCCTCTGTCAACT	100
	R: CGTTGGCCTCATTGTGCT	
18S	F: ATCAACCTGACAAGGCAACC	150
	R: CCTGCGGCTTAATTTGACTC	

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