A Dissertation

entitled

Enhanced Biomass and Lipid Productivities of Outdoor Alkaliphilic Microalgae Cultures through Increased Media Alkalinity

by

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Abstract

A major challenge to the economic viability of outdoor cultivation of microalgae is the high cost of CO₂ supply, even when microalgae farms are co-located with point sources of CO₂ emissions. In addition, the global capacity for algae biofuel generation is severely restricted when farm locations constrained by proximity to CO₂ sources along with the additional limitations of low slope lands and favorable climate. One potential solution to the impediments of CO₂ cost and availability is through the cultivation of microalgae in highly alkaline pH solutions (pH>10) that are effective at scavenging CO₂ from the atmosphere at high rates. The extreme alkaline pH media would also mitigate culture crashes due to microbial contamination and predators.

In this thesis, we report the indoor and outdoor cultivation of a microalgae isolate (Chlorella sp. 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 CO₂. Initial cultivation studies (both indoor and outdoor) resulted in biomass and lipid productivities that were comparable to those reported for other microalgae cultures.
cultivated in near-neutral media (pH 7-8.5). SLA-04 cultures also showed high lipid productivity and high glucose-to-lipid conversion efficiency when cultivated indoors mixotrophically in the presence of glucose as an organic carbon source.

Following this, experiments were performed to determine the effect of pH and bicarbonate (HCO$_3^-$) concentrations on biomass productivity of str. SLA-04. Increased HCO$_3^-$ concentrations in the culture medium resulted in an increase in the overall biomass productivity of str. SLA-04 (22 g-biomass∙m$^{-2}$∙d$^{-1}$). Simultaneously, the high medium pH (pH >10) led to increased mass transfer rates of CO$_2$ from the atmosphere. The improved CO$_2$ uptake rates from the atmosphere resulted in the replenishment of HCO$_3^-$ utilized during the growth of SLA-04. The quantum yields and photosynthetic parameters were also analyzed under high pH and HCO$_3^-$ conditions. Maximum quantum yield ($F_v/F_m$), an indicator of stress for microalgae, was > 0.65, suggesting that high pH conditions did not affect the photosynthetic activity of str. SLA-04. Further, high HCO$_3^-$ concentrations in the culture medium also improved the effective quantum yield ($Y_{II}$) and electron transfer rates (ETR). The increase in $Y_{II}$ and ETR would suggest that high cellular dissolved inorganic carbon (HCO$_3^-$) flux under high HCO$_3^-$ medium conditions could have driven the light-dependent reactions (in the thylakoid membrane) towards higher production of NADPH for use in carbon fixation.

Finally, large-scale cultivation studies were conducted to verify the reproducibility of biomass productivity of SLA-04 cultures under high pH and HCO$_3^-$ medium conditions. Our results showed that high biomass and lipid productivities (~20 g-biomass∙m$^{-2}$∙d$^{-1}$ and 2.3 g-FAMEs∙m$^{-2}$∙d$^{-1}$) were obtained and were similar to the results achieved in small raceway open ponds. Overall, our phototrophic cultivation studies demonstrate that
biomass and lipid productivities can be improved under high pH and HCO$_3^-$ conditions without additional concentrated CO$_2$ inputs.

Though high phototrophic productivities were achieved in the highly alkaline media, we recognized that achieving consistently high productivities throughout the year is difficult, especially during winter months due to shorter days and colder temperatures. One potential strategy to improve overall productivity and/or maintain consistently high productivity in the winter months is through mixotrophic cultivation. However, the high probability of rapid microbial contamination in outdoor reactor systems containing organic substrates have precluded the use of this approach by others. Since extreme alkaline pH media is expected to mitigate detrimental microbial contamination and predators, we assessed the feasibility of outdoor open pond mixotrophic cultivation of str. SLA-04 in a high pH growth medium (>10) supplemented with glucose. Three cultivation trials (of 10-12 d duration each) were performed in outdoor non-sterile raceway ponds. SLA-04 cultures grew well during all trials and no detrimental bacterial contamination was detected. The mixotrophically grown cultures showed significantly higher biomass and lipid productivities when compared to previously reported outdoor phototrophic cultivation studies. The lipid yield was measured to be 0.36 g-FAMEs·g-glucose$^{-1}$. To further improve the glucose-to-lipid conversion efficiency, a lipid-boost strategy was implemented wherein str. SLA-04 was first allowed to grow phototrophically until N-depletion followed by addition of glucose. The lipid-boost strategy significantly improved the lipid yields to 0.46 g-FAMEs·g-glucose$^{-1}$. Compared to the glucose conversion efficiencies for previously reported microalgae as well as oleaginous yeast, the conversion efficiencies obtained under both mixotrophic and lipid-
boost conditions were high. Overall, our results suggest that extreme growth conditions (pH>10) of str. SLA-04 enabled high-productivity mixotrophic and lipid-boost cultivation, even under non-sterile outdoor conditions.
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<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>CA</td>
<td>Carbonic anhydrase</td>
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<tr>
<td>CaCl₂·2H₂O</td>
<td>Calcium chloride dihydrate</td>
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<tr>
<td>CCM</td>
<td>Carbon concentrating mechanism</td>
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<td>CO₂</td>
<td>Carbon dioxide</td>
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<td>CO₃²⁻</td>
<td>Carbonate</td>
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<tr>
<td>CO₂*(aq)</td>
<td>CO₂ concentration at air-liquid interface</td>
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<td>CO₂bulk(aq)</td>
<td>CO₂ concentration in the bulk media</td>
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<tr>
<td>CuCl₂·2H₂O</td>
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<tr>
<td>DIC</td>
<td>Dissolved inorganic carbon</td>
</tr>
<tr>
<td>E</td>
<td>Enhancement Factor</td>
</tr>
<tr>
<td>ETRₘₐₓ</td>
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<td>Flame ionization detector</td>
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<td>HPLC</td>
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<td>Y NO</td>
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Chapter 1

1 Background and Significance

1.1 Algal biofuel challenges

Biofuels obtained from renewable sources have the potential to mitigate increasing carbon emissions and dependence on fossil energy [1, 2]. Microalgae with high lipid content are particularly attractive as feedstocks. Unlike other food crops, microalgae can be cultivated on marginal lands using low-quality water and nutrients from wastewaters [3, 4]. Studies also suggest that biofuels produced from microalgae could yield much more oil per land acre than other crops [5], at least partly due to their higher photosynthetic efficiency and ability to accumulate large quantities of storage lipids (usually triglycerides) [6, 7].

In spite of the promise, commercial production of microalgal biofuels is not in practice, at least partially, due to the inability to maintain desirable cultures with sustained high productivity over long periods [7]. In general, long-term productivity of open or closed cultivation systems is severely impacted by (i) contamination by competing species or predators such as zooplankton, (ii) inadequate mass transfer of CO₂, and (iii) low growth rates and/or lipid yields during outdoor mass production.
1.2 Carbon fixation by microalgae

Inorganic carbon uptake in aquatic photosynthetic organisms is a multiple-step mass transfer process. First CO$_2$ has to diffuse into the water phase around the cell (i.e. the growth media) and then permeate/transport into the cell. When the growth medium is low in alkalinity the inorganic carbon content of the media is intrinsically low and photosynthesis has to rely upon the rate of supply of CO$_2$ from the atmosphere. Since CO$_2$ diffusion rates and solubilities are low, most traditional algae cultivation methods sparge pure or enriched CO$_2$-containing gasses to provide sufficient DIC in solution [8-10]. While this approach certainly mitigates the C-limitations for photosynthesis, the cost of CO$_2$ supply to large areas (several hundred acres for large scale cultivation) is prohibitive. Terrestrial plants do not suffer from the same C-limitations as aquatic phototrophs since CO$_2$ has only to diffuse over short distances in the liquid phase of plant-leaf cells before becoming enzymatically fixed.

Photosynthetic C-fixation in both prokaryotes and eukaryotes is ubiquitously carried out by the RuBisCO (ribulose bisphosphate carboxylase and oxygenase) enzyme. RuBisCO is a part of the Calvin cycle that catalyzes the conversion of ribulose bisphosphate (RuBP) to 3-Phosphoglycerate (3-PGA) by incorporating CO$_2$ into the substrate as shown in Figure 1.1.
Figure 1.1: Shows the catalytic conversion of RuBP to 3PGA (by RUBisCO) in the Calvin cycle by incorporating CO$_2$.

It is important to note that RUBisCO has specific affinity for CO$_2$, but not for bicarbonate. However at pH > 8, HCO$_3^-$ is the dominant species (rather than CO$_2$). To overcome CO$_2$ limitations posed by pH driven chemical speciation, microalgae and cyanobacteria have evolved “carbon concentrating mechanisms” (CCMs). The CCM is primarily comprised of membrane-bound as well as intracellular carbonic anhydrase enzymes that convert HCO$_3^-$ to CO$_2$ [11-13].

A schematic of CCM for microalgae that grow at alkaline (pH > 8) conditions is shown in Figure 1.2. First, HCO$_3^-$ present in the media enters into the cytoplasm through an ATP-driven active transporter. The CAs present in cytoplasm then convert the HCO$_3^-$ into CO$_2$ in order to maintain a slightly lower pH (pH < 8) within the cell and also to allow passive diffusion of CO$_2$ (without ATP use) into the chloroplast. In the chloroplast, an inter-conversion of CO$_2$ and HCO$_3^-$ can again occur due to CA activity for cellular pH
regulation. Finally, the CO$_2$ and HCO$_3^-$ are transported into stroma where CAs again convert HCO$_3^-$ to CO$_2$ to deliver inorganic carbon to RuBisCO.

![Diagram of carbon concentrating mechanism]

**Figure 1.2:** Carbon concentrating mechanism of a photosynthetic organism. Under alkaline conditions, the predominant species, HCO$_3^-$, is diffused through active transportation and converted into CO$_2$ with the help of carbonic anhydrase enzymes. The inorganic carbon molecule was then transported into the chloroplast and finally into the stroma where RuBisCO enzyme utilizes it for the substrate conversion [11].

### 1.3 Influence of media alkalinity and pH on solution DIC and microalgae growth

In general, all aqueous solutions exposed to the atmosphere are driven towards equilibrium with atmospheric CO$_2$. When a mineral base (e.g. NaOH) is added to water, the acidic CO$_2$ from the atmosphere reacts with the base to form carbonates/bicarbonates.
Alternatively, when carbonate/bicarbonate salts (e.g. Na$_2$CO$_3$ or NaHCO$_3$) are directly added to water, they too undergo dissociation into ionic forms and equilibrate with atmospheric CO$_2$. Ultimately the speciation of available carbon in solution is decided by media pH as shown in the equation below.

$$CO_3^{2-} + H^+ \leftrightharpoons HCO_3^- + H^+ \leftrightharpoons H_2CO_3 \leftrightharpoons CO_2(\text{g})$$

(1)

At solution pH > 10.5, the dominant DIC species is CO$_3^{2-}$ and at pH 8.2, DIC in solution almost exclusively present as HCO$_3^-$. At lower pH (~ 6), H$_2$CO$_3$ (carbonic acid) is prevalent and finally at pH < 4.5, CO$_2(\text{L})$ is predominant. It is conceivable to achieve a nearly DIC-free solution below this pH due to the low concentration of CO$_2$ in the atmosphere and small Henry’s constant ($K_H$) for CO$_2$ solubility in water.

Thus, the net mass of DIC in aqueous media is driven by the extent of alkaline materials present in the solution. In other words, a high concentration of either base (OH$^-$) or added bicarbonate/carbonate salts would ultimately (after equilibrium with atmospheric CO$_2$) result in high solution DIC above a pH > 8. Conversely, DIC in solution is a function of [H$^+$] ions required to neutralize an alkaline solution to pH < 4.5 - also termed as “alkalinity.” More rigorously, total alkalinity (TA) can be written as:

$$TA = [HCO_3^-] + 2[CO_3^{2-}] + [OH^-]$$

(2)

The factor 2x for CO$_3^{2-}$ indicates that 2x [H$^+$] ions are required for its neutralization to carbonic acid.

When CO$_2$ is fixed at RuBisCO after uptake of HCO$_3^-$ by cell (Figure 1.2), a release of OH$^-$ ions into the media or uptake of positive ions (H$^+$ or Na$^+$) into the cell
must simultaneously occur to maintain electroneutrality as shown in Figure 1.3 [14]. It has been reported that for microalgae that rely on CCM for carbon transport, the stromal pH is close to 8 [15]. This suggests that the uptake of HCO$_3^-$ would be accompanied exclusively by the release of OH$^-$ ions since [H$^+$] is deficient at this pH.

![Figure 1.3: Shows that HCO$_3^-$ uptake under alkaline conditions is associated with either release of OH$^-$ ions or uptake of positive ions (H$^+$ or Na$^+$) to maintain electroneutrality.](image)

Irrespective of the actual charge balance mechanism (i.e. either H$^+$ uptake or OH$^-$ release), utilization of HCO$_3^-$ results in an increase in media pH. Thus, in a broad sense, HCO$_3^-$ is exchanged for OH$^-$ (mole for mole) leaving the media alkalinity remains unchanged (Eq. 2). However, the pH increase alters the DIC speciation (Eq. 1) and shifts the remaining DIC to CO$_3^{2-}$ - the DIC form that microalgae are unable to directly uptake. Thus, it would seem that as HCO$_3^-$ continues to get utilized, the culture media would quickly transition to a pH where only CO$_3^{2-}$ remains and photosynthetic C-fixation would cease. Further, previous studies speculated that increase in the CO$_3^{2-}$, a resultant of pH increase, could be toxic to microalgae cultures [16].
The disadvantages of neutralophilic and mesophilic microalgal cultures with regards to growth and culture stability encouraged us to investigate the growth and lipid accumulation kinetics of extreme alkaliphilic microalgae. Alkaliphiles are organisms that have the ability to survive and thrive at unusually high pH conditions (pH optima >9). The use of alkaliphilic microalgae can overcome many current limitations to large-scale algae production. Cultivation conditions that remain at extreme pH (>10) can allow sustained maintenance of desired alkaliphilic cultures since it is likely that contaminating populations will be less diverse in these harsh environments [16]. Further, since alkaline solutions scavenge CO$_2$ out of the atmosphere to maintain the carbonate equilibrium, dissolved inorganic carbon (DIC) for photosynthesis is also naturally more abundant at high pH [17]. Thus, costs associated with CO$_2$ sparging and distribution to large areas under microalgae cultivation can be avoided. In fact, natural alkaline lakes have been reported to have high aquatic photosynthetic CO$_2$ fixation rates [18]. Further, microalgae can be grown using low-quality alkaline water [19]. Commercial *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.

### 1.4 Research outline:

The goal of this study is to improve the biomass and lipid productivities of extreme alkaliphilic microalgae-SLA-04 without additional inputs of concentrated CO$_2$. Since this is the first study to report the use of extreme alkaliphilic microalgae (i.e. microalgae growing in media that predominantly remain above a pH value of 10) for biofuel production, several aspects associated with growth and lipid accumulation kinetics required thorough investigation. For instance, the following questions arise - how
well does the microalgae grow at extreme pH conditions, does the culture remain stable during prolonged exposure to the high pH and high alkalinity media, how does the inorganic carbon chemistry interact with the biology, would the cell composition be favorable for biofuel production? This research addresses several of these aspects and the results obtained are divided into three manuscript chapters.

The first chapter preliminary experiments to establish baseline growth and biofuel potential parameters and kinetics of SLA-04 cultures at high pH medium conditions (pH > 10) under indoor and outdoor conditions. Results showed that high pH conditions did not affect the growth of SLA-04 and kinetic parameters (i.e. biomass and lipid accumulation kinetics) were comparable with rates reported for neutralophilic microalgae. The fatty acids of lipid-enriched SLA-04 cultures comprised primarily of palmitic acid, oleic acid, linoleic acid and linolenic acid - a composition similar to cottonseed oil. However, the increase in the pH during the growth of SLA-04 cultures resulted in depletion of HCO$_3^-$ reserves in the culture medium suggesting that the medium was inadequately buffered to sustain prolonged (14-16 h) supply of bicarbonate at high concentrations.

Therefore, in the second the chapter we have focused on the improving the biomass productivities by increasing media alkalinity such that high HCO$_3^-$ concentrations were available even under high pH conditions. A series of experiments were conducted in small- (0.18 m$^2$) and large-scale ponds (3.68 m$^2$) over a period of 9 months. During this prolonged outdoor cultivation campaign, the cultures never experienced a “crash” or showed measurable signs of detrimental contamination. As hypothesized, increasing the media alkalinity prevented the depletion of HCO$_3^-$ and
ultimately resulted in a significant improvement in biomass productivity of str. SLA-04. In combination with high pH (10.1-10.2), the high alkalinity medium allowed transfer of atmospheric CO$_2$ at sufficient rates to sustain biomass growth without the need for inputs of concentrated CO$_2$.

The third chapter focuses on the mixotrophic cultivation of str. SLA-04 under outdoor non-sterile conditions. The goal of this chapter is to highlight the importance of extreme pH conditions that enabled mixotrophic cultivation of str. SLA-04 even under outdoor non-sterile conditions in the presence of glucose as an organic substrate. Cultivation studies were carried out under mixotrophic conditions over a 6 month period, and the results show that str. SLA-04 grew well with significantly improved biomass and lipid productivities. The organic carbon source (glucose) provided was utilized for both biomass and lipid production. Additionally, to improve the glucose to lipid conversion efficiency, a new “lipid-boost” strategy was employed wherein microalgae was first allowed to grow phototrophically followed by glucose addition to induce lipid accumulation and allow high yield glucose-to-lipid conversion.
Chapter 2

2 Cultivation of microalgae at extreme alkaline pH conditions – a novel approach for biofuel production

2.1 Background

Biofuels obtained from the renewable sources have the potential to mitigate increasing carbon emissions and dependence on fossil energy [20]. Microalgae with high lipid content are particularly attractive as feedstocks [21, 22] especially when cultivated on marginal lands using low-quality water (and nutrients) such as wastewater [23]. 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) the high cost of CO₂ delivery to (open or closed) cultivation systems [24-27], and (2) the 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 [28].

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 CO₂ dissolved in the aqueous growth medium. Since atmospheric CO₂ diffusion rate and solubility in water is low, pure or enriched CO₂-containing gases are sparged to enhance the availability of
dissolved CO₂. While this approach mitigates CO₂ limitations for microalgae growth, CO₂ supply over long distances is cost prohibitive [25, 26]. To lower the costs associated with CO₂ transportation, microalgae production facilities can be co-located with CO₂ sources such as power plants, cement industries or ethanol plants, but CO₂ delivery costs remain high [27]. The National Renewable Energy Laboratory’s (NREL) recent techno-economic report on microalgae biomass production [26] shows that even in co-located algal biorefineries, nearly 65% of cultivation-related variable operating costs are associated with recovery of CO₂ from flue gases and delivery to raceway ponds (of a total operating cost of $144 per ton dry algae, approximately $91 are attributable to CO₂ delivery to ponds). Further, co-location may be feasible with only a few of the available point sources of CO₂ due to land, water and climate constraints (e.g. northern temperate regions are unsuitable for algae cultivation) [27]. A recent study by Quinn et al. [25] 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 US 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 [24]. Finally, CO₂ sparged microalgae cultures that stay at near-neutral pH are prone to microbial contamination and predators (e.g. Daphnia) [28].

The use of extreme alkaliphilic microalgae can overcome many current limitations to large-scale algae production [29-33]. Extreme alkaliphiles are organisms that have the ability to survive and thrive at unusually high pH values (pH >10) [34]. Since aqueous solutions at pH>10 rapidly scavenge CO₂ [17], the supply rates of
dissolved inorganic carbon (DIC) from the atmosphere to such highly alkaline growth media is high, even in the absence of CO₂ sparging.

However, at pH > 9.5, HCO₃⁻ and CO₃²⁻ are the dominant DIC species while dissolved CO₂ concentrations are negligibly small due to the pH-dependent inorganic carbon equilibrium. Opportunely though, microalgae and cyanobacteria adapted to survive in alkaline solutions are able to sustain photosynthetic carbon fixation by utilizing HCO₃⁻ as the inorganic carbon source. It is now well established that phototrophic HCO₃⁻ utilization is facilitated through carbon concentrating mechanisms (CCMs) that are primarily comprised of membrane-bound as well as intracellular carbonic anhydrase enzymes that convert HCO₃⁻ to CO₂ within the cell [35]. RuBisCO then fixes the cellular CO₂ to organic carbon. High-pH growth media can thus provide a means for increased carbon uptake rates from the atmosphere as well as supply HCO₃⁻ as inorganic carbon for use by alkaliphilic microalgae. Not surprisingly, aquatic photosynthetic carbon fixation rates in natural alkaline lakes are high [18, 36, 37]. In engineered systems, cultivation of alkaliphilic cultures in high pH growth media could eliminate the requirement for co-location with CO₂ point sources [30-32].

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 [38]. 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 [39, 40]. 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 [30-32] and describe outdoor cultivation of an extreme alkaliphilic *Chlorella* sp., isolated from Soap Lake, Washington. 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 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.

### 2.2 Methods

#### 2.2.1 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 and contained the following: NaNO$_3$ (2.94 mM), KH$_2$PO$_4$ (1.43 mM), Na$_2$CO$_3$ (2.35 mM), NH$_4$Cl (0.93 mM), MgSO$_4$$\cdot$7H$_2$O (0.30 mM), CaCl$_2$$\cdot$2H$_2$O (0.17 mM), NaCl (0.42 mM), ferric ammonium citrate (10 mg·L$^{-1}$) and 1 mL·L$^{-1}$ trace metal solution. The trace metal solution contained - H$_3$BO$_3$ (9.7 mM), MnCl$_2$$\cdot$4H$_2$O (1.26 mM), ZnCl$_2$ (0.15 mM), CuCl$_2$$\cdot$2H$_2$O (0.11 mM), Na$_2$MoO$_4$$\cdot$2H$_2$O (0.07 mM), CoCl$_2$$\cdot$6H$_2$O (0.06 mM), NiCl$_2$·6H$_2$O (0.04 mM), V$_2$O$_5$ (0.01 mM) and KBr (0.08 mM). After addition of all components, the media pH was 10 ± 0.1. Solid media contained 15 g·L$^{-1}$ of agar in addition to the media components described above.

To isolate strains adapted to extreme pH conditions, water samples from Soap Lake in the State of Washington (USA) 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 [30].

DNA sequencing of SLA-04 was performed by the UTEX Culture Collection of Algae (Austin, TX). DNA was first extracted using a standard protocol [41] 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).

2.2.2 Indoor cultivation experiments

Initially, single colonies of str. 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·L⁻¹) as the
organic carbon source. The experimental set-up was similar to previously described systems [42]. 3 L Cytostir™ reactors (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" away from the vessel walls. Cultures were illuminated on one side which resulted in an optical path length of 6" 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.

Initially (day 0), two fluorescent lights were turned on and the incident irradiance levels were kept low to prevent photoinhibition [42] (incident intensity of 153 µmole·m⁻²·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 µmole·m⁻²·s⁻¹ for the remainder of the cultivation period. During the experiment, samples were periodically removed and analyzed as described in Section 2.3.

2.2.3 Outdoor cultivation experiments

Open raceway ponds with dimensions of 2’ × 1’ × 1’ (L × W × D) were constructed and used in these experiments (see photograph in Figure A1 of Appendix A). 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. Tap water available at the greenhouse facility was first filtered through a 10 µm 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 Section 2.2) were added to 7 L of freshly-prepared culture medium and allowed to grow in the ponds. 7 d old first-generation outdoor cultures were re-inoculated to fresh media and grown for two 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” (similar to indoor experiments, Section 2.2.3). During the experiment, samples were periodically removed and analyzed as described in Section 2.3.

2.3 Analytical Methods

2.3.1 Total suspended solids (TSS)

TSS measurements were performed to obtain biomass concentrations during growth. 20 mL of culture samples were filtered through a 0.45 µm glass fiber filter disc (Fisher Scientific, Pittsburgh, PA) and then rinsed with de-ionized water to remove media salts. The filter discs containing algal biomass were dried at 60 °C until a constant dry weight was obtained (~24 h). TSS was calculated by subtracting the weight of clean filter paper (before filtration of cultures) from the weight of filter containing dried biomass [43].

2.3.2 pH

During indoor cultivation studies (Section 2.2), pH was measured in the liquid samples using a hand-held pH meter (model: Orion A121, Thermo Scientific). pH of the
outdoor cultures (Section 2.3) was continuously monitored using Neptune APEX Lab Grade pH probes (Neptune Technology Group Inc.). pH probes were calibrated daily.

2.3.3 Soluble nitrogen

For indoor experiments (Section 2.2), total soluble N concentrations were measured using a colorimetric assay based on an alkaline persulfate digestion method. The assay was performed on culture supernatants (obtained from centrifuged cultures) by implementing a modified (scaled-down) Hach protocol (Persulfate Digestion TNT Method 10072, Hach Company, Loveland, CO). A more detailed description of the method is given in Section A1 of Appendix A.

For samples from outdoor cultivation experiments (Section 2.3), NO$_3^-$ concentrations in the supernatant (obtained from centrifuged cultures) were analyzed by an ion chromatograph (IC) equipped with an autosampler (Dionex ICS 3000, Thermo Fisher, Sunnyvale, CA). Analytes were separated on a Dionex IonPac™ CS12A anion-exchange column (maintained at 30°C) using a 30 mM potassium hydroxide eluent set at a flow rate of 1.0 mL·min$^{-1}$. Analytes were detected using a Dionex CD20 conductivity detector, and IC data were analyzed with the Chromeleon™ 7.0 software (Thermo Fisher). The instrument was calibrated using NaNO$_3$ standards of known concentrations (0-180 mg-NO$_3^-$·L$^{-1}$).

2.3.4 Dissolved inorganic carbon (DIC), HCO$_3$ and CO$_3^{2-}$

DIC was analyzed on an Innovox™ TOC analyzer equipped with an auto sampler (GE Analytical Instruments, Boulder, CO). Analysis was carried out by injecting 40 mL of filtered sample along with a 6 M HCl solution (vol. of acid/vol. of sample = 1%) and a 30% (w·v$^{-1}$) sodium persulfate solution (vol. of oxidizer/vol. of sample = 15%) into the
TOC analyzer. The acid converts the HCO$_3^-$ and CO$_3^{2-}$ in the sample to CO$_2$ while the organic carbon present in the sample is oxidized to CO$_2$ by the persulfate solution at supercritical conditions (T>350°C). DIC was measured based on CO$_2$ released upon acidification; calibrations were performed using NaHCO$_3$ standards.

HCO$_3^-$ and CO$_3^{2-}$ concentrations in the culture media were calculated from DIC and pH values using the DIC-pH equilibrium relationships based on the first ($K_1$) and second ($K_2$) dissociation constants of carbonic acid [44]. These relationships are given as:

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

and,

$$[CO_3^{2-}] = DIC \frac{K_1K_2}{[H^+]^2 + [H^+]K_1K_2}$$

where, $[H^+] = 10^{-pH}$ M, $K_1 = 10^{-6.3}$ M, and $K_2 = 10^{-10.25}$ M. The dissociation constants are for aqueous solutions with low (or no) salinity [45].

### 2.3.5 Total alkalinity

Total alkalinity (TA) of the culture medium was measured using a G20 compact titrator (Mettler-Toledo, Columbus, OH). The titrator is equipped with a 5 mL automatic burette to dispense titrant, a pH meter to continuously monitor pH and a stirrer to mix the samples while being titrated. 40 mL of sample was taken in a beaker and titrated with a 0.1 M HCl solution until the pH of the samples reached the titration endpoint of pH 4.5.

### 2.3.6 Fatty acid methyl esters (FAMEs)

Cellular lipids were recovered and quantified as fatty acid methyl esters (FAMEs) using an *in situ* transesterification method [25, 46]. Freeze dried biomass samples (10-20 mg) were weighed into clean 15 mL serum vials to which 1 mL of acidified methanol (5% H$_2$SO$_4 \cdot$v$^{-1}$) was added. The vials were crimped with Teflon-lined caps and
incubated at 90 °C for 90 min. Thereafter, the vials were cooled to room temperature, 4 mL hexane was added, and the mixture was incubated at 90°C for 15 min to extract FAMEs into hexane. After cooling down to room temperature, the hexane phase was recovered and analyzed using gas chromatography (GC). FAME standards (Sigma-Aldrich) were used to obtain calibration curves.

A Shimadzu 2010 GC equipped with an Rtx Bio-diesel column (15 m × 0.32 mm ID × 0.1 μm, Restek Corp., Bellefonte, PA) and a flame ionization detector (FID) was used to quantify FAMEs. The column oven temperature was programmed as follows – first, the temperature was held at 60 °C for 1 min. Thereafter, the temperature was increased to 370 °C at a ramp rate of 10 °C·min⁻¹. Finally, the temperature of 370 °C was maintained for 6 min at the end of the analysis run. Helium was used as a carrier gas (50 cm·s⁻¹). The injector and FID temperatures were maintained at 370 °C throughout the analysis.

2.3.7 Soluble sugar (glucose) analysis:

Samples from mixotrophic cultures were centrifuged and the supernatant was further filtered using a 0.2 μm filter. The sugars present in the filtered sample were analyzed on 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. 50 mM H₂SO₄ was used as the mobile phase at a flow rate of 0.6 mL·min⁻¹. The column was maintained at 65 °C. Glucose standards (Sigma-Aldrich) were used to obtain calibration curves.
2.3.8 Thermo-gravimetric analysis (TGA):

Qualitative estimates of triglyceride, starch and protein content in biomass were assessed using an SDT Q600 series (TA Instruments, New Castle, DE) simultaneous thermogravimetric analyzer (TGA) and differential scanning calorimeter (DSC). The instrument makes precise measurements of weight change (TGA) and differential heat flow (DSC) with an increase in temperature and/or time. Two identical alumina crucibles were placed on two horizontal beams and heated. For all measurements, 10 mg of sample was loaded in one crucible while another crucible was left empty as a reference. The TGA was programmed to heat the sample from room temperature to 600°C at a constant rate of 20 °C·min\(^{-1}\). \(\text{N}_2\) was continuously purged (100 mL·min\(^{-1}\)) through the TGA oven to maintain an inert atmosphere during analysis. The residual weight of the sample was recorded over time. Derivative weight loss was calculated from the temporal change in sample weight and is proportional to the degradation rate of the sample [47].

2.4 Results and discussion

2.4.1 Strain Identification

Microscopic examination of the cultures showed green spherical to ovoid cells with diameters ranging from 2-5 µm (see photograph in inset to Figure 2.1) – observations that are consistent with the morphology of \textit{Chlorella} sp. cells. Further, 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 \textit{Chlorella sorokiniana} str. UTEX 246 (see multiple sequence alignment data in Figure A2, Appendix A). The phylogenetic tree in Figure 2.1 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).

![Neighbor-Joining Distance Tree](image)

**Figure 2.1:** Neighbor-Joining Distance Tree (10,000 bootstrap trials – bootstrap values are shown at the branch nodes) showing the relationship of str. SLA-04 to similar sequences identified by BLAST. The phylogenetic tree was created with TreeView using multiple sequence alignment data generated by ClustalX 2.0.12. The scale bar represents substitutions per base pair. *Inset:* Microscopic image of SLA-04 showing green spherical to ovoid shaped microalgae cells. The scale bar in the image represents a 5µm length. The microscopic observations are consistent with *Chlorella sp.* cells.

### 2.4.2 Indoor phototrophic cultivation of str. SLA-04

SLA-04 cultures inoculated to an initial concentration of 0.1 g·L⁻¹ in a medium at pH 10 were cultivated indoors (triplicate reactors) under artificial illumination with
continuous air sparging. The phototrophic cultures grew well in the pH>10 medium (Figure 2.2a) with biomass productivities that compare favorably with other Chlorella cultures (Table 2.1) indicating that the extreme pH conditions are likely not inhibitory to str. SLA-04. In contrast, high pH conditions drastically diminish the growth of neutralophilic microalgae [16, 48, 49]. The literature information collated by Hansen et al. shows that media pH > 9.5 causes cessation of growth across several genera [16]. 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 [16]. While the mechanisms of growth cessation/inhibition in the neutralophilic microalgae 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 was relatively high – $58.6 \pm 2.92$ mg·L$^{-1}$·d$^{-1}$ during days 0-6 (Table 2.1). 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. 2 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.
Figure 2.2: (a) Biomass concentration and pH, (b) DIC and soluble N concentrations and (c) FAME concentrations and fatty acid profile 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.
Since the media pH was >10, it is likely that SLA-04 cultures initially grew by utilizing the available HCO$_3^-$ (estimated from Eq. 1 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 [44]. Generally, after DIC uptake, cellular carbonic anhydrases (part of the carbon concentrating mechanism) convert HCO$_3^-$ to CO$_2$ (Eq. 3), which is subsequently fixed by RuBisCO (Eq. 4) [35]. The utilization of HCO$_3^-$ by SLA-04 for photosynthetic carbon fixation (Eq. 3 and Eq. 4 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 by SLA-04 would also release OH$^-$ ions into the media (Eq. 5) [50].

\[
HCO_3^- \xrightarrow{\text{carbonic anhydrase}} CO_2, \text{cellular} + OH^- \quad (3)
\]
\[
CO_2, \text{cellular} + H_2O \xrightarrow{\text{RuBisCO}} [CH_2O]_{algae} + O_2 \quad (4)
\]
\[
NO_3^- + 2H_2O \rightarrow NH_3 + OH^- + 2O_2 \quad (5)
\]

However, since the cultures were air sparged, dissolution of ambient CO$_2$ would also simultaneously occur and neutralize the OH$^-$ ions in solution (Eq. 6) [17, 51]. The reactions shown in Eq. 6 are expected to be largely irreversible at pH values >10 due to the abundance of OH$^-$ ions.

\[
CO_{2(g)} \rightleftharpoons CO_{2(l), media} + OH^- \rightleftharpoons HCO_3^-, \text{media} \quad (6)
\]

In our experiments, the pH of the culture media was observed to increase during days 0-6 (see pH data in Figure 2.2a) suggesting that the rates of DIC addition into the media from atmospheric CO$_2$ (Eq. 6) were slower than rates of OH$^-$ generation from photosynthetic carbon fixation and nitrate assimilation (Eqs. 3-5). Accordingly, a decrease in media DIC was observed during days 0-5 (see DIC data in Figure 2.2b). An
additional consequence of the increase in pH is that the higher concentrations of OH\(^-\) in the media would drive the soluble DIC species towards a higher relative proportion of CO\(_3^{2-}\) (Eq. 7) [17, 51].

\[
HCO_3^- + OH^- \rightarrow CO_3^{2-} + H_2O \tag{7}
\]

The net result of the reaction in Eq. 7 occurring in conjunction with high rates of photosynthesis (Eqs. 3 and 4) would be a significant decrease in HCO\(_3^-\) concentration in the media. Using measured pH (Figure 2.2a) and DIC values (Figure 2.2b), we calculated the HCO\(_3^-\) and CO\(_3^{2-}\) concentrations in the culture media from Eqs. 1 and 2. Our estimates showed that the HCO\(_3^-\) concentrations in the media were significantly decreased by day 6 (see inset to Figure 2.2a). Further, the higher cell concentration (0.45 ± 0.02 g·L\(^{-1}\)) by day 6 would also have increased light attenuation within the culture. Thus, the reduced availability of HCO\(_3^-\) in combination with lower net photon flux density likely resulted in a decrease in culture productivity after day 6 (23.1 ± 2.83 mg·L\(^{-1}\)·d\(^{-1}\) (day 6-30)).

While CO\(_2\) fixation rates were low after day 6 (due to low availability of HCO\(_3^-\)), cells continued to uptake NO\(_3^-\) from the growth medium. In fact, the rate of NO\(_3^-\) uptake between days 0-3 was similar to uptake rate between days 6-10 (Figure 2.2b), although much lesser biomass was generated after day 6 (Figure 2.2a). Based on the measured values of NO\(_3^-\) utilized (Figure 2.2b) and biomass generated (Figure 2.2a), the putative biomass nitrogen content on day 10 (63.8 ± 7.3 mg·N·g-biomass\(^{-1}\)) was much higher than the nitrogen content on day 6 (42.4 ± 3.03 mg·N·g-biomass\(^{-1}\)). These results suggest that photosystem II (PS-II) was active even at very high pH conditions (pH>11) and the photosynthetically generated electrons were likely 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. 6) while the photosynthetic carbon fixation rates remained low (Eqs. 3 and 4). 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 2.2a). 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. 5). 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. 8) after day 10 allowed the final DIC and HCO$_3^-$ concentration in the medium to significantly exceed initial values (Figure 2.2b and inset to Figure 2.2a).

$$\text{TA} = [HCO_3^-] + 2[CO_3^{2-}] + [OH^-]$$

(8)

The depletion of soluble N in the media also led to the onset of lipid accumulation as is commonly observed with other microalgae [10, 43] (Figure 2.2c). Lipid content values (reported as fatty acid methyl ester (FAME)) of nearly 34% (w·w$^{-1}$) were measured and compare favorably with other mesophilic *Chlorella* strains cultivated in laboratory-scale indoor reactors with artificial illumination (Table 2.1). The increase in DIC concentration
Table 2.1: Comparison of biomass productivity and lipid content of indoor-grown Chlorella sorokiniana str. SLA-04 cultures with previously reported literature data for other Chlorella sp. cultivations under similar conditions.

<table>
<thead>
<tr>
<th>Cultivation scheme</th>
<th>Microalgae strain</th>
<th>Biomass productivity (mg-biomass·L⁻¹·day⁻¹)</th>
<th>FAME/Lipid content % (w·w⁻¹)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 L bottles</td>
<td>Chlorella vulgaris</td>
<td>10ᵃ</td>
<td>38¹</td>
<td>[52]</td>
</tr>
<tr>
<td></td>
<td>Chlorella vulgaris</td>
<td>41ᵇ</td>
<td>18²</td>
<td></td>
</tr>
<tr>
<td>2 L Bioreactor</td>
<td>Chlorella emersonii</td>
<td>28ᵇ</td>
<td>29²</td>
<td>[53]</td>
</tr>
<tr>
<td></td>
<td>Chlorella minutissima</td>
<td>32ᵇ</td>
<td>31²</td>
<td></td>
</tr>
<tr>
<td>2 L photobioreactors</td>
<td>Chlorella kessleri</td>
<td>65ᵇ</td>
<td>n. r.</td>
<td>[54]</td>
</tr>
<tr>
<td></td>
<td>Chlorella vulgaris</td>
<td>40ᵇ</td>
<td>28²</td>
<td></td>
</tr>
<tr>
<td>Tubular Bioreactor</td>
<td>Chlorella emersonii</td>
<td>41ᵇ</td>
<td>25²</td>
<td>[55]</td>
</tr>
<tr>
<td>Bioreactor</td>
<td>Chlorella vulgaris</td>
<td>104ᵇ</td>
<td>6.91²</td>
<td>[56]</td>
</tr>
<tr>
<td>Indoor ponds</td>
<td>Chlorella pyrenoidosa</td>
<td>27.9ᵃ</td>
<td>39.8³</td>
<td>[33]</td>
</tr>
<tr>
<td>3 L Cytostir® reactor</td>
<td>Chlorella sorokiniana str. SLA-04</td>
<td>58.7 ± 2.9ᵃᶜ</td>
<td>12⁴</td>
<td>Present study</td>
</tr>
<tr>
<td></td>
<td>Chlorella sorokiniana str. SLA-04</td>
<td>42 ± 4.1ᵃᵈ</td>
<td>34⁴</td>
<td></td>
</tr>
</tbody>
</table>

ᵃBiomass concentrations were directly measured as TSS.
ᵇBiomass 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.
ᶜProductivity calculated over days 0-6
ᵈProductivity calculated over days 0-30
¹Lipids were extracted using bead beating process and quantified gravimetrically.
²Lipids were quantified using Bligh and Dyer method.
³Lipids were quantified by in situ ¹H NMR spectroscopy.
⁴Lipids were quantified as FAME.

n. r. – not reported
due to increased TA would have provided more HCO$_3^-$ for the SLA-04 cultures during N-starvation and possibly enhanced lipid production [10].

The fatty acids of lipid-enriched SLA-04 cultures comprised primarily of palmitic acid (~30%), oleic acid (~20%), linoleic acid (~43%) and linolenic acid (7%) (Figure 2.2c) - a composition similar to cottonseed oil [57]. When recovered and converted to fatty acid methyl esters, the biodiesel produced from SLA-04 can be expected to meet ASTM D6751 fuel standards in the US [58].

2.4.3 Outdoor cultivation of SLA-04

After initial indoor tests (described in Section 3.1), we cultivated SLA-04 in outdoor raceway ponds to assess culture performance under more production-relevant conditions. Two cultivation trials were performed in duplicate ponds. Trial 1 was started in late August and continued through early September (Figure 2.4). Trial 2 was started in early December and continued through late December (Figure 2.3). Cultivations were started with an initial biomass concentration of nearly 0.2 g·L$^{-1}$. These concentrations were higher than initial concentrations used for indoor cultivation (0.1 g·L$^{-1}$) to prevent possible photoinhibition under natural daylight conditions [59]. The cultures were inoculated at night to allow for cell synchronization [60].
Figure 2.3: (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.
After inoculation, biomass concentrations increased monotonically along with a corresponding decrease in nitrate concentrations during both experimental trials (Figures 2.3a and 2.4a). A similar final biomass concentration of approximately 0.9 g·L⁻¹ was reached during both trials and visual observations of the cultures using light microscopy did not show any contaminating populations. The biomass productivity during Trial 1 was approximately 50 mg·L⁻¹·d⁻¹ and was nearly 20% higher than the productivity measured during Trial 2, likely due to a longer day cycle earlier in the year. The outdoor productivities were also lower than initial (day 0-6) productivities under indoor conditions, likely due to the relatively short duration of daylight (10-12 h) during experiments in Toledo (Ohio) (indoor experiments were continuously illuminated). A comparison of the outdoor raceway pond productivity of SLA-04 with other outdoor-grown 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 other studies reported in the literature (Table 2.2).
Table 2.2: Comparison of biomass productivity and lipid content of outdoor raceway pond-grown Chlorella sorokiniana str. SLA-04 cultures cultivated in the Fall season with previously reported literature data for other microalgae cultures similarly cultivated.

<table>
<thead>
<tr>
<th>Cultivation scheme</th>
<th>Microalgae strain</th>
<th>Biomass productivity (mg-biomass·L⁻¹·day⁻¹)</th>
<th>FAME/Lipid content % (w·w⁻¹)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raceway ponds</td>
<td><em>Scenedesmus acutus</em></td>
<td>39.5ᵃ</td>
<td>13.4</td>
<td>[61]</td>
</tr>
<tr>
<td>Raceway ponds</td>
<td><em>Scenedesmus acutus</em></td>
<td>66.6ᵇ</td>
<td>12.5</td>
<td>[62]</td>
</tr>
<tr>
<td>Raceway ponds</td>
<td><em>Pleurochrysis carterae</em></td>
<td>40ᶜ</td>
<td>n/a</td>
<td>[63]</td>
</tr>
<tr>
<td>Raceway ponds</td>
<td><em>Chlorella sorokiniana</em> str. SLA-04</td>
<td>48 ± 5.4ᵉ</td>
<td>16.1</td>
<td>Present study</td>
</tr>
<tr>
<td></td>
<td></td>
<td>39 ± 1.5ᶠ</td>
<td>10.3</td>
<td></td>
</tr>
</tbody>
</table>

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 Trial 1 cultivation carried out in August-September  
ᶠ Trial 2 cultivation carried out in December
pH measurements indicated a cyclic shift in pH values during the day (Figures 2.3b and 2.4b). Typically, pH varied between 10.8 and 9.5 during active growth (days 0-12). Uptake of inorganic carbon and nitrate for photosynthesis likely resulted in the release of OH\(^-\) and an increase in pH during the day (Eqs. 1 and 2) and was followed by a decrease during night due to CO\(_2\) transfer from the atmosphere and nighttime cell respiration (Eq. 3). 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 2.4b), as expected, due to consumption of nitrate [50]. By linearly interpolating between measured values of total alkalinity and using the real-time recorded values of pH, we estimated the temporal variation of media DIC and HCO\(_3^-\) (see Section A2 of Appendix A for the calculation procedure). These calculations showed a diurnal variation in DIC in a pattern that was the inverse of diurnal pH change – media DIC decreased during the day and increased at night (Figure 2.4c). In addition, due to the increase in media alkalinity, the average media DIC was also estimated to have increased over time. Bicarbonate concentrations, which depend on upon DIC values as well the pH-dependent DIC speciation (Eq. A7, Appendix A), also varied diurnally (Figure 2.4c). While HCO\(_3^-\) concentrations were estimated to decrease during the day, our calculations suggest that HCO\(_3^-\) was never completely depleted, and at least ~0.5-1mM 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 2.4c).
After the nitrate depletion from the media, 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 2.3c) and Trial 2 experiments, respectively. The greater accumulation of lipids during Trial 1 experiments was likely due to the greater daylight duration during these experiments. However, these values were significantly lower than the FAME content of indoor cultures (34% as discussed in Section 3.2). Previous studies have shown that lipid production increases when illumination levels are high [64, 65], likely due to the higher requirement of cellular reducing equivalents for lipid synthesis. Though the temperature was controlled in the greenhouse during outdoor cultivation of SLA-04 cultures, the short day periods likely did not provide sufficient photon flux for high levels of lipid accumulation. Similar observations were made in a recent study in which Olofsson et al. 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%) [66].
Figure 2.4: (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 the (a) and (b) are averages from duplicate runs. Error bars indicate one standard deviation from mean values.
2.4.4 Mixotrophic cultivation of SLA-04:

One approach for improving biomass and lipid productivities of microalgal cultures is through heterotrophic or mixotrophic cultivation [23]. 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 [23, 52]. To assess mixotrophic biomass and lipid productivities, indoor SLA-04 cultivation experiments were performed in a glucose-amended media in the presence of light. 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 [23, 67]. Higher glucose concentrations have been reported to cause substrate inhibition [52]. Similar to phototrophic cultivation, cultures inoculated at a concentration of 0.1 g·L$^{-1}$ into a pH 10 medium were incubated under artificial illumination and continuous air sparging.

Under mixotrophic conditions, cultures grew rapidly and nearly all of the supplied nitrate was consumed in the first 2 d (Figure 2.5a). During this period, 1.42 g of glucose was consumed (Figure 2.5b) 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 2.5a) 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 (Figures 2.5a and 2.5b). 1.42 g of glucose was also 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 2.5c), which typically corresponds to the thermal degradation of starch [68, 69]. Previous studies have shown that nearly 80% of biomass starch is thermally degraded over a temperature interval of 260-360 °C [68, 69]. A small mass of microalgae protein (~30%) is also volatilized over this temperature range [70]. From the weight loss data in Figure 2.5c, 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 2.5b).

Thermogravimetric analysis of day 10 samples (Figure 2.5d) showed a decrease in the magnitude of the 320 °C peak, but a significant increase in the peak corresponding to triglycerides degradation at 420 °C [47, 71]. Also, while FAME concentrations increased by nearly 0.7 g·L⁻¹ during days 4-10 (Figure 2.5b), the increase in biomass was only 0.2 g·L⁻¹ (Figure 2.5a). Culture pH also increased after day 4 (inset to Figure 2.5a, 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” (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 [67, 72, 73].
Figure 2.5: (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) show thermograms of day 4 and day 10 samples. Residual weight data are indicated by the dashed lines. Derivative weight loss data is represented by the solid lines.
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·d⁻¹. Fatty acid profiles of mixotrophic cultures (Figure A3 of Appendix A) were similar to those of phototrophic cultures (Figures 2.2c and 2.3c). 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. From Table 2.3, 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.
Table 2.3: 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 *Chlorella sp.* cultivations under similar conditions.

<table>
<thead>
<tr>
<th>Microalgaes strain</th>
<th>Initial glucose conc. (g·L⁻¹)</th>
<th>Final biomass conc. (g·L⁻¹)</th>
<th>Lipid content (%(w·w⁻¹))</th>
<th>Lipid productivity (g·L⁻¹·d⁻¹)</th>
<th>Lipid yield (g-lipid·g-glucose⁻¹)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Chlorella sp.</em> UTEX 259</td>
<td>10</td>
<td>1.70</td>
<td>21.0</td>
<td>0.03</td>
<td>0.04</td>
<td>[52]</td>
</tr>
<tr>
<td><em>Chlorella minutissima</em></td>
<td>10</td>
<td>0.38</td>
<td>11.8</td>
<td>0.004</td>
<td>0.004</td>
<td>[74]</td>
</tr>
<tr>
<td><em>Chlorella sorokiniana</em> UTEX1602</td>
<td>4</td>
<td>3.55</td>
<td>21.4</td>
<td>0.12</td>
<td>0.19</td>
<td>[23]</td>
</tr>
<tr>
<td><em>Chlorella sorokiniana</em> CCTCC M209220</td>
<td>5</td>
<td>1.20</td>
<td>47.3</td>
<td>0.05</td>
<td>0.12</td>
<td>[67]</td>
</tr>
<tr>
<td><em>Chlorella vulgaris</em></td>
<td>4</td>
<td>1.41</td>
<td>13.8</td>
<td>0.03</td>
<td>0.05</td>
<td>[75]</td>
</tr>
<tr>
<td><em>Chlorella sp.</em></td>
<td>2</td>
<td>1.38</td>
<td>26.0</td>
<td>0.04</td>
<td>0.18</td>
<td>[76]</td>
</tr>
<tr>
<td><em>Chlorella sorokiniana</em> SLA-04</td>
<td>4</td>
<td>2.25</td>
<td>36.7</td>
<td>0.08</td>
<td>0.21</td>
<td><em>Present study</em></td>
</tr>
</tbody>
</table>
2.5 Conclusions

This study has demonstrated that alkalophilic SLA-04 cultures were able to grow well at extreme pH (pH >10) without any additional supply of CO\textsubscript{2} under both indoor and outdoor conditions. The biomass productivities obtained under these conditions were comparable with productivities of previously reported mesophilic 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.
Chapter 3

3 High-productivity cultivation of microalgae without concentrated CO₂ input

3.1 Background

In spite of the potential of biofuels derived from microalgae to displace a significant volume of fossil fuels [77-80], their production is not yet commercially practiced largely due unfavorable process economics [81]. In particular, cultivation of microalgae has remained an expensive proposition even when high productivities are achieved due to frequent culture crashes [28, 82, 83] and the high cost of CO₂ supply [24, 84-86].

Generally, open raceway ponds (rather than photobioreactors) are widely considered as the most economical method for microalgae cultivation [87, 88] and cultures are usually grown in a near-neutral pH environment [89]. Since the mass transfer of CO₂ from the ambient atmosphere to pH-neutral growth media is slow, the availability of dissolved CO₂ in raceway ponds is traditionally improved by sparging concentrated CO₂ [88]. It is understood that when CO₂ from large emitters (e.g. fossil power plants, cement industries or ethanol fermentation facilities) are utilized for microalgae cultivation, the integrated process would mitigate CO₂ emissions from these industries.
Simultaneously, this approach would allow for large-scale production of microalgae and thus achieve scale-dependent cost savings for biofuel production.

However, CO_2 mass transfer rates from flue gas (containing < 15% CO_2) to neutral-pH ponds remain low, and it is estimated that only 10% of flue gas CO_2 is ultimately captured when sparged directly in ponds [90]. More efficient CO_2 capture can be accomplished through the use of microbubbles [91, 92], however at the expense of a higher energy penalty [93]. Alternately, more recently, use of pure/concentrated CO_2 has been proposed to improve the efficiency of CO_2 capture and to reduce the volume of gases transported to the cultivation ponds [94, 95]. It is assumed that in a commercial process, flue gas CO_2 would be captured at the fossil power plant into a chemical solvent (e.g. monoethanolamine) and subsequently released during solvent regeneration to yield a concentrated CO_2 stream that could be supplied to co-located cultivation ponds [96]. However, with this state-of-the-art approach, the costs of CO_2 delivery to ponds remain high. In a recent techno-economic report published by the National Renewable Energy Laboratory (NREL, Golden, Colorado, USA), nearly 65% of cultivation-related variable operating costs were assessed to be associated with CO_2 supply. Per the NREL report, of a total estimated operating cost of $144 per ton algae generated during cultivation, approximately $91 were attributed to the cost of CO_2 delivery to ponds [97].

In addition to high costs of CO_2 supply, a greater concern is the relevance of microalgae biofuels towards global fossil fuel displacement, if microalgae production is constrained by the proximate availability of flue gases or other high concentration CO_2 sources. Since fossil power plants are generally sited close to freshwater sources, the land surrounding the power plants is also usually suitable for agriculture or forestry. Large-
scale microalgae cultivation at these sites would likely not be an environmentally sustainable proposition. A recent study by Quinn et al., [86] estimated that microalgae cultivation systems that are constrained by the availability of flue gases (in addition to low-slope barren lands and favorable climates) could potentially produce <0.1 billion barrels of economically-viable biofuel per year. In contrast, the US consumes approximately 5 billion barrels of transportation fuel per year. The assessments of Quinn et al. thus indicate that microalgae biofuels that are sustainably produced in proximity to flue gas sources would, quite literally, be only a “drop in the US transportation fuel bucket”. However, global assessments of microalgae production potential based on meteorological data alone (without considering the proximity of flue gas resources) suggest that many regions can displace up to 30% of their current fuel use with microalgae-derived biofuels by utilizing the non-arable land. Thus, while the potential for meaningful displacement of fossil fuels with microagal biofuels exists, new cultivation technologies must be developed that achieve high productivities, yet do not rely on CO₂ from concentrated sources.

One potential approach to the cultivation of microalgae in the absence of concentrated CO₂ inputs is through utilization of highly alkaline pH solutions as the culture media since solutions at pH>10 can rapidly scavenge CO₂ from the ambient air [98]. Previously, we isolated and successfully cultivated an extreme alkaliphilic microalga (Chlorella sorokiniana SLA-04) in media at pH>10 (Chapter 2). Further, we demonstrated productivities of ~ 5 g·m⁻²·d⁻¹ during outdoor raceway-pond cultivation during autumn/winter months. However, we recognize that for commercial cultivation of this and other extreme alkaliphiles, much higher productivities are needed. As such,
based on a fundamental understanding of inorganic carbon chemistry and phototrophic carbon fixation, in this study, we have developed and demonstrated media conditions that allow for sustained high productivities in outdoor raceway ponds, even in the absence of supplemental CO$_2$ input.

3.2 Materials and Methods

3.2.1 Cultivation experiments

All experiments were carried out using a *Chlorella sorokiniana* str. SLA-04 that was isolated from Soap Lake in the State of Washington (USA) (see isolation and identification details in Chapter 2). A series of experiments were conducted from February to October 2015. First, the experiments were performed in small raceway ponds (total volume = 30 L, surface area = 0.18 m$^2$) and based on results, a second set of larger scale cultivations were performed (total volume = 750-1100 L, surface area = 3.68 m$^2$).

As previously described in Chapter 2, the 30 L ponds had dimensions of 2’ × 1’ × 1’ (L × W × D, see photograph in Figure B1 of Appendix B). These ponds were equipped with real-time temperature- and pH- monitoring and logging (Neptune APEX data logging systems, Neptune Technology Group Inc., Tallassee, AL). The cultures were mixed with paddlewheels at an average flow velocity of 6 cm·s$^{-1}$. Larger 3.68 m$^2$ ponds (see photographs in Figures B2 and B3, Appendix B) were purchased from Commercial Algae Professionals (Peachtree City, GA). Similar to 30 L ponds, the large ponds were also equipped with temperature, pH, and ambient light intensity monitoring devices. The data was recorded continuously using a YSI data logging system (YSI Incorporated, OH, USA). In larger ponds, the cultures were mixed using stainless steel helical/scissor bent-blade paddlewheels and an average flow velocity of 30 cm·s$^{-1}$ was achieved.
All ponds were placed in an outdoor greenhouse and tap water available at the greenhouse facility was first filtered through a 10 µm filter (to remove sediments) and used for media preparation without sterilization. Evaporation losses during the experiment were compensated with water addition based on the culture depth, and the samples were periodically removed and analyzed as described in Section 3.2.3.

3.2.2 Growth medium

Cultures were grown in a medium that comprised the following: KH$_2$PO$_4$ (0.3 mM), MgSO$_4$·7H$_2$O (0.3 mM), CaCl$_2$·2H$_2$O (0.17 mM), NaCl (0.42 mM), ferric ammonium citrate (10 mg·L$^{-1}$) and 1 mL trace metal solution. The trace metal solution comprised - H$_3$BO$_3$ (9.7 mM), MnCl$_2$·4H$_2$O (1.26 mM), ZnCl$_2$ (0.15 mM), CuCl$_2$·2H$_2$O (0.11 mM), Na$_2$MoO$_4$·2H$_2$O (0.07 mM), CoCl$_2$·6H$_2$O (0.06 mM), NiCl$_2$·6H$_2$O (0.04 mM), V$_2$O$_5$ (0.01 mM) and KBr (0.08 mM). NaHCO$_3$, Na$_2$CO$_3$, and NaNO$_3$ were supplemented to the medium as the inorganic carbon- and N- sources and varied based on the specific experiment goals (concentrations of each component are described along with the corresponding experimental data in the Results section)

3.2.3 Analytical

3.2.3.1 Wet sample analysis

3.2.3.1.1 Total suspended solids

Total solids (biomass) in the culture was determined by following the Laboratory Analytical Procedure outlined by NREL [99] with a slight modification. In brief, 20 mL culture samples were recovered from the ponds and their pH was first adjusted to a value of 8.2 (using 0.1 M HCl) to dissolve carbonate and phosphate precipitates present in the culture medium, if any. Thereafter, the samples were filtered through a 0.45 µm glass
fiber filter disc (Fisher Scientific, Pittsburgh, PA) and washed with de-ionized (DI). The filter discs containing biomass were dried at 40 °C until a constant dry weight was achieved. TSS value was obtained by subtracting the weight of the clean filter paper from the weight of the filter paper containing biomass.

### 3.2.3.1.2 Soluble Nitrogen

$\text{NO}_3^-$ concentrations in the supernatant (from samples centrifuged at 3000×g) were measured by an ion chromatograph (IC) equipped with an autosampler (Dionex ICS 3000, Thermo Fisher, Sunnyvale, CA). A Dionex IonPac™ CS12A anion-exchange column (maintained at 30 °C) was used to separate $\text{NO}_3^-$ from other anions in the sample using a 30 mM potassium hydroxide eluent set at a flow rate of 1.0 mL·min$^{-1}$. Dionex CD20 conductivity detector (maintained at 35 °C) was used for anion detection, and IC data were analyzed with the Chromeleon™ 7.0 software (Thermo Fisher). The instrument was calibrated using NaNO$_3$ standards of known concentrations (0-120 mg-NO$_3^-$·L$^{-1}$).

#### 3.2.3.1.3 Total alkalinity, dissolved inorganic carbon (DIC), carbonate and bicarbonate

Total alkalinity (TA) of the culture medium was measured using a G20 compact titrator (Mettler-Toledo, Columbus, OH). The titrator is equipped with a 5 mL automatic burette to dispense titrant, a pH meter to continuously monitor pH and a stirrer to mix the samples while being titrated. To determine the TA (referred as M-value), 40 mL of sample was taken in a beaker and titrated with a 0.1 M HCl solution until pH of the samples reached the titration endpoint of pH 4.5. To determine the carbonate concentration (referred as P-value), 5 mL of sample was titrated with a 0.1 M HCl to an endpoint pH value of 8.2. DIC was estimated as the difference between M- and P-values (Eq. 1), and
the HCO₃⁻ concentration was obtained by subtracting P-value from DIC concentration (Eq. 2).

\[
\text{DIC} = M - P
\]  

(1)

\[
\text{HCO}_3^- = \text{DIC} - P
\]  

(2)

To estimate TA values between measured experimental points, a linear interpolation was used, HCO₃⁻ concentrations of the interpolated values were estimated from real-time recorded values of pH. Detailed calculation procedure was shown in the Section B4 (Appendix B).

3.2.3.1.4 Pulse amplitude modulated (PAM) fluorometry

DUAL-PAM 100 Chlorophyll Fluorometer (Heinz Walz GmbH, Effeltrich, Germany) was used to measure the quantum yield and photosynthetic parameters. 3 mL of sample was taken in a quartz glass cuvette and incubated in dark for 5 minutes with continuous stirring to obtain the minimum fluorescence yield (F₀). Then a saturation pulse (10000 µmole·m⁻²·s⁻¹) of blue light was applied for 0.6 s to get maximum fluorescence yield (Fₘ). These fluorescence yield parameters (F₀ and Fₘ) were then used to estimate the maximum quantum yield (Fᵥ/Fₘ) with Fᵥ = Fₘ - F₀. Thereafter, a rapid light curve (RLC) was generated using 10 incremental actinic light levels (ranging from 11 to 840 µmole·m⁻²·s⁻¹) with 10s of dark incubation between each actinic “flash”. The fluorescence yield parameters obtained at each increment was used to determine the effective quantum yield (Y II), quantum yield of regulated energy dissipation (Y (NPQ)) and quantum yield of non-regulated energy dissipation (Y (NO)). The generated RLC was used to determine the maximum electron transfer rate (ETRₘₐₓ) [100]. A Dual PAM software (v 1.9) was used to record and analyze the data.
3.2.3.2 Dry sample analysis

The liquid samples collected were first centrifuged (3000×g) and the wet biomass pellet was rapidly frozen using liquid N$_2$ and freeze-dried using a Benchtop Freeze Dryer (Labconco, Kansas City, MO). The pressure and temperature of freeze drying system were maintained at 0.0025 mBar and -70 °C, respectively.

3.2.3.2.1 Moisture content

The moisture content of the freeze-dried samples were analyzed using an SDT Q600 series (TA Instruments, New Castle, DE) Thermogravimetric Analyzer (TGA). The instrument makes precise measurements of weight change with an increase in temperature and/or time. For all the experiments, nearly 10 mg of freeze-dried biomass sample was loaded in one crucible while another crucible was left empty as a reference. The TGA was programmed to first heat the sample from room temperature to 105 °C at a rate of 20 °C·min$^{-1}$ and then maintain isothermal conditions until a constant weight was achieved. Air was continuously purged (100 mL·min$^{-1}$) through the TGA oven during the analysis. The residual weight of the sample was recorded over time.

3.2.3.2.2 Fatty acid methyl esters (FAMEs)

Cellular lipids were recovered and quantified as fatty acid methyl esters (FAMEs) using an in situ transesterification method [25, 101]. Freeze dried biomass samples (30 mg) were weighed into clean 15 mL serum vials to which 1 mL of acidified methanol (5% H$_2$SO$_4$ v/v) was added. The vials were crimped with Teflon-lined caps and incubated at 90 °C for 90 min. After that, the vials were cooled to room temperature, 4 mL hexane was added, and the mixture was incubated at 90 °C for 15 min to extract FAMEs into hexane. After cooling down to room temperature, the hexane phase was recovered and
analyzed using gas chromatography (GC). FAME standards (Sigma-Aldrich) were used to obtain calibration curves.

A Shimadzu 2010 GC equipped with an Rtx Bio-diesel column (15 m × 0.32 mm ID × 0.1 µm, Restek Corp., Bellefonte, PA) and a flame ionization detector (FID) was used to quantify FAMEs. The column oven temperature was programmed as follows – first, the temperature was held at 60 °C for 1 min. Thereafter, the temperature was increased to 370 °C at a ramp rate of 10 °C·min⁻¹. Finally, the temperature of 370 °C was maintained for 6 min at the end of the analysis run. Nitrogen was used as a carrier gas (50 cm·s⁻¹). The injector and FID temperatures were maintained at 370 °C throughout the analysis.

3.2.3.2.3 Total carbohydrates

Total carbohydrates in the biomass samples were determined following the Laboratory Analytical Procedure outlined by NREL [102]. In brief, 50 mg of freeze-dried biomass was taken in an autoclavable 30 mL crimp-top glass tubes to which 0.5 mL of 72% H₂SO₄ was added. The glass tubes were then incubated at 30 °C for 60 min, and the samples were mixed every 5 min using a glass stir rod. After that, the acid concentration was adjusted to 4% by adding 13.89 mL of DI water. The vials were then sealed with Teflon-lined caps and autoclaved at 120 °C for 60 min. Samples were then cooled to room temperature, neutralized using CaCO₃ and finally filtered using a 0.2 µm filter.

The filtered samples were analyzed on 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. 50 mM H₂SO₄ was used as the mobile phase at a flow rate of 0.6 mL·min⁻¹. The column was maintained
at 65 °C. Glucose, xylose, galactose, arabinose and mannose standards (Sigma-Aldrich) were used to obtain calibration curves.

### 3.2.3.2.4 Elemental (CHN) analysis

Elemental analysis was carried out using a CHN analyzer (Flash 2000 series, CE Elantech Inc.) equipped with an autosampler and a thermal conductivity detector (TCD). 3-7 mg biomass samples were sealed in tin boats and were placed in the autosampler. The combustion temperature was set to 950 °C. Helium was used as the carrier gas (140 mL·min⁻¹) as well as the reference gas (100 mL·min⁻¹). Acetanilide standards were used to obtain calibration curves for C, H, and N.

### 3.2.3.2.5 Ash content

Ash content was determined using the Laboratory Analytical Procedure outlined by NREL [102] using an SDT Q600 series (TA Instruments, New Castle, DE) Thermogravimetric Analyzer (TGA). Two identical platinum crucibles were placed on two horizontal beams and heated. The instrument makes precise measurements of weight change with an increase in temperature and/or time. For all the experiments, 10 mg of washed and dried sample (free of media salts) was loaded in one crucible while another crucible was left empty as a reference. The TGA was programmed to first heat the sample from room temperature to 105 °C at a rate of 20 °C·min⁻¹ and maintain isothermal for 10 min to evaporate moisture, if any. Then, the sample was heated to 250 °C at a rate of 20 °C·min⁻¹ and maintained isothermal for 30 min. Finally, the sample was heated to 575 °C at a rate of 20 °C·min⁻¹ and maintained isothermal for 180 min. Air was continuously purged (100 mL·min⁻¹) through the TGA oven during analysis. The residual weight of the sample was recorded over time.
3.3 Results and Discussion

To improve the productivity of extreme alkaliphiles, it is important to understand the fundamentals of carbon uptake process under alkaline conditions. Therefore, we first describe the phototrophic carbon fixation process in alkaliphilic microalgae (Section 3.3.1). This is followed by a discussion of the rationale for increasing HCO$_3^-$ concentration and experimental results for cultivation of SLA-04 in the high alkalinity growth medium (Section 3.3.2). Thereafter, we have presented the rationale for using high medium pH in addition to high alkalinity and experimental results from studies in a pH~10 media that also contained a total alkalinity of 60-160mM are presented (Section 3.3.3). Based on the experimental data obtained from sections 3.3.2 and 3.3.3, large scale cultivations were carried out and the results were discussed in section 3.3.4.

3.3.1 Carbon uptake mechanism

The conversion of inorganic carbon to organic carbon using light energy (i.e. photosynthesis) takes place in two stages: light dependent and light independent reactions (Figure 3.1). The light dependent reactions occur in thylakoid region of cell and have two photosystems (PS I and PS II) that work in tandem to produce energy (ATP) and reducing equivalents (NADPH). When a photon strikes the chlorophyll within a photosystem, the energy gets absorbed and is either used for excitation of e$^-$ or dissipated as heat/fluorescence. The energized e$^-$ is then transported through an electron transport chain where it is used for nitrate reduction and chemical energy production (NADPH) (see the reactions below the dotted line, Figure 3.1) [105]. The NADPH thus produced is used in light independent reactions for carbon fixation process that first involves multiple steps for cellular DIC transport (see reactions above dotted line, Figure 3.1). Under
alkaline conditions where DIC primarily comprises of HCO\textsubscript{3}\textsuperscript{-}, the inorganic carbon uptake occurs through a carbon concentrating mechanism (CCM) involving a series of HCO\textsubscript{3}\textsuperscript{-} transporters and carbonic anhydrases (CA) as shown in Figure 3.1 [13, 106].

**Figure 3.1:** The light dependent reactions shows the flow of e\textsuperscript{-} generated during photosynthesis and the light independent reactions shows cellular DIC transport and carbon fixation mechanisms in alkaline medium conditions.

### 3.3.2 Rationale for increasing HCO\textsubscript{3}\textsuperscript{-} concentration in culture medium

Since DIC uptake process involves multiple steps, it can be kinetically limited depending on the external DIC concentrations. In fact, previous studies suggest that the external DIC concentration determines CA enzyme activity. Further, the Michaelis constant for CAAs is reported to be in the range of 10-20 mM [107, 108] and low DIC availability limits the kinetics of the enzyme. It has also been reported that under low HCO\textsubscript{3}\textsuperscript{-} concentrations, CAH1 enzyme expression is low [106, 109] which would
ultimately decrease the availability of \( \text{CO}_2 \) at RuBisCO. The decreased availability of \( \text{CO}_2 \) at RuBisCO site due to low external \( \text{HCO}_3^- \) concentrations could in turn affect the rates of the Calvin cycle where carbohydrate synthesis occurs using the chemical energy (NADPH) from the light dependent reactions. Therefore, it is reasonable to infer that high \( \text{HCO}_3^- \) medium conditions could increase both net DIC transport rates and CA enzyme activity which eventually could yield increased carbon fixation rates.

To test this hypothesis, a series of outdoor cultivation experiments (Trial 1) were conducted to determine the effect of media \( \text{HCO}_3^- \) concentration on biomass productivity. These experiments were performed in 30 L raceway ponds with working volume of 20 L, and a culture depth of 5” during February. SLA-04 cultures were inoculated to media with initial \( \text{HCO}_3^- \) concentrations ranging from 6-40 mM. In these experiments, the cultures were started at an initial pH of 8.7 such that nearly all of the available DIC was in the form of \( \text{HCO}_3^- \). Cultivations were started with an initial biomass concentration of nearly 0.2 g∙L\(^{-1}\) (Figure B5, Appendix B). Since the average daylight intensities were low in February in Ohio, natural light was supplemented with artificial illumination to ensure that the total incident irradiance was in the range 800 - 1000 µmole∙m\(^{-2}∙\)s\(^{-1}\). The TSS (Figure B5-1, Appendix B) and soluble N utilization data (Figure B5-2, Appendix B) indicates that biomass concentration increased with a corresponding decrease in soluble N concentration under all cultivations conditions.

The biomass productivities estimated for a 2-day cultivation period at each initial \( \text{HCO}_3^- \) concentration are shown in Figure 3.2a. One can observe that biomass productivities were improved as the \( \text{HCO}_3^- \) concentration in the culture medium was increased (Figure 3.2a). The overall biomass productivity of str. SLA-04 grown in the
medium with HCO$_3^-$ concentration of 6.3 mM (measured value) was $9.7 \pm 2.6$ g-biomass·m$^{-2}$·d$^{-1}$. When the medium HCO$_3^-$ concentration was increased to 39 mM (measured value), the biomass productivity increased by 2-fold and was measured to be $19.2 \pm 0.16$ g-biomass·m$^{-2}$·d$^{-1}$ (Figure 3.2a). The increase in biomass productivity at high DIC (HCO$_3^-$) medium concentrations indicates that the net transport rates (shown as filled ovals in Figure 3.1) were enhanced such that more DIC is available for CAs, consistent with our hypothesis at the beginning of these experiments.
Figure 3.2: Effect of HCO$_3^-$ concentration on (a) Biomass productivity, (b) pH of str. SLA-04 cultivated under outdoor conditions at an initial medium pH of 8.7 during February. Values shown in the graph are averages from duplicate runs. Error bars indicate one standard deviation from mean values.
Also, for cultures with low HCO₃⁻ concentration (6.3 mM), the increase in OH⁻ ions, a resultant of pH increase (Figure 3.2b) during the HCO₃⁻ uptake (Eq. 3), would have shifted the DIC equilibrium towards CO₃²⁻ (Eq. 4). The HCO₃⁻ uptake (Eq. 3) and DIC conversion (Eq. 4) during the growth of microalgae would have led to HCO₃⁻ depletion in the culture medium. Concomitantly, HCO₃⁻ concentrations (measured) indicate that HCO₃⁻ levels came to near depletion (shown in Figure B6, Appendix B).

\[ HCO_3^- \rightarrow CO_{2\text{fixed}} + OH^- \] (3)

\[ HCO_3^-, \text{media} + OH^- \rightleftharpoons CO_3^{2-}, \text{media} + H_2O \] (4)

On the other hand, with an increase in HCO₃⁻ concentration in the culture medium, it was observed that the relative net change in pH is low (Figure 3.2b) due to a stronger buffering action. Although smaller, the increase in pH even at high HCO₃⁻ concentrations would have led to an increase in CO₃²⁻ concentration which in turn resulted in depletion of HCO₃⁻ concentrations in the culture medium (Figure B6, Appendix B). Nonetheless, the high initial HCO₃⁻ concentration ensured that sufficient DIC is available during the growth period (Figure B6, Appendix B) that eventually resulted in increased biomass productivity (Figure 3.2a).
Table 3.1: Comparison of biomass productivity of outdoor grown SLA-04 cultures with previously reported literature data for other microalgae cultivations.

<table>
<thead>
<tr>
<th>Type of algae</th>
<th>Cultivation scheme</th>
<th>pH</th>
<th>Aeration</th>
<th>Biomass productivity (g·m⁻²·d⁻¹)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Staurosira sp.</td>
<td>Open ponds</td>
<td>8.2</td>
<td>CO₂</td>
<td>21.1</td>
<td>[84]</td>
</tr>
<tr>
<td>Desmodesmus sp.</td>
<td>Conventional ponds</td>
<td>7-7.5</td>
<td>CO₂</td>
<td>3.47ᵇ</td>
<td>[89]</td>
</tr>
<tr>
<td></td>
<td>ARID ponds</td>
<td></td>
<td></td>
<td>3.34ᵇ</td>
<td></td>
</tr>
<tr>
<td>Nanochloropsis Salina</td>
<td>Open ponds</td>
<td>8.5ᵃ</td>
<td>n/a</td>
<td>30.1ᶜ</td>
<td>[108]</td>
</tr>
<tr>
<td>Pleurochrysis carterae</td>
<td>Open ponds</td>
<td>7.7</td>
<td>CO₂</td>
<td>11.7ᵈ</td>
<td>[109]</td>
</tr>
<tr>
<td>Phaeodactylum tricornutum</td>
<td>Open ponds</td>
<td>n.r</td>
<td>CO₂</td>
<td>4.9ᵉ</td>
<td>[61]</td>
</tr>
<tr>
<td>Scenedesmus acuta</td>
<td>Open ponds</td>
<td>n.r</td>
<td>CO₂</td>
<td>6.62 ± 2.3ᶠ</td>
<td>[62]</td>
</tr>
<tr>
<td></td>
<td>(0.18 m²)</td>
<td>9.9ᵃ</td>
<td>n/a</td>
<td>34.7 ± 1.4ᵍᵃ</td>
<td>Present study</td>
</tr>
<tr>
<td></td>
<td>(3.68 m²)</td>
<td>10.05ᵈ</td>
<td>n/a</td>
<td>9.2 ± 0.67ᶠ</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10.16ᵈ</td>
<td></td>
<td></td>
<td>19.1ᶠ</td>
<td></td>
</tr>
</tbody>
</table>

ᵃ pH was not controlled and the value represents the initial pH of the culture system.
ᵇ Cultivation experiments were carried from January to April
ᶜ Cultivation experiments were carried during February
ᵈ Cultivation experiments were carried during Summer
ᵉ Cultivation experiments were carried from October to November
ᶠ Cultivation experiments were carried from February to May
ᵍ Cultivation experiments were carried in July
ʰ Maximum biomass productivity
ᵱ Overall biomass productivity
ᵵ Cultivation experiments were carried in September and at a culture depth of 10”
ᵶ Cultivation experiments were carried in October and at a culture depth of 7”
Further, when str. SLA-04 was cultivated under pH-controlled (pH ~ 8.7) conditions with an initial HCO$_3^-$ concentration of 17 mM, the biomass productivity obtained (16.5 g-biomass m$^{-2}$ d$^{-1}$, Figure 3.2a) is relatively higher (~20%) than the productivity obtained in pH-uncontrolled cultures inoculated with initial HCO$_3^-$ concentration of 19 mM (Figure 3.2a). The low biomass productivities associated with pH uncontrolled cultures would be due to depletion of soluble HCO$_3^-$ reserves (Figure B6, Appendix B) in the culture medium during the growth of microalgae (due to net HCO$_3^-$ depletion as indicated by Eqs. 3 and 4). Nonetheless, the biomass productivity of str. SLA-04 under pH uncontrolled conditions (i.e. without additional CO$_2$ supply) compare favorably with previously reported microalgae cultures cultivated with additional concentrated CO$_2$ inputs (Table 3.1). Overall, the results from Trial 1 experiments suggest that CO$_2$ supplementation can be eliminated and high yields can be achieved when alkaliphilic cultures are cultivated in the presence of high HCO$_3^-$ concentrations.

3.3.3 Inorganic carbon transfer and availability in extreme alkaliphilic media and cultures

CO$_2$ mass transfer rates into the alkaline media are governed by concentration gradient between the equilibrium CO$_2$ concentration at the gas-liquid interface ($CO_{2(aq)}^*$) and the CO$_2$ concentration in the bulk media ($CO_{2(aq)}^{bulk}$) – the net driving force is equal to $CO_{2(aq)}^* - CO_{2(aq)}^{bulk}$. The bulk CO$_2$ concentration ($CO_{2(aq)}^{bulk}$) is determined by the equilibrium established with HCO$_3^-$ and OH$^-$ ions present in the medium (Eq. 5).

$$CO_{2(bulk)(aq)} + OH^- \rightleftharpoons K \cdot HCO_3^-,\text{ media}$$ (5)
Since the disappearance of 1 mole each of $CO_{2\ bulk\ (aq)}$ and $OH^-$ results in appearance of 1 mole of $HCO_3^-$, Eq. 1 can be explained using a simple ordinary differential equations (ODE) which describes one dimensional mass transfer of $CO_{2\ bulk\ (aq)}$, $OH^-$, and $HCO_3^-$ (Eqs. 6 and 7).

\[
D_{CO_2} \cdot \frac{d^2 CO_2}{dx^2} + D_{HCO_3^-} \cdot \frac{d^2 HCO_3^-}{dx^2} = 0
\] (6)

\[
D_{OH^-} \cdot \frac{d^2 OH^-}{dx^2} + D_{HCO_3^-} \cdot \frac{d^2 HCO_3^-}{dx^2} = 0
\] (7)

Now, using the general solutions and boundary conditions reported by Danckwerts et al [112] for the ODEs (Eqs. 2 and 3), mass transfer rate of $CO_2$ was calculated and shown in Eq 8.

\[
r = K_L \cdot \left[ CO_{2\ (aq)}^* - CO_{2\ bulk\ (aq)} \right] \cdot \left[ 1 + \frac{D_{OH^-} \cdot D_{HCO_3^-} \cdot K \cdot [OH^-]}{D_{CO_2}(K \cdot CO_{2\ (aq)}^* \cdot D_{HCO_3^-} + D_{OH^-})} \right]
\] (8)

Where,

Mass transfer rate - $r$, moles·m$^{-2}$·d$^{-1}$

Mass transfer coefficient - $K_L$, m·d$^{-1}$

K, Equilibrium constant, m$^3$·mole$^{-1}$

$D_{CO_2}$, Diffusivity of $CO_2$, m$^2$·s$^{-1}$

$D_{OH^-}$, Diffusivity of $OH^-$, m$^2$·s$^{-1}$

$D_{HCO_3^-}$, Diffusivity of $HCO_3^-$, m$^2$·s$^{-1}$

Enhancement Factor - $E = \left[ 1 + \frac{D_{OH^-} \cdot D_{HCO_3^-} \cdot K \cdot [OH^-]}{D_{CO_2}(K \cdot CO_{2\ (aq)}^* \cdot D_{HCO_3^-} + D_{OH^-})} \right]$
Therefore, mass transfer rate of CO$_2$ represented in Eq. 4 is rewritten and shown below (Eq. 9).

\[
    r = K_L \cdot E \cdot \left[ CO_2^*_{(aq)} - CO_2^{bulk}_{(aq)} \right] \tag{9}
\]

Since the enhancement factor (E) is directly proportional to [OH\textsuperscript{-}] (Eq. 4), chemical parameters (shown in Table B1) \[99\] were used to compute E over a pH range of 9-10.5 (shown in upper section of Figure 3.3, filled triangles). In addition, a previously measured mass transfer coefficient ($K_L$) at a mixing velocity of 30 cm\textperiodcentered s\textsuperscript{-1} (0.1 m\textperiodcentered h\textsuperscript{-1}, \[113\]) was used and the product $K_L \cdot E$ (i.e. product of mass transfer coefficient and enhancement factor) was calculated (filled squares, Figure 3.3). Thereafter, using $CO_2^{bulk}_{(aq)}$ values from the DIC-pH equilibria and computing $CO_2^*_{(aq)}$ values at the air-media interface (from Henry’s Law using an atmospheric CO$_2$ concentration of 387 ppm), the air-media mass transfer driving force ($CO_2^*_{(aq)} - CO_2^{bulk}_{(aq)}$) was also estimated as a function of pH. These values are shown by the filled rhombus in Figure 3.3. Finally, pH-dependent concentrations of HCO$_3^-$ in a culture medium (maintained at an alkalinity of 140 mM) were calculated (filled circles, Figure 3.3).

It is interesting to note that below pH values of ~10, the mass transfer coefficient values are significantly low. Moreover, the air-liquid mass transfer driving force below the pH range of 9.6 have negative values indicating outgas of CO$_2$ at less alkaline pH conditions. Thus, outgas of CO$_2$ would result in utilization/depletion of HCO$_3^-$ in the growth medium. In fact our pH data (shown in Figure 3.2b) and HCO$_3^-$ concentration reserves in the culture medium (shown in Figure B6, Appendix B) from Trial 1 experiments suggests that HCO$_3^-$ utilized during the growth of microalgae was not replenished due to insufficient mass transfer rates of CO$_2$. However, at higher pH values
(>10), both mass transfer coefficient and driving force were improved. Therefore, if the pH is maintained above 10 during the cultivation of microalgae, the media allows for influx of atmospheric CO$_2$ into the media. Furthermore, previous studies have also suggested that extreme alkaline conditions (pH >10) would reduce contaminating populations and thereby allow sustained maintenance of microalgae culture [114]. Therefore, it is reasonable to conclude that high pH medium conditions (orange shaded area in Figure 3.3) would simultaneously improve both CO$_2$ mass transfer rate and culture stability.
Figure 3.3: Mass transfer process of CO$_2$ from the atmosphere into alkaliphilic microalgae cultivation media. The HCO$_3^-$ and mass transfer driving force values were calculated for cultivation medium with an alkalinity of 140 mM using pK$_1$ and pK$_2$ at temperature (T) 293 K and ionic strength (I) 0.19 mole·L$^{-1}$. 
In order to improve culture stability, atmospheric carbon uptake rate, and biomass productivity of str. SLA-04, a second set of cultivation experiments (Trial 2) were carried out in the month of July under high medium pH and HCO$_3^-$ conditions. SLA-04 cultures were inoculated to a medium at an initial pH of 9.9 and similar to Trial 1 experiments (discussed in section 3.4.2), cultivations were started with initial HCO$_3^-$ concentrations ranging from 4-30 mM. The initial high medium pH (~ 10) was attained through addition of CO$_3^{2-}$ to the medium (Figure 3.4b). The peak daylight intensities were measured to be approximately in the range 1000 - 1500 µmoles·m$^{-2}·$s$^{-1}$, and to prevent possible photoinhibition, cultivation depth was increased to 6” (working volume - 25 L) [63]. For neutralophilic microalgae, elevated medium pH (pH > 9.5) and the resulting high CO$_3^{2-}$ concentrations led to growth inhibition [16]. However, for SLA-04 cultures, an increase in the biomass concentration was observed (Figure B7, Appendix B), suggesting that high pH and elevated CO$_3^{2-}$ concentrations did not affect the metabolic activity of the cultures. The ability of SLA-04 to thrive under high pH conditions could be well attributed to its origin of isolation - Soap Lake. In addition, it was also observed that the maximum quantum yield (F$_{v}$/F$_{m}$) - an indicator of stress conditions [115], was > 0.65 (Table 3.2). The high F$_{v}$/F$_{m}$ value suggest that the photosynthetic activity of str. SLA-04 was not affected even under high pH and media DIC conditions [116].

As expected, the biomass productivities increased with increase in the HCO$_3^-$ concentration even under high pH conditions (Figure 3.4a). Further, the productivities under all cultivation conditions were observed to be high during the first day (henceforth referred as maximum biomass productivity), likely due to greater penetration of light in the relatively dilute cultures initially. Additionally, the maximum biomass productivity
was measured to be high in cultures with 29 mM HCO$_3^-$ concentration (34.7 g-biomass•m$^{-2}$•d$^{-1}$) (Figure 3.4a). The productivity obtained was higher than the other neutralophilic microalgae cultures cultivated in open raceway ponds (Table 3.1). As the cultures grew, biomass concentration increased (see Figure B7, Appendix B) which in turn might have led to light attenuation. This resulted in reduced productivity on the second day, thereby reducing the overall productivity (Figure 3.4a). Nonetheless, the overall productivity of str. SLA-04 inoculated at HCO$_3^-$ concentration of 29 mM was measured to be 22.8 g-biomass•m$^{-2}$•d$^{-1}$ which is comparable with the previously reported literature data (Table 3.1).
Figure 3.4: Effect of pH and HCO$_3^-$ concentration on (a) Biomass productivity, (b) pH of str. SLA-04 cultivated under outdoor conditions at an initial medium pH of 9.9 during July. Values shown in the graph are averages from duplicate runs. Error bars indicate one standard deviation from mean values.
Further, it can be observed that both maximum and overall biomass productivities of str. SLA-04 inoculated at initial pH 9.9 were higher (~15%) than the productivities obtained in control experiments (initial pH 8.2, Figure 3.4a). Our experimental data suggest that HCO$_3^-$ replenishment during the night was greater for cultures inoculated at an initial pH of 9.9 (Figure B8, Appendix B). Therefore, the availability of soluble HCO$_3^-$ in the culture medium is high in the cultures inoculated at an initial pH 9.9 because of which the biomass productivities would have improved.

**Table 3.2:** Effect of HCO$_3^-$ concentrations on the quantum yield parameters of str. SLA-04 cultures grown under outdoor cultivation conditions.

<table>
<thead>
<tr>
<th>Quantum Yield</th>
<th>Parameter</th>
<th>HCO$_3^-$ concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>6.3</td>
</tr>
<tr>
<td>Maximum quantum yield</td>
<td>$F_{\text{V}}/F_{\text{M}}$</td>
<td>0.698</td>
</tr>
<tr>
<td>Effective quantum yield</td>
<td>$Y_{\text{II}}$</td>
<td>0.209</td>
</tr>
<tr>
<td>Quantum yield of energy dissipation</td>
<td>$Y_\text{(NPQ)}$</td>
<td>0.094</td>
</tr>
<tr>
<td>Quantum yield of non-regulated energy dissipation</td>
<td>$Y_\text{(NO)}$</td>
<td>0.697</td>
</tr>
</tbody>
</table>

In addition, the quantum yield and photosynthetic parameters were also quantified to determine the effect of cultivation conditions (i.e. pH and HCO$_3^-$ concentration) on str. SLA-04 (Table 3.2). It was observed that the fraction of energy absorbed that gets converted into chemical energy (effective quantum yield ($Y_{\text{II}}$)) improved under high HCO$_3^-$ concentrations (Table 3.2). Since the sum of all the quantum yields equals to unity ($Y_{\text{II}} + Y_{\text{(NPQ)}} + Y_{\text{(NO)}} = 1$) [117], an increase in $Y_{\text{II}}$ would have resulted in decreased $Y_{\text{(NPQ)}}$ and $Y_{\text{(NO)}}$ (Table 3.2). The low $Y_{\text{(NPQ)}}$ and $Y_{\text{(NO)}}$ values suggest that the energy loss associated with heat and fluorescence decreased under high...
HCO$_3^-$ conditions. In addition, since electron transfer rate (ETR) is a function of Y II (Eq. 10), an increase in Y II could also result in increased ETR [118, 119]. This is confirmed with our data associated with ETR$_{\text{max}}$ (Figure 3.5).

\[
ETR = PAR \times Y \ II \times 0.5 \times AF
\]  

(10)

The increase in ETR$_{\text{max}}$ and Y II would suggest that high cellular DIC flux under high alkalinity conditions could have driven the light-dependent reactions (shown in Figure 3.1) towards higher production of NADPH for use in carbon fixation. Overall, these results indicate that SLA-04 cultures when grown under high HCO$_3^-$ concentrations, led to improved biomass productivity and quantum yield parameters and high pH medium conditions resulted in increased atmospheric carbon uptake rates.

**Figure 3.5:** Effect of HCO$_3^-$ concentration on the electron transfer rate (ETR$_{\text{max}}$). The regression analysis (p-value < 0.05) indicates the ETR$_{\text{max}}$ is dependent on the HCO$_3^-$ concentration.
3.3.4 Large-scale cultivation of str. SLA-04

Based on Trial 2 experiment results (section 4.3), larger-scale cultivation studies (Trial 3) were started in the month of September. The average daylight intensities were measured to be in between 360 – 1160 µmoles·m$^{-2}$·s$^{-1}$ (Figure B9, Appendix B). Figure 3.6a shows the pH and biomass of SLA-04 cultures inoculated into 10" deep ponds. The medium alkalinity was measured to be 118.9 ± 3.3 mM and corresponding DIC concentration was 79 ± 1.8 mM (see inset to Figure 3.6a). Since the cultures were inoculated during night, initial CO$_2$ uptake from atmosphere resulted in an initial decrease in pH (Figure 3.6a). As the day cycle started, uptake of HCO$_3^-$ via photosynthesis resulted in the release of OH$^-$ (Eq. 3) and a concomitant increase in pH was observed (Figure 3.6a). Although there is an increase in pH during the day cycle, a steady growth in SLA-04 microalgae cultures was observed until N-depletion (see Figure 3-6a for growth data and Figure 3.6b for soluble N data) due to sufficient HCO$_3^-$ (39.1 ± 0.2 mM) concentration in the high alkalinity culture medium. During days 0-5, the biomass productivity was measured to be 9.6 ± 0.45 g-biomass·m$^{-2}$·d$^{-1}$ on ash free dry weight basis.
Figure 3.6: (a) Biomass concentration and pH, (b) Soluble-N utilized and FAMEs and total carbohydrate concentration profiles of SLA-04 microalgae cultures grown in 3.68 m² open ponds at a culture depth of 10” and completed in the month of September. Insert to (a) shows the total alkalinity (TA) and dissolved inorganic carbon (DIC) concentrations of the culture medium. Values shown in the graph are averaged from two replicate runs. Error bars indicate one standard deviation from mean values.
In addition, lipid content (reported as fatty acid methyl esters (FAMEs)) was analyzed (Figure 3.6b) and the lipid productivity during the growth phase (days 0-5) was measured to be $0.58 \pm 0.2 \text{ g-FAMEs}\cdot\text{m}^{-2}\cdot\text{d}^{-1}$. Further, since the onset of lipid accumulation is a resultant of nutrient deficient conditions [9, 120], SLA-04 cultures were grown for another 5 days after the soluble-N was depleted from culture medium (Figure 3.6b). It was observed that the lipid concentration significantly increased (Figure 3.6b) which then resulted in an increase in the overall lipid productivity ($1.92 \pm 0.03 \text{ g-FAMEs}\cdot\text{m}^{-2}\cdot\text{d}^{-1}$, days 0-10). The biomass and lipid productivities of SLA-04 were considerably higher in comparison with reported lipid productivities of \textit{Scenedesmus Sp.} [62], \textit{Nanochloropsis Salina} [90], and \textit{Scenedesmus acuta} [63] cultures grown in outdoor raceway ponds. Thus, our results suggest that by maintaining high pH and alkalinity, both biomass and lipid productivities can be improved without any additional supply of CO$_2$.

However, the biomass productivity of SLA-04 microalgae was low when compared to the experimental data obtained from small raceway open ponds (results discussed in section 3.3.2 and 3.3.3). Since cultivations were conducted in September and at a culture depth of 10", low biomass productivity could be due to increased light attenuation. Similar findings were reported [63, 110] wherein the productivities were reduced at increased culture depths during the autumn season. Nonetheless, it has been reported that reduced culture depths resulted in improved biomass and lipid productivities [110]. Hence, an additional set of cultivation experiments (Trial 4) were performed at a culture depth of 7".
Figure 3.7: (a) Biomass concentration and pH., (b) shows Soluble-N utilized and FAMEs and total carbohydrate concentration profiles, (c) measured ETRmax at culture depths of 10” and 7” during the month of October. Insert to (a) shows the total alkalinity (TA) and dissolved inorganic carbon (DIC) concentrations of the culture medium.
Trial 4 experiments were conducted during October and the average daylight intensities varied from 270 – 920 µmoles·m⁻²·s⁻¹ (Figure B10, Appendix B). SLA-04 microalgae cultures were inoculated to an initial concentration of 0.32 ± 0.01 g·L⁻¹ (Figure 3.7a). The initial media DIC concentration was 112 mM and the corresponding medium alkalinity was measured to be 158.7 mM (see inset to Figure 3.7a). As shown in Figure 3.7a, a maximum biomass concentration of 0.72 g·L⁻¹ was obtained and the biomass productivity during the growth phase (until N-depletion, day 3) was measured to be 19.1 g-biomass·m⁻²·d⁻¹ on an ash free dry weight basis. The productivity obtained under these conditions was comparable with previously reported literature data (Table 3.1). Also, lipid content was analyzed during the growth phase (days 0-3, Figure 3.7b) and the productivity was measured to be 1.8 g-FAMEs·m⁻²·d⁻¹. Further, similar to Trial 3 experiments, incubation of str. SLA-04 after the soluble N depletion (after day 3, Figure 3.7b) resulted in a significant increase in the FAME concentration. The overall FAME productivity has increased by 25% (2.3 g-FAMEs·m⁻²·d⁻¹) when compared to FAME productivity obtained during the growth phase.

Since starch is another storage carbon molecule during the growth of microalgae [73], the amount of starch accumulated (in both Trial 3 and 4 experiments) was also analyzed and reported as total carbohydrates (Figures 3.6b and 3.7b). Similar to previously reported literature data [73, 121], starch accumulation continued even after the soluble N depletion (Figure 3.6b and 3.7b). The overall productivities were measured to be 2.9 ± 0.5 g-total carbohydrates·m⁻²·d⁻¹ (days 0-10) and 1.9 g-total carbohydrates·m⁻²·d⁻¹ (days 0-12) for Trial 3 and Trial 4 experiments respectively. The summative mass of
closure, based on measured biochemical composition, is able to account for 85-95% of the measured cell mass (Table 3.3).

The increased biomass and lipid productivities during the growth phase at low culture depth (7”, Trial 4 experiments) could be explained on the basis of maximum electron transfer rate (ETR_{max}) measurements. From Figure 3.7c, one can observe that measured ETR_{max} at lower culture depth (7”, Trial 4 experiments) was higher than the ETR_{max} obtained at culture depth of 10”. The increase in ETR_{max} would have resulted in higher production of NADPH (light dependent reactions shown in Figure 3.1). Thus, increased NADPH along with high DIC medium concentrations would have resulted in increased carbon fixation rate and therby biomass productivity at lower culture depth. Further, it was reported that energy (ATP) and reducing equivalents (NADPH) required for fatty acid synthesis is relatively high when compared to starch synthesis [122]. Therefore, we speculate that because of increased ATP and NADPH production, a resultant of increased ETR_{max}, carbon is stored predominantly in the form of lipids rather than starch during the growth of SLA-04 under low culture depth conditions (culture depth - 7”). This would have resulted in improved lipid productivities when compared to SLA-04 cultures grown at a culture depth of 10”.

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Table 3.3: Biochemical composition of str. SLA-04 cultivated in 3.68 m² open ponds.

<table>
<thead>
<tr>
<th>Cultivation method</th>
<th>Cultivation depth</th>
<th>Time period</th>
<th>FAMEs (%(w⋅w⁻¹))</th>
<th>Total carbohydrates (%(w⋅w⁻¹))</th>
<th>Protein * (%(w⋅w⁻¹))</th>
<th>Nucleic acids ** (%(w⋅w⁻¹))</th>
<th>ASH content (%(w⋅w⁻¹))</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10&quot;</td>
<td>Day 0</td>
<td>7.8 ± 0.6</td>
<td>33.7 ± 2</td>
<td>20.5</td>
<td>5.0</td>
<td>18.1 ± 0.5</td>
<td>85.1 ± 3.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Day 10</td>
<td>17 ± 0.15</td>
<td>42.9 ± 1</td>
<td>17.4 ± 0.03</td>
<td></td>
<td>7.5 ± 0.5</td>
<td>88.8 ± 1.2</td>
</tr>
<tr>
<td>Phototrophic</td>
<td>7&quot;</td>
<td>Day 0</td>
<td>7.1</td>
<td>20.3</td>
<td>43.6</td>
<td></td>
<td>18.7</td>
<td>94.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Day 5</td>
<td>14.6</td>
<td>20.1</td>
<td>38.1</td>
<td>5.0</td>
<td>9.5</td>
<td>87.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Day 12</td>
<td>21.8</td>
<td>25.0</td>
<td>32.7</td>
<td></td>
<td>8.8</td>
<td>93.3</td>
</tr>
</tbody>
</table>

*Protein content was estimated using a conversion factor of 5.99.

**Nucleic acid content was obtained from literature [84].
3.3.5 Mass transfer rates of CO$_2$: Calculations based on theoretical and experimental data

Mass transfer rates of CO$_2$ in Trial 2, 3, and 4 experiments were calculated using both literature and experimental data. First, using the chemical parameters (shown in Table B1, Appendix B), enhancement factor (E) for absorption of CO$_2$ in a medium pH of 10.2 is calculated to be 39.8. Further, with the mass transfer coefficient (0.1 m·h$^{-1}$) from Weissman et al. [113] and using the empirical formula for mass transfer coefficient prediction (Eq. 11), we computed the mass transfer coefficients (K$_L$, m·h$^{-1}$) for our cultivation systems operated at mixing velocities of 6 cm·s$^{-1}$ and 30 cm·s$^{-1}$. The K$_L$ values for Trial 2, 3, and 4 were 0.04 m·h$^{-1}$, 0.09 m·h$^{-1}$ and 0.13 m·h$^{-1}$ respectively. Finally, using the enhancement factor (E), and computed K values, K$_L$·E (i.e. product of mass transfer coefficient and enhancement factor) is determined

$$K_L = 9.4 \cdot U^{0.65} \cdot H^{-1.85}$$

Alternatively, using the pH and DIC data from Trial 1 and 2 experiments (discussed in chapter 2), we have computed rate constants associated with mass transfer rates of CO$_2$ over a pH range of 10.2-10.1. In brief, K$_L$·E is calculated by dividing the rate of increase in DIC (mmole·m$^{-2}$·d$^{-1}$) with the driving force established between surface and bulk CO$_2$ concentration ((CO$_2^{*}(aq) - CO_2^{bulk}(aq)$), mmole·m$^{-3}$). The rate constant (K$_L$·E) was found to be 1.98 ± 0.19 m·h$^{-1}$. Since the enhancement factor (E) at pH 10.2 is 39.8, K$_L$ is calculated and found to be 0.05 ± 0.005 m·h$^{-1}$. Detailed calculations are shown in Tables B11-1 and B11-2 (Section B11, Appendix B). Finally, using this K$_L$ value and empirical formula (Eq.11), we have computed the K$_L$ for Trial 2 (0.04 ± 0.004m·h$^{-1}$), Trial 3 (0.1 ± 0.01 m·h$^{-1}$) and Trial 4 (0.14 ± 0.013 m·h$^{-1}$) experiments (Shown in Table 3.4).
With the mass transfer coefficients \((K_L)\) obtained from literature and experimental data, \(CO_2\) absorption rates for Trial 2, 3 and 4 experiments were calculated (shown in Table 3.4). One can observe that the predicted and experimentally determined \(CO_2\) absorption rates achieved were in close correspondence suggesting that the model developed for predicting the mass transfer rates using Danckwerts gas-liquid reactions (Eq. 8) is in concurrence with the experimentally determined mass transfer rates. It was observed that in Trial 2 experiments carbon accumulated in biomass \((9.12 \text{ g-C} \cdot \text{m}^{-2} \cdot \text{d}^{-1}, \text{Table 3.4})\) is greater than the inorganic carbon transfer into the culture medium \((6.18 \pm 0.61 \text{ g-C} \cdot \text{m}^{-2} \cdot \text{d}^{-1}, \text{Table 3.4})\). This would have resulted in depletion of \(HCO_3^-\) reserves in the culture medium. In fact, experimental data associated with pH from Trial 2 experiments (Figure 3.2b) suggest that the carbon transfer rates from the atmosphere were not sufficient because of which pH increased.

Further, it is interesting to note that when the mixing velocity was increased to 30 cm\(\cdot\)s\(^{-1}\) in Trial 3 experiments, the mass transfer rates of \(CO_2\) have significantly improved \((8.63 \pm 0.85 \text{ g-C} \cdot \text{m}^{-2} \cdot \text{d}^{-1}, \text{Table 3.4})\). Similar, findings were reported wherein increased mixing rates resulted in improved mass transfer coefficients and thereby the \(CO_2\) absorption rates [113]. However, since experiments were carried out at a culture depth of 10\("\), increased light attenuation has resulted in reduced carbon fixation in SLA-04 \((3.91 \pm 0.56 \text{ g-C} \cdot \text{m}^{-2} \cdot \text{d}^{-1}, \text{Table 3.4})\).

Nonetheless, when the cultivations were carried out at a culture depth of 7\("\) and mixing velocity of 30 cm\(\cdot\)s\(^{-1}\) (Trial 4), not only were the inorganic carbon transfer rates from the atmosphere \((8.25 \pm 0.81 \text{ g-C} \cdot \text{m}^{-2} \cdot \text{d}^{-1})\) improved but because of reduced culture depth, the organic carbon accumulated has also significantly improved \((7.73 \text{ g-C} \cdot \text{m}^{-2} \cdot \text{d}^{-1})\).
Furthermore, one can observe that the mass transfer rates of CO$_2$ were higher than the organic carbon accumulated (Trial 3 and 4). This high mass transfer rates would have resulted in retaining the HCO$_3^-$ concentrations. Indeed, the pH data from Trial 3 and 4 (Figures 3.6a and 3.7a) suggest that the HCO$_3^-$ utilized by SLA-04 during the day cycle was replenished during the night. Overall from Trial 4 experiments, we can conclude that by maintaining high pH and high HCO$_3^-$ medium conditions, mass transfer rates of CO$_2$ can be improved and thus improved biomass productivities can be obtained without additional CO$_2$ inputs.
Table 3.4: Predicted and experimentally determined mass transfer coefficients and mass transfer rates of CO₂ associated with Trial 2, 3 and 4 experiments.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Predicted</th>
<th>Experimental</th>
<th>Organic carbon accumulated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_L$ (m·h⁻¹)</td>
<td>$K_L$·E (m·h⁻¹)</td>
<td>Mass transfer rate (g-C·m⁻²·d⁻¹)</td>
</tr>
<tr>
<td>Trial 2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.04</td>
<td>1.51</td>
<td>5.25</td>
</tr>
<tr>
<td>Trial 3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.09</td>
<td>2.48</td>
<td>7.80</td>
</tr>
<tr>
<td>Trial 4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.13</td>
<td>4.70</td>
<td>7.46</td>
</tr>
</tbody>
</table>

<sup>a</sup> Experiments were carried out in July at a culture depth of 6˝ and mixing velocity 6 cm·s⁻¹.

<sup>b</sup> Experiments were carried out in September at a culture depth of 10˝ and mixing velocity 30 cm·s⁻¹.

<sup>c</sup> Experiments were carried out in October at a culture depth of 7˝ and mixing velocity 30 cm·s⁻¹.

<sup>d</sup> Mass transfer coefficients were predicted for Trials 2, 3, and 4 using transfer coefficients from literature [111] and empirical formula (Eq. 11).

<sup>e</sup> Using the pH data from Trial 2, 3, and 4 experiments, enhancement factors (E) are estimated and $K_L$·E values are computed.

<sup>f</sup> Mass transfer rates were computed using $K_L$·E and mass transfer driving force established in Trial 2, 3, and 4 experiments.

<sup>g</sup> Mass transfer coefficients were experimentally determined for cultivations carried out in September and December (calculations shown in Section B11, Appendix B) and used to compute rate constants for Trial 2, 3, and 4 experiments.

<sup>h</sup> Organic carbon accumulated in Trial 2, 3, and 4 experiments are calculated using CHN data.
3.4 Conclusion

This study has successfully demonstrated phototrophic cultivation of alkaliphilic SLA-04 microalgae in ~1000L open ponds without additional inputs of concentrated CO₂. High HCO₃⁻/CO₃²⁻ buffered growth medium provided sufficient inorganic carbon for the microalgae to grow without any carbon limitations even at high pH (>10) conditions. As a result, both biomass and lipid productivities were high. In addition, our calculations associated with mass transfer rates of CO₂ suggested that high pH conditions resulted in improved carbon uptake rates from the atmosphere and thereby allowed replenishment of HCO₃⁻ in the culture medium that was utilized during the growth of microalgae. Further, the ability of alkaliphilic SLA-04 microalgae cultures to use bicarbonate as an inorganic carbon source which can be continually recycled with harvesting methods that do not rely on chemical addition (e.g. flocculation) could encourage the use of inexpensive inorganic carbon sources that could eventually result in a decrease in the overall operating costs. Overall, phototrophic cultivation studies suggest that productivities of extreme alkaliphilic SLA-04 can be improved through increased media alkalinity (DIC) and pH conditions without a need for co-location of algae biorefineries with CO₂ point sources.
Chapter 4

4 Novel approach for mixotrophic cultivation of microalgae under outdoor non-sterile conditions.

4.1 Background

Microalgae are a promising feedstock for sustainable production of renewable biofuels, especially since they can be cultivated on marginal/non-arable lands using low-quality water resources (e.g. brackish water, sea water or municipal wastewater) [121, 122]. Since microalgae are aquatic, phototrophic growth requires the utilization of dissolved inorganic carbon (DIC) available in the culture medium. At circumneutral pH conditions, the dissolved CO\(_2\) concentration in the culture medium is commonly increased by sparging with either pure or CO\(_2\)-rich flue gases [94, 123]. However, this approach requires co-location of algae facilities with CO\(_2\) point sources to minimize the cost burden of transporting CO\(_2\) over long distances. As a result, a majority of arid lands are not useful for algae cultivation due to unavailability of proximate CO\(_2\) sources [124, 125]. More recently, we have demonstrated that the use of extreme alkaline pH media and cultures adapted to high pH (extreme alkaliphiles) allows high productivity cultivation of microalgae even in the absence of concentrated CO\(_2\) inputs due to the high rates of atmospheric CO\(_2\) transfer into alkaline media (Chapter 3 and refs [30-32]).
In addition to overcoming DIC limitations, a second challenge is achieving consistently high biomass and lipid productivity in winter months when solar irradiance levels are low and days are short [66]. Inconsistent and low productivity poses challenges in terms of sizing downstream conversion equipment. A possible strategy to attain consistent (and high) productivities throughout the year is by supplementing the growth medium with organic substrates (e.g. glucose or other sugars) as carbon and energy sources [75, 76, 126-130]. Indeed, the sugar-to-lipid approach has been extensively studied in the literature, most commonly under dark fermentation (heterotrophic) conditions with oleaginous yeast [126-129]. Lipid yields can potentially be improved by cultivating cultures with organic substrates in the presence of light (mixotrophic cultivation) where the irradiance serves as an additional source of energy. While inapplicable for yeasts that are unable to perform photosynthesis, mixotrophic cultivation is a viable strategy for microalgae. However, mixotrophic microalgae cultivation has thus far been limited to lab-scale studies since it is nearly impossible to prevent bacterial and/or fungal contamination in unsterile outdoor conditions. High media pH conditions (>10), however, may allow outdoor cultivation of extreme alkaliphiles with a lower risk of contamination due to the relatively low microbial diversity in these harsh environments [38]. Further, the photosynthetic activity (alongside heterotrophy) during mixotrophic cultivation is likely to keep the culture medium at a high pH value (due to the release of OH\(^-\) during photosynthesis) and also provide additional O\(_2\) for heterotrophy.

In this report, we extend our previous work on indoor mixotrophic cultivation (chapter 2 and ref [31]) and describe outdoor mixotrophic cultivation of the extreme
alkaliphile *Chlorella sorokiniana* str. SLA-04, isolated from Soap Lake, Washington. In addition to mixotrophic cultivation, a lipid-boost strategy was also tested wherein str. SLA-04 was first allowed to grow phototrophically and subsequently amended with glucose (after nutrient depletion) to induce rapid lipid accumulation from the assimilation of the sugars. Culture concentration, nutrient utilization, lipid and total carbohydrate content were monitored during cultivation to estimate biomass-, lipid- and carbohydrate-productivities. Energy balance and process economics associated with mixotrophic and lipid-boost cultivation were also assessed. To our knowledge, this is the first report to demonstrate mixotrophic and lipid-boost cultivation strategies of microalgae under non-sterile outdoor conditions.

### 4.2 Materials and Methods

#### 4.2.1 Cultivation experiments

All experiments used *Chlorella sorokiniana* str. SLA-04 that was isolated from Soap Lake in the State of Washington (USA). A series of experiments were conducted from July to October 2015. Experiments were performed in both small raceway ponds (total volume = 30 L, surface area = 0.18 m²) and larger-scale cultivations ponds (total volume = 1100 L, surface area = 3.68 m²).

As previously described (Chapters 2 and 3), the 30 L ponds had dimensions of 2’ × 1’ × 1’ (L × W × D, see photograph in Figure C1 of Appendix C). These ponds were equipped with real-time temperature- and pH- monitoring and logging (Neptune APEX data logging systems, Neptune Technology Group Inc., Tallassee, AL). The cultures were mixed with paddlewheels at an average flow velocity of 6 cm·s⁻¹. 3.68 m² ponds (see photographs in Figures C2 and C3 of Appendix C)) were purchased from Commercial
Algae Professionals (Peachtree City, GA). Similar to 30 L ponds, the larger ponds were also equipped with temperature, pH, and ambient light intensity monitoring devices. Sensor data was collected and stored using a YSI data logging system (YSI Incorporated, OH, USA). In larger ponds, the cultures were mixed using stainless steel helical/scissor bent-blade paddlewheels at an average flow velocity of 30 cm·s⁻¹.

All ponds were placed in an outdoor greenhouse and tap water available at the greenhouse facility was first filtered through a 10 µm filter (to remove sediments) and used for media preparation without sterilization. Evaporation losses during the experiment were compensated with daily water addition based on the culture depth. Culture samples were periodically removed and analyzed as described in Section 4.2.3.

4.2.2 Growth medium

Cultures were grown in a medium that comprises the nutrients: NaHCO₃ (40 mM), Na₂CO₃ (40 mM), NaNO₃, KH₂PO₄ (0.3 mM), MgSO₄.7H₂O (0.3 mM), CaCl₂.2H₂O (0.17 mM), NaCl (0.42 mM), ferric ammonium citrate (10 mg·L⁻¹) and 1 mL trace metal solution. The trace metal solution comprised - H₃BO₃ (9.7 mM), MnCl₂.4H₂O (1.26 mM), ZnCl₂ (0.15 mM), CuCl₂.2H₂O (0.11 mM), Na₂MoO₄.2H₂O (0.07 mM), CoCl₂.6H₂O (0.06 mM), NiCl₂.6H₂O (0.04 mM), V₂O₅ (0.01 mM) and KBr (0.08 mM).

4.2.3 Analytical

4.2.3.1 Wet sample analysis

4.2.3.1.1 Total suspended solids

Total solids (biomass) in the culture was determined by following the Laboratory Analytical Procedure outlined by NREL with a slight modification [99]. In brief, 20 mL
culture samples were recovered from the ponds and their pH was first adjusted to a value of 8.2 (using 0.1 M HCl) to dissolve carbonate and phosphate precipitates, if any, present in the culture medium. Thereafter, the samples were filtered through a 0.45 µm glass fiber filter disc (Fisher Scientific, Pittsburgh, PA) and washed with de-ionized (DI) water. The filter discs containing biomass were dried at 40 °C until a constant dry weight was achieved. A total suspended solids (TSS) value was obtained by subtracting the weight of the clean filter paper from the weight of the filter paper containing biomass.

4.2.3.1.2 Soluble Nitrogen

NO$_3^-$ concentrations in the supernatant (from samples centrifuged at 3000×g) were measured by an ion chromatograph (IC) equipped with an autosampler (Dionex ICS 3000, Thermo Fisher, Sunnyvale, CA). A Dionex IonPac™ CS12A anion-exchange column (maintained at 30 °C) was used to separate NO$_3^-$ from other anions in the sample using a 30 mM potassium hydroxide eluent set at a flow rate of 1.0 mL·min$^{-1}$. Dionex CD20 conductivity detector (maintained at 35 °C) was used for anion detection, and IC data were analyzed with the Chromeleon™ 7.0 software (Thermo Fisher). The instrument was calibrated using NaNO$_3$ standards of known concentrations (0-120 mg-NO$_3^-$·L$^{-1}$).

4.2.3.1.3 Total alkalinity and Dissolved inorganic carbon (DIC)

Total alkalinity (TA) of the culture medium was measured using a G20 compact titrator (Mettler-Toledo, Columbus, OH). The titrator is equipped with a 5 mL automatic burette to dispense titrant, a pH meter to continuously monitor pH and a stirrer to mix the samples while being titrated. To determine the TA (referred as M-value), 40 mL of sample was taken in a beaker and titrated with a 0.1 M HCl solution until pH of the samples reached the titration endpoint of pH 4.5. To determine the carbonate concentration
(referred as P-value), 5 mL of sample was titrated with a 0.1 M HCL to an endpoint pH value of 8.2. DIC was estimated as the difference between M- and P-values (Eq. 1).

\[
DIC = M - P
\]  
(Eq 1)

### 4.2.3.1.4 Pulse amplitude modulated (PAM) fluorometry

DUAL-PAM 100 Chlorophyll Fluorometer (Heinz Walz GmbH, Effeltrich, Germany) was used to measure the quantum yield and photosynthetic parameters. 3 mL of sample was taken in a quartz glass cuvette and incubated in dark for 5 minutes with continuous stirring to obtain the minimum fluorescence yield \(F_o\). Then, a saturation pulse (10000 µmole·m\(^{-2}\)·s\(^{-1}\)) of blue light was applied for 0.6s to get maximum fluorescence yield \(F_m\). These fluorescence yield parameters \(F_o\) and \(F_m\) were then used to estimate the maximum quantum yield \(F_v/F_m\) [131], with \(F_v = F_m - F_o\). A Dual PAM software (v 1.9) was used to record and analyze the data.

### 4.2.3.1.5 Soluble organic carbon

Samples containing sugars were first centrifuged, and pH of the supernatant obtained was adjusted to 7. Then the supernatant was filtered using a 0.2 µm size filter. The sugars present in the filtered samples were then analyzed by an Agilent 1100 HPLC (Agilent Technologies Inc., Santa Clara, CA) using Shodex SH1011 ion exchange column with refractive index (RI) detector. The flow rate was set at 0.6 mL·min\(^{-1}\) with 50 mM sulfuric acid as the mobile phase at 50 °C. Glucose standards were used to obtain calibration curves.

### 4.2.3.2 Dry sample analysis

The liquid samples collected were first centrifuged \((3000\times g)\) and the wet biomass pellet was rapidly frozen using liquid N\(_2\) and freeze-dried using a Benchtop Freeze Dryer
Labconco, Kansas City, MO). The pressure and temperature of freeze drying system were maintained at 0.0025 mBar and -70 °C, respectively.

4.2.3.2.1 Fatty acid methyl esters (FAMEs)

Cellular lipids were recovered and quantified as fatty acid methyl esters (FAMEs) using an in situ transesterification method [25, 46]. Freeze dried biomass samples (30 mg) were weighed into clean 15 mL serum vials to which 1 mL of acidified methanol (5% H₂SO₄ v/v) was added. The vials were crimped with Teflon-lined caps and incubated at 90 °C for 90 min. Thereafter, the vials were cooled to room temperature, 4 mL hexane was added, and the mixture was incubated at 90 °C for 15 min to extract FAMEs into hexane. After cooling down to room temperature, the hexane phase was recovered and analyzed using gas chromatography (GC). FAME standards (Sigma-Aldrich) were used to obtain calibration curves.

A Shimadzu 2010 GC equipped with an Rtx Bio-diesel column (15 m × 0.32 mm ID × 0.1 µm, Restek Corp., Bellefonte, PA) and a flame ionization detector (FID) was used to quantify FAMEs. The column oven temperature was programmed as follows – first, the temperature was held at 60 °C for 1 min. Thereafter, the temperature was increased to 370 °C at a ramp rate of 10 °C·min⁻¹. Finally, a temperature of 370 °C was maintained for 6 min at the end of the analysis run. Nitrogen was used as a carrier gas (50 cm·s⁻¹). The injector and FID temperatures were maintained at 370 °C throughout the analysis.

4.2.3.2.2 Total carbohydrates

Total carbohydrates present in the freeze dried microalgae was determined by following the Laboratory Analytical Procedure outlined by NREL [132]. In brief, 50 mg
of biomass was taken in an autoclavable 30 mL crimp-top glass tubes to which 0.5 mL of 72% H$_2$SO$_4$ was added. The glass tubes were then incubated at 30 °C for 60 min, with samples mixed using a glass stir rod in 5 min intervals. After that, the acid concentration was adjusted to 4% by adding 13.89 mL of DI water. The vials were then sealed with Teflon-lined caps and autoclaved at 120 °C for 60 min. Samples were then cooled to room temperature, neutralized using CaCO$_3$ and finally filtered using a 0.2 µm filter.

The filtered samples were analyzed on 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. 50 mM H$_2$SO$_4$ was used as the mobile phase at a flow rate of 0.6 mL·min$^{-1}$. The column was maintained at 65 °C. Glucose, xylose, galactose, arabinose and mannose standards (Sigma-Aldrich) were used to obtain calibration curves.

4.2.3.2.3 Elemental (CHN) analysis

Elemental analysis was carried out using a CHN analyzer (Flash 2000 series, CE Elantech Inc.) equipped with an autosampler and a thermal conductivity detector (TCD). 3-7 mg samples were sealed in tin boats and placed in the autosampler for combustion followed by analysis of the flue gases. The combustion temperature was set to 950 °C with helium as both carrier gas (140 mL·min$^{-1}$) and reference gas (100 mL·min$^{-1}$). Acetanilide standards were used to obtain calibration curves for C, H, and N.

4.2.3.2.4 Ash content

Ash content was determined using the Laboratory Analytical Procedure outlined by NREL [102] using an SDT Q600 series (TA Instruments, New Castle, DE) Thermogravimetric Analyzer (TGA). Two identical platinum crucibles were placed on
two horizontal beams and heated. The instrument makes precise measurements of weight change (TGA) with an increase in temperature and/or time. For all the experiments, 10 mg of washed and dried sample (free of media salts) was loaded in one crucible while another crucible was left empty as a reference. The TGA was programmed to first heat the sample from room temperature to 105 °C at a rate of 20 °C·min⁻¹ and maintain isothermal for 10 min to evaporate moisture, if any. Then, the sample was heated to 250 °C at a rate of 20 °C·min⁻¹ and maintained isothermal for 30 min. Finally, the sample was heated to 575 °C at a rate of 20 °C·min⁻¹ and maintained isothermal for 180 min. Air was continuously purged (100 mL·min⁻¹) through the TGA oven during analysis. The residual weight of the sample was recorded over time.

### 4.2.4 Energy balance and economic analysis

The energy balance and economic analysis assume cultivation of *str. SLA-04* in a 1 Ha open raceway pond with paddlewheels at a culture depth of 7” (corresponding to a volume of 1.78 × 10⁶ L). Further, two scenarios were assessed wherein cultivation periods of 12 days (scenario 1) and 5 days (scenario 2) were fixed. The total mass of inputs (nutrients) required and products (FAMEs and carbohydrates) obtained were estimated for the two scenarios using nutrient utilization data and biochemical composition of *SLA-04* (see Table C3, Appendix C). Since *str. SLA-04* can grow without additional CO₂ supply, energy utilization and costs related to CO₂ sparging and transportation were not considered.

To estimate the net energy content, difference of energy inputs (i.e. energy associated with paddlewheels and glucose) and output fuel energy (i.e. energy content in FAMEs and carbohydrates) was used. In detail, the energy content in glucose was
estimated using the calorific value of glucose (15.57 kJ∙g⁻¹) and paddlewheel energy consumption was obtained from the literature [133]. The output fuel energy of FAMEs was calculated using calorific values of palmitic acid and oleic acid mixed in a ratio of 1:3 since the FAME profile showed that C16 and C18 fatty acids were present in this ratio. The energy content of cellular carbohydrates was assessed as the calorific value of glucose.

Similar to net energy content, net profit was also estimated using operating costs and output fuel price. The operating costs were mainly associated with paddlewheel energy consumption and nutrient utilization. Average industrial electricity price (6.7 cents∙kWh⁻¹, US EIA) was used to calculate costs associated with paddlewheel energy consumption. N, P, and Fe costs were computed based on 100% replacement costs associated with N and 50% replacement costs associated with P and Fe (assuming some nutrient recycle). For the output fuel prices, first, the products obtained (FAMEs and carbohydrates) were converted into gasoline gallon equivalents assuming biodiesel and ethanol as the liquid fuels produced. A fuel price of 3$∙GGE⁻¹ was used to determine the overall revenue from fuel sales [26].

4.3 Results and Discussion

Our previous studies under sterile indoor conditions demonstrated the feasibility of mixotrophic growth and high lipid productivity of SLA-04 cultures with glucose as the organic substrate (Chapter 2 and ref [31]). However, due to the low alkalinity (and thereby low buffer capacity) of the medium used in our previous study, the medium pH decreased from an initial value of ~10 to <8 during cultivation, likely as a result of CO₂ evolution during respiration of the organic substrate (Chapter 2 and ref [31]). Therefore,
in this study, to maintain high medium pH conditions throughout cultivation and thereby prevent culture contamination, experiments were performed in a high alkalinity medium (120-150 mM).

4.3.1 Mixotrophic cultivation

First, experiments were conducted in the month of September at a culture depth of 10" with glucose as the organic substrate. The average daylight intensities were measured to be between 360 – 1160 µmoles·m⁻²·s⁻¹ (Figure C4, Appendix C). As shown in Figure 4.1a, cultures were inoculated to an initial concentrations of 0.26 g·L⁻¹ into a pH 10 medium. The initial medium alkalinity was measured to be 112 ± 0.2 mM (see Figure C5 of Appendix C) and the initial glucose concentrations was measured to be 1.51 ± 0.01 g·L⁻¹ (shown in the inset to Figure 4.1a). The increase in biomass concentration (Figure 4.1a) suggests that glucose supplemented SLA-04 cultures grew without any measurable signs of detrimental contamination even under non-sterile open-pond conditions.

Additionally, our measurements of photosystem II (PSII) have verified that cultures exhibited a high photosynthetic efficiency (Fv/Fm) > 0.65 (Figure C6, Appendix C). The photosystem II parameters suggests that str. SLA-04 was not under stress and maintained a high photosynthetically activity.

Further, due to the organic carbon and reducing power (NADPH) provided in the form of glucose, the soluble-N utilization rate was high (see Figure 4.1a) [134]. During days 0-1, 0.38 ± 0.02 g·L⁻¹ of the glucose provided was utilized and a biomass concentration of 0.49 ± 0.1 g·L⁻¹ was obtained (see Figure 4.1a). The biomass yield during this period was calculated to be 0.59 ± 0.23 g-biomass·g-glucose⁻¹ and the biomass productivity was 62 ± 27.6 g-biomass·m⁻²·d⁻¹. After the soluble-N was depelted from the
medium, SLA-04 cultures were incubated for additional 9 days. It was observed that 1.17 ± 0.12 g·L⁻¹ of the total glucose provided was utilized over the entire cultivation period of 10 d and a biomass concentrations of 0.91 ± 0.04 g·L⁻¹ was obtained (Day 10, Figure 4.1a) with an overall biomass yield of 0.55 ± 0.02 g-biomass·g-glucose⁻¹. Additionally, while the pH decreased slightly during cultivation (see pH data, Figure 4.1a), likely from release of CO₂ during glucose metabolism [135], the culture pH remained above values of 9.7 due to highly buffered medium conditions (Figure C5, Appendix C).
Figure 4.1: (a) Biomass concentration, Soluble-N utilized and pH of SLA-04 microalgae cultures grown mixotrophically at a culture depths of 10". Inset in (a) shows glucose utilization during the cultivation period, and (b) FAMEs and total carbohydrate concentration profiles. Experiments were carried out in the month of September and the values shown in both the graphs are averages from duplicate runs. Error bars indicate one standard deviation from mean values.

In addition to growth and glucose utilization, lipids and starch accumulated (reported as FAMEs and total carbohydrates) were also quantified (Figure 4.1b). Carbohydrate accumulation was initially more rapid than lipid accumulation suggesting a sequential accumulation of starch followed by lipids as previously noted in the literature.
for other microalgae cultures [73]. The total carbohydrate content increased to 0.35 ± 0.02 g·L⁻¹ (49.2 ± 2 % w·w⁻¹), day 4) and then steadily decreased. FAME concentrations increased to 0.37 ± 0.02 g·L⁻¹ (41 ± 0.2 % w·w⁻¹) over the 10 d cultivation period. The corresponding lipid productivity was estimated to be 9.5 ± 0.62 g-FAMEs·m⁻²·d⁻¹ with an overall lipid yield of 0.3 ± 0.01 g-FAMEs·g-glucose⁻¹.
Table 4.1: Comparison of lipid yield and net energy content of various oleaginous yeast with str. SLA-04 grown under mixotrophic and lipid-boost cultivation conditions.

<table>
<thead>
<tr>
<th>Cultivation strategy</th>
<th>Strain</th>
<th>Volume (L)</th>
<th>FAMEs (%(w-w⁻¹))</th>
<th>Yield (g·g⁻¹)</th>
<th>Energy investedd (kJ)</th>
<th>Energy return (kJ)</th>
<th>Net Energy content (kJ)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heterotrophic</td>
<td>YL-ad9</td>
<td>2</td>
<td>67</td>
<td>0.234</td>
<td>7.78</td>
<td>-7.82</td>
<td>[129]</td>
<td></td>
</tr>
<tr>
<td>Heterotrophic</td>
<td><em>R. turoloides</em> DSMZ 4444</td>
<td>0.3</td>
<td>59</td>
<td>0.29</td>
<td>9.64</td>
<td>-5.96</td>
<td>[128]</td>
<td></td>
</tr>
<tr>
<td>Heterotrophic</td>
<td><em>C. curvatus</em></td>
<td>2</td>
<td>53</td>
<td>0.14</td>
<td>4.66</td>
<td>-10.95</td>
<td>[127]</td>
<td></td>
</tr>
<tr>
<td>Heterotrophic</td>
<td><em>R. turoloides</em></td>
<td>15</td>
<td>67</td>
<td>0.23</td>
<td>7.65</td>
<td>-7.95</td>
<td>[126]</td>
<td></td>
</tr>
<tr>
<td>Mixotrophic</td>
<td>SLA-04</td>
<td>1000</td>
<td>41 ± 0.2</td>
<td>0.3 ± 0.01</td>
<td>12.2 ± 0.6</td>
<td>-3.63 ± 0.1</td>
<td>Present study</td>
<td></td>
</tr>
<tr>
<td>Mixotrophic</td>
<td>SLA-04</td>
<td>750</td>
<td>42.6 ± 1.5</td>
<td>0.36 ± 0.003</td>
<td>14 ± 0.2</td>
<td>-1.63 ± 0.24</td>
<td>Present study</td>
<td></td>
</tr>
<tr>
<td>Lipid-boost</td>
<td>SLA-04</td>
<td>25</td>
<td>30.2</td>
<td>0.5</td>
<td>18.56</td>
<td>2.96</td>
<td>Present study</td>
<td></td>
</tr>
<tr>
<td>Lipid-boost</td>
<td>SLA-04</td>
<td>750</td>
<td>33</td>
<td>0.46</td>
<td>20.03</td>
<td>4.43</td>
<td>Present study</td>
<td></td>
</tr>
</tbody>
</table>

aCultivations were carried out at a culture depth of 10” and in the month of September.

bCultivations were carried out at a culture depth of 7” and in the month of October.

cCultivations were carried out in small raceway ponds (culture depth of 6”) and in the month of July.

dEnergy invested was calculated solely based on calorific value of glucose and energy associated with operations was not considered.

eEnergy return was calculated using energy content from both carbohydrates and lipids accumulated.
The yield obtained is relatively higher than that of the previously reported oleaginous yeast (Table 4.1) and other microalgae (Chapter 2). However, the net energy balance showed a negative net energy return of -3.63 ± 0.1 kJ (Table 4.1) suggesting that heterotrophic organic carbon utilization (rather than phototrophy) was a much higher proportion of the mixotrophy. Understandably, this was related to the relatively high pond depth of 10" used in this study. Higher light attenuation (low light conditions) in the deep ponds would have likely mitigated phototrophy.

To improve the phototrophic contribution to overall growth, a second set of mixotrophic experiments were next performed with a lower culture depth of 7" during the month of October. Average daylight intensities were varied between 270 – 920 µmoles·m$^{-2}$·s$^{-1}$ during this experiment (Figure C7, Appendix C). Similar to the previous 10" study, cultures were inoculated at an initial concentration of 0.4 ± 0.04 g·L$^{-1}$ in a medium at pH 10.2 and alkalinity of 115.7 ± 3.3 mM (Figure C8 of Appendix C). The initial glucose concentrations was measured to be 1.46 ± 0.03 g·L$^{-1}$ (shown in the inset to Figure 4.2a).

In this experiment also, the cultures grew without detrimental contamination. All the supplied N was consumed within one day with a biomass yield of 0.92 ± 0.13 g-biomass·g-glucose$^{-1}$ at a productivity of 57.5 ± 4.5 g-biomass·m$^{-2}$·d$^{-1}$. To allow lipid accumulation, cultures were incubated for an additional 10 days. The final biomass concentration was 0.98 ± 0.02 g·L$^{-1}$ (day 11) with an overall biomass yield of 0.54 ± 0.03 g-biomass·g-glucose$^{-1}$.
Starch and lipids were also quantified and are shown in Figure 4.2b. The concentration profile indicates that the total carbohydrate content initially reached 30.7 ± 2.3 % w·w⁻¹ (day 3) and later steadily decreased, similar to the starch content of cultures grown in 10" deep ponds. However, it was also observed the lipid content of the cultures in the 7" ponds was 12% higher (Figure 4.2b) than in cultures grown at a culture depth of 10". The overall lipid yield (days 0-11) in the 7" ponds was estimated to be 0.36 g-FAMEs·g-glucose⁻¹ utilized and the overall lipid productivity was 7.2 ± 0.23 g-FAMEs·m⁻²·d⁻¹. Increased biomass and lipid yields in the 7" ponds suggest that improved light penetration at lower culture depth likely allowed greater phototrophy than in 10" ponds.

Since starch accumulation rapidly occurred during the initial mixotrophic cultivation period followed by a decrease later, it can be inferred that that glucose was first stored in the form of starch which was later reapportioned into lipids (long term storage). Though lipid yields and net energy return were clearly improved at lower culture depths, the net energy content remained negative (see Table 4.1). A plausible reason for the unfavorable energy returns is the utilization of glucose for initial culture growth and synthesis of low energy protein and starch. Based on these observations, we hypothesized that if the initial culture growth could be achieved solely via photosynthesis followed by organic carbon addition, a greater portion or the organic substrates may be converted to lipids. This led us to implementation of a lipid-boost strategy wherein, SLA-04 microalgae cultures were first grown phototrophically and amended with glucose only after growth stopped (i.e. post- N depletion).
Figure 4.2: (a) Biomass concentration, Soluble-N utilized and pH of SLA-04 microalgae cultures grown mixotrophically at culture depths of 7". Inset in (a) shows glucose utilization during the cultivation period, and b) FAMEs and total carbohydrate concentration profiles. Experiments were carried out in the month of October and the values shown in both the graphs are averages from duplicate runs. Error bars indicate one standard deviation from mean values.
4.3.2 Lipid-boost strategy

Initially, to test our hypothesis of improving the yields through lipid-boost strategy, experiments were carried out in small raceway open ponds (with a working volume of 25 L) during the month of July. In these studies, we also assessed acetate as an organic carbon source (in addition to glucose) since previous reports suggest that acetate can be utilized by microalgae for the cellular synthesis of acetyl-CoA, a precursor for fatty acid synthesis [136]. Both organic carbon sources (i.e. glucose and acetate) were added after nitrogen was depleted. The photosystem II (PSII) measurements verified that cultures incubated in the presence of glucose and acetate remained photosynthetically active (Fv/Fm > 0.65; Figure C9 of Appendix C). These studies showed that although some acetate was utilized, the FAME productivities were significantly higher in the glucose amended medium (4.11 g-FAMEs·m⁻²·d⁻¹) when compared to acetate (2.42 g-FAMEs·m⁻²·d⁻¹) (Table C1, Appendix C), suggesting that glucose is a more favorable substrate for lipid synthesis by SLA-04. Further, the lipid yield and net energy content were also high for glucose amended lipid-boost cultures and significantly improved when compared to glucose-amended mixotrophic cultures (Table 4.1).

To validate these results at larger scales, lipid-boost experiments were performed with glucose in 750 L ponds maintained at a culture depth of 7" since better glucose-to-lipid conversion was observed at this culture depth during mixotrophic experiments (see Section 4.3.1). The cultures were inoculated to an initial concentration of 0.31 g·L⁻¹ at a medium pH of 10 (Figure 4.3a) and grown phototrophically for 2 d. Glucose (see inset to Figure 4.3a) was added just before the soluble-N was depleted in the phototrophic cultures (day 2, dotted line in Figure 4.3a represents the point at which the glucose was
added) and cultures were incubated for nine more days to allow lipid accumulation. Overall, the cultures reached a biomass concentration of 0.93 g·L⁻¹ and 0.6 g·L⁻¹ glucose was utilized. Based on FAME analysis data (Figure 4.3b), it appears that there was no lag between glucose addition and the onset of lipid accumulation (day 3, Figure 4.3a). A FAME concentration of 0.31 g·L⁻¹ was obtained after the 12 day experiment with an overall FAMEs productivity of 4.7 g-FAMEs·m⁻²·d⁻¹. More importantly, the lipid yield (0.46 g-FAMEs·g-glucose⁻¹) was significantly improved when compared to mixotrophic cultures (Table 4.1). The increase in lipid yield clearly suggests a greater proportion of glucose utilized was directed towards lipid synthesis, since cell replication was likely arrested due to N-deprivation prior to glucose addition. Overall, this approach resulted on a net positive return on energy invested suggesting that the lipid-boost cultivation strategy could be a more environmentally sustainable option relative to mixotrophic or heterotrophic cultivation strategies (Table 4.1). Finally, mass balance calculations typically showed a > 90% summative mass closure (See Table C2, Appendix C) validating the lipid and carbohydrate measurements made during our experiments.
Figure 4.3: (a) Biomass concentration, Soluble-N utilized and pH of SLA-04 microalgae cultures grown under lipid-boost strategy at culture depths of 7”. Inset in (a) shows glucose utilization during the cultivation period. The dotted line indicates the time period at which glucose was added, and (b) FAMEs and total carbohydrate concentration profiles.
Furthermore, as observed in mixotrophic cultivation studies, glucose metabolism was accompanied by a decrease in pH due to the release of CO$_2$ even under lipid-boost cultivation strategy (Figure 4.3a). The CO$_2$ released could either escape into atmosphere or remain in the system depending on driving force established between CO$_2$ at air-liquid interface ($CO^*_2(aq)$) and bulk CO$_2$ concentrations in the aqueous culture media ($CO^{bulk}_2(aq)$). If $CO^*_2(aq)$ is greater than $CO^{bulk}_2(aq)$, the mass transfer of CO$_2$ would occur from atmosphere into the culture system and vice-versa. ($CO^*_2(aq)$) is determined by the concentration of CO$_2$ in air and the Henry’s constant for CO$_2$. In the bulk, the aqueous CO$_2$ concentration ($CO^{bulk}_2(aq)$) is determined by the equilibrium established with HCO$_3^-$, CO$_3^{2-}$ and OH$^-$ ions present in the medium. The equilibrium constant values are from Cents et al. [98] and detailed calculations were shown in Section C9 (Appendix C).

Figure 4.4a and 4.4b shows the $CO^*_2(aq)$ and $CO^{bulk}_2(aq)$ concentration profiles of SLA-04 microalgae cultures grown under mixotrophic and lipid-boost cultivation strategies, respectively. It is evident the $CO^{bulk}_2(aq)$ exceeded $CO^*_2(aq)$ (Figure 4.4a) under mixotrophic conditions suggesting that the CO$_2$ released during glucose metabolism would have escaped into the atmosphere. However, in the lipid-boost cultivation experiments, though there is an increase in the $CO^{bulk}_2(aq)$, it never exceeded the $CO^*_2(aq)$ (Figure 4.4b). Thus, lipid-boost cultivation strategy would result in both improved yields as well as simultaneously eliminate the carbon loss during glucose metabolism.
Figure 4.4: (a) CO₂ concentration at surface and bulk CO₂ concentration for mixotrophic SLA-04 microalgae cultures inoculated at culture depth of 7″ and (b) CO₂ concentration at surface and bulk CO₂ concentration for SLA-04 microalgae cultures cultivated under lipid-boost strategy and inoculated at culture depth of 7″.
4.3.3 Net energy content and process economics

Net energy content were determined (as described in section 4.2.4) using the biochemical composition of SLA-04 cultures (shown in Table C3, Appendix C), assuming that the capital costs would be same under all cultivation conditions (i.e. phototrophic, mixotrophic and lipid-boost) for a 1Ha pond. The energy content of protein was not considered in these calculations since it was assumed that the protein-rich residue would be used for animal feed or for nutrient recycling. For a 12-day cultivation cycle (Table 4.2, Scenario 1), the net energy return calculated for phototrophic cultivation (+319 GJ·Ha⁻¹·yr⁻¹) is much higher when compared to net energy return from mixotrophic and lipid-boost cultivation strategies. Further, the net energy return calculated for mixotrophic conditions showed negative values (-32 GJ·Ha⁻¹·yr⁻¹) suggesting that the amount of energy provided in the form of glucose was more than the energy return through the fuel products obtained. The negative net energy return is likely due to insufficient fuel product yields (Table 4.1). However, due to an increase in glucose-to-lipid conversion efficiency, the lipid-boost strategy (Table 4.1) resulted in a net positive energy return (+140 GJ·Ha⁻¹·yr⁻¹). Assessments for a 5-day cultivation cycle (Table 4.2, Scenario 2), made using day 5 biochemical composition (Table C3, Appendix C), showed that the net energy content under both phototrophic (517 GJ·Ha⁻¹·yr⁻¹) and lipid-boost (369 GJ·Ha⁻¹·yr⁻¹) cultivation strategies was significantly improved when compared to the 12-day cultivation cycle scenario (Scenario 1). In contrast, net energy return for mixotrophic cultivation was estimated to be worse for Scenario 2 relative to Scenario 1. These net energy calculations suggest that lipid-boost cultivation strategy is likely more sustainable than mixotrophic cultivation, and perhaps comparable to phototrophic
cultivation in terms of energy recovery, especially when applied over short cultivation cycles.

A preliminary economic estimate was also performed to determine the net revenue with respect to operational costs associated with cultivation (Table 4.3). For a 12-day cultivation cycle (Table 4.3, Scenario 1), these calculations suggest that the net revenue obtained under phototrophic conditions (10,220 $\cdot$ Ha$^{-1} \cdot$ yr$^{-1}$) is approximately 52% and 20% higher than the revenue obtained from mixotrophic (5,411 $\cdot$ Ha$^{-1} \cdot$ yr$^{-1}$) and lipid-boost (8,342 $\cdot$ Ha$^{-1} \cdot$ yr$^{-1}$) cultivation. For a 5-day cultivation cycle (Table 4.3, Scenario 2), our calculations suggest a nearly 2-fold higher revenue for the lipid-boost strategy (16,839 $\cdot$ Ha$^{-1} \cdot$ yr$^{-1}$) when compared to Scenario 1. In fact, the revenue from lipid-boost could exceed the revenues from phototrophic cultivation at a glucose purchase price of 0.35 $\cdot$ kg$^{-1}$. Revenues from mixotrophic cultivation are much lower. The net revenue under lipid-boost and mixotrophic cultivation strategies primarily depends on the cost of glucose. For example, if glucose cost is reduced by 50% (0.175 $\cdot$ kg$^{-1}$), the net profit under lipid-boost strategy would be 22,512 $\cdot$ Ha$^{-1} \cdot$ yr$^{-1}$ and is approximately 25% higher than the net revenue obtained when glucose price was fixed at 0.35$\cdot$kg$^{-1}$ (16,839 $\cdot$ Ha$^{-1} \cdot$ yr$^{-1}$). Therefore, it is possible to achieve high net revenues under lipid-boost cultivation strategies if a low-cost sugar is used as an organic carbon source [137].
Table 4.2: Net energy content in str. SLA-04 grown under phototrophic, mixotrophic and lipid-boost cultivation conditions.

**Scenario 1: 12-day cultivation period**

<table>
<thead>
<tr>
<th>Cultivation strategy</th>
<th>Paddlewheel energy (^a) (GJ·Ha(^{-1})·Yr(^{-1}))</th>
<th>Energy in glucose (^b) (GJ·Ha(^{-1})·Yr(^{-1}))</th>
<th>Energy content in lipids (^c) (GJ·Ha(^{-1})·Yr(^{-1}))</th>
<th>Energy content in carbohydrates (^b) (GJ·Ha(^{-1})·Yr(^{-1}))</th>
<th>Total (GJ·Ha(^{-1})·Yr(^{-1}))</th>
<th>Net energy (GJ·Ha(^{-1})·Yr(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phototrophic</td>
<td>n/a</td>
<td>250</td>
<td>134</td>
<td>384</td>
<td>319</td>
<td></td>
</tr>
<tr>
<td>Mixotrophic lipid-boost</td>
<td>65</td>
<td>934</td>
<td>754</td>
<td>967</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>515</td>
<td>523</td>
<td>720</td>
<td>140</td>
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</tr>
</tbody>
</table>

**Scenario 2: 5-day cultivation period**

<table>
<thead>
<tr>
<th>Cultivation strategy</th>
<th>Paddlewheel energy (^a) (GJ·Ha(^{-1})·Yr(^{-1}))</th>
<th>Energy in glucose (^b) (GJ·Ha(^{-1})·Yr(^{-1}))</th>
<th>Energy content in lipids (^c) (GJ·Ha(^{-1})·Yr(^{-1}))</th>
<th>Energy content in carbohydrates (^b) (GJ·Ha(^{-1})·Yr(^{-1}))</th>
<th>Total (GJ·Ha(^{-1})·Yr(^{-1}))</th>
<th>Net energy (GJ·Ha(^{-1})·Yr(^{-1}))</th>
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</thead>
<tbody>
<tr>
<td>Phototrophic</td>
<td>n/a</td>
<td>354</td>
<td>229</td>
<td>583</td>
<td>517</td>
<td></td>
</tr>
<tr>
<td>Mixotrophic lipid-boost</td>
<td>65</td>
<td>1,592</td>
<td>1,017</td>
<td>1,517</td>
<td>-140</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>505</td>
<td>545</td>
<td>939</td>
<td>369</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Paddlewheel energy consumption was obtained from literature.

\(^b\) Energy for glucose and carbohydrates were calculated based on calorific value of glucose.

\(^c\) Energy for lipids was calculated based on calorific value of palmitic and oleic acids.
Table 4.3: Cultivation process economics of str. SLA-04 grown under phototrophic, mixotrophic and lipid-boost cultivation conditions.

<table>
<thead>
<tr>
<th>Scenario 1: 12-day cultivation period</th>
<th>Cost IN ($/Ha/yr)</th>
<th>Cost OUT ($/Ha/yr)</th>
<th>Net Profit ($∙Ha⁻¹∙Yr⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cultivation method</strong></td>
<td><strong>Paddlewheels</strong></td>
<td><strong>Nutrients</strong></td>
<td><strong>FAMEs</strong></td>
</tr>
<tr>
<td></td>
<td>$1,216</td>
<td>$904</td>
<td>$20,989</td>
</tr>
<tr>
<td></td>
<td>$11,574</td>
<td>$13,060</td>
<td>$8,975</td>
</tr>
<tr>
<td>Phototrophic</td>
<td>n/a</td>
<td>$6,245</td>
<td>$6,095</td>
</tr>
<tr>
<td>Mixotrophic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>lipid-boost</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Scenario 2: 5-day cultivation period</th>
<th>Cost IN ($/Ha/yr)</th>
<th>Cost OUT ($/Ha/yr)</th>
<th>Net Profit ($∙Ha⁻¹∙Yr⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cultivation method</strong></td>
<td><strong>Paddlewheels</strong></td>
<td><strong>Nutrients</strong></td>
<td><strong>FAMEs</strong></td>
</tr>
<tr>
<td></td>
<td>$1,216</td>
<td>$2,173</td>
<td>$35,780</td>
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<tr>
<td></td>
<td>$11,346</td>
<td>$13,603</td>
<td>$17,970</td>
</tr>
<tr>
<td>Phototrophic</td>
<td>n/a</td>
<td>$8,832</td>
<td>$10,423</td>
</tr>
<tr>
<td>Mixotrophic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>lipid-boost</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\( ^a \) Costs associated with Paddlewheel energy consumption was estimated using 6.5 cents∙kWh⁻¹.

\( ^b \) Costs associated with nutrient utilization was estimated using bulk price of N, P and Fe.

\( ^c \) Costs associated with glucose utilization was estimated using 0.35$∙kg⁻¹.
4.4 Conclusions

This study has demonstrated that extreme pH and alkalinity medium in conjunction with an extreme alkaliphilic microalgae strain allowed successful implementation of mixotrophic and lipid-boost cultivation strategies with high biomass and lipid productivities under non-sterile conditions. In addition, it was also observed that cultivation depth has a significant effect on fuel yields from organic substrates. Reducing the culture depth favored greater phototrophy and decreased the utilization of organic carbon as an energy source, and this improved the conversion of the organic carbon substrate towards fuel products. Further, our data suggests that the glucose utilized by SLA-04 was either lost to respiration or first stored in the form of starch which later got reapportioned into lipids. On the other hand, with the lipid-boost cultivation strategy, it appears that there was less organic carbon lost to respiration and carbohydrate storage and a greater proportion was converted to cellular lipids. The better utilization of glucose for lipid production under lipid-boost cultivation strategy resulted in an improved glucose to lipid conversion efficiency and ultimately to more favorable net energy return and process economics.
Appendix A

A. Supplementary Information: Cultivation of microalgae at extreme alkaline pH conditions – a novel approach for biofuel production

Figure A1: Photograph of raceway ponds during the cultivation of SLA-04 cultures.
Section A1: Description of the modified (scaled-down) Hach Persulfate Digestion TNT Method 10072.

Three reagents were prepared separately - (1) a persulfate reagent was made by adding one “Hach persulfate” powder pillow to 8 mL of DI water, (2) a sodium metabisulfite reagent was made by adding one “Hach Reagent A” pillow to 0.75 mL of DI water and (3) a chromotropic acid solution was made by adding one “Hach Reagent B” powder pillow to 0.75 mL of DI water. First, 100 µL of the recovered supernatant was mixed with 100 µL of the persulfate reagent in a Teflon-capped GC vial and digested at 105 °C for 60 min. Thereafter, the sample was allowed to cool to ambient temperature and 65 µL of the digested sample was mixed with 21 µL of the sodium metabisulfite reagent in a 1.5 mL acid-resistant polystyrene cuvette (BrandTech Scientific Inc., Essex, CT) and incubated for 3 min. Then, 21 µL of the chromotropic acid solution was added to the cuvette and incubated for 2 min. Finally, 800 µL of “Hach Reagent C” (acid) was added to the cuvette and incubated for 5 min for the color formation to occur. Absorbance was measured at 410 nm (Model UV1800, Shimadzu Inc., Colombia, MD). N concentration in the supernatant (soluble total N) was estimated from a calibration curve generated by applying the same protocol as the samples to NaNO₃ solutions of known concentrations.
**Table A1**: Identities of the strain sequences used for the multiple sequence analysis shown in Figure S2.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Strain</th>
<th>NCBI GenBank Accession Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>Chlorella Sorokiniana</em> UTEX 246</td>
<td>KP645223</td>
</tr>
<tr>
<td>2</td>
<td><em>Chlorella sorokiniana</em> isolate 34-2</td>
<td>KU948993</td>
</tr>
<tr>
<td>3</td>
<td><em>Chlorella sp.</em></td>
<td>KF731759</td>
</tr>
<tr>
<td>4</td>
<td><em>Chlorella thermophila</em> ITBB HTA1-65</td>
<td>KJ002639</td>
</tr>
<tr>
<td>5</td>
<td><em>Chlorella sp.</em> TISTR 8990</td>
<td>JX683125</td>
</tr>
<tr>
<td>6</td>
<td><em>Chlorella sorokiniana</em></td>
<td>LK021940</td>
</tr>
<tr>
<td>7</td>
<td><em>Chlorella vulgaris</em> UTEX2714</td>
<td>KJ676110</td>
</tr>
<tr>
<td>8</td>
<td><em>Chlorella sp.</em> IFRPD 1014</td>
<td>AB260897</td>
</tr>
<tr>
<td>9</td>
<td><em>Chlorella sp.</em> ZJU0204</td>
<td>JX097068</td>
</tr>
<tr>
<td>10</td>
<td><em>Chlorella sp.</em> ZJU0205</td>
<td>JX097072</td>
</tr>
</tbody>
</table>
Figure A2: Alignment of 5.8s rDNA region, ITS1 and ITS2 amplified sequences of str. SLA-04 with other eukaryotic microalgae. In the Figure, the numerical entries on the left correspond to the sequences shown in Table A1.
Section A2: Estimation of HCO₃⁻ and CO₃²⁻ concentrations from DIC.

Bicarbonate concentration is a function of total dissolved inorganic carbon (DIC) and pH and is given by equation (1) which is also shown below

\[
[HCO_3^-] = \frac{[H^+]K_1^'}{[H^+]^2+\left[H^+\right]K_1^'+K_1^'K_2}
\]  

(A1)

At any given pH value, the fractional term on the right hand side of Eq. (1) is constant, Eq. (1) can be re-written as

\[
[HCO_3^-] = DIC \cdot \alpha
\]

where,

\[
\alpha = \frac{[H^+]K_1^'}{[H^+]^2+\left[H^+\right]K_1^'+K_1^'K_2}
\]

Further, DIC is sum of bicarbonate and carbonate concentrations present in the culture medium

\[
DIC = [HCO_3^-] + [CO_3^{2-}]
\]

(A3)

From Eqs. S1 and S3,

\[
[HCO_3^-] = \left[[HCO_3^-] + [CO_3^{2-}]\right] \cdot \alpha
\]

(A4)

Or,

\[
[CO_3^{2-}] = \frac{1-\alpha}{\alpha} [HCO_3^-]
\]

(A5)

Now, the total alkalinity (TA) of a solution can be given as

\[
TA = [HCO_3^-] + 2[CO_3^{2-}] + [OH^-]
\]

(A6)

Substituting the expression for \([CO_3^{2-}]\) from Eq. S5 into the right hand side of Eq. S6 and rearranging terms, we get

\[
[HCO_3^-] = (TA - [OH^-]) \cdot \frac{\alpha}{2-\alpha}
\]

(A7)

Eq. A7 allows the estimation of \([HCO_3^-]\) in a solution of given media alkalinity and pH.
Figure A3: FAME content and fatty acid profile of mixotrophically grown SLA-04 cultures.
Appendix b

B. Supplementary Information: High-productivity cultivation of microalgae without concentrated CO$_2$ input

Figure B1: Photograph of 30 L raceway ponds during the cultivation of SLA-04 cultures.
Figure B2: Photograph of 1100 L raceway ponds during the cultivation of SLA-04 cultures operated at a culture depth of 10".
Figure B3: Photograph of 1100 L raceway ponds during the cultivation of SLA-04 cultures operated at a culture depth of 7".
Section B4: Estimation of $\text{HCO}_3^-$ and $\text{CO}_3^{2-}$ concentrations from DIC.

The reactions that occur in bicarbonate/carbonate solutions are given below:

\[\text{CO}_2(L) + \text{OH}^- \rightleftharpoons \text{HCO}_3^-, \quad K_1 = 4.5 \times 10^7 \frac{L}{\text{mol.}}\]

\[k_1 = \frac{[\text{HCO}_3^-]}{[\text{CO}_2(L)][\text{OH}^-]} \quad \text{(B1)}\]

\[[\text{HCO}_3^-] = K_1 \left[\text{CO}_2(L)\right] [\text{OH}^-] \quad \text{(B2)}\]

\[\text{HCO}_3^- + \text{OH}^- \rightleftharpoons \text{CO}_3^{2-} + \text{H}_2\text{O}, \quad K_2 = 4.9 \times 10^3 \frac{L}{\text{mol.}}\]

\[K_2 = \frac{[\text{CO}_3^{2-}]}{[\text{HCO}_3^-][\text{OH}^-]} \quad \text{(B3)}\]

\[[\text{CO}_3^{2-}] = K_2 [\text{HCO}_3^-] [\text{OH}^-] \quad \text{(B4)}\]

By substituting Eq. B2 in Eq. B4 and rearranging, we get,

\[[\text{CO}_3^{2-}] = K_1 K_2 [\text{CO}_2(L)] [\text{OH}^-]^2 \quad \text{(B5)}\]

At any given pH, DIC is sum of dissolved CO2, bicarbonate and carbonate concentrations present in the culture medium

\[\text{DIC} = [\text{HCO}_3^-] + [\text{CO}_3^{2-}] + [\text{CO}_2(L)] \quad \text{(B6)}\]

By substituting Eqs. B2 and B5 in B6 and rearranging, we get,

\[[\text{HCO}_3^-] = \text{DIC} \frac{K_1 [\text{OH}^-]}{1 + K_1 [\text{OH}^-] + K_1 K_2 [\text{OH}^-]^2} \quad \text{(B7)}\]

At any given pH value, the fractional term on the right hand side of Eq. (1) is constant, Eq. (1) can be re-written as

\[[\text{HCO}_3^-] = \text{DIC} \cdot \beta \quad \text{(B8)}\]

Where,

\[\beta = \frac{K_1 [\text{OH}^-]}{1 + K_1 [\text{OH}^-] + K_1 K_2 [\text{OH}^-]^2} \quad \text{(B9)}\]

Further, at high pH conditions, dissolved CO2 concentrations are low and DIC is sum of bicarbonate and carbonate concentrations present in the culture medium

\[\text{DIC} = [\text{HCO}_3^-] + [\text{CO}_3^{2-}] \quad \text{(B10)}\]

From Eqs. B8 and S10,
\[ [HCO_3^-] = [[HCO_3^-] + [CO_3^{2-}]] \cdot [\beta] \quad \text{(B11)} \]

Or,
\[ [CO_3^{2-}] = \frac{1-\beta}{\beta} [HCO_3^-] \quad \text{(B12)} \]

Now, the total alkalinity (TA) of a solution can be given as
\[ TA = [HCO_3^-] + 2[CO_3^{2-}] + [OH^-] \quad \text{(B13)} \]

Substituting the expression for \([CO_3^{2-}]\) from Eq. B5 into the right hand side of Eq. S6 and rearranging terms, we get
\[ [HCO_3^-] = (TA - [OH^-]) \cdot \frac{\beta}{2-\beta} \quad \text{(B14)} \]

Eq. B14 allows the estimation of \([HCO_3^-]\) in a solution of given media alkalinity and pH.
Figure B 5-1: (a), (b), and (c) Biomass concentration profiles of str. SLA-04 when cultivated at an initial medium pH of 8.7 and under varied HCO$_3^-$ concentrations during February.

Figure B 5-2: (a), (b), and (c) Soluble N utilization by str. SLA-04 when cultivated at an initial medium pH of 8.7 and under varied HCO$_3^-$ concentrations during February.
Figure B6: HCO$_3^-$ concentrations (measured) in the culture medium during the growth of str. SLA-04 inoculated at an initial medium pH 8.7.
Table B1: Chemical parameters at 297 K for the absorption of CO$_2$ in a 0.5 M bicarbonate/0.5 M carbonate solution

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Equilibrium constant, K, m$^3$ mole$^{-1}$</td>
<td>$4.50 \times 10^{10}$</td>
</tr>
<tr>
<td>Diffusivity of CO$<em>2$, $D</em>{CO_2}$, m$^2$ s$^{-1}$</td>
<td>$1.59 \times 10^{-9}$</td>
</tr>
<tr>
<td>Diffusivity of OH$^-$, $D_{OH^-}$, m$^2$ s$^{-1}$</td>
<td>$5.17 \times 10^{-9}$</td>
</tr>
<tr>
<td>Diffusivity of HCO$<em>3^-$, $D</em>{HCO_3^-}$, m$^2$ s$^{-1}$</td>
<td>$1.18 \times 10^{-9}$</td>
</tr>
</tbody>
</table>

Figure B7: Biomass concentration profiles of str. SLA-04 when cultivated at an initial medium pH of 9.9 and under varied HCO$_3^-$ concentrations during February. The data associated with filled diamonds represents the cultures inoculated at an initial medium pH of 8.2.
**Figure B8:** Estimated $\text{HCO}_3^-$ concentrations in the culture medium during the growth of str. SLA-04 inoculated at an initial medium pH 9.9.

**Figure B9:** Sunlight intensities for the cultures grown in the month of September. The average light intensities were measured to be in between $360 - 1160 \mu\text{moles} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ with a day length of $\sim 12.5$ h.
Figure B10: Sunlight intensities for the cultures grown in the month of October. The average light intensities were measured to be in between 270 – 920 μmoles·m⁻²·s⁻¹ with a day length of ~11 h.
Section B11:

Table B11-1: Detailed calculations for mass transfer rates of DIC (shown as $\Delta$DIC).

<table>
<thead>
<tr>
<th>Time (d)</th>
<th>pH</th>
<th>Temperature (ºC)</th>
<th>Ionic strength (I)</th>
<th>$pK_1$</th>
<th>$pK_2$</th>
<th>TA (mM)</th>
<th>HCO$_3^-$ (mM)</th>
<th>CO$_3^{2-}$ (mM)</th>
<th>DIC (mM)</th>
<th>$\Delta$DIC (mM)</th>
<th>$\Delta$DIC (mmol·m$^{-2}$·d$^{-1}$)</th>
<th>$\Delta$DIC (g-C·m$^{-2}$·d$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>1.21</td>
<td>10.2</td>
<td>17.8</td>
<td>6.33</td>
<td>10.15</td>
<td>2.4</td>
<td>2.7</td>
<td>5.2</td>
<td>0.23</td>
<td>688.1</td>
<td>8.3</td>
<td></td>
</tr>
<tr>
<td>Day 2</td>
<td>4.33</td>
<td>10.2</td>
<td>24.0</td>
<td>6.29</td>
<td>10.09</td>
<td>2.2</td>
<td>2.9</td>
<td>5.4</td>
<td>0.22</td>
<td>635.3</td>
<td>7.6</td>
<td></td>
</tr>
<tr>
<td>Day 3</td>
<td>5.29</td>
<td>10.2</td>
<td>21.8</td>
<td>6.30</td>
<td>10.11</td>
<td>2.3</td>
<td>2.8</td>
<td>5.1</td>
<td>0.22</td>
<td>641.1</td>
<td>7.7</td>
<td></td>
</tr>
<tr>
<td>Day 4</td>
<td>6.29</td>
<td>10.2</td>
<td>20.9</td>
<td>6.31</td>
<td>10.12</td>
<td>2.3</td>
<td>2.8</td>
<td>5.1</td>
<td>0.22</td>
<td>657.6</td>
<td>7.9</td>
<td></td>
</tr>
<tr>
<td>Day 5</td>
<td>10.38</td>
<td>10.1</td>
<td>20.6</td>
<td>6.34</td>
<td>10.16</td>
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<td>2.7</td>
<td>5.2</td>
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<td>8.2</td>
<td></td>
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<tr>
<td>Day 6</td>
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<td>10.2</td>
<td>16.6</td>
<td>6.35</td>
<td>10.18</td>
<td>2.5</td>
<td>2.7</td>
<td>5.2</td>
<td>0.24</td>
<td>690.1</td>
<td>8.3</td>
<td></td>
</tr>
<tr>
<td>Day 7</td>
<td>11.38</td>
<td>10.1</td>
<td>14.8</td>
<td>6.35</td>
<td>10.18</td>
<td>3.0</td>
<td>2.5</td>
<td>5.5</td>
<td>0.22</td>
<td>645.0</td>
<td>7.7</td>
<td></td>
</tr>
<tr>
<td>Day 8</td>
<td>11.42</td>
<td>10.2</td>
<td>15.1</td>
<td>6.35</td>
<td>10.18</td>
<td>3.0</td>
<td>2.5</td>
<td>5.5</td>
<td>0.22</td>
<td>645.0</td>
<td>7.7</td>
<td></td>
</tr>
<tr>
<td>Day 9</td>
<td>15.25</td>
<td>10.2</td>
<td>23.2</td>
<td>6.29</td>
<td>10.09</td>
<td>2.2</td>
<td>2.8</td>
<td>5.1</td>
<td>0.22</td>
<td>645.0</td>
<td>7.7</td>
<td></td>
</tr>
<tr>
<td>Day 10</td>
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<td>22.9</td>
<td>6.30</td>
<td>10.10</td>
<td>2.6</td>
<td>2.6</td>
<td>5.3</td>
<td>0.22</td>
<td>645.0</td>
<td>7.7</td>
<td></td>
</tr>
</tbody>
</table>

Set 1

| Day 1   | 0.99 | 10.2  | 17.8 | 6.33 | 10.16 | 1.4 | 1.5 | 2.9 | 0.14 | 548.3 | 6.6 |
| Day 2   | 1.97 | 10.2  | 18.9 | 6.33 | 10.15 | 1.3 | 1.5 | 2.8 | 0.14 | 617.0 | 7.4 |
| Day 3   | 2.95 | 10.2  | 19.7 | 6.32 | 10.14 | 1.3 | 1.5 | 2.8 | 0.14 | 618.3 | 7.4 |
| Day 4   | 3.97 | 10.2  | 19.2 | 6.32 | 10.15 | 1.3 | 1.5 | 2.8 | 0.14 | 615.5 | 7.4 |
| Day 5   | 4.99 | 10.2  | 18.1 | 6.33 | 10.15 | 1.6 | 1.4 | 3.0 | 0.14 | 547.4 | 6.6 |
| Day 6   | 5.80 | 10.1  | 19.7 | 6.32 | 10.14 | 1.6 | 1.4 | 3.0 | 0.14 | 492.7 | 5.9 |
| Day 7   | 6.72 | 10.2  | 20.8 | 6.31 | 10.13 | 1.3 | 1.5 | 2.8 | 0.14 | 558.4 | 6.7 |

Set 2

| Day 1   | 0.99 | 10.2  | 17.8 | 6.33 | 10.16 | 1.4 | 1.5 | 2.9 | 0.14 | 548.3 | 6.6 |
| Day 2   | 1.97 | 10.2  | 18.9 | 6.33 | 10.15 | 1.3 | 1.5 | 2.8 | 0.14 | 617.0 | 7.4 |
| Day 3   | 2.95 | 10.2  | 19.7 | 6.32 | 10.14 | 1.3 | 1.5 | 2.8 | 0.14 | 618.3 | 7.4 |
| Day 4   | 3.97 | 10.2  | 19.2 | 6.32 | 10.15 | 1.3 | 1.5 | 2.8 | 0.14 | 615.5 | 7.4 |
| Day 5   | 4.99 | 10.2  | 18.8 | 6.33 | 10.15 | 1.6 | 1.4 | 3.0 | 0.14 | 547.4 | 6.6 |
| Day 6   | 5.80 | 10.1  | 19.7 | 6.32 | 10.14 | 1.6 | 1.4 | 3.0 | 0.14 | 492.7 | 5.9 |
| Day 7   | 6.72 | 10.2  | 20.8 | 6.31 | 10.13 | 1.3 | 1.5 | 2.8 | 0.14 | 558.4 | 6.7 |

* Data obtained from the experiments carried out during December 2013
* Data obtained from the experiments carried out during September 2013
* Cultivations were carried out in small raceway ponds (surface area = 0.18 m$^2$) and the rate of increase in DIC was calculated using this surface area.
Table B11-2: Mass transfer coefficients calculated using the mass transfer rates (reported in Table B11-1)

<table>
<thead>
<tr>
<th>ΔDIC (mmol·m(^{-2})·d(^{-1}))</th>
<th>Mass transfer driving force</th>
<th>K(_L)·E (m·h(^{-1}))</th>
<th>K(_L) (m·h(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ΔDIC (mmol·m(^{-3}))</td>
<td>CO(_2^*(aq))</td>
<td>CO(<em>2)(</em>\text{bulk}(aq))</td>
</tr>
<tr>
<td>688.1</td>
<td>0.23</td>
<td>12.97</td>
<td>2.21 0.06</td>
</tr>
<tr>
<td>635.3</td>
<td>0.18</td>
<td>13.02</td>
<td>2.03 0.05</td>
</tr>
<tr>
<td>641.1</td>
<td>0.20</td>
<td>13.00</td>
<td>2.05 0.05</td>
</tr>
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<td>657.6</td>
<td>0.21</td>
<td>12.99</td>
<td>2.11 0.05</td>
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<td>0.24</td>
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<td>2.19 0.06</td>
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<td>0.26</td>
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<td>617.0</td>
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<td>1.98 0.05</td>
</tr>
<tr>
<td>618.3</td>
<td>0.19</td>
<td>13.01</td>
<td>1.98 0.05</td>
</tr>
<tr>
<td>615.5</td>
<td>0.19</td>
<td>13.01</td>
<td>1.97 0.05</td>
</tr>
<tr>
<td>547.4</td>
<td>0.19</td>
<td>13.01</td>
<td>1.75 0.04</td>
</tr>
<tr>
<td>492.7</td>
<td>0.19</td>
<td>13.01</td>
<td>1.58 0.04</td>
</tr>
<tr>
<td>558.4</td>
<td>0.18</td>
<td>13.02</td>
<td>1.79 0.04</td>
</tr>
</tbody>
</table>
Additional Data:

**Figure B12**: Photosynthetic efficiencies ($F_v/F_m$) of SLA-04 microalgae cultivated during the months of (a) February, (b) July, (c) September, and (d) October.

**Figure B13**: a) shows the nitrogen utilized for SLA-04 microalgae cultures grown at a culture depth of 10" and slope value represents the utilization rate (1.28 mg-N·L$^{-1}$·d$^{-1}$) and b) shows the nitrogen utilized for SLA-04 microalgae cultures grown at a culture depth of 7" and slope value represents the utilization rate (4.32 mg-N·L$^{-1}$·d$^{-1}$).
Appendix C

C. Supplementary Information: Novel approach for outdoor mixotrophic cultivation of microalgae

Figure C1: Photograph of 30 L raceway ponds during the cultivation of SLA-04 cultures.
Figure C2: Photograph of 1100 L raceway ponds during the cultivation of SLA-04 cultures operated at a culture depth of 10".
Figure C3: Photograph of 1100 L raceway ponds during the cultivation of SLA-04 cultures operated at a culture depth of 7”
Figure C4: Sunlight intensities for the cultures grown in the month of September. The average light intensities were measured to be in between $360 - 1160 \, \mu\text{moles} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ with a day length of 12.5 hr.

Figure C5: shows the total alkalinity (TA) and dissolved inorganic carbon (DIC) for mixotrophic SLA-04 microalgae cultures grown at a culture depth of 10"
**Figure C6**: Photosynthetic efficiency ($F_v/F_m$) of SLA-04 microalgae cultivated at a culture depth of 10". $F_v/F_m$ values suggest that the photosynthetic activity of SLA-04 cultures was not compromised even in the presence of organic carbon.

**Figure C7**: Sunlight intensities for the cultures grown in the month of October. The average light intensities were measured to be in between 270 – 920 µmoles·m⁻²·s⁻¹ with a day length of 11 hr.
Figure C8: Total alkalinity (TA) and dissolved inorganic carbon (DIC) of mixotrophic SLA-04 microalgae cultures grown at a culture depth of 7”.

Table C1: Lipid yields and productivities of SLA-04 cultures grown in raceway open ponds (culture volume = 25 L) under lipid-boost strategy.

<table>
<thead>
<tr>
<th>Cultivation strategy</th>
<th>Organic carbon source</th>
<th>Organic carbon utilized (g·L⁻¹)</th>
<th>Lipid (%(w·w⁻¹))</th>
<th>Lipid productivity yield (g·g⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipid-boost</td>
<td>Glucose</td>
<td>0.43</td>
<td>30</td>
<td>4.11</td>
</tr>
<tr>
<td></td>
<td>Acetate</td>
<td>0.26</td>
<td>20.5</td>
<td>2.42</td>
</tr>
</tbody>
</table>
Figure C9: a) Biomass concentration of SLA-04 cultures grown mixotrophically in open raceway ponds with a working volume of 25 L. The mixotrophic cultures were supplemented with glucose and acetate after nitrogen depletion, b) shows the photosynthetic efficiency (Fv/Fm) of SLA-04 microalgae cultures. Fv/Fm values suggest that the photosynthetic activity of SLA-04 cultures was not compromised even in the presence of organic carbon.
Table C2: Biochemical composition of str. SLA-04 grown under mixotrophic and lipid-boost cultivation conditions at a culture depth of 7".

<table>
<thead>
<tr>
<th>Cultivation method</th>
<th>Time period</th>
<th>FAMEs (%(w·w⁻¹))</th>
<th>Total carbohydrates (%(w·w⁻¹))</th>
<th>Protein (^a) (%(w·w⁻¹))</th>
<th>Nucleic acids (^b)</th>
<th>ASH content (%(w·w⁻¹))</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mixotrophic</td>
<td>Day 0</td>
<td>8.04</td>
<td>27.73</td>
<td>32.28</td>
<td></td>
<td>20.36</td>
<td>93.41</td>
</tr>
<tr>
<td></td>
<td>Day 3</td>
<td>17.54</td>
<td>30.71</td>
<td>28.23</td>
<td></td>
<td>5.79</td>
<td>87.26</td>
</tr>
<tr>
<td></td>
<td>Day 11</td>
<td>42.58</td>
<td>25.68</td>
<td>20.48</td>
<td></td>
<td>3.51</td>
<td>97.24</td>
</tr>
<tr>
<td>2-Stage</td>
<td>Day 0</td>
<td>9.13</td>
<td>20.93</td>
<td>39.60</td>
<td></td>
<td>17.78</td>
<td>92.44</td>
</tr>
<tr>
<td></td>
<td>Day 12</td>
<td>32.93</td>
<td>26.52</td>
<td>19.20</td>
<td></td>
<td>8.16</td>
<td>91.80</td>
</tr>
</tbody>
</table>

\(^a\)Protein content was estimated using a conversion factor of 5.99.

\(^b\)Nucleic acid content was obtained from literature.
Section C9: Determination of liquid-phase CO$_2$ and bulk CO$_2$

Liquid-phase concentration of CO$_2$ ($CO_2^{aq}$) is determined by the concentration of CO$_2$ in air and the Henry’s constant for CO$_2$ (Eq C1).

$$CO_2^{aq} = \frac{P}{K_h}$$

(C1)

Where $P$ is the partial pressure of CO$_2$ and $K_h$ is Henry’s constant for CO$_2$.

The values for $CO_2^{bulk}$ are calculated using HCO$_3^-$ and CO$_3^{2-}$ concentration that were determined experimentally. The detailed calculation procedure is given below:

$$CO_2^{bulk \quad (aq)} + OH^- \overset{k_1}{\rightarrow} HCO_3^{-, \quad media}$$

(C2)

From Eq. C1,

$$k_1 = \frac{[HCO_3^-]}{[CO_2][OH^-]}$$

(C3)

$$HCO_3^{-, \quad media} + OH^- \overset{k_2}{\leftrightarrow} CO_3^{2-, \quad media} + H_2O$$

(C4)

From Eq. C2,

$$k_2 = \frac{[CO_3^{2-}]}{[HCO_3^-][OH^-]}$$

(C5)

Now using Eqs. C3 and C4, $CO_2^{bulk \quad (aq)}$ can be determined.

$$CO_2^{bulk \quad (aq)} = \frac{k_2}{k_1} \times \frac{[HCO_3^-]^2}{CO_3^{2-}}$$

(C5)

The equilibrium constants were obtained from the literature [98]

$$\frac{k_2}{k_1} = 1.08 \times 10^6$$
Table C3: Biochemical composition of str. SLA-04 grown under phototrophic, mixotrophic and lipid-boost cultivation conditions.

<table>
<thead>
<tr>
<th>Cultivation method</th>
<th>Time period</th>
<th>Biomass concentration (g·L⁻¹)</th>
<th>FAMEs (%(w⁻¹))</th>
<th>Total carbohydrates (%(w⁻¹))</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Phototrophic</strong></td>
<td>Day 0</td>
<td>0.32</td>
<td>7.1</td>
<td>20.3</td>
</tr>
<tr>
<td></td>
<td>Day 5</td>
<td>0.64</td>
<td>14.6</td>
<td>20.1</td>
</tr>
<tr>
<td></td>
<td>Day 12</td>
<td>0.72</td>
<td>21.8</td>
<td>25</td>
</tr>
<tr>
<td><strong>Mixotrophic</strong></td>
<td>Day 0</td>
<td>0.4</td>
<td>8.04</td>
<td>27.73</td>
</tr>
<tr>
<td></td>
<td>Day 5</td>
<td>0.9</td>
<td>26.84</td>
<td>28.20</td>
</tr>
<tr>
<td></td>
<td>Day 11</td>
<td>0.98</td>
<td>42.58</td>
<td>25.68</td>
</tr>
<tr>
<td><strong>Lipid-boost</strong></td>
<td>Day 0</td>
<td>0.31</td>
<td>9.13</td>
<td>20.93</td>
</tr>
<tr>
<td></td>
<td>Day 5</td>
<td>0.77</td>
<td>17.37</td>
<td>26.90</td>
</tr>
<tr>
<td></td>
<td>Day 12</td>
<td>0.93</td>
<td>32.93</td>
<td>26.52</td>
</tr>
</tbody>
</table>

*Phototrophic cultivation was obtained from previous cultivation studies (Chapter 3)
Appendix D

D. Laboratory-scale demonstration of algae de-watering using temperature-sensitive hydrogels

Introduction

Biofuels produced from microalgae, at an estimated oil content of 40%, promise 10 to 100 times greater yield per land acre than other crops. Currently, a major hurdle for commercial-scale production of fuels from microalgae is recovering algal biomass from growth media with minimal cost and energy input. When cultivated in open raceway ponds, microalgae concentrations are very low – typically 0.1% (w·w⁻¹). For fuel production, the challenge is therefore to harvest these dilute cultures efficiently and economically. Also, commercial-scale harvesting methods should also allow for recycling of water and unutilized water-soluble nutrients – critical for environmental sustainability of microalgae production.

The current commercial methods for harvesting microalgae include centrifugation, filtration and flocculation followed by settling or dissolved air flotation. Centrifugation can produce thick pastes with high solids concentration (~20% (w·w⁻¹)) and this method also allows for recovery of uncontaminated culture media for recycle. Recently, microalgae-specific low-speed centrifuges have been developed by Evodos™
that require less energy (8 kWh/m$^3$ of media processed) than more conventional centrifuges used in the biotechnology and fermentation industries. Cell lysis is also be prevented in the Evodos™ models. Nonetheless, the capital costs associated with centrifugation are unappealing.

Cross flow filtration is another method that has primary use in biotechnology/fermentation but could also be applied for separation of microalgae. With this technique, reasonably high biomass concentrations can be achieved (8 % (w·w$^{-1}$)), and since no chemicals are added in this process, the recovered permeate can be recycled. When solids concentrations are low, such as in microalgae cultures from open raceway ponds, the energy use is also low due to small cake resistance (approximately 2 kWh/m$^3$). However, the primary concern with cross flow filtration is the cost associated with membrane replacement and membrane cleaning. Due to the presence of exo-polysaccharides in many algal cells, irreversible fouling of membranes is unavoidable in commercially available 0.22µm cross flow filtration modules that would necessitate frequent membrane replacements. Fouling issues could be avoided, at least in part, by use of membranes with larger pore sizes (3-5 µm). However, low-cost large-pore membrane modules are not commercially available and may be challenging to manufacture since conventional polymer membrane supports have sub-micron pore sizes.

Flocculation followed by settling or dissolved air flotation, adapted from wastewater treatment, can also be used for harvesting microalgae. With this technique too, reasonably high slurry concentrations can be achieved (5 – 10 % (w·w$^{-1}$)) with energy consumption varying between 10-20 kWh/m$^3$. However, addition of flocculants precludes the recycle of growth media for microalgae re-cultivation. Also, the carryover
of flocculants with the harvested biomass could negatively impact downstream conversion processes and fuel quality.

Historical observations have shown that some microalgae species are also able to “autoflocculate” under alkaline conditions. Recent investigations to elucidate the mechanism for this occasional spontaneous settling of microalgal cells show that the process might actually be a result of chemical flocculation due to precipitation of calcium and magnesium hydroxides (from Ca and Mg salts in the growth media) when solution pH is high. Further, this process results in only a small increase in concentration of cells (up to 0.1 to 0.5%). Secondary harvesting methods would therefore still be needed to increase concentrations to levels compatible with conversion processes (~10% (w\text{-}w^{-1})). In any case, auto flocculation appears to be species-specific and this method is likely applicable only to a few select strains.

As discussed above, it is clear that while harvesting methods from other industries may be applied to microalgae cultures, they are not particularly well-suited. Cross flow filtration and centrifugation are best suited for biotechnology applications with high cell concentrations of fermentation cultures and high value of the products. Flocculation works well in wastewater treatment since recyclability of water and quality of recovered biomass are not of primary concern. Clearly, transformative alternatives for dewatering dilute microalgae slurries are needed.

Hydrogels are superabsorbent cross-linked polymers that can absorb large quantities of water and “swell” in aqueous solutions. Stimuli-sensitive hydrogels can also “de-swell” (i.e. release the absorbed water) based on an external stimulus. Temperature-sensitive polymers such as poly(N-isopropyl acrylamide) (PNIPAAm) swell at room temperature
but show a large volume change (deswell) as they undergo a phase transition when heated above their lower critical solution temperatures (LCST). The LCST for PNIPAAm is 32°C, but can be increased or lowered based on co-polymerization with hydrophilic or hydrophobic co-monomers. The swelling kinetics of PNIPAAm gels can also be improved through intercalation of additional polymers such as poly(vinyl alcohol) into the PNIPAAm gel structure.

**Swelling kinetics of poly-N-isopropylacrylamide (PNIPAAm) hydrogels in the presence and absence of microalgae**

To prepare PNIPAAm hydrogels, first, 7.92g of N-isopropylacrylamide, 80mg of N, N′-methylenebisacrylamide and 5mg of ammonium persulfate were dissolved in 100mL of de-ionized (DI) water. This solution was then transferred to a glass tube (2.5cm dia. and 15cm long) with one end sealed, and flushed with N2 for 5min to purge the dissolved O2. Then, 5mg of sodium meta-bisulphate was added and the solution was again flushed with N2. At this stage, the other end of the glass tube was also sealed and polymerization reaction was allowed to proceed for 1h at room temperature.

Once the polymerization reaction was completed, a clear transparent gel was formed in the tube. The gel was removed from the tube and washed for 48h with DI water in order to remove any unreacted monomer and cross-linker. After washing, the gel was cut into 10mm×8mm×5mm pieces which were frozen for 24h at -20°C. The frozen gel pieces were then thawed and dried in an oven, before examining their swelling/deswelling kinetics.

*Chlorella sp.* cultures for dewatering experiments were cultivated under continuous illumination, stirring, and air sparging in 3L Cytostir® spinner flask reactors.
(Kimble/Kontes, Vineland, NJ, USA). The growth medium contained NaNO₃ (0.25 g·L⁻¹), NH₄Cl (0.05 g·L⁻¹), MgSO₄ • 7H₂O (0.075 g·L⁻¹), CaCl₂ • 2H₂O (0.025 g·L⁻¹), NaCl (0.025 g·L⁻¹), ferric ammonium citrate C₆H₃⁺₄yFe₃N₇O₇ (0.01 g·L⁻¹), K₂HPO₄ (0.25 g·L⁻¹), Na₂CO₃ (0.25 g·L⁻¹), and trace element solution (1mL/1L-medium). Biomass concentration during culture growth was determined by measuring total suspended solids (TSS) concentration (described later).

After cultures reached stationary phase (concentration of 1.6 g·L⁻¹), a portion of the cultures was centrifuged (3000×g) and the obtained pellet was re-suspended in fresh media to obtain slurries of various concentrations.

Hydrogels were swollen in these variously concentrated microalgae slurries as well as in cell-free media. 0.5 g of dry hydrogel was added to the aqueous solutions and incubated at room temperature (20 °C). Swelling kinetics of hydrogels incubated in cell-free media were measured directly though gravimetric analysis by periodically removing the hydrogels and measuring their weight. In addition, for the experiments that incubated hydrogels with microalgae slurries, the concentrations of microalgae biomass in free aqueous solution (not absorbed by the hydrogels) were also periodically monitored. Due to the uptake of aqueous media by the hydrogels, the suspended cell concentrations in these cultures increased over time. The volume of media absorbed by the hydrogels was then estimated based on the increase in cell concentrations. Thereafter, hydrogel swelling ratios were calculated by dividing the mass of absorbed media by the dry gel weight. The changes in swelling ratio of the hydrogels incubated in the presence and absence of microalgae cells is shown in Figure 2. From these results, it can be seen that the hydrogel swelling rates in dilute (1.6 g·L⁻¹) as well as concentrated cultures (80.4 g·L⁻¹) were
estimated to be very similar and also closely matched with the measured swelling ratios in cell-free media. These results suggested that gel performance was not affected by the presence of algae and occlusion of gel pores by algal cells, if any, is likely insignificant. Also, after slurries were dewatered, final biomass concentrations in all cases were increased by a factor of approximately 2.15 as evident from the slope of the linear correlation shown in the inset to Figure 1. These results further confirm that absorption of aqueous solutions by the hydrogels was not affected by the presence of algae cells over the wide biomass concentration range of 1 g·L⁻¹ to 160 g·L⁻¹.

**Figure 1:** Swelling performance of hydrogels in microalgae slurries. Inset shows the correlation between cell concentrations before and after incubation of microalgae slurries with hydrogels. Results show that the rates of water absorption were not influenced by the presence of microalgal cells even at very high concentrations of >80 g·L⁻¹.
PNIPAAm swelling and deswelling performance during cyclic reuse

For these experiments, the PNIPAAm hydrogels were prepared using a protocol similar to Example 1, but the gels were cut into smaller pieces (3mm×3mm×3mm). To determine swelling kinetics, dry gels were added to water or algae growth media and incubated at room temperature for 24h. Uptake of growth media by hydrogels was quantified by gravimetrically monitoring swollen gel weights. Before starting the experiment, prepared hydrogel pieces were dehydrated in an oven at 40°C. 0.5g of dry gel pieces were weighed at room temperature, placed on a sheet of nylon mesh (approximately 6 cm in diameter) of known weight and incubated in fresh growth medium (composition of the growth medium is described later). As the hydrogels swelled, the nylon mesh along with the enclosed hydrogels was periodically removed and weighed. Before weighing, the free liquid (around the hydrogel pieces) was allowed to drain by gravity and the outside of the mesh was dabbed with moist paper towels to remove any additional external liquid. Swollen gel weights were measured using an analytical balance (Shimadzu AUW120D, Kyoto, Japan; max: 120g, min: 1mg; accuracy: d=0.1mg). Hydrogels were allowed to swell until nearly-constant gel weight was achieved.

Thereafter, the hydrogels were recovered and incubated in an oven at 37°C for 4h to deswell the gels and release the absorbed aqueous media. During the deswelling process also, gels were periodically weighed until constant weight was achieved.

As a result of a larger surface area (due to smaller-sized gels) in contact with the solution, these gels swelled at a faster rate than the hydrogels that were previously used for experiments described in Example 1 (compare swelling rates in Figure 2(a) with
Figure 1). Highest swelling rates were obtained during the initial period of incubation and swelling ratios of nearly 20g-absorbed solution/g-dry gel were obtained in 0-2h. Equilibrium swelling ratios of nearly 35g-absorbed solution/g-dry gel were obtained after much longer incubation (>12h). These results indicated that the harvesting process would be most expeditious if short swelling periods are employed (2h or less) to rapidly absorb the aqueous solutions, rather than await equilibrium. Longer residence times would allow lower use of gel per stage, but savings in gel cost may not be significant in this approach if gel recycle is effective. In addition, the short swelling period would also match the deswelling time to release most of the absorbed media (approx. 2h; see deswelling curve in Figure 2(a)) such that swelling and deswelling operations could be carried out in parallel akin to commercial pressure-swing adsorption.

To assess consistency in gel performance over prolonged reuse, we performed repeated swelling and deswelling of hydrogel samples over multiple cycles. Hydrogels were swelled in microalgae growth media for 2h at 20°C, following which hydrogels were recovered and deswollen in an oven (37°C) for 2h. The recovered gels were then reused to absorb fresh media and then deswelled again; this cyclic process was repeated 10 times. Swollen and deswollen gel weights were recorded during each cycle and were used to calculate swelling ratios. From Figure 2(b), the swelling ratios remained close to 20g-absorbed solution/g-dry gel for each of the swelling cycle demonstrating that reuse of hydrogels did not influence their absorption performance. These results indicate that hydrogels could likely be used for prolonged periods without replacement by new gel material.
Figure 2: (a) Swelling and deswelling kinetics of PNIPAAm hydrogels in DI water and microalgae culture media. (b) Aqueous media uptake and release performance of PNIPAAm hydrogels when subjected to successive swelling-deswelling cycles. The solid and dashed lines in (b) represent the swelling and deswelling portions of each cycle, respectively.

Stage-wise concentration of microalgae cultures using PNIPAAm hydrogels.

In these experiments, we tested the feasibility of using PNIPAAm hydrogels for harvesting and microalgae cultures by implementing a stagewise dewatering approach. In each stage, the added hydrogels absorbed a fraction of the growth media to cause a partial increase in culture concentrations. In order to keep the absorption rate high, the
gels were allowed to swell partially only (and rapidly absorb the aqueous growth media) for approximately 2h. The mass of dehydrated hydrogels (absorbent) added to each stage was such that the volume of swollen gel (at the end of the 2h incubation) was equal or less than the volume of free liquid remaining at the end of the stage operation. Adequate availability of free liquid at the end of the stage incubation period was necessary to keep all gels submerged for the entire duration of the incubation such that the absorption capability of all gels were utilized. In addition, the free liquid remaining at the conclusion of the stage operation permitted a quick and easy flow of algae-rich slurries through the mesh used during recovery of swollen gels and also helped to “wash down” the cells that might be physically adhered to gel surfaces. Mass of dehydrated hydrogel to add to each stage was assessed from the volume of culture fed to a stage, the expected swelling ratio of 20g-absorbed media/g-dry-gel during the 2h stage-residence time and the constraint of retaining at least half of the feed liquid unabsorbed in each stage.

Biomass concentrations in the aqueous streams were determined by measuring total suspended solids (TSS). For TSS estimates, culture samples were filtered through a pre-weighed glass fiber filter. Subsequently, the filter paper containing the algae cake was washed with pure water to dissolve inorganic precipitates, if any. Thereafter, the filter paper was carefully removed and dried in an oven at 50°C until a constant weight was obtained. Dry weight of algae biomass collected on the filter paper was calculated as the difference in weight of the filter paper before and after filtration. The concentration was calculated by dividing the measured algae mass by the filtered culture volume.

During each stage, cultures at room temperature were placed in a beaker and exposed to shrunken hydrogels that had been previously deswelled at 40°C. A stir bar was
used for keeping the suspension well mixed. At the end of the incubation period, the suspension was passed through a nylon mesh to separate the swollen gel pieces from the concentrated culture. The swollen gels collected on the mesh were deswelled in an oven to recover the absorbed growth medium. During deswelling, the oven temperature was maintained at 37-38°C to prevent microalgal cells from losing viability. The volume of concentrated culture collected was measured with a measuring cylinder. A part of the concentrated culture was set aside for TSS measurements and the remaining volume was transferred to the next dewatering stage.

The results of the two experimental runs performed for stagewise implementation of microalgae harvesting using PNIPAAm hydrogels are shown in Figures 3 and 4. These Figures show a detailed material balance for each stage of the process. As expected, an approximately 2-fold increase in concentration was achieved after each stage. Starting from an initial culture volume of 500mL, we were able to remove >99% of the aqueous media from the dilute feed such that <2.5mL of the feed volume remained at the of the 8-stage process. 90% of the culture media was recovered in only 3 stages. The concentrated cultures were approximately 100 g·L⁻¹. The stage-wise increase in concentrations obtained during the implementation of this process is shown in Figure 5. These experiments demonstrate that concentrations of 100 g·L⁻¹ or more can be achieved starting from dilute microalgae cultures.
Figure 3: Stage-wise concentration of microalgae cultures using PNIPAAm hydrogels – Experimental run #1.
Figure 4: Stage-wise concentration of microalgae cultures using PNIPAAm hydrogels – Experimental run #2.
**Figure 5**: Slurry concentrations at each stage of the stagewise concentration of microalgae cultures. Values shown in the graph are averaged from two replicate runs. Error bars indicate on standard deviation from mean values.
References


