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# Cultivation of Microalgae at Extreme Alkaline pH Conditions: A Novel Approach for Biofuel Production

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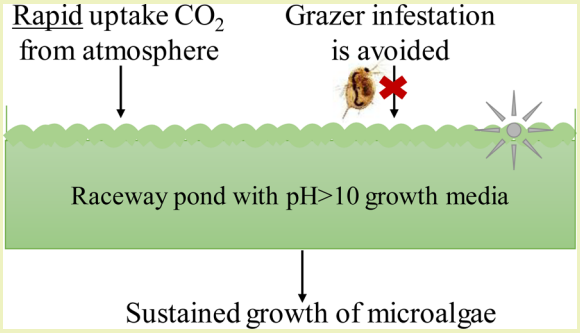
Supporting Information

**ABSTRACT:** A major challenge to the economic viability of outdoor cultivation of microalgae is the high cost of $CO_2$ supply, even when microalgae farms are co-located with point sources of $CO_2$ emissions. In addition, the global capacity for algae biofuel generation is severely restricted when microalgae farm locations are constrained by proximity to $CO_2$ sources along with the additional limitations of low slope lands and favorable climate. One potential solution to the impediments of $CO_2$ supply cost and availability is through cultivation of microalgae in highly alkaline pH solutions (pH >10) that are effective at scavenging $CO_2$ from the atmosphere at high rates. The extremely alkaline pH media would also mitigate culture crashes due to microbial contamination and predators. In this study, we report the indoor and outdoor phototrophic cultivation of a microalgae isolate (*Chlorella sorokiniana* str. SLA-04) adapted to grow in unusually high-pH environments. The isolate was cultivated in a growth medium at pH >10 without any inputs of concentrated $CO_2$. Both indoor and outdoor studies showed biomass and lipid productivities that were comparable to those reported for other microalgae cultures cultivated in near-neutral pH media (pH 7−8.5) under similar conditions. SLA-04 cultures also showed high lipid productivity and high glucose-to-lipid conversion efficiency when cultivated mixotrophically in the presence of glucose as an organic carbon source. From the energy content (calorific value) of the lipids produced and glucose consumed, a relatively high amount of lipid calories (0.62) were produced per glucose calorie consumed. In conclusion, our results demonstrate the feasibility of microalgae cultivation in extremely high-pH media (pH >10) as a novel strategy for biofuel production without dependence on concentrated $CO_2$ inputs.




**KEYWORDS:** *Microalgae, Biofuel, Alkaliphilic, Phototrophic, Mixotrophic, Lipid, Outdoor cultivation, Raceway ponds, Photobioreactor, $CO_2$*

## BACKGROUND

Biofuels obtained from the renewable sources have the potential to mitigate increasing carbon emissions and dependence on fossil energy.[1] Microalgae with high lipid content are particularly attractive as feedstocks[2,3] especially when cultivated on marginal lands using low-quality water (and nutrients) such as wastewater.[4] In spite of the promise, commercial production of microalgal biofuels is not in practice, at least partially, due to the following two major challenges in microalgae cultivation: (1) high cost of $CO_2$ delivery to (open or closed) cultivation systems[5−8] and (2) inability to maintain desirable cultures with sustained high productivity over long periods due to contamination by competing microbial species (bacteria, viruses, and other microalgae) or predators such as zooplankton.[9]

During cultivation, the growth rates of microalgae are strongly influenced by the availability of dissolved inorganic carbon (DIC) in the culture medium. Typically, microalgae cultivated at circumneutral pH conditions uptake and fix soluble $CO_2$ present in the aqueous growth medium. Since atmospheric $CO_2$ diffusion rates and solubility in water are low, pure or enriched $CO_2$-containing gases are sparged to increase dissolved $CO_2$ concentrations. While this approach mitigates $CO_2$ limitations for microalgae growth and allows utilization/recycle of industrial waste $CO_2$, supply of $CO_2$ over long distances is cost prohibitive.[6,7] To lower the costs associated with $CO_2$ transportation, microalgae production facilities can be co-located with $CO_2$ sources such as power plants, cement industries, or ethanol plants, but $CO_2$ delivery costs remain high.[8] The National Renewable Energy Laboratory's (NREL) recent techno-economic report on microalgae biomass production[7] shows that even in co-located algal biorefineries nearly 65% of cultivation-related variable operating costs are associated with recovery of $CO_2$ from flue gases and delivery to raceway ponds (of a total operating cost of $144 per ton dry algae, approximately $91 are attributable to $CO_2$ delivery to ponds). Furthermore, co-location may be feasible with only a few of the available point sources of $CO_2$ due to land, water and climate constraints (e.g., northern temperate regions are

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unsuitable for algae cultivation).[8] A recent study by Quinn et al.[6] estimated that microalgae cultivation systems that are simultaneously constrained by availability of flue gases, low-slope barren lands, and favorable climates would achieve less than 10% of the U.S. Department of Energy's (DOE) 2030 advanced fuel targets. Flue gas contaminants (e.g., heavy metals from coal combustion) could also negatively impact the quality of microalgae produced.[5] Finally, $CO_2$ sparged microalgae cultures that stay at near-neutral pH are prone to microbial contamination and predators (e.g., *Daphnia*).[9]

The use of extreme alkaliphilic microalgae can overcome many current limitations to large-scale algae production.[10−14] Extreme alkaliphiles are organisms that have the ability to survive and thrive at unusually high pH values (pH >10).[15] Since aqueous solutions at pH >10 rapidly scavenge $CO_2$,[16] the supply rates of dissolved inorganic carbon (DIC) from the atmosphere to such highly alkaline growth media is high, even in the absence of $CO_2$ sparging.

However, at pH >10, $HCO_3^-$ and $CO_3^{2-}$ are the dominant DIC species, while dissolved $CO_2$ concentrations are negligibly small due to the pH-dependent inorganic carbon equilibrium. Opportunely, microalgae and cyanobacteria adapted to survive in alkaline solutions are able to sustain photosynthetic carbon fixation by utilizing $HCO_3^-$ as the inorganic carbon source. It is now well-established that phototrophic $HCO_3^-$ utilization is facilitated through carbon-concentrating mechanisms (CCMs) that are primarily comprised of membrane-bound and intracellular carbonic anhydrase enzymes that convert $HCO_3^-$ to $CO_2$ within the cell.[17] RuBisCO then fixes the cellular $CO_2$ to organic carbon. High-pH growth media can thus provide a means for increased carbon uptake rates from the atmosphere as well as supply $HCO_3^-$ as inorganic carbon for use by alkaliphilic microalgae. Not surprisingly, aquatic photosynthetic carbon fixation rates in natural alkaline lakes are high.[18−20] In engineered systems, cultivation of alkaliphilic cultures in high-pH growth media could eliminate the requirement for co-location with $CO_2$ point sources.[11−13]

In addition, cultivation conditions that remain at extreme pH (>10) can allow sustained maintenance of desired alkaliphilic cultures, due to the relatively low microbial diversity in these harsh environments.[21] Previous reports also suggest that grazer infestations are less likely in alkaline environments. For example, *Daphnia* eggs lose viability when pH values exceed 10−10.5.[22,23] In commercial practice, *Spirulina* production is successful, at least partly, due to the high-pH growth conditions that enable prolonged maintenance of these cyanobacterial species in low-cost open ponds.

In this report, we extend our previous work on cultivation of alkaliphilic microalgae[11−13] and describe outdoor cultivation of an extreme alkaliphilic *Chlorella sorokiniana* str. SLA-04 (henceforth referred to as SLA-04), isolated from Soap Lake, WA. After initial indoor cultivation with artificial illumination, SLA-04 cultures were grown under natural sunlight in open raceway ponds (22 L) in media at pH >10. Mixotrophic growth was also evaluated. Culture concentration, nutrient utilization, and lipid content were monitored during cultivation to estimate biomass and lipid productivities. Kinetic parameters of extreme alkaliphilic microalgae cultivation are compared with rates reported for neutralophilic microalgae (i.e., microalgae that normally grow at circumneutral pH). To our knowledge, this is the first report of microalgae cultivation studies in media at pH >10.

## METHODS

**Strain Isolation, Growth Medium, and rDNA Sequencing.** The culture media composition for the studies reported here was based on Bold's original recipe with minor modifications[24] (a detailed media recipe is given in Supporting Information). To isolate strains adapted to extreme pH conditions, water samples from the alkaline Soap Lake, WA were inoculated on sterile agar plates using the spread-plate technique. A single microalgal colony that grew most rapidly on the solid media was further purified using a streak-plate technique. Unialgal colonies were confirmed through microscopic observation and the strain was designated SLA-04.[11]

DNA sequencing of SLA-04 was performed by the UTEX Culture Collection of Algae (Austin, TX). DNA was first extracted using a standard protocol[25] and amplified using primers designed to amplify the 5.8S rDNA region and both internal transcribed spacer regions (ITS1 and ITS2). The amplified product was sequenced and then assembled using the Geneious software package. The amplified small subunit (SSU) rDNA (5.8S) and internally transcribed spacer (ITS1 and ITS2) regions were identified using Basic Local Alignment Search Tool (BLAST) queries. Thereafter, a multiple sequence alignment was performed using the ClustalX 2.0.12 program. ClustalX was set to exclude positions with gaps and correct for multiple substitutions. A phylogenetic tree was created with TreeView using the multiple sequence alignment data generated by ClustalX 2.0.12 (10 000 bootstrap trials).

**Indoor Cultivation Experiments.** Initially, single colonies of SLA-04 (from agar plates) were inoculated into 50 mL of liquid media contained in 250 mL Erlenmeyer flasks and grown on an illuminated shaker table. Cultures were subsequently scaled to 500 mL (in 1 L Erlenmeyer flasks) which served as inoculum for the indoor cultivation experiments that are described below.

For indoor cultivation studies, autoclaved media (121 °C, 30 min) was used and all experiments were performed at room temperature (20 °C) under aseptic conditions. For mixotrophic growth, the medium was supplemented with glucose (4 $g \cdot L^{-1}$) as the organic carbon source. The experimental setup was similar to previously described systems.[26] Cytostir reactors (3 L, Kimble Chase, Vineland, NJ) were placed on stir plates and illuminated using a bank of four Ecolux Starcoat 54W fluorescent tubes (GE Lighting, Cleveland, OH) set on a frame such that the lights were 3 in. away from the vessel walls. Cultures were illuminated on one side which resulted in an optical path length of 6 in. through the culture (equal to the diameter of the Cytostir reactors). The reactors were stirred at a speed of 120 rpm, sparged with ambient air, and continuously illuminated (see photograph in Figure S1a).

Initially (day 0), two fluorescent lights were turned on and the incident irradiance levels were kept low to prevent photoinhibition[26] (incident intensity of 153 $\mu mol \cdot m^{-2} \cdot s^{-1}$ measured using model LI-250A light meter, Li-Cor Biosciences, Lincoln, NE). As the culture concentrations increased, two additional fluorescent lamps were turned on (at the end of day 2), resulting in the cultures being illuminated at an incident irradiance of 294 $\mu mol \cdot m^{-2} \cdot s^{-1}$ for the remainder of the cultivation period. During the experiment, samples were periodically removed and analyzed as described in the analytical methods section.

**Outdoor Cultivation Experiments.** Open raceway ponds with dimensions of 2 ft. × 1 ft. × 1 ft. ($L \times W \times D$) were constructed and used in these experiments (see photograph in Figure S1b). The ponds were equipped with real-time temperature and pH monitoring and logging (Neptune APEX data logging systems, Neptune Technology Group Inc., Tallassee, AL) and were placed in an outdoor temperature-controlled greenhouse. The thermostat in the greenhouse was set to 25 °C, and the greenhouse vents automatically open/shutoff according to the set temperature. Tap water available at the greenhouse facility was first filtered through a 10 $\mu$m filter (to remove sediments) and used for media preparation without sterilization. To adapt SLA-04 cultures to the outdoor environment (diel light cycle and temperature), 3 L of indoor-grown SLA-04 cultures (grown in spinner flasks as described in the "indoor cultivation experiments" section) were added to 7 L of freshly prepared culture medium and


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allowed to grow in the ponds. First-generation outdoor cultures (7 days old) were reinoculated to fresh media and grown for 2 more growth cycles to adequately adapt the cultures to the outdoor conditions. Finally, outdoor cultivation studies were performed by inoculating 5 L of the third-generation outdoor SLA-04 cultures to 17 L of a freshly prepared culture medium. The total culture volume of 22 L in the ponds resulted in an optical path length of approximately 6 in. (similar to indoor experiments). Three outdoor cultivation trials were performed: Trial 1 in September, Trial 2 in December, and Trial 3 in March. Trials 1 and 2 experiments were performed without any pH control. In Trial 3, culture pH was controlled during the day (to match with the photosynthetically active period) using an automated $CO_2$ addition system. A 5% (v/v) $CO_2$–$N_2$ mixture was used for pH control. During each experiment, samples were periodically removed and analyzed as described in the "Analytical Methods" section.

**Analytical Methods.** Analytical methods are described briefly here. Detailed descriptions of all the analytical methods are given in the Supporting Information.

*Wet Sample Analysis.* Biomass concentrations in cultures were measured as total suspended solids (TSS) by following the Laboratory Analytical Procedure outlined by NREL.[27] The TSS obtained was then used to calculate the productivity using the relationship given below:

$$\text{Biomass productivity} = \frac{\text{TSS}_f - \text{TSS}_i}{t_f - t_i} \quad (1)$$

where $\text{TSS}_f$ and $\text{TSS}_i$ represent the final and initial TSS values on days $t_f$ and $t_i$, respectively.

For indoor cultivation studies, pH was measured periodically (model: Orion A121, Thermo Scientific). For outdoor cultures, pH was continuously monitored (Neptune APEX Lab grade pH probes, Neptune Technology Group Inc.). pH probes were calibrated daily.

Media DIC was analyzed on an Innovox TOC analyzer equipped with an auto sampler (GE Analytical Instruments, Boulder, CO). From DIC and pH values, $HCO_3^-$ and $CO_3^{2-}$ concentrations in the culture media were calculated using the DIC–pH equilibrium relationships based on the first ($K_1$) and second ($K_2$) dissociation constants of carbonic acid.[28] These relationships are given as

$$[HCO_3^-] = \text{DIC}\frac{[H^+]K_1}{[H^+]^2 + [H^+]K_1 + K_1K_2} \quad (2)$$

and

$$[CO_3^{2-}] = \text{DIC}\frac{K_1K_2}{[H^+]^2 + [H^+]K_1 + K_1K_2} \quad (3)$$

where $[H^+] = 10^{-pH}$ (M). $K_1$ and $K_2$ were estimated from previously reported correlations of the equilibrium constants with temperature.[29,30]

Total alkalinity (TA) of the culture medium was measured using a G20 compact titrator (Mettler-Toledo, Columbus, OH); a 40 mL sample was taken in a beaker and titrated with a 0.1 M HCl solution until the pH of the samples reached the titration end point of pH 4.5. Total soluble N concentrations for indoor experiments were measured on supernatants of centrifuged samples using a colorimetric assay based on an alkaline persulfate digestion method. For samples from outdoor cultivation experiments, $NO_3^-$ concentrations in the supernatant were analyzed by an ion chromatograph (IC) equipped with a Dionex IonPac CS12A anion-exchange column and a conductivity detector (Dionex ICS 3000, Thermo Fisher, Sunnyvale, CA). For mixotrophic cultures, glucose analysis was carried out using an Agilent 1100 HPLC (Agilent Technologies Inc., Santa Clara, CA) equipped with a Shodex SH1011 column (Showa Denko America Inc., New York, NY) and a refractive index (RI) detector.

A DUAL-PAM 100 Chlorophyll Fluorometer (Heinz Walz Gmbh, Effeltrich, Germany) was used to measure the photosynthetic quantum yield. A 3 mL sample was taken in a quartz glass cuvette and incubated in the dark for 5 min with continuous stirring to obtain the minimum fluorescence yield ($F_0$). Then, a saturation pulse (10 000 $\mu$mole·m$^{-2}$·s$^{-1}$) of blue light was applied for 0.6 s to get the maximum fluorescence yield ($F_m$). These fluorescence yield parameters ($F_0$ and $F_m$) were then used to estimate the maximum quantum yield ($F_v/F_m$) where $F_v = F_m - F_0$.

*Dry Sample Analysis.* Cellular lipids were quantified as fatty acid methyl esters (FAMEs) using an *in situ* transesterification method.[31] Qualitative estimates of triglyceride, starch, and protein contents in biomass were also assessed using a thermogravimetric analyzer (SDT Q600 series, TA Instruments, New Castle, DE).[32]

## RESULTS AND DISCUSSION

**Strain Identification.** Microscopic examination of the cultures showed 2–5 $\mu$m sized cells (see photograph in inset to Figure S2) consistent with the morphology of *Chlorella* sp. cells. Furthermore, multiple sequence analysis results comparing the amplified genomic regions of str. SLA-04 with other closely related sequences in the NCBI database confirmed that str. SLA-04 has the highest percent identity to *Chlorella sorokiniana* str. UTEX 246 (Figure S3). The phylogenetic tree in Figure S2 shows the close relation of str. SLA-04 with str. UTEX 246 as well as phylogenetic proximity with other members of the genus *Chlorella*. The nucleotide sequence of str. SLA-04 has been submitted to the NCBI GenBank database (accession number KX260111).

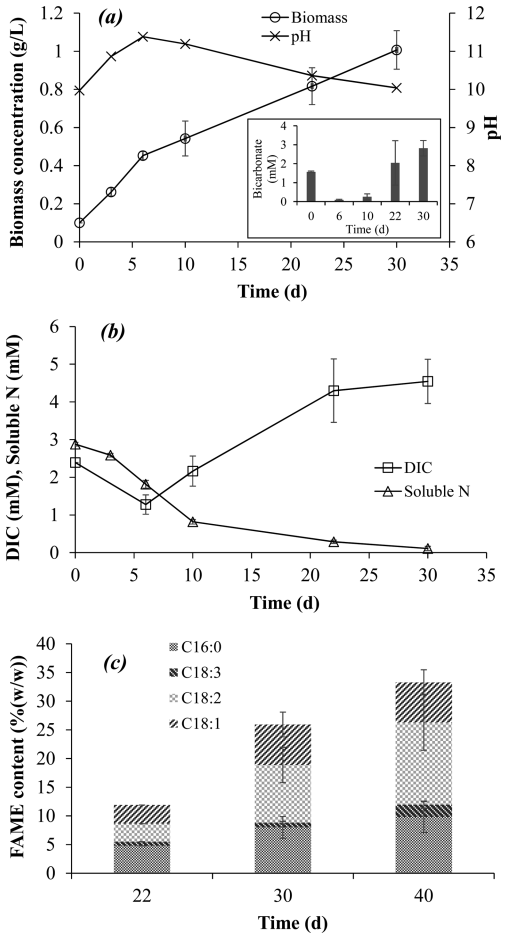


**Figure 1.** (a) Biomass concentration and pH, (b) DIC and soluble N concentrations, and (c) increase in the overall FAMEs content and fatty acid profiles of SLA-04 cultures during phototrophic cultivation in 3 L Cytostir reactors. Inset in (a) shows the bicarbonate concentrations in the medium during cultivation. Values shown in the graph are averages from triplicate runs. Error bars indicate one standard deviation from mean values.



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**Table 1. Comparison of Biomass Productivity and Lipid Content of Indoor-Grown *Chlorella sorokiniana* str. SLA-04 Cultures with Previously Reported Literature Data for Other Neutralophilic *Chlorella* sp. Cultivations under Similar Illumination Conditions**

| cultivation scheme | microalgae strain | biomass productivity (mg-biomass·$L^{-1}$·$day^{-1}$) | FAME/lipid content % (w·$w^{-1}$) | ref |
|---|---|---|---|---|
| 2 L bottles | *Chlorella vulgaris* | 10[a] | 38[e] | 49 |
| | *Chlorella vulgaris* | 41[b] | 18[f] | |
| 2 L bioreactor | *Chlorella emersonii* | 28[b] | 29[f] | 58 |
| | *Chlorella minutissima* | 32[b] | 31[f] | |
| 2 L photobioreactors | *Chlorella kessleri* | 65[b] | n.r.[i] | 59 |
| tubular bioreactor | *Chlorella vulgaris* | 40[b] | 28[f] | 60 |
| | *Chlorella emersonii* | 41[b] | 25[f] | |
| bioreactor | *Chlorella vulgaris* | 104[b] | 6.91[f] | 61 |
| indoor ponds | *Chlorella pyrenoidosa* | 27.9[a] | 39.8[g] | 14 |
| 150 mL Erlenmeyer flasks | *Chlorella vulgaris* | 50[b] | 9.2[f] | 51 |
| 3 L Cytostir reactor | *Chlorella sorokiniana* str. SLA-04 | 58.7 ± 2.9[a,c] | 12[h] | present study |
| | *Chlorella sorokiniana* str. SLA-04 | 42 ± 4.1[a,d] | 34[h] | |

[a]Biomass concentrations were directly measured as TSS. [b]Biomass concentrations were estimated from optical density measurements and correlation with TSS. It is possible that some values may be overestimates due to excess pigment production during cultivation under low irradiance levels. [c]Productivity calculated over days 0–6 [d]Productivity calculated over days 6–30. [e]Lipids were extracted using bead beating process and quantified gravimetrically. [f]Lipids were quantified using Bligh and Dyer method. [g]Lipids were quantified by in situ $^1$H NMR spectroscopy. [h]Lipids were quantified as FAME. [i]n.r.: not reported.

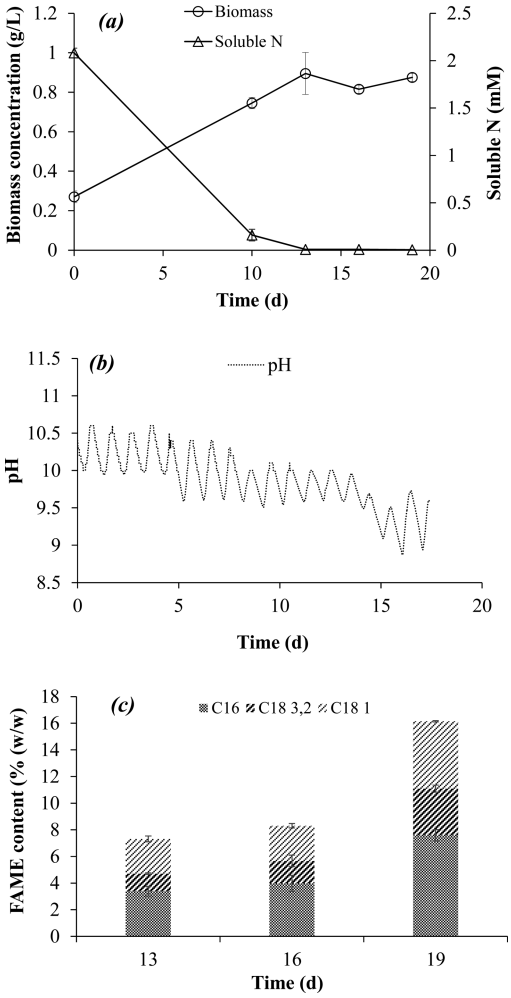


**Figure 2.** (a) Biomass and soluble N concentrations, (b) pH, and (c) FAME concentrations and fatty acid profiles from Trial 1 outdoor raceway pond experiments performed during August–September. Values shown in the graph are averages from duplicate runs. Error bars indicate one standard deviation from mean values.

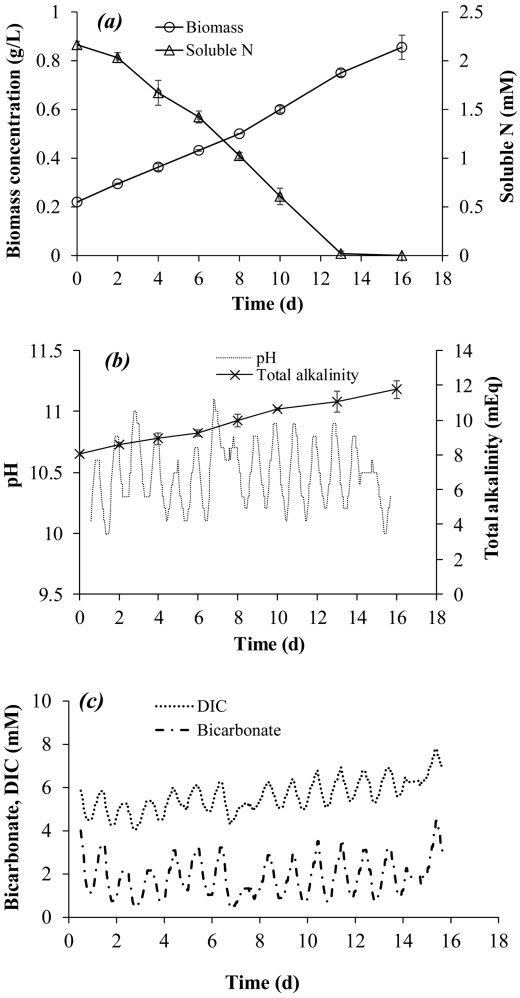


**Figure 3.** (a) Biomass and soluble N concentrations, (b) pH and total alkalinity, and (c) estimated DIC and bicarbonate concentrations from Trial 2 outdoor raceway pond experiments performed during December. Values shown in (a) and (b) are averages from duplicate runs. Error bars indicate one standard deviation from mean values.



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**Table 2. Comparison of Biomass Productivity and Lipid Content of Outdoor Raceway Pond-Grown *Chlorella sorokiniana* str. SLA-04 Cultures Cultivated in Autumn−Winter with Previously Reported Literature Data for Other Neutralophilic Microalgae Cultures Cultivated in Similar Reactor Systems**

| cultivation scheme | microalgae strain | biomass productivity (mg-biomass·$L^{-1}$·$day^{-1}$) | FAME/lipid content % (w·$w^{-1}$) | ref |
|---|---|---|---|---|
| raceway ponds | *Scenedesmus acutus* | 39.5[a] | 13.4 | 62 |
| raceway ponds | *Scenedesmus acutus* | 66.6[b] | 12.5 | 63 |
| | | 40[c] | n.r.[k] | |
| raceway ponds | *Pleurochrysis carterae* | 52[d] | 33.9 | 64 |
| raceway ponds | *Nannochloropsis Oceanica* | 20[e] | 11.2 | 65 |
| raceway ponds | *Chlorella sorokiniana* str. SLA-04 | 48 ± 5.4[f] | 16.1 | present study |
| | | 39 ± 1.5[g] | 10.3 | |
| raceway ponds | *Chlorella sorokiniana* str. SLA-04 | 74 ± 2.1[h,i] | n.r. | present study |
| | | 67 ± 0.7[h,j] | | |

[a]Cultivation experiments were carried from February to March. [b]Cultivation experiments were carried in December. [c]Cultivation experiments were conducted in January. [d]Cultivation experiments were conducted in Autumn. [e]Cultivation experiments were conducted in Winter 2014 and 2015. [f]Trial 1 cultivation carried out in August−September. [g]Trial 2 cultivation carried out in December. [h]Trial 3 cultivation carried out in March. [i]pH was controlled at 8.2 by sparging with 5% $CO_2$. [j]pH is controlled at 10 by sparging with 5% $CO_2$. [k]n.r.: not reported

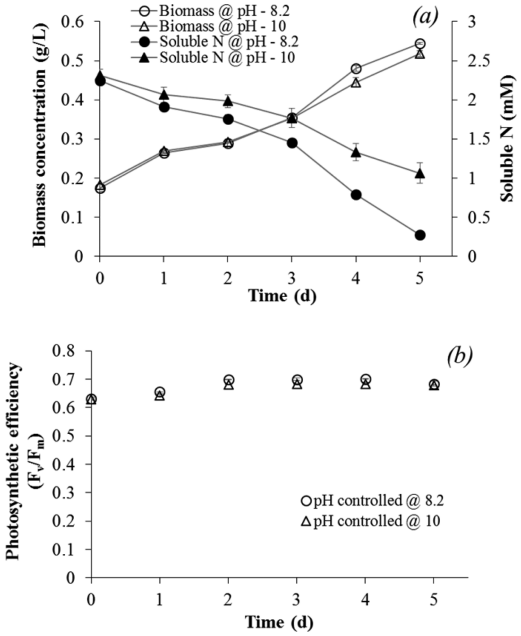


**Figure 4.** (a) Biomass and soluble N concentrations and (b) photosynthetic efficiencies ($F_v/F_m$) from Trial 3 outdoor raceway pond experiments performed during March. Values shown in (a) and (b) are averages from duplicate runs. Error bars indicate one standard deviation from mean values.

**Indoor Phototrophic Cultivation of str. SLA-04.** Indoor SLA-04 cultures grew well in the pH >10 medium with biomass productivities that compare favorably with values reported for other *Chlorella* cultures cultivated in circumneutral-pH media (Figure 1a and Table 1). In contrast, high-pH conditions have been reported to drastically diminish the growth of neutralophilic microalgae.[33−35] The literature information collated by Hansen et al. shows that media pH >9.5 causes cessation of growth across several genera.[35] Of the 35 microalgae strains investigated by Hansen et al., 28 strains did not grow at all in media at pH >9.5, while the growth of the remaining 7 cultures was significantly inhibited.[35] While the mechanisms of growth cessation/inhibition were not fully identified, Hansen and co-workers speculated that the high-pH conditions could have denatured cell proteins or that the high $CO_3^{2-}$ concentrations could be toxic via unknown mechanisms.

In our experiments, the biomass productivity of alkaliphilic SLA-04 cultures (calculated using eq 1) was relatively high (−58.7 ± 2.9 mg·$L^{-1}$·$day^{-1}$ during days 0−6, Table 1). As such, the cultures did not appear to be inhibited by high media pH (>10), and the relatively high $CO_3^{2-}$ concentration in the media (estimated from eq 3 to be 1.5 mM initially and higher later due to increase in pH during growth). It is likely that SLA-04, on account of its origin in Soap Lake, was well-adapted to thrive in an unusually high-pH environment.

Since the media pH was >10, it is likely that SLA-04 cultures initially grew by utilizing the available $HCO_3^-$ (estimated from eq 2 to be nearly 1.6 mM at the start of the experiment), rather than dissolved $CO_2$, since dissolved $CO_2$ concentrations in the medium (in equilibrium with ambient $CO_2$) at these pH values are exceedingly small.[28] Photosynthetic autotrophs that utilize $HCO_3^-$ employ carbon concentrating mechanisms with cellular carbonic anhydrases that convert $HCO_3^-$ to $CO_2$ (eq 4);[17] $CO_2$ is subsequently fixed by RuBisCO (eq 5).[17] The utilization of $HCO_3^-$ by SLA-04 for photosynthetic carbon fixation (eqs 4 and 5 together) would thus be expected to release $OH^-$ ions into culture medium. To a lesser extent, $NO_3^-$ uptake and reduction ($NO_3^-$ to $NH_3$) for amino acid synthesis would also release $OH^-$ ions into the media (eq 6).[36]

$$HCO_3^- \xrightarrow{\text{carbonic anhydrase}} CO_{2,\text{cellular}} + OH^- \quad (4)$$

$$CO_{2,\text{cellular}} + H_2O \xrightarrow{\text{RuBisCO}} [CH_2O]_{\text{algae}} + O_2 \quad (5)$$

$$NO_3^- + 2H_2O \rightarrow NH_3 + OH^- + 2O_2 \quad (6)$$

However, since the cultures were air sparged, dissolution of ambient $CO_2$ would also simultaneously occur and neutralize the $OH^-$ ions in solution (eq 7).[16,37] The reactions shown in eq 7 are expected to be largely irreversible at pH values >10 due to the abundance of $OH^-$ ions.

$$CO_{2(g)} \rightleftharpoons CO_{2(l),\text{media}} + OH^- \rightleftharpoons HCO_{3,\text{media}}^- \quad (7)$$

In our experiments, the pH of the culture medium was observed to increase during days 0−6 (see pH data in Figure 1a), suggesting that the rates of DIC addition into the media from atmospheric $CO_2$ (eq 7) were slower than rates of $OH^-$ generation from photosynthetic carbon fixation and nitrate assimilation (eqs 4−6). Accordingly, a decrease in media DIC was observed during days 0−5 (see DIC data in Figure 1b). An


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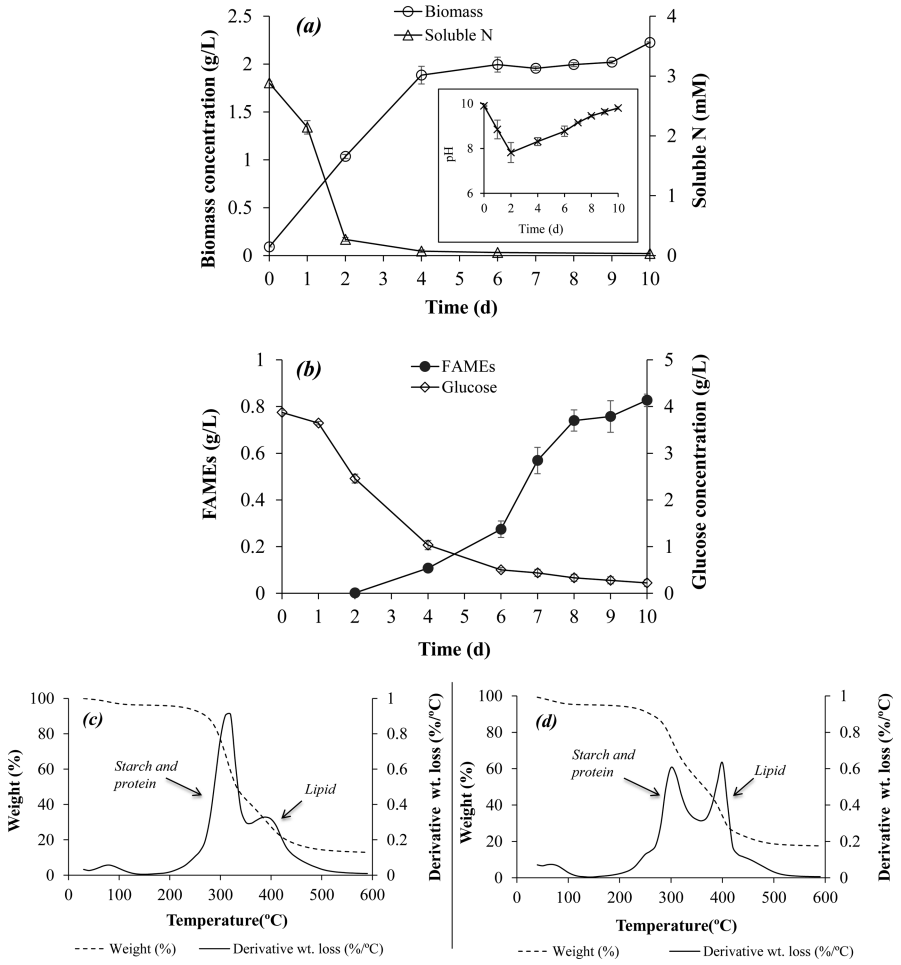


**Figure 5.** (a) Biomass and soluble N concentrations, and (b) FAME and glucose concentrations of SLA-04 cultures during mixotrophic cultivation in 3 L Cytostir reactors. Values shown in (a) and (b) are averages from triplicate runs. Error bars indicate one standard deviation from mean values. (c) and (d) Thermograms of day 4 and 10 samples. Residual weight data are indicated by the dashed lines. Derivative weight loss data are shown as solid lines.

additional consequence of the increase in pH is that the higher concentrations of $OH^-$ in the media would drive the soluble DIC species toward a higher relative proportion of $CO_3^{2-}$ (eq 8).[16,37]

$$HCO_3^- + OH^- \leftarrow CO_3^{2-} + H_2O \quad (8)$$

Reaction 8 occurs in parallel with high rates of photosynthesis (eqs 4 and 5) and results in significant decrease in $HCO_3^-$ concentration in the media. Using measured pH and DIC values (Figure 1), we estimated that $HCO_3^-$ concentrations (eq 2) in the media were <0.1 mM on day 6 (see inset to Figure 1a). The low availability of $HCO_3^-$ in combination with increased light attenuation within the culture (due to culture growth) likely resulted in a decrease in culture productivity after day 6 (23.1 ± 2.83 mg·$L^{-1}$·$day^{-1}$ (calculated using eq 1, over days 6–30).

While $CO_2$ fixation rates were low after day 6, cells continued to uptake $NO_3^-$ from the growth medium. In fact, the rate of $NO_3^-$ uptake between days 3–6 was similar to uptake rate between days 6–10 (Figure 1b), although much less biomass was generated after day 6 (Figure 1a). These results suggest that photosystem II (PS-II) was active even at the very high pH conditions (pH >11) and that the photosynthetically generated electrons appear to be utilized for $NO_3^-$ reduction when $HCO_3^-$ availability was limited.

After day 10, the rate of abiotic addition of DIC to the media (from ambient $CO_2$) was likely high due to elevated media pH (eq 7) while the photosynthetic carbon fixation rates remained low (eqs 4 and 5) in the optically dense cultures. As a result of the higher influx of $CO_2$ into the medium relative to photosynthetic uptake, a decrease in pH was observed after day 10 (Figure 1a). In addition, $NO_3^-$ assimilation for amino acid synthesis by SLA-04 would have increased the net mass of alkaline materials present in solution due to the addition of $OH^-$ to the media in lieu of $NO_3^-$ (eq 6). Since the net mass of DIC present in aqueous media exposed to the atmosphere is determined by the extent of alkaline materials present in the solution, the increased total alkalinity (TA, see eq 9) after day 10 allowed the final DIC and $HCO_3^-$ concentration in the medium to significantly exceed initial values (Figure 1b and inset to Figure 1a).

$$TA = [HCO_3^-] + 2[CO_3^{2-}] + [OH^-] \quad (9)$$

The depletion of soluble N in the media also led to the onset of lipid accumulation as is commonly observed with other microalgae[38,39] (Figure 1c). Lipid content values (reported as fatty acid methyl ester (FAME)) of nearly 34% (w·$w^{-1}$) were measured and compare favorably with other indoor neutralo-


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Table 3. Comparison of Lipid Content, Lipid Productivity, and Lipid Yields of Mixotrophically Grown *Chlorella sorokiniana* str. SLA-04 Cultures with Previously Reported Literature Data for Other Neutralophilic *Chlorella* sp. Cultivations under Similar Illumination Conditions

| microalgae strain | initial glucose conc. ($g{\cdot}L^{-1}$) | final biomass conc. ($g{\cdot}L^{-1}$) | lipid content (% ($w{\cdot}w^{-1}$)) | lipid productivity ($g{\cdot}L^{-1}{\cdot}day^{-1}$) | lipid yield ($g\text{-lipid}{\cdot}g\text{-glucose}^{-1}$) | ref |
|---|---|---|---|---|---|---|
| *Chlorella* sp. UTEX 259 | 10 | 1.70 | 21.0 | 0.03 | 0.04 | 49 |
| *Chlorella vulgaris* | 7 | 4.19 | 10.6 | 0.04 | 0.07 | 51 |
| *Chlorella minutissima* | 10 | 0.38 | 11.8 | 0.004 | 0.004 | 66 |
| *Chlorella sorokiniana* UTEX1602 | 4 | 3.55 | 21.4 | 0.12 | 0.19 | 4 |
| *Chlorella sorokiniana* CCTCC M209220 | 5 | 1.20 | 47.3 | 0.05 | 0.12 | 50 |
| *Chlorella vulgaris* | 4 | 1.41 | 13.8 | 0.03 | 0.05 | 67 |
| *Chlorella* sp. | 2 | 1.38 | 26.0 | 0.04 | 0.18 | 68 |
| *Chlorella sorokiniana* SLA-04 | 4 | 2.25 | 36.7 | 0.08 | 0.21 | present study |

philic *Chlorella* cultures (Table 1). The increase in DIC concentration due to increased TA would have provided more $HCO_3^-$ for the SLA-04 cultures during N-starvation and possibly enhanced lipid production.[39]

The fatty acid composition of lipid-enriched SLA-04 cultures (Figure 1c) is similar to cottonseed oil.[40] When recovered and converted to FAMEs, the biodiesel produced from SLA-04 can be expected to meet ASTM D6751 fuel standards in the United States.[41] Furthermore, since linolenic acid (C18:3) is a small fraction of the total FAMEs (~2.5%, Figure 1c), the biodiesel produced from SLA-04 can also be expected to meet European standards for biodiesel (EN 14214).

**Outdoor Cultivation of SLA-04 without pH Control.** Following indoor tests, we cultivated SLA-04 in outdoor raceway ponds to assess culture performance under more production-relevant conditions. The results for two outdoor autumn/winter cultivation trials (designated Trials 1 and 2) are shown in Figures 2 and 3. Cultivations were started with a relatively high initial biomass concentration of nearly 0.2 $g{\cdot}L^{-1}$ to prevent possible photoinhibition under natural daylight conditions.[42] The cultures were inoculated at night to allow for cell synchronization.[43] The temperature profiles for both the cultivation trials are provided in Figure S4a,b.

A similar final biomass concentration of approximately 0.9 $g{\cdot}L^{-1}$ was reached during both trials (Figures 2a and 3a), and visual observations of the cultures using light microscopy did not show contaminating populations. The biomass productivity during Trial 1 (August−September) was approximately 50 $mg{\cdot}L^{-1}{\cdot}day^{-1}$ and was nearly 20% higher than the productivity measured during Trial 2 (December). Since, the temperatures under both cultivation trials (Trials 1 and 2) were similar (Figure 5a,b), it seems likely that increased productivities in Trial 1 experiments were due to a longer day cycle earlier in the year. The outdoor productivities were also lower than the initial productivities (day 0−6) under indoor conditions, likely due to the relatively short daytime (10−12 h) during experiments in Toledo, OH (indoor experiments were continuously illuminated). A comparison of the outdoor raceway pond productivity of SLA-04 with other outdoor-grown neutralophilic microalgae cultures cultivated for a similar experimental duration (2−3 weeks) shows that the biomass productivities and lipid content values measured during this study are similar to those of other studies reported in the literature (Table 2). In the studies referenced in Table 2, cultures was maintained at moderate values (8.2−8.7) through addition of concentrated $CO_2$. In contrast, in our study, high-pH SLA-04 cultures were incubated without any inputs of concentrated $CO_2$; rather, the SLA-04 cultures relied solely on atmospheric $CO_2$. Despite the lack of concentrated $CO_2$ additions, SLA-04 cultures achieved productivities similar to those reported by others.

pH measurements indicated a cyclic shift in pH values during the day (Figures 2b and 3b). Typically, pH varied between 10.8 and 9.5. Uptake of inorganic carbon and nitrate for photosynthesis likely resulted in the release of $OH^-$ and an increase in pH during the day (eqs 4 and 6) and was followed by a decrease during night due to $CO_2$ transfer from the atmosphere and nighttime cell respiration (eq 7). This cyclic pH shift is a good indication that SLA-04 cultures continued active photosynthesis under natural daylight even when media pH was high (pH >10). Total alkalinity measurements during Trial 2 indicated an increase in these values over time (Figure 3b), as expected, due to consumption of nitrate.[36] By linearly interpolating between measured values of total alkalinity and


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using the real-time recorded values of pH, we estimated the temporal variation of media DIC and $HCO_3^-$ (Figure 3c; see Section S2 of the Supporting Information for the calculation procedure). Due to the increase in media alkalinity, the media DIC at the end of the 12 day cultivation trials was higher than initial values. Bicarbonate concentrations, which depend on upon DIC values as well the pH-dependent DIC speciation (eq S7), varied diurnally (Figure 3c). While $HCO_3^-$ concentrations were estimated to decrease during the day, our calculations suggest that $HCO_3^-$ was never completely depleted, and at least ~0.5−1 mM $HCO_3^-$ remained in solution and would permit phototrophic carbon fixation throughout the day. The decrease in pH during the night allowed for replenishment of the media bicarbonate (Figure 3c).

After nitrate depletion, SLA-04 cultures were incubated for 5 additional days to allow for lipid accumulation. The average FAME content on day 19 was estimated to be 16 and 10% (g-FAME·g-biomass$^{-1}$) for Trial 1 (Figure 2c) and Trial 2 experiments, respectively. These values were significantly lower than the FAME content of indoor cultures (34% as discussed in the section "Indoor Phototrophic Cultivation of str. SLA-04"). Previous studies have shown that lipid production increases when illumination levels are high[44,45] due to the higher requirement of cellular reducing equivalents for lipid synthesis. Low photon flux levels in autumn/winter possibly limited lipid accumulation in outdoor cultures. Olofsson et al. recently observed that the lipid content of December-grown *Nannochloropsis oculata* cultures were much lower (11%) than the lipid content of cultures grown during July (~30%).[46]

**Outdoor Cultivation of SLA-04 with pH Control.** While Trials 1 and 2 experiments showed that SLA-04 cultures maintained good productivity in extreme alkaline media (Figures 2b and 3b), we sought to systematically assess the dependence of growth performance on media pH. As such, we performed a new set of outdoor cultivation experiments at (i) moderate pH (controlled at pH 8.2) and (ii) high pH (controlled at pH 10.0), and the results of these experiments are shown in Figure 4. The growth curves in Figure 4a show that the growth rates of cultures controlled at pH 8.2 were similar to the growth rates of cultures maintained at pH 10.0. It was noted that while overall biomass productivity remained unchanged with pH, the consumption of nitrate was higher at pH 8.2 than that at pH 10.0 suggesting a metabolic control toward greater protein synthesis in moderate-pH conditions.

Furthermore, to assess if photosynthetic activity was influenced by pH, we measured photosynthetic quantum yield ($F_v/F_m$), an indicator of stress in photosynthetic organisms,[47] in the pH 8.2 and 10.0 cultures. $F_v/F_m$ values were initially ~0.6 and increased to values >0.65 in both moderate- and high-pH cultures (Figure 4b) indicating that that the photosynthetic activity of str. SLA-04 was not negatively impacted by high-pH conditions.[48] The biomass productivities were estimated using eq 1 at the end of the experiment. Overall, the results from Trial 3 experiments suggest that strain SLA-04 has a broad pH optima and functions similarly well at moderate and extremely alkaline pH conditions.

To assess contamination resistance due to the high media pH, we challenged the moderate- and high-pH cultures to external contaminating populations. In these experiments, we deliberately introduced a live population of *Daphnia magna*, a well-documented microalgae grazer,[22] into both moderate- and high-pH SLA-04 cultures and assessed the viability of the predatory zooplankton. When introduced into the pH 10 culture, the *D. magna* population became instantaneously inactive and lost motility indicating organism death[23] (see Video S1). In contrast, the *D. magna* populations continued to remain active in cultures that were at pH 8.2 (see Video S2). These results clearly indicate that the high-pH environment is acutely detrimental, at least to some common infesting populations.

**Mixotrophic Cultivation of SLA-04.** One approach for improving biomass and lipid productivities of microalgal cultures is through heterotrophic or mixotrophic cultivation.[4] While heterotrophy solely relies on organic carbon as the energy source, mixotrophic cultures can derive additional energy from photosynthesis. As a result, glucose-to-lipid conversion efficiencies during mixotrophic cultivation have been shown to be higher than heterotrophic cultures.[4,49] To assess mixotrophic biomass and lipid productivities, indoor SLA-04 cultivation experiments were performed in a glucose-amended media with continuous illumination and air sparging. Glucose concentration in the media was 4 g·L$^{-1}$ based on previous reports which have shown that the best substrate utilization occurred in media containing <10 g·L$^{-1}$ glucose.[4,50,51] Higher glucose concentrations have been reported to cause substrate inhibition.[49]

Under mixotrophic conditions, cultures grew rapidly and nearly all of the supplied nitrate was consumed in the first 2 days (Figure 5a). During this period, 1.42 g of glucose was consumed (Figure 5b), and 0.95 g of biomass was generated (biomass yield relative to glucose used = 0.67 g·g$^{-1}$). Media pH also decreased significantly (see inset to Figure 5a) indicating glucose respiration and $CO_2$ release. During days 2−4, glucose continued to be consumed at high rates concurrently with a rapid increase biomass concentrations, despite depletion of nitrate from the medium (Figure 5a,b). A total of 1.42 g of glucose was consumed, and 0.85 g of biomass was produced during this period. Thermogravimetric analysis of the biomass samples recovered on day 4 showed a large derivative weight loss peak at 320 °C (Figure 5c), which typically corresponds to the thermal degradation of starch.[52,53] Previous studies have shown that nearly 80% of biomass starch is thermally degraded over a temperature interval of 260−360 °C.[52,53] A small mass of microalgae protein (~30%) is also volatilized over this temperature range.[54] From the weight loss data in Figure 5c, it can be observed that nearly 55% of cell mass is volatilized over the temperature interval of 260−360 °C, suggesting that biomass samples from day 4 had a high starch content, approximately 40%. It seems likely that glucose consumed during days 2−4 was utilized for starch accumulation. After day 4, the rates of glucose utilization and biomass generation were significantly decreased, but a high rate of lipid synthesis was observed (see FAME data in Figure 5b). Thermogravimetric analysis of day 10 samples (Figure 5d) showed a decrease in the magnitude of the 320 °C peak and a significant increase in the peak corresponding to triglycerides degradation at 420 °C.[32,55] Also, while FAME concentrations increased by nearly 0.7 g·L$^{-1}$ during days 4−10 (Figure 4b), the increase in biomass was only 0.2 g·L$^{-1}$ (Figure 5a). Culture pH also increased after day 4 (inset to Figure 4a, indicating a decrease in external glucose respiration rates. Taken together, the biomass, FAME and thermogravimetry measurements suggest that SLA-04 cultures first accumulated glucose as starch (days 2−4) and subsequently converted the intracellular carbohydrates to storage lipids. This mechanism of carbon "reapportionment"


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(rather than *de novo* lipid synthesis from external glucose) is consistent with previous hypotheses for post N-depletion lipid synthesis for phototrophic and mixotrophic cultures.[50,56,57]

Mixotrophic SLA-04 cultures had an overall glucose-to-lipid conversion efficiency of 0.21 g-lipid·g-glucose$^{-1}$ and a lipid productivity of 0.08 g·L$^{-1}$·day$^{-1}$. Fatty acid profiles of mixotrophic cultures (Figure S5) were similar to those of phototrophic cultures (Figures 1c and 2c). In terms of carbon conversion efficiency, 39% of glucose-C was converted into lipid-C during mixotrophic growth. From the energy content (calorific value) of the lipids produced and glucose consumed, the net energy efficiency of glucose-to-lipid conversion was 0.62 lipid calories produced per glucose calorie consumed. While high lipid productivities would decrease the capital expense of a production facility, the lipid yields relative to glucose would most significantly impact the operating costs due to the high price of glucose. Nonetheless, alternative organic carbon sources such as cellulosic materials and sugars from industrial and agricultural waste could be used to reduce the overall operating costs. From Table 3, it can be seen that in terms of both lipid productivity and lipid yields, the extremophilic str. SLA-04 compares favorably with other neutralophilic cultures.

## CONCLUSIONS

This study has demonstrated that alkaliphilic SLA-04 cultures were able to grow well at extreme pH (pH >10) without any additional supply of $CO_2$ under both indoor and outdoor conditions. The biomass productivities obtained under these conditions were comparable with productivities of previously reported neutralophilic cultures. In addition, SLA-04 cultures were able to grow mixotrophically in glucose-supplemented media and achieved high lipid productivity and relatively high efficiencies of glucose conversion to lipids. In more buffered alkaline systems, it might be possible to maintain a high pH during mixotrophic cultivation which would permit supplementation of glucose to SLA-04 cultures in low-cost raceway pond. The fatty acid composition of SLA-04 cultures (obtained under both phototrophic and mixotrophic conditions) is also favorable for biofuel production. Overall, our results demonstrate the feasibility of a novel strategy of microalgae cultivation in extremely high-pH media.

## ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssuschemeng.7b01534.

Photographs of indoor and outdoor cultivation setup, media composition and analytical methods, identities of strains used for multiple sequence analysis, phylogenetic tree and light microscope image of SLA-04, alignment of 5.8s rDNA region, ITS1 and ITS2 amplified sequences of str. SLA-04 with other eukaryotic microalgae, estimation of $HCO_3^-$ and $CO_3^{2-}$ concentrations from DIC values, FAME content and fatty acid profile of mixotrophically grown SLA-04, temperature profiles of Trials 1−3 experiments, and an example calculation showing $HCO_3^-$, $CO_3^{2-}$ and *DIC* values as a function of pH (PDF)

Video S1 shows *D. magna* in high-pH media (MPG)

Video S2 shows *D. magna* in moderate pH media (MPG)

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### Notes

The authors declare no competing financial interest.

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