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ARTICLE

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The Impact of Elevated CO_2 Concentration on the Quality of Algal Starch as a Potential Biofuel Feedstock

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ABSTRACT: Cultured microalgae are viewed as important producers of lipids and polysaccharides, both of which are precursor molecules for the production of biofuels. This study addressed the impact of elevated carbon dioxide (CO_2) on *Chlorella sorokiniana* production of starch and on several properties of the starch produced. The production of *C. sorokiniana* biomass, lipid and starch were enhanced when cultures were supplied with 2% CO_2. Starch granules from algae grown in ambient air and 2% CO_2 were analyzed by transmission electron microscopy (TEM) and scanning electron microscopy (SEM). The granules from algae grown in 2% CO_2 were disk-shaped and contained mainly stromal starch; granules from cultures grown in ambient air were cup-shaped with primarily pyrenoid starch. The granules from cells grown in 2% CO_2 had a higher proportion of the accumulated starch as the highly branched, amylopectin glucan than did granules from cells grown in air. The rate of hydrolysis of starch from 2% CO_2-grown cells was 1.25 times greater than that from air-grown cells and 2–11 times higher than the rates of hydrolysis of starches from cereal grains. These data indicate that culturing *C. sorokiniana* in elevated CO_2 not only increases biomass yield but also improves the structure and composition of starch granules for use in biofuel generation. These modifications in culture conditions increase the hydrolysis efficiency of the starch hydrolysis, thus providing potentially important gains for biofuel production.

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Introduction

Microalgae are attractive candidates for alternative energy feedstocks because of the high efficiency by which they convert CO_2 to lipid and polysaccharide. Algal lipid is usually the primary target for generating biofuels; however, in many algal species studied, starch also accumulates, and biofuels from both lipids and starch will influence the potential of microalgae for bioenergy production (Radakovits et al., 2010). It has been suggested that emissions of otherwise harmful industrial CO_2 might be harvested and delivered to algae for generating biofuels as part of a strategy that reduces the rate of CO_2 accumulation in the atmosphere (Gao and McKinley, 1994; Kadam, 1997; Patil et al., 2008). If algal storage molecules are to be a part of the future energy picture, it will be important to understand the impact of the changed CO_2 environment on biofuel feedstocks.

This report focuses primarily on microalgal starch production. Starch is an osmotically inert, semi-crystalline polymer of glucose that is readily digested to its constituent glucoses (Smith, 2008). Current bioethanol production in the US uses maize starch as a feedstock (Smith, 2008) but microalgal-derived starch may be more economical than that from terrestrial plants (Dragone et al., 2010). Some studies have examined the impacts of cell culture conditions on synthesis and accumulation of starch by algae (Brányiková et al., 2011; Dragone et al., 2011). In addition to changes in the quantity of starch produced by algae, there also is interest in how the culture environment affects starch characteristics that impact the efficiency of starch hydrolysis (Dragone et al., 2010). Many highly interacting factors determine the rate and extent of breakdown of starch granules by hydrolytic enzymes including starch granule size and morphology, crystalline structure, the ratio of amylose to amylopectin, and

the association of starch granules with other cellular molecules such as lipids and proteins (Svihus et al., 2005).

Hydrolysis of starch polymers to glucose is an important step in the production of bioethanol and other biofuels (Dragone et al., 2010). Average granule size, size distribution and morphology affect the specific surface area upon which amylolytic enzymes act; generally the rate of starch hydrolysis decreases as granule volume increases (MacGregor and Morgan, 1980; Stevnebø et al., 2006). The starch granule amylose: amylopectin ratio is important to consider if algal starch is to be used for industrial processes. Although there are exceptions, the hydrolysis of native, amylose-rich starches proceeds at a slower rate than those with more amylopectin (Tester et al., 2006). Because of the interconnectivity of the structural and compositional features of starch mentioned previously, ascribing differences in hydrolysis rate to specific factors remains uncertain (Tester et al., 2006).

While several studies have highlighted the importance of CO_2 supplementation on large-scale production of algae, there is limited information about how the starch accumulation and granule morphology, size and composition are affected if algae are grown in an elevated CO_2 environment. Here we report on the impact of elevated CO_2 on these particular granule characteristics. We determined whether the properties of the granules from algae grown in ambient air versus in elevated CO_2 affect the rate of starch hydrolysis. The use of high CO_2 in cultures of algal feedstocks could affect the utility of algal starch in biofuel generation and provide a means of recycling CO_2 and slowing the increase in atmospheric CO_2.

Materials and Methods

Algal Strain and Growth Condition

Chlorella sorokiniana (UTEX2805) was purchased from the Culture Collection of Algae at the University of Texas (UTEX, Austin, TX). The cells were cultured autotrophically in inorganic N8 medium (Mandalam and Palsson, 1998; Vonshak, 1986). Fresh cultures were initiated by adding a sufficient volume of cells from a settled stock culture in an exponential growth phase to give an OD_{550} of 0.250, indicating $5–6 \times 10^6$ cells/mL. Cells were grown in 250 mL cylindrical glass tubes that were submerged vertically in a water-filled rectangular glass aquarium tank to minimize temperature fluctuations from 25°C. Magnetic stirrers placed below the tank kept the cultured cells uniformly distributed in the tubes. Cultures were aerated at 250 mL/min via 3.18 mm ID tubing connected to a luer lock fitting at the cylinder's top and then extended to the cylinder's bottom. Cultures were aerated with ambient air (0.038% CO_2) or 2% ("high") CO_2. The bubbling of gas also contributed to the mixing of the cells and medium in the tubes. Cultures were irradiated with fluorescent lights at ~10,000 Lux, with 16 h of light and 8 h of dark per day. Cultures were harvested at four phases of growth (late lag, middle logarithmic, late log and early senescence phases; days 2, 4, 6, and 8 of culturing, respectively) by centrifugation at 5,000×*g*.

Lipid and Starch Extraction

Total lipids were extracted from freeze-dried *C. sorokiniana* cells, as described in Cheng et al. (2011a). Briefly, 1.5 mL of Folch solvent (a 2:1 (v/v) mixture of chloroform and methanol) was added to 15–20 mg of freeze-dried algae with 0.75 mL Zirconia/Silica beads (0.5 mm, Biospec Products, Inc., Bartlesville, OK). Cells were disrupted by bead beating (Fastprep System 101, MP Biomedicals LLC, Solon, OH) at 6.5 m/s for 20 s, for 6 cycles with 30 s intervals on ice. Phase separation was achieved by adding 1.2 mL 0.9% $NaCl_2$ (w/v), vortexing and centrifuging at 6,000×*g* for 5 min. The lower phase (chloroform and lipid) was used for total lipid analysis. The pellets remaining after lipid extraction contain starch and cell wall polysaccharides (S/CW). The S/CW samples were washed three times each with 1 mL acetone and water. The pellets were suspended in 1 mL of sterilized water and the starch granules were gelatinized by heating at 80°C for 30 min. One milliliter of a starch hydrolyzing mixture of enzymes (6U of amyloglucosidase [Roche Applied Science, Indianapolis, IN] and 15U of α-amylase [MP Biomedicals] in 100 mM sodium acetate buffer [pH 5] containing 0.04% NaN_3, w/v) was added and incubated at 37°C with shaking at 150 rpm (Steadyshake 757 Benchtop Incubator Shaker, Amerex Instruments, Inc., Lafayette, CA) overnight. The incubated samples were then centrifuged at 12,000×*g* for 10 min to pellet insoluble cell wall residues and the supernatants (starch digests, SD) were collected for starch content analysis.

Lipid Analysis

Total lipid determination was carried out with the colorimetric method described by Cheng et al. (2011b), with some modifications. Briefly, 30 μL of lipid extraction samples (above) were loaded into the 96-well plate in triplicate along with corn oil standards that contained 5–120 μg lipids. Chloroform was evaporated off at 90°C. One hundred microliter of concentrated sulfuric acid was added to each well and mixed thoroughly. The plate was incubated at 90°C for 20 min and then cooled to room temperature. Background absorbance was read at 540 nm using a microplate reader (Softmax v 2.43, Vmax, Molecular Device, Sunnyvale, CA). Then, 50 μL of vanillin-phosphoric acid reagent (0.2 mg vanillin/mL 17% (v/v) phosphoric acid) was added to each well and mixed. After incubation at room temperature for 10 min the absorbance was again measured at 540 nm. The net absorbance was calculated by subtracting the corresponding background absorbance.

Starch Content Analysis

The amount of starch in the SD samples was determined using the phenol-sulfuric acid method adapted to a 96-well

microplate, according to Masuko et al. (2005). Briefly, 150 μl of concentrated sulfuric acid was added to 50 μl of each SD sample in a 96-well microplate. After thorough mixing, 30 μl of 5% phenol (w/w, in water) was added and mixed in. After incubation for 5 min at 90°C and allowing the samples to cool, the absorbance at 490 nm of each well was measured. Glucose, at concentrations 0.05–0.5 mg/mL, was used as the standard for the measurement of carbohydrate in the SD samples. The starch hydrolysis enzyme mixture was used as a blank. All tests were performed in triplicate.

Purification of Starch Granules

Starch granules were prepared from algal cells grown in ambient and elevated CO_2 at different growth phases, as described by Delrue et al. (1992), with some modifications. Cells were harvested and suspended in lysis buffer (20 mM Tris-HCl [pH 7.5], 5 mM EDTA, 1 mM dithiothreitol) and then disrupted using a bead-beater. The disrupted cells were then centrifuged at 10,000×*g* for 20 min. The pellets (starch granules and cell fragments) were resuspended in cold sterile water and centrifuged twice through a Percoll gradient at 10,000×*g* for 20 min to separate the high density starch granules from the lower density cell debris. The purified starch pellets were then washed two times with sterile water and two times with acetone and allowed to dry in a fume hood overnight.

Starch Granule Size Analysis

Purified starch granule size measurement was performed on three biological replicates from high and ambient CO_2 cultures at day 4 of culturing by using an LS 200 laser diffraction Particle Size Analyzer (Beckman Coulter). The frequencies of detection of granules of different sizes were recorded.

Scanning Electron Microscopy of Starch Granules

Isolated starch granules were washed two times with distilled water and acetone, and then dried at room temperature. The dried starch samples were dusted onto carbon double stick discs and these then were mounted on stubs and coated with gold. The morphologies of the starch granule samples were examined under a scanning electron microscope (model XL 30 SFEG; Phillips Electronics NV, Eindhoven, The Netherlands).

Transmission Electron Microscopy of Algal Cells

Cells from high and ambient CO_2 at day 4 of culturing were fixed in Karnovsky's fixative using a microwave, as described by Russin and Trivett (2001). Samples were then post-fixed with 1% (w/v) osmium tetroxide for 2 h. The samples were dehydrated rapidly through an alcohol series (70–100% v/v) alcohols containing 2% (w/v) uranyl acetate). Dehydration was continued with 1:1 and then 1:3 ethanol:propylene oxide, 10 min for each step. Pure epoxy resin (Epon/Araldite) replaced the final ethanol step and was infiltrated overnight (Heumann, 1992). Cells were then embedded and cut with a diamond knife (Diatome, Switzerland, EMS USA distributor). Cells were viewed and images were taken using a Philips CM120 Biotwin TEM (FEI Company, Hillsboro, OR). Cell preparation and imaging were completed in the Electron Microscopy Laboratory at the Department of Pathology and Laboratory Medicine, School of Medicine, University of California at Davis.

Amylose/Amylopectin Ratio

The amylose and amylopectin contents of *C. sorokiniana* starch were determined with three biological replicates of starch granules purified from high CO_2- and ambient air-cultured cells harvested at day 4 of culturing using an assay kit (K-AMYL Kit, Megazyme, Ireland), according to the manufacturer's instructions.

Enzymatic Hydrolysis of Algal and Various Cereal Grain Starches

Algal starch hydrolysis measurements were performed on three biological replicates of purified starch granules from high and ambient CO_2-cultures at day 4 of culturing. Purified maize, rice and wheat starches were purchased from Megazyme. Starch samples (5 mg) were digested with α-amylase isolated from *Aspergillus niger* (MP Biomedicals) at a final concentration of 50 U/mg starch. The 1 mL digestion volume contained 250 U of enzyme in 100 mM sodium acetate buffer (pH 5) containing 0.04% (w/v) NaN_3. The reaction mixture was incubated at 37°C with shaking at 150 rpm. Aliquots of the incubation mixture (0.1 mL) were taken every 30 min from 0 to 3 h and centrifuged at 15,000×*g* for 5 min. The supernatant was incubated at 90°C for 5 min to inactivate the enzyme and the reducing sugar was determined using the dinitrosalicylic acid reagent, as described by Doehlert et al. (1982). A substrate control (buffer replacing enzyme) was subtracted from the experimental values and glucose concentrations 0.5–5 mg/mL were used as the standard to construct a calibration curve.

Results and Discussion

The Effect of CO_2 Concentration on Cell Growth and Biomass Productivity

The rate of growth of *C. sorokiniana* was greater when cultures were grown in elevated (2%) CO_2 compared to ambient air. The cultures in elevated CO_2 reached the stationary phase after day 5. Air-grown cultures did not reach the 5-day cellular mass of the high CO_2-grown cultures until day 7 and reached the stationary phase after 8 days (Fig. 1A). Biomass productivity, that is the accumulation in cell mass (expressed as mg increase in biomass per liter of culture per day) was significantly greater in the high CO_2-grown cultures than in the air-grown cultures throughout the culture cycle. Under

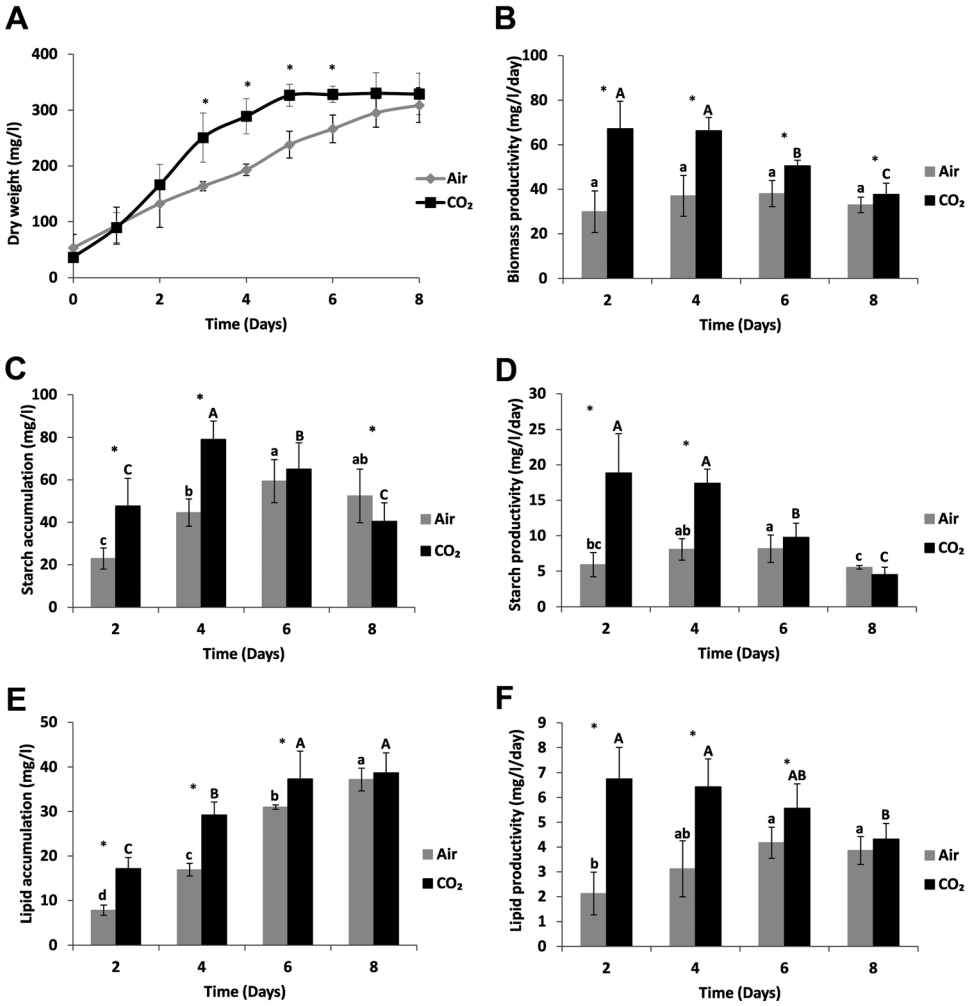

Figure 1. Dry weight, starch, and lipid accumulation (**A**, **C**, and **E**) and biomass, starch, and lipid productivity (**B**, **D**, and **F**) of *C. sorokiniana* grown in 2% CO_2 and air. Values are shown as the means ± SD of three independent replicates. *Statistically significant difference between CO_2 and air at $P \leq 0.05$. Different letters indicate statistically significant differences between sampling times (ANOVA-Tukey test, $P \leq 0.05$).

high CO_2, biomass productivity was highest at days 2 and 4 (67 mg/L/day), and decreased thereafter. Under ambient air, biomass productivity was similar at all stages of the culture period (Fig. 1B). de Morais and Costa (2007) reported that biomass productivity of *Chlorella kessleri* was 87 mg/L/day at 6% CO_2. However, Chiu et al. (2009) found that the biomass productivity of *Nannochloropsis oculata* NCTU-3 cultured in 2% CO_2 was 480 mg/L/day. They used a semi-continuous system in which fresh culture medium was replaced every 24 or 72 h. The lower productivity of *C. sorokiniana* cultures may have been due to the differences in algal strains and the efficiency of the heterotrophic semi-continuous system used.

The Effect of CO_2 Concentration on Lipid and Starch Accumulation and Productivity

The accumulation of starch by *C. sorokiniana* grown in 2% CO_2 was twice that of cells grown in air at day 2 of sampling (Fig. 1C). Cells grown in 2% CO_2 accumulated starch at the beginning of the cell cycle reaching about 80 mg/L at day 4 of culturing; the starch levels began to decrease near the end of the culture period. In contrast, when grown in air, *C. sorokiniana* cells slowly accumulated starch, which reached the highest concentration at day 6 (60 mg/L). No difference in starch accumulation was observed between air and high CO_2 grown cells at day 6. However, after 8 days of growth in ambient CO_2, the accumulation of starch was significantly higher than when the cells were grown in high CO_2 (Fig. 1C). Izumo et al. (2007) estimated that the amount of starch per cell of *Chlorella kessleri* 11 h from ambient CO_2 cells was higher than that of cells grown in high CO_2; however, the starch accumulation per milliliter culture was similar between the two CO_2 environments. This conclusion contrasts with our study, which found that the amount of starch for 2% CO_2-grown *C. sorokiniana* cells was higher than that of cells grown in ambient air (supplemental data). In a subsequent study,

Izumo et al. (2011) reported that when *Chlamydomonas reinhardtii* 137C was grown under 5% CO_2 or under 5% CO_2 until the log phase and then transferred to ambient air for 6 h the cells accumulated 4.2 mg starch/L at the end of 6 h in air. In contrast, cells cultured continuously in 5% CO_2 had 5.3 mg starch/L. Both of these levels were ca. 10% of what we have observed in cultures of *C. sorokiniana*.

Productivity also varied between CO_2 treatments. Cells grown in elevated CO_2 had the greatest starch productivity (18.85 mg/L/day) at day 2. However, the maximum productivity (8.16 mg/L/day) of cells grown in air was observed at day 6 (Fig. 1D). Ho et al. (2012) performed additional analyses of data presented by Sydney et al. (2010) and reported starch productivities of 4.93, 16, 20, and 21.65 mg/L/day for *Botryococcus braunii* SAG-30.81, *Spirulina platensis* LEB-52, *Dunaliella tertiolecta* SAG-13.86 and *Chlorella vulgaris* LEB-104, respectively. This suggests that starch productivity of *C. sorokiniana* is less like that of *B. braunii* and more like that of the other species studied by Sydney et al. (2010), but the difference in "high" levels of CO_2 in that study and ours leaves some uncertainty in this comparison.

The trends in lipid accumulation for air- and 2% CO_2-grown cultures paralleled those observed for starch accumulation. Cultures showed a steady increase in lipid accumulation over the entire 8-day culture period (Fig. 1E). Lipid accumulation in the 2% CO_2- and air-grown cells reached similar maximum levels (38 and 37 mg/L, respectively) at day 8. Like the accumulation of starch, lipid accumulation and productivity were significantly greater in 2% CO_2-grown cells than in air-grown cells through the first 6 days of the culture period (Fig. 1E and F). The highest lipid productivity (6.75 mg/L/day) was achieved in cells grown in elevated CO_2 at day 2, but the maximum lipid productivity in air-grown cultures (4.17 mg/L/day) was reached at day 6 (Fig. 1F). Lipid productivity of *C. sorokiniana* when grown in air was similar to that reported for air-grown *C. vulgaris* (4.5 mg/L/day) (Widjaja et al., 2009). However, they reported lipid productivities under elevated CO_2 of 9.5 and 13.3 mg/L/day at 0.33 and 0.83% CO_2, respectively, values 1.5–2 times greater than what we observed for 2% CO_2-grown *C. sorokiniana* cultures. The lipid and starch productivities observed in our study may differ from other reports because of differences between the strains of algae studied and because CO_2 levels used in those studies were 15–30% of the levels we investigated. Differences in the metabolic capacities of algae to adapt to different levels of elevated CO_2 during growth in culture have been observed. For example, Salih (2011) reported differences in the tolerance of microalgal strains to increased CO_2: maximum levels tolerated were 15% for *Chlamydomonas* sp., 40% for *Chlorella* sp. and 100% for *Cyanidium caldarium*. These differences suggest that maximum starch and lipid productivities may be constrained by both CO_2 concentration and the tolerance of microalgae of elevated CO_2. In addition, a metabolic trade-off between starch and lipid productivity may occur when the concentration of CO_2 is modified in cultures (Cheng et al., 2013); the accumulation of starch reached 18.85 mg/L/day in this study while lipid reached 6.75 mg/L/day. Although *C. sorokiniana* cultures yielded moderate starch and lipid productivities in our hands, this species may be used in other situations and could be affected by environmental factors not considered in our work. For instance, de-Bashan et al. (2008) reported that *C. sorokiniana* used for water bioremediation grew faster and accumulated more ammonium when co-cultivated with the bacterium *Azospirillum brasilense* and cells were maintained under high temperature (40–42°C) and light intensity (2,500 μmol/m^2/s) for 5 h daily than when cells were maintained under moderate conditions (28°C and 60 μmol/m^2/s). These results suggest that the cell production environment must also be considered when comparing the accumulation of lipid and starch by *C. sorokiniana*.

Algal Starch Granule Size

Starch granules were purified from cells collected during the middle-log phase of culturing (4 days). The size distributions of the starch granules were determined by laser diffraction particle size analysis (Table I) and granule morphologies were examined by SEM (Fig. 2). There were significant differences in the distribution of granule sizes for cells grown in air and 2% CO_2. Cells grown in air had few small granules and more granules in the larger size classes; in contrast, granules from cells grown in 2% CO_2 were mostly small in size. The average granule size from cells grown in 2% CO_2 was 0.96 μm, significantly smaller than that of granules from air-grown cells (1.27 μm) (Table I). This result superficially agrees with granule size data reported for *C. kessleri* 11 h in which cells grown in 3% CO_2 were shifted to ambient CO_2 and starch granule size increased (Izumo et al., 2007); the culture growth conditions and history were not precisely the same in our study and theirs.

Table I. Sizes and amylose contents of starch granules purified from cells at day 4 of culturing under air or 2% CO_2.

Condition	Average granule size (μm)	% of smallest granules (<0.7 μm)	% of mid-size granules (0.7–1.4 μm)	% of large granules (1.4–2.1 μm)	% of very large granules (>2.1 μm)	Amylose content (%)
Air	1.27 ± 0.12^a	51.2 ± 2.85^b	42.57 ± 2.60^a	4.50 ± 0.51^a	1.07 ± 0.33^a	28.99 ± 0.95^a
2% CO_2	0.96 ± 0.09^b	62.85 ± 0.39^a	34.64 ± 0.15^b	2.08 ± 0.28^b	0.42 ± 0.25^b	23.40 ± 0.75^b

Values are mean ± standard errors of three different biological replicates. Means with different letters within the same column show statistically significant differences ($P \leq 0.05$) by Tukey's test ($n = 3$) while means with the same letter do not.

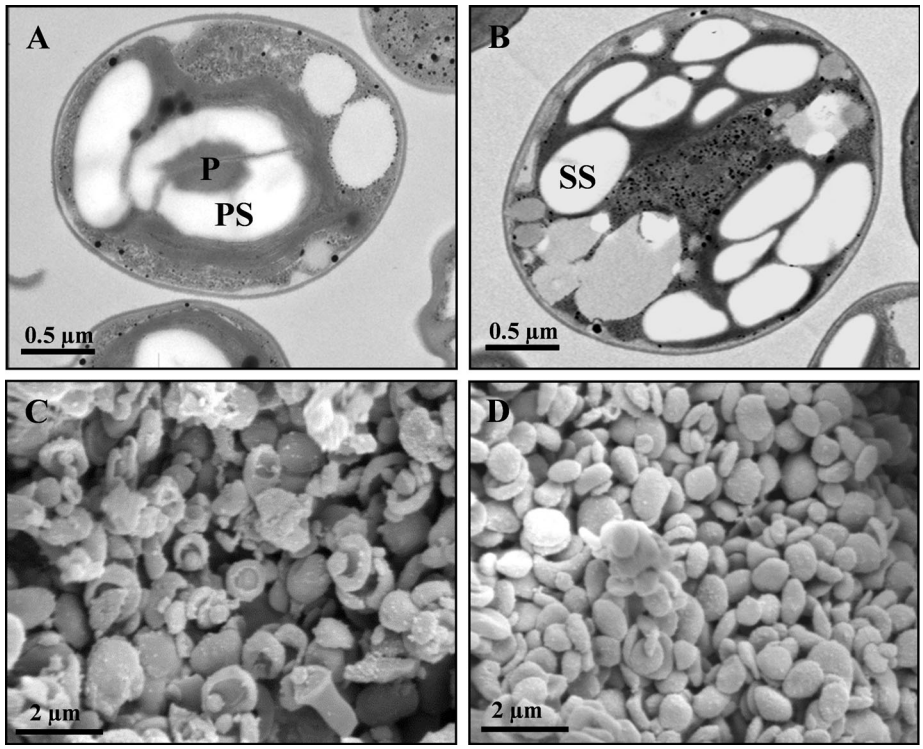

Figure 2. TEM observation of *C. sorokiniana* cells at 4 days of culture under air and 2% CO_2 (**A** and **B**, respectively). SEM observation of *C. sorokiniana* starch granules isolated at 4 days of culture under air and 2% CO_2 (**C** and **D**, respectively). The bars represent 0.5 and 2 m, respectively. P, pyrenoid; PS, pyrenoid starch; and SS, stromal starch.

Algal Starch Amylose Content and Morphology

The ratio of amylose and amylopectin in starch granules is a characteristic that has important impacts on options for the end uses of granules derived from higher plants and algae. Therefore, the relative amounts of amylose and amylopectin in the starch granules purified from *C. sorokiniana* cells grown in air and 2% CO_2 were determined. The amylose (i.e., unbranched α-1,4-glucan) contents of *C. sorokiniana* algal starches are shown in Table I. Granules from cells grown under 2% CO_2 showed significantly lower amylose content than did granules from cells grown in air. This may be related to the difference in granule size and morphology of cells from the two culture conditions. Blanshard et al. (1991) reported that amylose is preferably accumulated in the amorphous zones of starch granules and there is often a positive relationship between amylose content and granule size; however, this relationship is not absolute (MacGregor and Morgan, 1980). For example, Stevnebø et al. (2006) showed that high- and normal-amylose barley varieties had a higher proportion of small granules than low-amylose varieties, but reported no significant granule size differences between the high- and normal-amylose accumulating lines. This, and several other studies illustrate that starch granule architecture and composition can be determined by many interacting factors (Tester et al., 2006).

The SEM observations indicate that starch granules from *C. sorokiniana* cells grown in 2% CO_2 were disk-shaped, while those from air-grown cells were cup-shaped (Fig. 2). Izumo et al. (2007), who compared the sizes and morphologies of *C. kessleri* 11 h starch granules in cells from the linear to stationary phases of cultures grown in air and 3% CO_2, observed similar differences. However, in another study, Izumo et al. (2011) reported that the morphology of *Chlamydomonas reinhardtii* 137C starch granules may be altered mid-culture. Cells grown in 5% CO_2 had disk-shaped starch granules, but when grown in elevated CO_2 until log phase and then transferred to ambient CO_2 for 6 h, the granules became cup-shaped, morphological characteristics similar to those of the granules from air-grown cultures in our study (Fig. 2). It should be noted that in our experiment starch granules were purified from cells harvested during the middle of the logarithmic phase.

Algal Starch Hydrolysis

The morphology, surface area-to-volume ratio and relative amylose:amylopectin composition of starch granules are determinants of granule hydrolysis rate, a key process dictating the efficiency of starch as a biofuel feedstock (Svihus et al., 2005; Tester et al., 2006). Native starch granules isolated from cultured *C. sorokiniana* cells and starch samples

from maize, rice and wheat were incubated with *A. niger* α-amylase, an inexpensive and ubiquitous enzyme that efficiently hydrolyses starch without an energy-intensive pre-gelatinization step (Tester et al., 2006). The subsequent rates of starch hydrolysis, based on the generation of glucose reducing equivalents, were compared. Interestingly, after 30 min of hydrolysis, starch granules from both the air- and 2% CO_2-grown algal cells were significantly more susceptible to hydrolysis than the cereal starch samples and this difference in the extent of digestion persisted through 180 min of incubation ($P \leq 0.05$) (Fig. 3). Furthermore, the starch granules from cells cultured in high CO_2 were digested 1.2 times more rapidly than those from air-grown cells throughout the 180 min incubation period. In addition, the initial hydrolysis rates of algal starch were significantly higher than those of maize, rice and wheat starches and rates for starch granules from 2% CO_2-grown cells were significantly higher than were the rates for granules from air-grown cells (Table II). Our analysis does not support a more specific conclusion about how relative granule amylose and amylopectin contents and sizes affect hydrolysis rate. For these assays, we have used starch granules from 4-day air and 2% CO_2 cultures. The compositions and size distributions of the two granule populations are not distinctly different, rather they are overlapping (Table I).

Several characteristics of stored starch have been investigated as factors affecting starch hydrolysis. Skrabanja et al. (1999) investigating pea and Noda et al. (2003) investigating rice starches, agreed that starch granules from genotypes with low-amylose content were more susceptible to digestion than those from genotypes with a normal amylose content. Sharma et al. (2010) and Yangcheng et al. (2013) studied bioethanol production from corn starch and reported that corn sources that accumulated starch reserves with a lower amylose content were more efficiently converted to ethanol than those that accumulated starches with a higher amylose content. In our study, when the rates of hydrolysis for the three maize starch samples were compared, increasing amylose content correlated with decreased digestion (Table II).

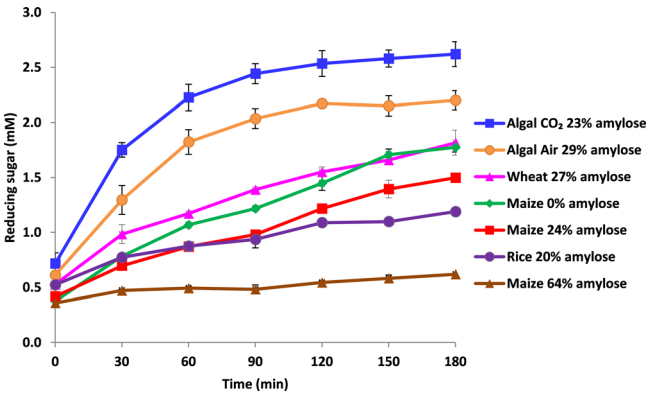

Figure 3. Enzymatic hydrolysis of purified algal starch at day 4 of culturing and maize, rice, and wheat starches containing different percentages of amylose. Values are mean ± standard errors of three replicates.

Table II. Hydrolysis rates of various starches containing different percentages of amylose.

Source of starch	Amylose percentage	Initial rate of starch hydrolysis (mM reducing sugar/min)	R^2
Algal starch Air	29	2.02 ± 0.11^{b}	0.99
Algal starch CO_2	23	2.52 ± 0.13^{a}	0.96
Maize	0	1.16 ± 0.02^{c}	0.99
Maize	24	0.76 ± 0.08^{d}	0.97
Maize	64	0.22 ± 0.03^{e}	0.85
Rice	20	0.58 ± 0.02^{d}	0.93
Wheat	27	1.07 ± 0.03^{c}	0.93

Values are mean ± standard errors of three replicates. Means with different letters within the same column show statistically significant differences ($P \leq 0.05$) by Tukey's test ($n = 3$) while means with the same letter do not.

Vasanthan and Bhatty (1996) reported that in addition to the amylose contents of granules, granule size also influenced the rate of starch hydrolysis and this could be a factor that affected algal starch granule digestion in our study. Algal cells grown in air were smaller on a percentage volume basis by laser diffraction analysis (LDA; Table II). This method rapidly determines the size distribution of millions of particles in a solution but suffers from the drawback in that it assumes all particles are spherical (Wilson et al., 2006). The pyrenoid starch, although larger by LDA, is cup-shaped and likely has a greater surface area to volume ratio for depolymerisation by α-amylase than the discoid stromal starch (Fig. 2).

Our studies indicate that *C. sorkiniana* cells grown in high CO_2 accumulated smaller starch granules with a lower percentage of amylose than did cells grown in air and that the smaller granules were more effectively digested by α-amylase. Although these observations suggest a relationship between the granule characteristics we have measured and the more efficient hydrolysis of their starch glucans, other factors, including the compactness of the starch as affected by the distribution of amylopectin glucan chains (Luengwilai et al., 2010), the possible accumulations of lipids or proteins on the granule surfaces (Stevnebø et al., 2006; Svihus et al., 2005), and the presence and density of pores on the surfaces of the granules (Dhital et al., 2010) should be studied before definitive conclusions can be drawn.

Conclusion

We have demonstrated that culturing *C. sorokiniana* cells in 2% CO_2 enhances both lipid and starch accumulation and affects the structural properties of the starch that is produced. No trade-off between starch and lipid accumulation was noted in 2% CO_2-treated cultures through the first 4 days of the culture period when the dry weight of cells was increasing rapidly. In 2% CO_2, starch granules were disk-shaped and contained mainly stromal starch; granules from air-cultured

cells were cup-shaped, containing primarily pyrenoid starch and were larger than granules from cells grown in 2% CO_2. The granules from cells grown in high CO_2 contained more amylopectin than granules from air-grown cells and were more readily hydrolyzed. Thus, several of the starch granule properties that were influenced by the CO_2 in the culture environment could be utilized to enhance the efficiency and economics of using microalgal starch for biofuel production. Because the productivity of other microalgal species and strains has been shown to be enhanced by tests involving various elevated levels of CO_2, it now is important to determine what CO_2 level is optimal for *C. sorokiniana*'s starch and lipid production. This will be of value as commercial strategies for microalgal production of biofuel precursors such as starch and lipids evolve.

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References

Blanshard J, Jaroszkiewicz E, Gidley M. 1991. The structure and behavior of the starch granule as studied by NMR. NMR applications in biopolymers. New York: Springer Plenum Press. p 155–173.

Brányiková I, Maršálková B, Doucha J, Brányik T, Bišová K, Zachleder V, Vítová M. 2011. Microalgae-novel highly efficient starch producers. Biotechnol Bioeng 108(4):766–776.

Cheng YS, Zheng Y, Labavitch JM, VanderGheynst JS. 2011a. The impact of cell wall carbohydrate composition on the chitosan flocculation of *Chlorella*. Process Biochem 46(10):1927–1933.

Cheng YS, Zheng Y, VanderGheynst JS. 2011b. Rapid quantitative analysis of lipids using a colorimetric method in a microplate format. Lipids 46(1):95–103.

Cheng Y-S, Zheng Y, Labavitch JM, VanderGheynst JS. 2013. Virus infection of *Chlorella variabilis* and enzymatic saccharification of algal biomass for bioethanol production. Bioresour Technol 137:326–331.

Chiu S-Y, Kao C-Y, Tsai M-T, Ong S-C, Chen C-H, Lin C-S. 2009. Lipid accumulation and CO_2 utilization of *Nannochloropsis oculata* in response to CO_2 aeration. Bioresour Technol 100(2):833–838.

de Morais MG, Costa JAV. 2007. Isolation and selection of microalgae from coal fired thermoelectric power plant for biofixation of carbon dioxide. Energy Convers Manage 48(7):2169–2173.

de-Bashan LE, Trejo A, Huss VAR, Hernandez J-P, Bashan Y. 2008. *Chlorella sorokiniana* UTEX 2805, a heat and intense, sunlight-tolerant microalga with potential for removing ammonium from wastewater. Bioresour Technol 99(11):4980–4989.

Delrue B, Fontaine T, Routier F, Decq A, Wieruszeski JM, Van Den Koornhuyse N, Maddelein ML, Fournet B, Ball S. 1992. Waxy *Chlamydomonas reinhardtii*: Monocellular algal mutants defective in amylose biosynthesis and granule-bound starch synthase activity accumulate a structurally modified amylopectin. J Bacteriol 174(11): 3612–3620.

Dhital S, Shrestha AK, Gidley MJ. 2010. Relationship between granule size and in vitro digestibility of maize and potato starches. Carbohydrate Polymers 82(2):480–488.

Doehlert DC, Duke SH, Anderson L. 1982. Beta-amylases from alfalfa (*Medicago sativa* L.) roots. Plant Physiol 69(5):1096–1102.

Dragone G, Fernandes BD, Vicente AA, Teixeira JA. 2010. Third generation biofuels from microalgae. In: Vilas AM, editor. Current research, technology and education topics in applied microbiology and microbial biotechnology, volume 2. Badajoz: Formatex Research Center. p 1355–1366.

Dragone G, Fernandes BD, Abreu AP, Vicente AA, Teixeira JA. 2011. Nutrient limitation as a strategy for increasing starch accumulation in microalgae. Appl Energy 88(10):3331–3335.

Gao K, McKinley KR. 1994. Use of macroalgae for marine biomass production and CO_2 remediation: A review. J Appl Phycology 6(1): 45–60.

Heumann HG. 1992. Microwave-stimulated glutaraldehyde and osmium tetroxide fixation of plant tissue: Ultrastructural preservation in seconds. Histochemistry 97(4):341–347.

Ho S-H, Chen C-Y, Chang J-S. 2012. Effect of light intensity and nitrogen starvation on CO_2 fixation and lipid/carbohydrate production of an indigenous microalga *Scenedesmus obliquus* CNW-N. Bioresour Technol 113(0):244–252.

Izumo A, Fujiwara S, Oyama Y, Satoh A, Fujita N, Nakamura Y, Tsuzuki M. 2007. Physicochemical properties of starch in *Chlorella* change depending on the CO_2 concentration during growth: Comparison of structure and properties of pyrenoid and stroma starch. Plant Sci 172(6):1138–1147.

Izumo A, Fujiwara S, Sakurai T, Ball SG, Ishii Y, Ono H, Yoshida M, Fujita N, Nakamura Y, Buléon A, and others. 2011. Effects of granule-bound starch synthase I-defective mutation on the morphology and structure of pyrenoidal starch in *Chlamydomonas*. Plant Sci 180(2):238–245.

Kadam KL. 1997. Power plant flue gas as a source of CO_2 for microalgae cultivation: Economic impact of different process options. Energy Convers Manage 38:S505–S510.

Luengwilai K, Tananuwong K, Shoemaker CF, Beckles DM. 2010. Starch molecular structure shows little association with fruit physiology and starch metabolism in tomato. J Agric Food Chem 58(2):1275–1282.

MacGregor A, Morgan J. 1980. Hydrolysis of large and small starch granules from normal and waxy barley cultivars by alpha-amylases from barley malt. Cereal Chem 57(6):397–402.

Mandalam RK, Palsson B. 1998. Elemental balancing of biomass and medium composition enhances growth capacity in high-density *Chlorella vulgaris* cultures. Biotechnol Bioeng 59(5):605–611.

Masuko T, Minami A, Iwasaki N, Majima T, Nishimura S, Lee YC. 2005. Carbohydrate analysis by a phenol-sulfuric acid method in microplate format. Anal Biochem 339(1):69–72.

Noda T, Nishiba Y, Sato T, Suda I. 2003. Properties of starches from several low-amylose rice cultivars. Cereal Chem J 80(2):193–197.

Patil V, Tran K-Q, Giselrød HR. 2008. Towards sustainable production of biofuels from microalgae. Int J Mol Sci 9(7):1188–1195.

Radakovits R, Jinkerson RE, Darzins A, Posewitz MC. 2010. Genetic engineering of algae for enhanced biofuel production. Eukaryotic Cell 9(4):486–501.

Russin W, Trivett C. 2001. Vacuum-microwave combination for processing plant tissues for electron microscopy. In: Giberson R, Demaree R Jr, editors. Microwave techniques and protocols. Totowa, New Jersey: Humana Press. p 25–35.

Salih FM. 2011. Microalgae tolerance to high concentrations of carbon dioxide: A review. J Environ Prot 2:648–654.

Sharma V, Rausch KD, Graeber JV, Schmidt SJ, Buriak P, Tumbleson M, Singh V. 2010. Effect of resistant starch on hydrolysis and fermentation of corn starch for ethanol. Appl Biochem Biotechnol 160(3):800–811.

Skrabanja V, Liljeberg HG, Hedley CL, Kreft I, Björck IM. 1999. Influence of genotype and processing on the in vitro rate of starch hydrolysis and resistant starch formation in peas (*Pisum sativum* L.). J Agric Food Chem 47(5):2033–2039.

Smith AM. 2008. Prospects for increasing starch and sucrose yields for bioethanol production. Plant J 54(4):546–558.

Stevnebø A, Sahlström S, Svihus B. 2006. Starch structure and degree of starch hydrolysis of small and large starch granules from barley varieties with varying amylose content. Animal Feed Sci Technol 130(1–2):23–38.

Svihus B, Uhlen AK, Harstad OM. 2005. Effect of starch granule structure, associated components and processing on nutritive value of cereal starch: A review. Animal Feed Sci Technol 122(3–4):303–320.

Sydney EB, Sturm W, de Carvalho JC, Thomaz-Soccol V, Larroche C, Pandey A, Soccol CR. 2010. Potential carbon dioxide fixation by industrially important microalgae. Bioresour Technol 101(15):5892–5896.

Tester RF, Qi X, Karkalas J. 2006. Hydrolysis of native starches with amylases. Animal Feed Sci Technol 130(1–2):39–54.

Vasanthan T, Bhatty RS. 1996. Physicochemical properties of small-and large-granule starches of waxy, regular, and high-amylose barleys. Cereal Chem 73(2):199–207.

Vonshak A. 1986. Laboratory techniques for the cultivation of microalgae. Handbook of microalgal mass culture. Boca Raton, Florida: CRC Press. p 117–145.

Widjaja A, Chien C-C, Ju Y-H. 2009. Study of increasing lipid production from fresh water microalgae *Chlorella vulgaris*. J Taiwan Inst Chem Eng 40(1):13–20.

Wilson JD, Bechtel DB, Todd TC, Seib PA. 2006. Measurement of wheat starch granule size distribution using image analysis and laser diffraction technology. Cereal Chem J 83(3):259–268.

Yangcheng H, Jiang H, Blanco M, Jane J-L. 2013. Characterization of normal and waxy corn starch for bioethanol production. J Agri Food Chem 61(2):379–386.

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