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Developing Microalgae as Production Platforms for Natural Products

A Thesis submitted in partial satisfaction of the requirements
for the degree Master of Science

in

Chemistry

by

Nathan Schoepp

Committee in charge:

      Professor Michael Burkart, Chair
      Professor Stephen Mayfield
      Professor Nathan Gianneschi

2013

The Thesis of Nathan Schoepp is approved, and it is acceptable in quality and form for

publication on microfilm and electronically:

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Chair

The University of California, San Diego

2013

DEDICATION

I dedicate this thesis to my parents, Scott and Julie Schoepp, and to Rotem Fishel for the

support and teaching you have all shown.

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ABSTRACT OF THE THESIS

Developing Microalgae as Production Platforms for Natural Products

by

Nathan Schoepp

Master of Science in Chemistry & Biochemistry

University of California, San Diego, 2013

Professor Michael Burkart, Chair

Microalgae and cyanobacteria have recently reemerged as a potential crop in the effort to abate continually increasing issues of sustainability in the production of food and fuel. In a field where substantial discrepancies consistently exist between laboratory and outdoor cultivation, gains in the development of laboratory technologies have in general not been paralleled with field demonstrations. For these reasons, we developed a low-maintenance system and process for research-scale outdoor cultivation of a variety of both freshwater and marine eukaryotic microalgae and cyanobacteria. *Scenedesmus*, *Chlorella*, *Chlamydomonas*, *Arthrospira*, *Anabaena*, *Porphyridium*, *Nannochloropsis*,

Dunaliella, and *Phaeodactylum* were tested in the system, and demonstrate the growth of both model and commercial-production organisms. Biomass densities ranging from 0.30 g/L – 0.73 g/L were obtained in 100 L hanging polybags, with similar densities of 0.16 g/L – 0.82 g/L achieved during outdoor autotrophic growth in 800 L airlifted stock-ponds. Total capital expenses of the stock-pond system were under $4000 dollars, with ongoing production costs ranging from 0.04 $/g – 0.27 $/g. Depending on the strain cultivated, hundreds to thousands of grams of dry biomass could be produced in a single growth cycle in the stock-ponds, suitable for a variety of uses including inoculum generation, recombinant protein production, natural product isolation, and biofuel applications. Following testing in the stock-ponds, *Scenedesmus dimorphus* (UTEX 1237) was grown semi-continuously in an 8,000 L airlift-driven raceway, yielding over eight kilograms of dry biomass.

Introduction

  The need for sustainable substitutes of petroleum-based products will continually increase as petroleum reserves dwindle. It is clear that even in the midst of periodic surges in petroleum supply, a broad range of industries must begin utilizing sustainable feedstocks as replacements for those derived from fossil reserves. Reliance on petroleum reaches beyond fuels and plastics into human, animal, and aquaculture feed markets, which are now closely linked to the price of crude oil (Hardy, 2010; Trostle, 2010), and becoming equally stressed. Utilizing photosynthetically derived substitutes has the potential to reduce carbon outputs and dramatically increase sustainability. Production of such replacements for food (Ibanez & Cifuentes, 2013) and fuel (Jones & Mayfield, 2012) has been proposed in a number of biological production platforms, including microalgae. From an agricultural point of view, microalgae can serve as one of the most efficient photosynthetic crops for the production of raw biomass (DOE, 2010) without direct competition with food crops. In order for the potential of any bio-product industry such as microalgae to be realized, efficient biomass production is necessary to meet feedstock demands, and test production of new commodities. While the idea of large-scale (>50 L), high-rate cultivation has been proposed as early as the 1950s (Cook, 1951), recent renewed interest in algae's potential has led to a surge in the development of laboratory-scale algal culturing systems, genetic techniques, and related technologies. However, academic research remains lacking in large-scale production systems that can be operated safely and reliably, yet are flexible enough to grow a variety of strains. These systems are a necessity in evaluating the potential vs. realized productivity of

microalgae, elucidating issues of crop protection, and establishing baselines for future crop improvement both in the laboratory and outdoors.

To date, only certain genera of microalgae such as *Arthrospira*, *Haematococcus*, *Chlorella*, and *Dunaliella* have been cultivated as economically viable crops due to various native characteristics such as protein content, lipid accumulation, and pigment production (Raja et al., 2007). Industrial production has likely been realized in part due to the FDA's approval of these strains as feed supplements (Lorenz & Cysewski, 2000). Early and immediate promise as valuable producers led to their development as production strains serving niche markets such as production of the food colorant astaxanthin. On the other hand, several genera such as *Nannochloropsis*, *Scenedesmus*, and *Porphyridium* have been suggested as potential bio-fuel or bio-product candidates based on laboratory studies (Arad & Levy-Ontman, 2010; Gouveia & Oliveira, 2009). Genetically malleable model alga and cyanobacteria also exist, such as *Chlamydomonas reinhardtii*, *Anabaena* sp. strain PCC 7120, and *Phaeodactylum tricornutum* continue to be developed as recombinant protein production platforms (Chaurasia & Apte, 2011; Mayfield et al., 2007; Zaslavskaia et al., 2000), and biofuel candidates (Yu et al., 2011). Lacking from this ever-increasing body of knowledge are large-scale production studies with these and other strains of microalgae not yet established as industrial crops, likely as a result of the lack of systems described in detail towards this purpose. Several fields require biomass sources on the order of hundreds of grams including natural product characterization, animal feed trials, lipid extraction and biofuel production, and bio-product proof of concept studies. In each of these cases production on the order of

hundreds of liters or more is a critical middle ground in the establishment of a reliable production platform. Work in systems at this scale will ultimately determine which uses of microalgae find a permanent place in our future.

In any system designed for high-rate outdoor autotrophic growth of microalgae, five critical parameters must be addressed: light, pH, temperature, carbon dioxide supplementation, and nutrient requirements (Pulz, 2001). The majority of these factors are closely linked. For example, the fact that light is a critical system parameter introduces issues such as temperature control, material choice, and overall system geometry; factors of less concern when growing heterotrophically. Other parameters, such as continuous carbon dioxide fertilization, are unique to the cultivation of photosynthetic organisms, and can present challenges such as pH control. Furthermore, when working with volumes above 50 L, several laboratory techniques become unfeasible, including the most common forms of media pretreatment, mixing, and temperature control. It is inconceivable to imagine any single academic facility housing a specialized growth system for all potential production strains. For these reasons, we developed a single system capable of growing a wide range of microalgae in both a greenhouse and outdoor environment that is feasible to construct in an academic setting, and requires minimal resource inputs.

Materials and Methods

Maintenance of Stock Cultures

 Scenedesmus dimoprhus (*Sd*) (UTEX 1237), *Chlorella vulgaris* (*Cv*) (UTEX 259), *Arthrospira platensis* (*Ap*) (UTEX LB 2340), *Porphyridium purpureum* (*Pp*) (UTEX LB 2757), and *Dunaliella tertiolecta* (*Dt*) (UTEX LB 999), were obtained from the University of Texas at Austin Culture Collection. *Chlamydomonas reinhardtii* (*Cr*) (CC-1690) was obtained from the Chlamydomonas Resource Center, University of Minnesota, USA. *Nannochloropsis salina* (*Ns*) (NCMA 1776) was obtained from the National Center for Marine Algae and Microbiota, Maine, USA. *Anabaena* sp. PCC 7120 (*Asp*) was provided courtesy of the Golden lab, University of California, San Diego. *Phaeodactylum tricornutum* (*Pt*) was provided courtesy of the Hildebrand lab, Scripps Institute of Oceanography.

 Freshwater microalgae (Table 1) were maintained on 1.5% agar plates of Sueoka's High Salt Medium (HSM) (Sueoka, 1960), pH 7.0, containing 5.3 mM KH_2PO_4, 8.3 mM K_2HPO_4, 9.3 mM NH_4Cl, 0.08 mM $MgSO_4.7H_2O$, 0.07 mM $CaCl_2.2H_2O$, and trace nutrients (Kropat et al., 2011). When necessary plate stocks were passed through 1.5% agar plates of HSM containing 500 mg/L ampicillin, 100 mg/L cefotaxime, and 40 mg/L carbendazim (Kan & Pan, 2010). *Chlorella vulgaris* was found to be sensitive to carbendazim, and was maintained on HSM plates free of antibiotics and antifungals.

 Arthrospira platensis was maintained in 50 mL liquid cultures of modified Zarrouk's Medium (MZM) (Raoof et al., 2006) containing 160 mM $NaHCO_3$, 2.9 mM KH_2PO_4, 30 mM $NaNO_3$, 5.7 mM K_2SO_4, 0.8 mM $MgSO_4.7H_2O$, 17 mM NaCl,

0.25 mM $CaCl_2.2H_2O$, and trace nutrients (Kropat et al., 2011) with the addition of H_3BO_3 and $CoCl_2.6H_2O$ (Supplemental Information). All liquid cultures were kept in an atmosphere of 1% CO_2. *Arthrospira platensis* was also maintained on 1.5% agar plates of UTEX Spirulina Medium under ambient atmosphere.

Anabaena sp. PCC 7120 was maintained on 1.5% agar plates of modified HSM (MHSM1) media containing 25 mM $NaHCO_3$, 1.0 mM K_2HPO_4, 9.3 mM $NaNO_3$, 0.08 mM $MgSO_4.7H_2O$, 0.07 mM $CaCl_2.2H_2O$, and trace nutrients (Kropat et al., 2011) with the addition of H_3BO_3 and $CoCl_2.6H_2O$ (Supplemental Information). All stock plates were kept in an atmosphere of 1% CO_2.

Marine microalgae were maintained on 1.5% agar plates of artificial seawater (ASW), pH 8.0, containing 380 mM NaCl, 9.5 mM $CaCl_2.2H_2O$, 44.0 mM $MgCl_2.6H_2O$, 22.0 mM Na_2SO_4, 10.0 mM KCl, 0.2 mM $NaHPO_4$, 3.0 mM $NaNO_3$, trace elements (Kropat et al., 2011) with the addition of H_3BO_3 and $CoCl_2.6H_2O$ (Supplemental Information), and buffered with 15 mM tricine. When necessary, plate stocks were passed through ASW plates containing 250 mg/L ampicillin, 50 mg/L cefotaxime, and 20 mg/L carbendazim.

Plate stocks maintained under ambient conditions were kept at 24 ˚C under 6500 K fluorescent lights at an irradiance of 60 μmol $m^{-2} s^{-1}$. Strains maintained under CO_2 were kept at 27.0 ˚C under 6500 K fluorescent lights at an irradiance of 95 μmol $m^{-2} s^{-1}$.

Scale-up of Liquid Cultures

A two-step scale-up was used for the growth of all freshwater species (Figure 1), with the exception of *Ap* and *Asp*. 50 mL cultures in 125 mL Erlenmyer flasks were started from plates by scraping a portion of the plate and re-suspending in 1 mL of media before addition to the flask. Following growth, 50 mL cultures were passed directly into 1.5 L cultures in 2 L Erlenmeyer flasks. A modified tris-acetate-phosphate (TAP) (Supplemental Information) media was used for the scale-up of all freshwater species.

For marine species and cyanobacteria, a three-step scale-up was used for all growths. 50 mL cultures were started from plates followed by passage into 200 mL in 500 mL Erlenmeyer flasks, before being passed into 1.5 L cultures. *Pt* was found to be sensitive to the elevated temperature of the CO_2 box used during scale-up of strains. In the case of *Pt*, 50 mL cultures were grown on a rotary shaker at 140 rpm under ambient atmosphere, and used to inoculate 1.5 L cultures. 1.5 L cultures of *Pt* were bubbled with air at a flow rate of 2 liters/minute (lpm) and supplemented with 1% CO_2. All marine strains were scaled in ASW. Cyanobacteria were scaled in MHSM1.

All media was autoclaved prior to use. All liquid cultures with the exception of *Pt* were grown on a rotary shaker at 140 rpm in a CO_2 box (Supplemental Information) under an atmosphere of 1% CO_2, at a temperature of 27.0 ˚C and an irradiance of 80 $\mu mol\ m^{-2}\ s^{-1}$. During scale-up, cultures were grown to an optical density at 750 nm of 0.6-1.0 before being passed. A single 1.5 L culture was used to inoculate one 100 L hanging polybag in the strain's respective medium. In the greenhouse and outdoors marine strains were grown in supplemented natural seawater

(SNSW) using sand-filtered coastal seawater obtained from Scripps pier (Scripps Institute of Oceanography, California) supplemented with 0.2 mM $NaHPO_4$, 3.0 mM $NaNO_3$, trace elements (Kropat et al., 2011), and 25 mM $NaHCO_3$ to increase the natural buffering capacity. Cobalt, boron, and vitamins were not added for any large-scale growths.

Growth in 100 L Hanging Polybags

All growths in the greenhouse were conducted in 100 L hanging polybags (Figure 2). The bags were suspended in a v-shape, creating two connected bubble columns (Laing & Britain, 1991; Martínez-Jerónimo & Espinosa-Chávez, 1994; Trotta, 1981). Polybags were constructed using 10-mil high-density polyethylene (HDPE) purchased from Landsberg Company (item #1084412), San Diego, CA. Each polybag was suspended by four 4-inch folds at both ends, secured with duct tape, and hung from an 18-inch length of 1/2-inch metal conduit (Supplemental Information). Air and CO_2 were supplied to the system via separate PVC lines, converging via HDPE tubing before entering the polybag. Air was supplied via a regenerative blower (Republic HRB201). Polybag air-lines were constructed out of HDPE tubing. Air was supplied and maintained to each culture at a flow rate of approximately 10 lpm by manually adjusting a PVC ball valve such that both columns had gentle mixing. Minor variations in air flow-rate did not manifest in observable growth differences for any cultures. CO_2 was supplied via 50 lb. compressed cylinders fitted with regulators at an output pressure of 10 psi, and delivered through the bag air-lines. CO_2 output

pressure was adjusted if needed based on daily pH readings. Cultures were dosed with CO_2 every half hour for five minutes using solenoids controlled by a timer.

The pH of cultures grown in HSM was maintained between 6.0-7.0 for the entirety of the growths by manually adjusting CO_2 flow after daily pH readings. The pH of cultures grown in MHSM1 was maintained between 7.2-7.8. Cultures of *Ap* grown in MZM were maintained at a pH between 9.0-9.6. Deionized water was used for all freshwater growths to avoid problems with residual chlorine in municipal water. It was found that filtering municipal water through activated charcoal filters (Purewaterproducts, Denton, TX) dechlorinated the water sufficiently, but excess hardness often resulted in precipitates in the final medium. The pH of cultures grown in ASW was maintained between 7.2-7.8.

Growths for all polybag cultures were conducted over three culture generations. Culture media for first generation growths was sterile filtered in-line using a 0.2 μM filter cartridge (GE Memtrex MNY921EGS) and a peristaltic pump (Cole Parmer, EW-77410-10 drive with EW-77601-10 pump head) in order to minimize variability in initial conditions between cultures. Filter and tubing were sterilized by cycling 95% ethanol for five minutes, and flushed with water before use. Following each generation of growth, 80 L of culture were removed and the polybags refilled with the strains respective media. At the start of the second and third generation of growth, media was prepared and added to the culture untreated, after removal of 80 L of culture, in order to observe strain robustness and overall longevity in the presence of potential contamination. Samples were withdrawn through the vent

holes in the top of the polybags using a 60 mL syringe fitted with a 3-foot length of 5/16-inch vinyl tubing.

Growth in 800 L Air-lifted Stock-Ponds

Following growth in the polybags, all strains except *Asp* and *Pt* were tested outdoors in open 800 L air-lifted stock-ponds (Figure 3). Air was supplied via a single Republic HRB201 regenerative blower. CO_2 was supplied from 50 lb. compressed cylinders fitted with regulators at an output pressure of 10 psi, and delivered through a separate CO_2 loop constructed from 1/4-inch porous irrigation soaker hose. CO_2 output pressure was adjusted if needed based on daily pH readings. Cultures were dosed with CO_2 every half hour for five minutes using solenoids controlled by a timer. Stock-ponds were purchased from US plastics (Item #9437). Lifters were constructed out of 1.5-inch PVC, so that the total length was 18 inches, corresponding to the depth of the ponds when filled to 800 L. Air was delivered to the base of the lifters through 5/16-inch clear vinyl tubing, and carried from the blower via PVC and HDPE irrigation tubing.

All medias were identical to those used in polybag growths. A single generation of culture was grown in the stock ponds for each of the strains tested in order to observe strain health in an open outdoor environment and compare productivities between strains. Samples were withdrawn by dipping a 50 mL conical vial six inches below the surface of the pond. Ponds were manually stirred prior to collection to ensure representative sampling. Minor settling was observed during some growths.

In addition to single generation growths in the stock-ponds, *Scenedesmus dimorphus* and *Porphyridium purpureum* were selected for continuous production testing based on their high productivities and overall robustness in the polybags. All continuous cultivation tests were carried out in the same fashion as single generation growths. Harvesting and nutrient dosing took place at regular intervals to prevent light and nutrient limitation, respectively.

Growth in 8,000 L Air-lift Driven Raceway

Following scale-up in the stock-ponds Scenedesmus dimorphus was grown in a single 8,000 L air-lift driven raceway constructed out of standard cinder blocks mortared, filled with cement, and placed on a base of compacted decomposed granite. Two raceways were constructed and lined using black EPDM liner, each circulated with two airlift arrays constructed out of 2-inch PVC (Supplemental Information), driven by two combined Republic HRB 201-1 regenerative blowers, and operated at a depth of 11.5 inches. The arrays were placed six feet from the end of each turn. CO_2 was delivered to the raceway in front of each lifter array using two loops identical to those used in the stock-ponds. CO_2 was supplied from 50 lb. cylinders fitted with regulators at an output pressure of 10 psi and controlled using a Milwaukee MC122 pH controller connected to a solenoid. Each straightaway measured 4 x 30 feet, with each turn measuring four feet in radius.

Sd was grown in MHSM1. Three stock-ponds of *Sd* in MHSM1 were grown to an optical density at 750 nm of 0.75 and a total of 2000 L used for inoculation of the raceway. Samples were withdrawn by dipping a 50 mL conical vial four inches

below the surface of the pond at the middle of the straightaway and just after the turn.

Measurements were averaged from these two points.

Culture Monitoring

For polybag, stock-pond, and raceway growths, culture samples were

collected daily between 8 am and 12 pm. Optical density at 750 nm was measured

daily using a Bausch and Lomb Spectronic 20 spectrometer. 13 x 100 mm round test

tubes were used as cuvettes. Culture pH was measured daily using a Milwaukee

SMS120 pH meter. Temperature was read daily using a generic infrared thermometer.

Cell counts were taken every other day using a Hausser Scientific brightfield

hemacytometer (catalog #3110). Visual observations were also made during cell

counts. No crop protective actions were taken during any of the growths. Dry weights

were obtained during mid or late exponential phase using fritted glass filter

assemblies (ChemGlass 1424 assembly) and 4.7 cm Whatman GF-B or GF-F glass

fiber filter discs, depending on strain size. Filters were prewashed five times using

10-15 mL of DI water and dried overnight in a 105°C oven. Filters were allowed to

cool in a desiccator before weighing. Weights were taken on an analytical balance

readable to 0.1 mg. Optical density-dry weight correlation curves (Supplemental

Information) were created for each strain by measuring a series of dilutions created

from mid or late exponential phase cultures.

Culture Harvesting and Storage

All cultures were harvested using a Lavin 12-413V continuous flow

centrifuge. Cultures were pumped into the centrifuge using a Cole-Parmer peristaltic pump (EW-77410-10 drive with EW-77601-10 pump head). Concentrated biomass was then stored frozen in plastic bags at -20˚C. For growths of non-motile strains (*Sd*, *Cv*, *Ap*, *Pp*, *Ns*, and *Pt*) in the stock-ponds, air was turned off and cultures were left overnight to settle before centrifugation. During continuous production testing of *Sd* and *Pp*, effluent from the centrifuge was returned directly to the pond without treatment. For each harvest of *Sd* grown in the raceway, 4,000 L was pumped into five empty stock-ponds, and allowed to settle overnight before centrifuging. In these cases, residual media was discarded before harvesting the concentrated biomass. The volume removed was then replaced with fresh media.

Results

100 L Polybag Performance

   With the current protocols used for bag making (Supplemental Information), bag failure and significant leaking were not observed. Small leaks would occasionally develop in the crease at the base of polybags, but this was in all cases repairable by patching with duct tape. Air and CO_2 delivery remained constant throughout all growths following minor adjustments after hanging or removing polybags. Equal mixing in each of the two columns was also maintained. The system was flexible with regard to the number of bags being grown, and no problems were observed with 3-48 polybags grown simultaneously. Over the course of each growth in the polybags, no more than 2 L was lost to evaporation. Bag integrity was maintained over three generations for all strains, and previous growths (not shown) have demonstrated bag longevity as long as eight weeks before cultures were removed for other reasons. Capital and time costs for the systems are listed in Table 2.

800 L Air-lifted Stock-Pond Performance

   Simple design of the stock-ponds allows for rapid exchange of degraded or broken parts, however the materials used have proved durable over two years. In all growths, air-lifts provided adequate mixing and only slight settling was observed in the center of the pond. Based on daily pH readings, CO_2 delivery was also adequate, and per liter of culture more efficient than in the polybags. This was most likely due to increased dissolution of the CO_2 into the media as a result of the small bubble size generated using the soaker hose loops.

8,000 L Air-lift Driven Raceway Performance

Growth in the 8,000 L air-lift driven raceway was similar to that in the stock-ponds. Little maintenance was needed, and significant fouling was not observed. Initial leaks in the seams were identified using a 1% fluorescein solution by dropping a small volume of solution at each corner and crease and watching for suction. Leaks were patched with silicone.

Localized settling before each raceway turn was observed during growth, but overall mixing was adequate for sustained growth. Growth in the raceway provided the most representative mimic of current industrial practices for large-scale cultivation of algae. The relatively few reported raceway growths of any strain of microalgae or cyanobacteria highlight the need for more established systems, and are a testament to the issues of scale-up, culture transfer, crop protection, and consistency often observed during outdoor growth.

Strain Performance

Over the course of the growths in the polybags and stock-ponds, significant differences in strain robustness and consistency were observed. Biomass accumulation and productivity varied widely between strains (Figure 4). Minimal media and system optimization was carried out with regard to a single strain, although when single strains were cultivated in multiple medias, differences in strain longevity were observed (Supplemental Information). The goal of these experiments was to demonstrate the use of a single optimized system with generic medias to effectively

culture a suite of production and laboratory model organisms in order to produce biomass consistently that could be used for studies on the use of microalgae as a source of bio-products.

Scenedesmus dimorphus was the most productive and robust of the freshwater strains cultivated, reaching the highest average biomass concentration at 0.82 g/L and the highest average productivity at 0.09 g/L/day of all strains during outdoor growth in the stock-ponds. This productivity translates to a total harvestable biomass level of 72 g/day for each stock-pond during peak growth. During growth in the raceway, productivity averaged 0.06 g/L/day yielding 480 g/day of total harvestable biomass. *Sd* was tolerant of the abiotic stresses of light, temperature, and pH variation as well as typical observed biotic stresses such as grazers, fungal infection, and bacterial competition (Table 3) when compared to other strains cultivated. *Sd* also demonstrated rapid settling once mixing was stopped, shortening total harvest time significantly. No culture crashes have been observed during routine culturing with *Sd*. Lastly, Sd was the only strain able to maintain a similar level of productivity in both the polybag and stock-pond systems.

Porphyridium purpureum was the most productive and robust of the marine strains cultivated. *Pp* reached its maximum average productivity at 0.06 g/L/day during outdoor growth, corresponding to a total harvestable biomass level of 50 g/day for each stock-pond. *Porphyridium* showed tolerance to typical abiotic and biotic stresses. *Pp* showed the most rapid settling of the nine strains grown after mixing was stopped. Above average settling was observed early in stock-pond growths.

In addition to differences in productivities, notable differences in the affects of biotic and abiotic stressors were observed between strains (Table 3). *Chlorella vulgaris* showed a marked sensitivity to rotifers, while *Chlamydomonas reinhardtii* and *Dunaliella tertiolecta* were susceptible to contamination by ciliates. In the case of *Dt*, rapid culture crashing was observed in the polybags once ciliates were present. *Phaeodactylum tricornutum* and *Anabaena* sp. PCC 7120 were inconsistent during large-scale growth, and several cultures crashed likely due to light-stress, with no visible biological contamination present. Aside from these two cases, abiotic factors did not appear to play a major role in culture stress. No noticeable affects such as photo-bleaching, temperature shock, or pH sensitivity were observed as a result of light variation, environmental temperature variation and transfer of cultures, or temporary pH spikes resulting from lack of CO_2 for short periods of time.

Continuous and Semi-continuous Production

Continous production was tested using both *Scenedesmus dimorphus* and *Porphyridium purpureum*. In both tests triplicate stock-ponds were successfully cultivated for approximately 20 days (supplemental information), after which culture health deteriorated likely as a result of nutrient depletion. Healthy cultures of each strain were maintained over the course of 4 harvests for *Sd*, and 3 harvests for *Pp*. *Sd* produced a net of 2.0 kilograms of dry biomass over the course of the 23 day growth based on dry weight measurements of the cultures before and after each harvest. *Pp* produced a net of 0.4 kilograms of dry biomass over 23 days. After the initial media preparation, a single nutrient addition was performed but was not based on

measurement of nutrient levels (Supplemental Information). Clumping was observed during the growths of *Pp*, but did not appear to negatively affect the culture, and stayed homogenous throughout the growth. Foam formation was observed on the surface of the stock-pond, and likely contributed to light limitation during continuous cultivation of *Pp*. During this study, the system performed well, and no problems such as bio-fouling or component degradation were observed that might prevent continuous cultivation for much longer periods of time.

Growth of *Sd* in the raceway was preformed in a semi-continuous fashion, with harvested culture being replaced with fresh media, as opposed to effluent from the harvest process. Over the 27 days of growth, *Sd* in the raceway yielded approximately 8.0 kilograms of dry biomass according to dry weight measurements before and after harvesting. No problems were observed that would prevent growth for longer periods.

Discussion

Strain Performance and Relevance to Industrial Production

Scenedesmus dimorphus showed the highest average accumulation (0.82 g/L) and productivity (0.09 g/L/day) of all strains tested in the stock-ponds (Figure 4), demonstrating the strain's overall vigor and potential as an industrial crop. *Sd* also showed consistent growth when cultivated continuously in the stock-ponds for 23 days and semi-continuously for 27 days (Supplemental Information), generating over 8 kg of dry biomass during growth in the raceway. Demonstration of continuous cultivation is a highly relevant test for large-scale outdoor cultivation experiments, and one of the most important factors in industrial agriculture. The full spectrum of outdoor conditions and variables is exceedingly difficult to replicate in a laboratory or other indoor system, and large discrepancies can exist between the two settings. Despite production of a range of products including bioethanol (Ho et al., 2013; Miranda et al., 2012), biogas (Yen & Brune, 2007), and biofuel (El-Sheekh et al., 2013) from *Scenedesmus* spp., very few reports of large-scale outdoor or greenhouse growth exist. Miranda (Miranda et al., 2012) reported growth of *Scenedesmus obliquus* in an open outdoor raceway agitated by a paddle wheel with a biomass concentration of 0.81 g/L, achieving similar productivities even under different environmental conditions, again highlighting the robustness of *Sd* during outdoor growth, even under differing environmental conditions.

Porphyridium purpureum proved to be a similarly productive and robust strain, with several additional characteristics making it a potentially viable industrial strain. Productivity of *Pp* was second only to *Sd* during growth in the stock-ponds,

with an average productivity of 0.06 g/L/day. Generation of a number of valuable

products including sulfated extracellular polysaccharides (Arad & Levy-Ontman,

2010), pigments (Kathiresan et al., 2007), and protein (Safi et al., 2013) has been

described in *Pp*. Bio-products like these, whether produced directly, or as co-products

are an important economic asset to the developing microalgae industry. However, for

the economic value of these products to be leveraged, a demonstration of consistent

large-scale cultivation is needed. We have demonstrated that *Pp* can be grown both in

a batch-wise and continuous manner outdoors at a scale larger than any performed

previously. During continuous production, growth lasted over 20 days and several

hundred grams of dry biomass were generated. Other potential limiting factors such

as harvesting efficiency will also play a role in establishment of commercial

microalgal crops. Both *Sd* and *Pp* showed rapid settling once mixing was stopped,

reducing centrifugation time by 75%. While a minor factor at the scale, and under the

conditions tested here, difficulty of harvest can add significant costs to production

and must be factored into a strain's overall potential.

In addition to *Sd* and *Pp*, *Arthrospira platensis* and *Nannochloropsis salina*

also demonstrated consistent growth in the system, reaching respective densities of

0.32 g/L and 0.35 g/L in the stock-ponds. *Arthrospira* as a genus has a long history of

large-scale production and globally is one of the most established algal crops for

protein and feed production (Ahsan et al., 2008; Vonshak & Richmond, 1988).

Likewise, *Nannochloropsis* is well established for its ability to accumulate oil under

various culture conditions, and is one of the most discussed genera for biofuel

production (Moazami et al., 2012). Daily biomass production in the stock-ponds was

similar to or higher than previously reported outdoor growths (Boussiba et al., 1987; Radmann et al., 2007; Vonshak & Guy, 1992), again demonstrating the flexibility of the system to produce biomass from a wide range of microalgae and cyanobacteria.

Not all strains were consistent producers. *Chlorella vulgaris* and *Dunaliella tertiolecta* both demonstrated acute sensitivity to grazers. In each case, repeated culture crashes were observed due to contamination by rotifers and ciliates respectively. The sensitivity of these strains, both of which are commonly used as autotrophic producers of carbohydrates (Brányiková et al., 2011), carotenoids, and lipids (Borowitzka & Borowitzka, 1990; Widjaja et al., 2009), illustrates the necessity for crop protective strategies for microalgae, as well as demonstrates an additional need for outdoor screening of potential industrial strains in order to discover strain vulnerabilities. Ciliate contamination of varying lethality has been reported previously for both strains (Moheimani, 2013; Moreno-Garrido & Canavate, 2001). In our study, neither strain was able to be cultured consistently for three generations in the polybags as a result of repetitive grazer contamination following addition of unsterilized media. Previous reports of long term cultivation of *Cv* with mention of ciliate contamination do exist, and productivities were similar to those reported here (0.08 g/L/day). Ultimately, it is highly unlikely that any strain of microalgae or cyanobacteria is invulnerable to contamination when grown at the artificially high concentrations necessary for industrial production while simultaneously being exposed to the full gamut of pests present in the natural environment. Even strains as robust as *Sd* have shown susceptibility to predation (Letcher et al., 2013) during outdoor growth. As the number of species cultivated outdoors increases, parallel

gains in crop protection strategies such as media development, chemical control, or biological methods must be made. This cannot be realized without consistent examination outside the artificial laboratory environment.

Chlamydomonas reinhardtii, *Anabaena* sp. PCC 7120, and *Phaeodactylum ticornutum* are laboratory models representing three major classes of algae, and recombinant protein expression has been leveraged in all three strains (Chaurasia & Apte, 2011; Mayfield et al., 2007; Radakovits et al., 2011) for a variety of purposes including enhanced biofuel production. As is the case with naturally produced bio-products, the proposed benefits of recombinant protein expression in microalgae and cyanobacteria are often derived from the assumption that large-scale photosynthetic production will be possible. A system for large-scale growth of these model strains is therefore critical. The growths conducted here demonstrate the often-ignored reality that laboratory models and production strains differ significantly in their performance outdoors. While growth was achieved for all three strains in the polybags, successful pond cultivation was only realized with *Cr*, and productivities were low (0.02 g/L/day) compared to other strains. Additionally, ciliate contamination was observed in *Cr* cultures, again demonstrating the compounding difficulties of mass cultivation of laboratory model strains. As genetic tools continue to be developed, it will become increasingly important to have established large-scale methods for biomass production in order to test high-value, therapeutic, and industrially relevant proteins produced from photosynthetic microorganisms.

System Performance

The polybag, stock-pond, and ADR systems were designed with the goal of consistent large-scale biomass production. Several aspects of outdoor cultivation systems, such as culture turnover, consistency, and safety were considered and refined during preliminary testing of the system.

System sterilization or turnover is often desired when growing at scale. At massive scales of production this often becomes economically unfeasible, but for a large majority of systems it is crucial to prevent issues such as increasing biofilm formation, build-up of contaminant levels, and cross-culture contamination. As crop protection strategies continue to be developed, growing in a batch-wise fashion to avoid contamination issues remains an economical option if the biomass contains a valuable product. For example, *Cv* and *Dt* could both be cultivated successfully for a single generation, generating significant amounts (140 g and 90 g respectively) of dry biomass from three bags. Theoretically, biomass could be continuously produced if cultures were continuously restarted. However, to efficiently cultivate any strain in this manner requires an ability to clean the system, which is often difficult in fixed, complex photobioreactors (Lee, 2001). All three systems described here could be cleaned rapidly, and no cross-contamination was observed in the stock-ponds. In the case of the polybags, bags were removed and discarded upon completion of growth.

System repair and maintenance must also be considered in large outdoor systems exposed to the elements, especially in the often remote and harsh locations where large-scale cultivation is feasible. Our system contains no mechanical parts other than the blower, and is constructed entirely of readily available consumer

materials. The mixing systems described could easily be adapted to different containers, and used in a range of settings.

A final consideration addressed in the designs was safety. No moving parts were present in the system, minimizing the risk of accidents when working with the systems. Additionally, blowers and CO_2 tanks could be centralized away from the polybags and stock-ponds themselves and easily plumbed to the cultures.

Cost Analysis

The compiled capital and time costs are intended to serve as a relative baseline for comparison to other systems and discussions of improvement. Thorough life-cycle assessments and the boundaries set for such can vary widely making comparison of two separate systems difficult (Cherubini & Strømman, 2011). However, an estimated capital expense along with system productivities can allow basic comparisons to be made even between different systems by normalizing productivity to either monetary or time costs. The goal of these estimates is not to gauge the economic viability of the system, but to serve as a comparison to other systems in an academic setting. As new systems are designed, constructed, and reported, such basic comparisons will be crucial, but are currently not often reported, even in cases where the economics of the system are discussed (Rodolfi et al., 2009).

Conclusion

A reliable, and simple system and method for kilogram-scale cultivation of microalgae has been developed and tested with a wide variety of strains. Three genera of freshwater green algae, two genera of cyanobacteria, and four genera of marine microalgae have been successfully cultivated. Productivities were in almost every case comparable to those previously reported for autotrophic growth of individual strains at a similar scale. Monetary and time costs have been included to serve as a baseline for comparison to other systems.

It is hoped that the descriptions here will serve as a reliable platform for future production of microalgae biomass in a variety of settings, as well as for technological improvements in the field. Over the course of this study, automation of systems such as CO_2 control and data collection, as well as crop protection strategies were realized as areas with potential for technology improvement. Automation has the potential to improve system reliability and accuracy, and decrease labor costs. Crop protection is closely linked with continuous production and will likely play a large role in the development of microalgae as an industrial crop, and may potentially be the ultimate dictator of what strains can be grown. The systems described here can serve as a crucial bridge between the laboratory and fully realized industrial production as microalgae continue to be developed as a bio-product platform, and the struggle to find a suitable replacement for petroleum continues.
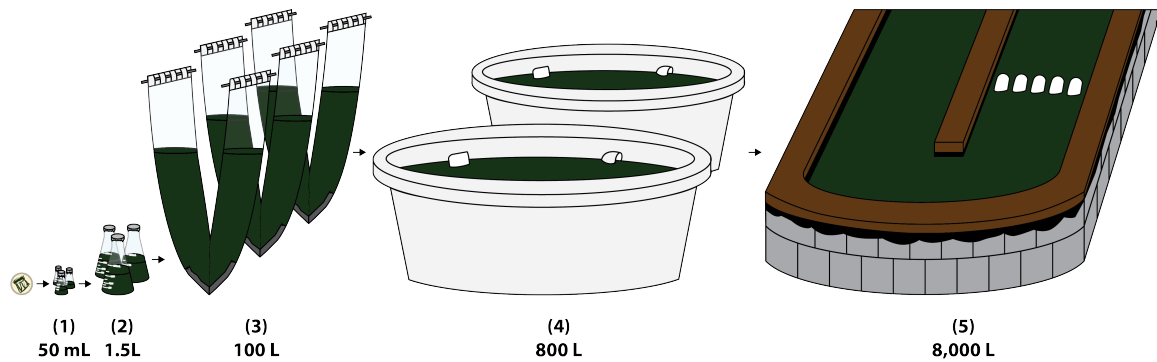
Figure 1
Scale-up steps used for the cultivation of the nine strains tested. Triplicate 50 mL liquid cultures (1) were started from plate stocks by scraping and resuspending a portion of the plate. 50 mL cultures were either used to inoculate 250 mL cultures (in the case of marine and cyanobacteria strains) or used directly to inoculate triplicate 1.5 L cultures (2) in the case of freshwater species. When growing marine and cyanobacteria strains, 250 mL cultures were used to inoculate 1.5 L cultures. 1.5 L cultures were used to inoculate 100 L hanging polybags (3). For seven of the strains tested, polybag cultures were then used to inoculate outdoor air-lifted stock ponds (4). In the case of *Scenedesmus dimorphus*, outdoor ponds were used to inoculate a single 8,000 L raceway (5).
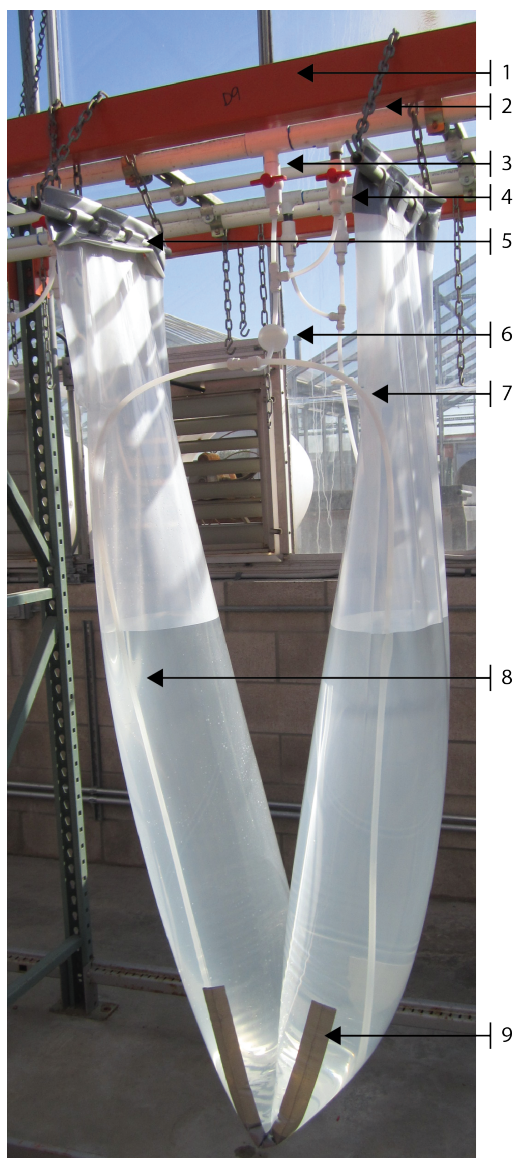
Figure 2
Picture of a 100 L hanging polybag (polybag) filled with water. Polybags were used for either greenhouse production or scale-up. All strains tested showed growth in the polybags. Readily available materials were used to construct the system. (1) pallet rack, (2) 3/16-inch support chain, (3) 1/2-inch PVC air valve, (4) 1/2-inch PVC CO_2 valve, (5) support folds secured with duct tape, (6) 0.2 uM air filter, (7) slit to allow air/CO_2 loop to enter bag, (8) air/CO_2 loop constructed from 3/8-inch HDPE tubing, (9) preventative tape at polybag crease.

Figure 3
Picture of 800 L airlifted stock pond (stock-pond) filled with water. The system contains no moving parts, and is easily cleaned and maintained. (1) 1.5-inch PVC lifter, (2)/(3) weight ring for lifters/CO_2 loop constructed out of 1/2-inch irrigation tubing filled with sand, (4) airstone connected to air line for additional agitation, (5) air line constructed from 1/2-inch irrigation tubing, (6) 1/4-inch CO_2 loop constructed of porous soaker hose, (7) 5/16-inch clear vinyl tubing used to deliver air to base of lifters.
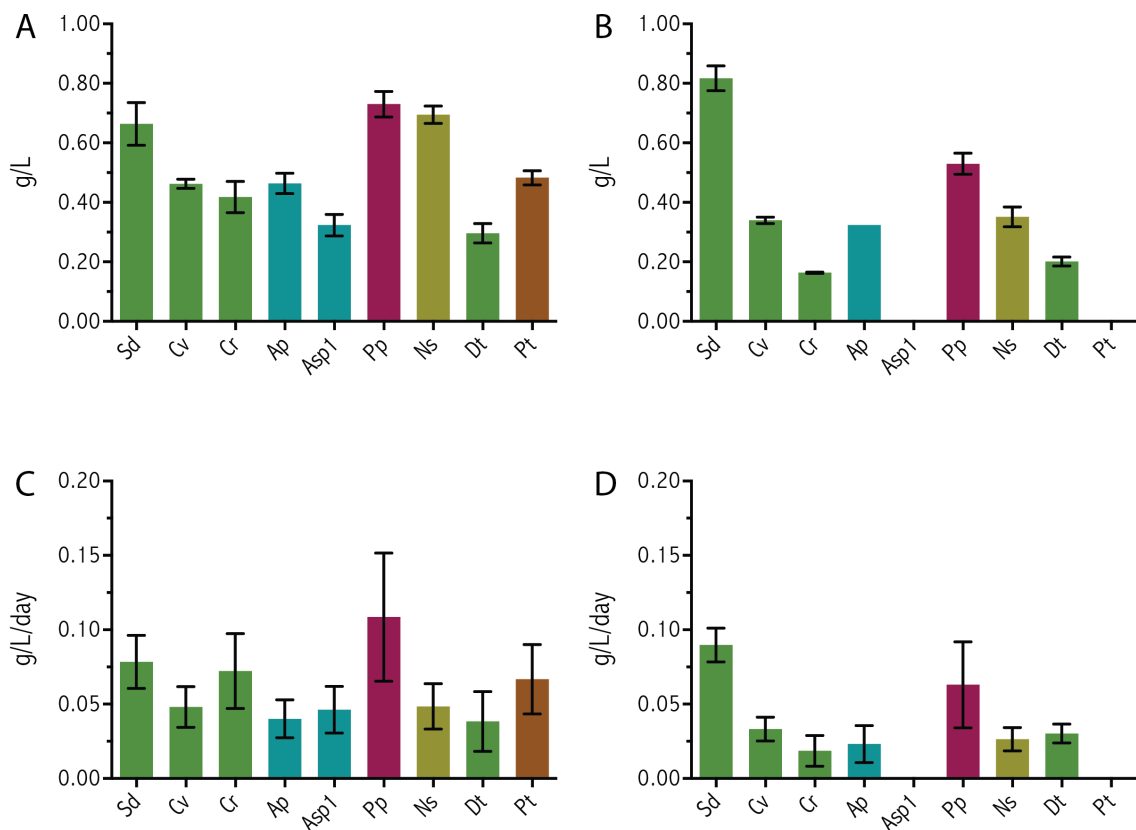
Figure 4
Maximum volumetric density and average productivity for strains grown in the polybags and stock-ponds. (A) Maximum volumetric density obtained during polybag growth. (B) Maximum volumetric density obtained during air-lifted stock pond growths. (C) Average volumetric productivity obtained in the polybags. (D) Average volumetric productivity obtained in airlifted stock pond growths. For volumetric densities, error bars represent error of the average maximum for cultures on the same day. For average daily productivities, error bars represent error of average daily productivities of cultures grown during the same period.

Table 1
Strains cultivated listed with media used at each stage, reason for testing, and bio-product potential. Nine strains across six phylums were successfully cultivated in the polybags (polybags). Of those, seven strains were successfully cultivated in outdoor airlifted stock ponds (stock-ponds). Additional medias that have been tested successfully with each strain are listed. Media recipes are available in the supplemental information.

Environment	Taxa	Genus/species	Abbr.	Outdoor Growth Media	Reason for Testing	Primary Bio-product Potential
Freshwater	Chlorophytes	*Scenedesmus dimorphus (UTEX 1237)*	*Sd*	HSM, MHSM1	Commercial produciton potential	Lipid
		Chlorella vulgaris (UTEX 259)	*Cv*	HSM	Commercial produciton model	Lipid
		Chlamydomonas reinhardtii (CC-1690)	*Cr*	HSM, MHSM1	Genetic model	High-value recombinant proteins
	Cyanobacteria	*Arthrospira platensis (UTEX LB2340)*	*Ap*	MZM	Commercial produciton model	Feed protein
		Anabaena sp. PCC 7120	*Asp1*	MHSM1	Genetic model, nitrogen fixer	High-value recombinant proteins
Marine	Rhodophyte	*Porphyridium purpureum (UTEX LB2757)*	*Pp*	SNSW	Commercial produciton potential	Protein, pigments, sulfated polysaccharides
	Eustigmatophyte	*Nannochloropsis salina (NCMA 1776)*	*Ns*	SNSW	Commercial produciton potential	Lipid
	Chlorophyte	*Dunaliella tertiolecta (UTEX LB 999)*	*Dt*	SNSW	Commercial produciton model	Lipid, pigments
	Diatom	*Phaeodactylum tricornutum*	*Pt*	SNSW	Genetic model	High-value recombinant proteins

Table 2
Total capital and production costs for both systems, as well as approximate time costs for basic culturing activities. Production costs vary depending on strain and media used, as well as the volume of culture grown due to varying process efficiencies in each system. Process time requirements vary accordingly.

	Polybags (12 bag system)	Airlifted Stock Ponds (6 pond system)	Raceway
Capital ($/unit)	190.00	500.00	10000.00
Capital (total system cost, $)	2300.00	3000.00	10000.00
Production in HSM ($/kg biomass)	22.00 - 35.00	44.00 - 200.00	ND
Production in MHSM1 ($/kg biomass)	26.00 - 40.00	35.00	40.00
Production in MZM ($/kg biomass)	440.00	270.00	ND
Production in SNSW ($/kg biomass)	21.00 - 40.00	50.00 - 95.00	ND
Set-up/Inoculation (hrs./person/unit)	1.25	0.75	3.00
Harvest/Take Down (hrs./person/unit)	0.75	3.50	8.00

Table 3

Summary of productivities and tolerance to stresses among strains tested, reported as both volumetric and areal measurements. Maximum densities represent the maximum average densities for a single day between replicate bags or ponds. Average productivities represent the average of the productivities between replicate bags or ponds during exponential growth.

| | Volumetric | | | | Areal | | | | | |
| | PBs | | ASPs | | PBs | | ASPs | | | |
Strain	Maximum Density (g/L)	Average Productivity (g/L/day)	Maximum Density (g/L)	Average Productivity (g/L/day)	Maximum Density (g/m^2)	Average Productivity (g/m^2/day)	Maximum Density (g/m^2)	Average Productivity (g/m^2/day)	Tolerance to Abiotic Stressors	Tolerance to Biotic Stressors
Sd	0.664	0.087	0.817	0.090	179.46	23.51	195.69	21.56	+	+
Cv	0.462	0.048	0.339	0.033	124.86	12.97	81.31	7.90	-	-
Cr	0.418	0.072	0.164	0.019	112.97	19.46	39.27	4.55	+	-
Ap	0.464	0.040	0.324	0.023	125.41	10.81	77.60	5.51	+	+
Asp1	0.324	0.044	ND	ND	87.60	11.89	ND	ND	-	-
Pp	0.730	0.109	0.530	0.063	197.30	29.46	126.95	15.09	+	+
Ns	0.694	0.048	0.351	0.026	187.57	12.97	84.07	6.23	+	+
Dt	0.296	0.038	0.202	0.030	80.00	10.27	48.38	7.19	+	-
Pt	0.483	0.067	ND	ND	130.54	18.11	ND	ND	-	-

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