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1 **TITLE:**
2 Cultivation of Green Microalgae in Bubble Column Photobioreactors and an Assay for Neutral
3 Lipids
4
5 **AUTHORS AND AFFILIATIONS:**
6 Qichen Wang, Haixin Peng, Brendan T. Higgins
7
8 Department of Biosystems Engineering, Auburn University, Auburn, AL, USA
9
10 **Corresponding Author:**
11 Brendan T. Higgins (bth0023@auburn.edu)
12
13 **Email Addresses of Co-authors:**
14 Qichen Wang (qsw0002@auburn.edu)
15 Haixin Peng (hzp0033@auburn.edu)
16
17 **KEYWORDS:**
18 Microalgae; photobioreactor; cultivation; lipid extraction; neutral lipids; biofuel
19
20 **SUMMARY:**
21 Here, we present a protocol to construct lab-scale bubble column photobioreactors and use them
22 to culture microalgae. It also provides a method for the determination of the culture growth rate
23 and neutral lipid content.
24
25 **ABSTRACT:**
26 There is significant interest in the study of microalgae for engineering applications such as the
27 production of biofuels, high value products, and for the treatment of wastes. As most new
28 research efforts begin at laboratory scale, there is a need for cost-effective methods for culturing
29 microalgae in a reproducible manner. Here, we communicate an effective approach to culture
30 microalgae in laboratory-scale photobioreactors, and to measure the growth and neutral lipid
31 content of that algae. Instructions are also included on how to set up the photobioreactor system.
32 Although the example organisms are species of *Chlorella* and *Auxenochlorella*, this system can be
33 adapted to cultivate a wide range of microalgae, including co-cultures of algae with non-algae
34 species. Stock cultures are first grown in bottles to produce inoculum for the photobioreactor
35 system. Algae inoculum is concentrated and transferred to photobioreactors for cultivation in
36 batch mode. Samples are collected daily for the optical density readings. At the end of the batch
37 culture, cells are harvested by centrifuge, washed, and freeze dried to obtain a final dry weight
38 concentration. The final dry weight concentration is used to create a correlation between the
39 optical density and the dry weight concentration. A modified Folch method is subsequently used
40 to extract total lipids from the freeze-dried biomass and the extract is assayed for its neutral lipid
41 content using a microplate assay. This assay has been published previously but protocol steps
42 were included here to highlight critical steps in the procedure where errors frequently occur. The
43 bioreactor system described here fills a niche between simple flask cultivation and fully-
44 controlled commercial bioreactors. Even with only 3-4 biological replicates per treatment, our

45 approach to culturing algae leads to tight standard deviations in the growth and lipid assays.
46
47 **INTRODUCTION:**
48 The application of microalgae in engineering and biotechnology has attracted great interest in
49 recent years. Microalgae are being studied for use in wastewater treatment[1-4], biofuel
50 production[5-8], and the production of nutraceuticals and other high value products[9,10]. Algae are
51 also being genetically modified at greater rates in an effort to improve their fitness for specific
52 engineering applications[11,12]. Consequently, there is great interest in experimentation with
53 industrially relevant organisms in controlled settings. The purpose of this method is to
54 communicate an effective approach to culture microalgae in a controlled laboratory
55 environment, and to measure the growth and neutral lipid content of that algae. Improving
56 growth rates and neutral lipid content of microalgae have been identified as two key bottlenecks
57 toward commercialization of algal biofuels[13].
58
59 A wide range of approaches have been used to culture algae for experimental purposes. In
60 general, these approaches can be divided between large-scale outdoor cultivation and small-
61 scale indoor cultivation. Outdoor cultivation in photobioreactors and open ponds is appropriate
62 for experimentation aimed at scaling up processes that have already been proven at laboratory
63 scale (*e.g.,* to test scale-up of a new high-lipid strain of algae)[14]. However, indoor small-scale
64 cultivation is appropriate when developing new or improved algae strains or performing
65 experiments aimed at understanding biological mechanisms. In these latter cases, a high-degree
66 of experimental control is required to tease out subtle changes in biological behavior. To that
67 end, axenic cultures are often required in order to minimize the complex biotic factors associated
68 with other organisms (*e.g.* bacteria, other algae) that inevitably grow in large-scale outdoor
69 systems. Even when studying interactions among algae and other organisms, we have found that
70 use of highly-controlled experimental conditions is helpful when examining molecular exchange
71 among organisms[15-17].
72
73 Within the category of small-scale indoor algae cultivation, a range of approaches have been
74 used. Perhaps the most common approach is to grow algae in Erlenmeyer flasks on a shaker table
75 beneath a light bank[18,19]. Exchange of oxygen and CO_2 takes place by passive diffusion through a
76 foam plug in the top of the flask. Some researchers have improved this set-up through active
77 aeration of the flasks[20]. Another approach is to cultivate algae in bottles, mixed by stir bar and
78 active aeration. Despite their simplicity, we have found that the use of flasks and bottles often
79 leads to inconsistent results among biological replicates. Presumably this is due to position
80 effects – different positions receive different amounts of light, which also affect internal reactor
81 temperatures. Daily rotation of reactors to new positions can help but does not alleviate the
82 problem because certain stages of algae growth (*e.g.,* early exponential) are more sensitive to
83 positional effects than others (*e.g.,* log phase).
84
85 On the opposite side of the spectrum of technological sophistication are fully-controlled
86 commercial photobioreactors. These systems continuously monitor and adjust conditions in the
87 reactor to optimize algae growth. They have programmable lighting, real-time temperature
88 control, and pH control. Unfortunately, they are expensive and typically cost several thousand

89 dollars per reactor. Most scientific and engineering journals require biological replication of
90 results, necessitating the purchase of multiple bioreactors. We present here a bubble column
91 reactor system that bridges the divide between the simple (flask) and sophisticated (fully-
92 controlled bioreactor) approaches for lab-scale algae cultivation. Bubble columns use rising gas
93 bubbles to facilitate gas exchange and mix the reactor. This approach provides some degree of
94 control over the lighting and temperature but does so in a way that is cost-effective. Moreover,
95 we have found this system to yield highly consistent results among biological replicates, reducing
96 the required number of biological replicates needed in order to obtain statistically significant
97 results when compared to the flask or bottle approach. We have also used this system to
98 successfully cultivate mixtures of algae and bacteria[21]. In addition to algae cultivation, we outline
99 a procedure for measuring the neutral lipid content in the cultured algae. The latter method has
100 been published elsewhere[22], but we include the procedure here to provide step-by-step
101 instructions for how to employ it successfully.
102
103 **PROTOCOL:**
104
105 **1. Setup of Bubble Column Photobioreactors**
106
107 1.1) Construct a set of vented lids from the plastic lids that came with the 1 L glass bottles and
108 hybridization tubes (see **Figure 1** for schematic and photos). Construct lids for the humidifier,
109 mixing trap, each air lift photobioreactor, and each bottle reactor.
110
111 1.1.1) Drill ¼” holes in the lid: 2 holes are needed for bioreactor and humidifier lids; 3 holes are
112 needed for the mixing trap.
113
114 1.1.2) Slip a ¼” O-ring over the threads of a 1/8” panel mount Luer fitting, and slide this into the
115 ¼” hole drilled in the lid (**Figure 1A**).
116
117 1.1.3) Slip a second ¼” O-ring over the threads so that the lid is sandwiched between the two O-
118 rings. Slip a locknut onto the threads and tighten to fix the panel mount Luer in place.
119
120 1.1.4) Snap lock rings onto the exposed male Luer projecting from the lid. Repeat steps 1.1.2-
121 1.1.4 for each hole in the lid.
122
123 1.1.5) For lids that will be used on bubble column and bottle reactors, attach 1/8” female Luer to
124 barb fittings to 1.5” pieces of 1/8” ID PVC tubing. Attach these to each of the exposed male Luer
125 fittings on the lid.
126
127 1.1.6) Connect a check valve (pointing away from the lid) to the free end of one of the 1/8” pieces.
128
129 NOTE: This will serve as the exhaust port for the bioreactor.
130
131 1.1.7) Connect a male Luer to barb fitting to the second piece of 1/8” tubing projecting from the
132 lid. Click the rotating lock ring into place and fasten a 0.2 µm air filter to this.

133
134 NOTE: This will serve as the inlet port for the reactor.
135
136 [Place **Figure 1** here]
137
138 1.2) Assemble the air delivery system (see **Figure 2A and 2B** for a schematic and photo).
139
140 1.2.1) Attach 1/8” NPT thread to barb fittings to the inlets and outlets on the back of each
141 rotameter.
142
143 NOTE: 200-2500 cm^3/min rotameters are for air pressure modulation upstream of the humidifier,
144 100-1000 cm^3/min rotameters are for bottle reactors, 50-500 cm^3/min rotameters are for air lift
145 bioreactors, and 5-50 cm^3/min rotemeters are for CO_2 flow regulation. It is recommended to
146 mount rotameters to a fixed surface (*e.g.* plastic sheet) so they do not fall over during operation.
147
148 1.2.2) Shut off the compressed air source, then connect ¼” ID flexible PVC tubing to the
149 compressed air source with a hose clamp. Step down the hose diameter to 1/8” ID PVC flexible
150 tubing using a ¼” female to barb fitting and a 1/8” male to barb fitting.
151
152 1.2.3) Connect the free end of the 1/8” ID tubing to the inlet of a 200-2500 cm^3/min rotameter.
153
154 NOTE: The outlet of this rotameter will feed the humidifier bottle via 1/8” ID tubing.
155
156 1.2.4) Connect the 1/8” tubing to an inlet to a vented lid (use a female Luer to barb fitting to
157 make the connection). Then connect a second piece of 1/8” tubing to the inside of the panel
158 mount fitting.
159
160 NOTE: This piece will hang down into the humidifier and bubble air through the water.
161
162 1.2.5) Attach 1/8” female Luer to barb fittings to each end of a piece of 1/8” ID tubing and use
163 this piece to connect the outlet of the humidifier to the inlet of the mixing trap.
164
165 1.2.6) In the same manner as 1.2.5, connect the outlet of the CO_2 regulator to a second port on
166 the mixing trap.
167
168 1.2.7) Construct a manifold using 1/8” tubing and 1/8” multiport barb (see **Figure 2C**) to feed air
169 into the rotameter banks.
170
171 NOTE: These rotameters will be used to supply the bioreactors. Avoid constructing more than 6
172 rotameters in series. Instead, use parallel banks of rotameters to expand the system. Ensure that
173 the total flow demand for all reactors is less than 2500 cm^3/min (or else a larger rotameter will
174 be needed upstream of the humidifier).
175
176 [Place **Figure 2** here]

1.2.8) Connect the outlet (3rd port) of the mixing trap to the newly constructed rotameter banks using 1/8” tubing and a 1/8” female to barb Luer.

1.2.9) Connect sufficiently long 1/8” tubing to the outlets of each rotameter in the rotameter bank to supply air to the bioreactors. Label the ends of the tubing as well as the rotameters in the bank.

1.2.10) Apply water-proof silicone around all ports on the humidifier and mixing trap lids to ensure they are air tight.

1.3) Set up fish tanks, stir plates, and lights (**Figure 3**).

CAUTION: This system requires a large number of outlets and sufficient circuit capacity to support all components. Avoid stringing together multiple power strips and extension cords in a daisy-chain fashion because this is an electrical hazard. Use of GFI type outlets and power strips is highly encouraged due to the presence of water in the system.

1.3.1) Arrange the low-profile magnetic stirrers on a level surface that is strong enough to hold the weight of water-filled fish tanks.

1.3.2) Place small wooden or plastic blocks (that are slightly taller than the stir plates) around the perimeter of the stir plates to support the weight of the fish tanks.

CAUTION: Avoid placing the fish tanks directly on the stir plates as the weight will crush them.

1.3.3) Place the fish tanks over the stir plates and support blocks and fill the tanks with water.

1.3.4) Cut a piece of a stiff plastic sheet to fit on top of the fish tank as a lid. Cut holes in this cover to slide the hybridization tubes in and out. Also cut a hole for the fish tank heater.

1.3.5) Arrange the fluorescent light banks next to the fish tank to provide horizontal illumination of the bioreactors. Plug the light bank into a light timer to set a day/night cycle.

[Place **Figure 3** here]

2. Preparation of Microalgae Inoculum

2.1) Obtain microalgae inoculum from a cryo-preserved, plated, or liquid culture.

NOTE: It is recommended that cryopreserved organisms be plated prior to the use as inoculum to ensure that cells are viable and that the resulting culture is axenic. Agar medium (*e.g.*, ATCC #5 sporulating agar)[21] is a rich medium that works well for reviving species of *Chlorella* and *Auxenochlorella* from cryo-storage.

221
222 2.2) Prepare 2.4 L of mineral medium that is appropriate for the particular microalgae species.
223
224 NOTE: Examples include N8 medium[23] for species of *Chlorella*, N8-NH_4 medium[21] for species of
225 *Auxenochlorella*. Use of a medium appropriate for the algae strain is one of the most important
226 steps toward ensuring robust algae growth.
227
228 2.3) Aliquot 2.4 L of mineral medium equally into three 1 L glass bottles, add stir bars to each
229 bottle and assemble the vented lids (**Figure 1**) for each bottle. Double check that the aeration
230 tube is on the inlet side and each bottle has a stir bar in it.
231
232 2.4) Autoclave the stock bottles using a liquid sterilization cycle (121 °C) for 30 min. Autoclave
233 100 mL of deionized water (dH_2O) and some 1.5 mL tubes at the same time, which will later be
234 used for plating. Allow the medium to cool overnight. Alternatively, cool the reactor to room
235 temperature and then aerate for 2 h prior to inoculation.
236
237 2.5) In a biosafety cabinet (BSC), inoculate microalgae from a plate or axenic liquid culture into
238 the stock bottles. Use sterile technique to maintain axenic cultures in the following steps.
239
240 2.5.1) Add 20 mL of autoclaved dH_2O to a sterile 50 mL centrifuge tube. Use a sterile 10 µL
241 disposable loop to pick several single colonies from the plate from step 2.1. Dip the loop into the
242 50 mL tube and wash the algae cells into the 20 mL of autoclaved dH_2O. Shake the 50 mL tube to
243 make a homogenous microalgae solution.
244
245 2.5.2) Pipette 6 mL of microalgae solution into each stock bottle with a 10 mL sterile serological
246 pipette. Swirl the bottle to mix the microalgae evenly into the medium.
247
248 2.6) Use a 2 mL sterile serological pipette to draw 1 mL samples from each stock bottle and
249 transfer into sterile 1.5 mL tubes.
250
251 NOTE: Micropipettes are not recommended for this step due to the risk of contamination.
252 Tighten the vented lids on stock bottles.
253
254 2.7) Place the stock bottles on stir plates (~150 rpm) and adjust air flow rate, CO_2, and lighting
255 levels as appropriate for the species. Rotate the stock bottle position every day.
256
257 2.8) Dilute the 1 mL samples obtained during step 2.6 (100-fold dilution in sterile water usually
258 works well) and spread plate onto a rich agar medium.
259
260 NOTE: These plates can be used to check for instances of contamination as well as serve as a
261 source of future algae inoculum for further experiments.
262
263 2.9) Take samples from the bottles (in the BSC) every two days to check the microalgae growth.
264 Place samples in a 96-well microplate in triplicate (200 µL) and measure optical density (OD) at

265 550 nm and 680 nm every two days until OD reaches 0.2-0.3 (which typically requires 5-7 days).
266
267 2.10) Stop the incubation and place the stock bottles on a bench for 24-48 h to allow the algae
268 cells to settle by gravity.
269
270 NOTE: The settled cells will be used next to inoculate the bubble column photobioreactors. If a
271 more rapid cell collection is desired, cells may be centrifuged at no more than 1,000 x g to collect
272 cells.
273
274 **3. Cultivation of Microalgae in Bubble Column Photobioreactors**
275
276 3.1) The day before bioreactor inoculation, prepare appropriate media and transfer 200 mL (or
277 desired volume) to the bubble column photobioreactor tubes (hybridization tubes). Autoclave
278 tubes with media and vented lids in place.
279
280 NOTE: If using wastewater as a growth medium, autoclave the empty bioreactors and add sterile-
281 filtered wastewater (if axenic culture is desired).
282
283 3.2) Concentrate the settled microalgae stock by removing the supernatant using a vacuum
284 pump. Leave less than 100 mL of medium in each bottle but avoid removing settled algae.
285
286 NOTE: Conduct this procedure inside a BSC and follow sterile technique. A simple vacuum
287 apparatus can be constructed using either a vacuum flask or bottle. Fit a sterile serological pipette
288 onto the end of the tubing.
289
290 3.3) Suspend and transfer the algae slurry to sterile 50 mL centrifuge tubes. Centrifuge at 1,000
291 x g for 5 min to further concentrate algae.
292
293 3.4) In the BSC, remove enough supernatant to achieve a total volume of ~80 mL of algae
294 concentrates for 12 photobioreacters. Avoid vacuuming out the pellet. Transfer the algae
295 concentrate to a sterile container (or the used algae stock bottle).
296
297 3.5) Add 6 mL of algae slurry into each photobioreactor with a sterile 10 mL serological pipette.
298
299 3.6) Sterile filter (0.2 µm syringe or vacuum filter) and add appropriate amounts of any other
300 compounds (*e.g.* vitamin stocks) which cannot be autoclaved.
301
302 3.7) Swirl bioreactors to mix algae into the medium.
303
304 3.8) Draw a 2 mL sample from each bioreactor using a serological pipette and transfer to a 2 mL
305 tube. Collect a 2 mL sample (in a BSC) every 24 h to monitor culture progress. Check the sample
306 for pH using test strips and adjust the reactor as needed with either 3 M NaOH or 3 M HCl.
307
308 3.9) Tighten the bioreactor lids and place all bioreactors into the fish tank water bath. Adjust the

309 aeration, CO_2, and lighting to the appropriate level for the species. Rotate the bioreactor position
310 each day after sampling (Step 3.8).
311
312 3.10). Apply 200 µL of each culture sample in triplicate to wells of a 96 well microplate. Measure
313 optical density (OD) at 550 nm and 680 nm.
314
315 3.10.1) On the last day of the culture period, measure OD under different dilution factors (*e.g.,* a
316 1x, 2x, 4x, 8x, 16x and 32x) to establish a correlation between the OD and the actual dry weight
317 after harvest (step 4).
318
319 3.11) Centrifuge the 2 mL sample tube at 12,000 x g for 5 min.
320
321 3.12) Filter the supernatant through 0.2 µm non-sterile syringe filter and store the supernatant
322 (and pellet if needed) at no higher than -20 °C for long term storage and later analyses of changes
323 in media composition.
324
325 **4. Harvest and Freeze Drying of Microalgal Biomass**
326
327 4.1) Measure a fixed volume of algae culture from each bioreactor with a graduated cylinder
328 (*e.g.,* 160 mL from a bioreactor that originally contained 200 mL of medium) and transfer into
329 centrifuge bottles. Rinse the graduated cylinder with dH_2O in between each measurement.
330
331 4.2) Centrifuge at 4,696 x g for 5 min. Discard the supernatant by carefully vacuuming it out.
332
333 4.3) Transfer the pellets to labeled 50 mL tubes. Rinse the centrifuge bottles with dH_2O, and
334 transfer contents to the 50 mL tubes. Ensure the total tube volume does not exceed 45 mL.
335
336 4.4) Wash the algae pellets with dH_2O to remove salts.
337
338 4.4.1) Centrifuge the 50 mL tubes at 4,696 x g for 5 min and discard the supernatant.
339
340 4.4.2) Add 40 mL dH_2O to each 50 mL tube; vortex to mix. Centrifuge again at 4,696 x g for 5 min
341 and discard supernatant.
342
343 4.4.3) Repeat step 4.4.2 again.
344
345 4.5) Label and weigh empty 15 mL centrifuge tubes on a 4-decimal balance (label both the lid and
346 tube and weigh them together). Weigh one tube per algae culture. Weigh each 15 mL tube twice
347 to minimize error.
348
349 4.6) After the last wash, discard the supernatant, and add 7.5 mL of dH_2O to each 50 mL tube.
350 Vortex and transfer the algae slurries into the pre-weighed 15 mL tubes. Rinse the 50 mL tubes
351 with additional dH_2O and transfer liquid to the 15 mL tubes. Avoid exceeding 12 mL of total
352 volume in the 15 mL tubes.

353
354 4.7) Centrifuge the 15 mL tubes at 4,696 x g for 5 min and decant the supernatant. Freeze the
355 tubes with pellets at -80 °C for at least 30 min in preparation for freeze-drying.
356
357 4.8) Freeze dry overnight or until dried.
358
359 4.9) Weigh and record the freeze dried 15 mL tubes with algae.
360
361 **5. Lipid Extraction using a Modified Folch method[24]**
362
363 5.1) Weigh out 20 mg of freeze-dried algal biomass into a 2 mL screw cap polypropylene tube
364 (check manufacturer label to ensure product is suitable for bead extractions).
365
366 5.2) Add 1.5 mL of Folch solvent (2:1 chloroform/methanol) to each 2 mL tube (which contains
367 20 mg of freeze-dried algae). Pour ~0.5 mL zirconia/silica beads (0.5 mm) into each tube until
368 liquid level in tube reaches 2 mL.
369
370 CAUTION: Handle chloroform and methanol in a fume hood and avoid breathing fumes or skin
371 contact.
372
373 5.3) Homogenize the algae samples in a bead mill for 20 s at a speed of 6.5 m/s. Transfer tubes
374 to ice for 30 s to chill samples. Repeat five more times to fully extract lipids.
375
376 5.4) Filter the homogenate through a 5 mL syringe containing a stainless-steel wire mesh disk
377 (#60 mesh) to strain out the beads, collecting filtrate in a 15 mL tube.
378
379 5.5) Wash the beads with 1.5 mL of Folch solvent, pushing liquid through with the syringe as
380 necessary. Repeat this wash two more times and collect all filtrate in the 15 mL tube, yielding a
381 final volume of approximately 6 mL.
382
383 5.6) Add 1.2 mL of 0.9% (w/v) NaCl solution to the Folch extract in the 15 mL tube and vortex to
384 mix well.
385
386 NOTE: If necessary, more Folch solvent can be used to wash beads (use 0.2x the total wash
387 volume of 0.9% NaCl solution to induce phase separation).
388
389 5.7) Centrifuge the 15 mL tubes at 6,000 x g for 5 min. Record the bottom chloroform (green)
390 phase volume to the nearest 0.1 mL using lines on the side of the 15 mL tube. Transfer the bottom
391 phase to a glass vial (with the lid) using a glass Pasteur pipette.
392
393 5.8) Store the lipid at -20 °C or (-80 °C if there are plans to use this extract for fatty acid analysis).
394
395 **6. Neutral Lipid Assay using a Microplate Method (adapted from Higgins *et al.* 2014[22])**
396

6.1) Prepare stock solutions. Prepare 10 mL 1 mg/mL vegetable oil standard in chloroform and store at -20 °C.

NOTE: Any vegetable oil may be used in this assay because it is not sensitive to the types of fatty acids. Prepare 10 mL of 200 µg/mL Nile Red solution in dimethyl sulfoxide (DMSO) and store in the dark at room temperature.

6.2) Pre-heat dry microplate block to 55 °C in a fume hood. While this is heating, dilute the lipid extracts and vegetable oil standard 3-fold with methanol.

NOTE: This dilution can be altered based on the lipid content of the algae, but this level works well for most *Chlorella*.

6.3) For each diluted sample, add 80 µL to a 96 well polypropylene microplate in quadruplicate.

CAUTION: Use of polystyrene plasticware is not recommended for use with organic solvents.

6.4) For the solvent blank, apply 80 µL of 2:1 methanol/chloroform in quadruplicate. For standards, add 10, 30, 60, 90, and 120 µL of the diluted vegetable oil standard in quadruplicate.

6.5) Place the microplate in a dry block heater at 55 °C for 20-30 min until all solvent has evaporated. While the solvent evaporates, prepare the working Nile Red solution (need 200 µL of 1 µg/ml solution per plate well). As an example, twelve samples and a full set of standards requires 16 mL of 1.0 µg/mL solution; prepare by dissolving 80 µL of the 200 µg/mL stock (in DMSO) into 16 mL dH_2O.

6.6) Remove the microplate from the heating block and let cool to room temperature. Add 30 µL of isopropyl alcohol to each well and mix by pipetting up and down. Ensure all pipette channels are mixing the solution and resuspending the lipids, yielding a homogeneous green liquid.

6.7) Add 200 µL of Nile Red solution (1 µg/mL) to each well, pipette up/down 10 times to mix. Incubate the plate for 5 min at room temperature. While waiting, prepare a 50% bleach solution by mixing bleach (6% hypochlorite) with dH_2O. 20 µL per well are needed. Preparation of 3 mL of 50% bleach is sufficient for 12 samples and a full set of standards.

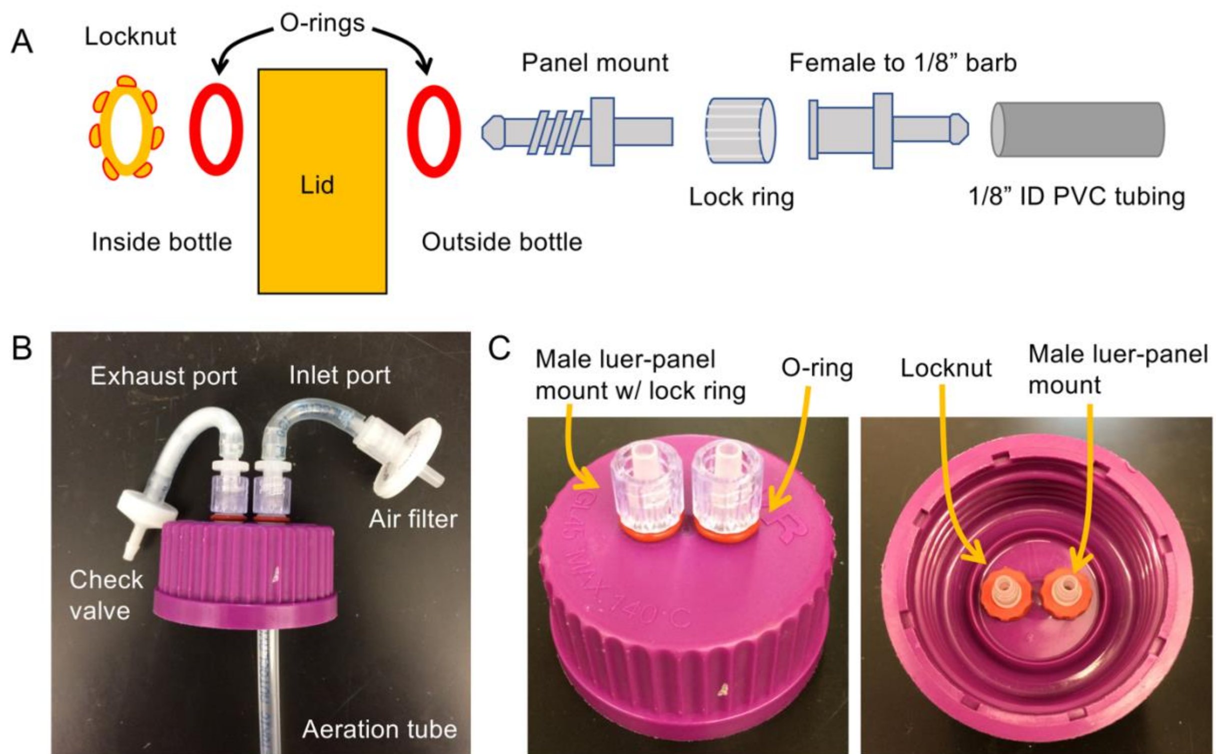
6.8) Add 20 µL of bleach solution to each microplate well and pipette up and down 5 times to mix well. Incubate 30 min at room temperature.

6.9) After 30 min, read fluorescence in the samples every 5-10 min at 530 nm excitation/575 nm emission with auto cutoff set to 570 nm until the signal from algae samples stabilizes. Typically, 60 min of total incubation is sufficient.

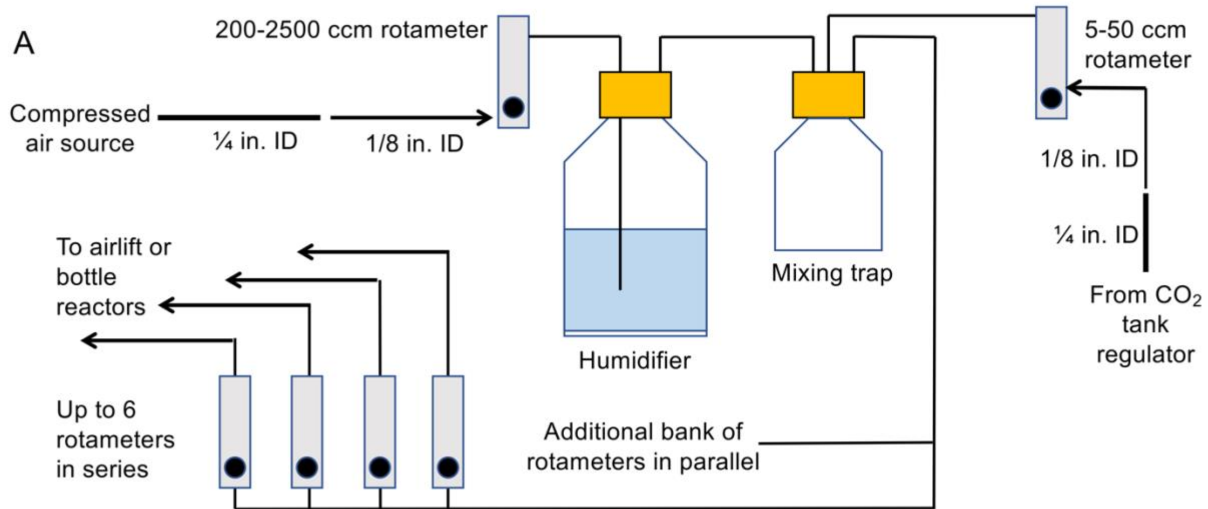
6.10) Create a calibration curve for the vegetable oil standards (in the range of 0-40 ng oil/well).

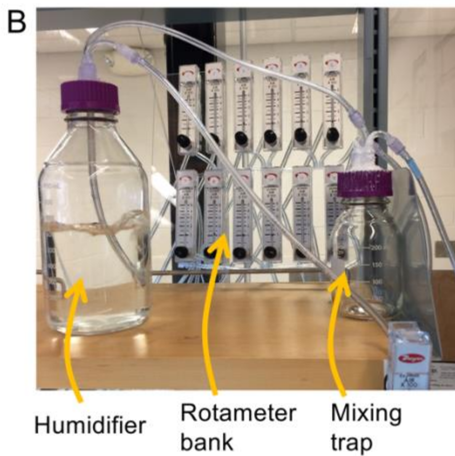
441 NOTE: A linear fit works well for low (<30 ng/well) oil concentrations and a polynomial fit may be
442 used if the standard exceeds 30 ng/well. Use this correlation to quantify the neutral lipid in the
443 sample wells.
444
445 **REPRESENTATIVE RESULTS:**
446 This procedure yields a time course of algal optical density data at OD 550 nm (**Figure 4A**). The
447 optical density and dry weight concentration data can be correlated (**Figure 4B**). This is
448 accomplished by first calculating the final dry weight algae concentration after the freeze-drying
449 step. Next, the optical density of the culture serial dilution (performed on the last day of
450 sampling) and the actual dry weight concentrations can be correlated. For low cell
451 concentrations, a linear correlation can be used whereas for higher cell concentrations, a second
452 order polynomial correlation may be used. It is recommended to create a separate correlation
453 for each culture condition. Finally, the correlation can be applied to the time course optical
454 density data to obtain a dry weight growth curve (**Figure 4C**). In this example experiment,
455 *Auxenochlorella protothecoides* (UTEX 2341[25]) was cultured under four conditions: axenic control
456 cultures grown on fresh N8-NH_4 medium[21], in co-culture with the bacteria *Azospirillum*
457 *brasilense*, in medium supplemented with 50 mg/L of indole-3-acetic acid, and on spent medium
458 from *A. brasilense*. *A. brasilense* is known to produce indole-3-acetic acid, a plant growth
459 promoting hormone, that also promotes growth in some microalgae. However, at 50 mg/L, the
460 IAA treatment completely inhibited *A. protothecoides* growth. Consequently, optical density data
461 was available, but the quantity of algae was insufficient to obtain an accurate dry weight
462 concentration. In this case, the correlation for the control culture may be applied or optical
463 density data may be reported directly. Because OD data was collected at both 550 nm and 680
464 nm absorbance, either dataset may be used for the correlation between OD and dry weight.
465 Typically, OD 550 is used because it almost completely excludes the absorbance of chlorophyll[26],
466 thus suppressing bias from changes in chlorophyll content. In contrast, OD 680 includes the
467 absorbance of chlorophyll and high ratios of OD 680/550 indicate high chlorophyll content in the
468 algae. **Figure 4D** shows a set of twelve algae cultures growing in the bubble column
469 photobioreactors. Even with only three biological replicates per treatment, tight standard
470 deviations were achieved, allowing for high sensitivity to differences among treatments.
471
472 [Place **Figure 4** here]
473
474 Neutral lipid data is shown for two example experiments in **Figure 5**. This assay has been shown
475 to correlate well with neutral lipid content, particularly triacyglycerol (TAG) content. This can be
476 seen by comparing the neutral lipid assay (**Figure 5A**) to a corresponding thin layer
477 chromatography plate (**Figure 5B**). The same trend can be seen in the second experiment (**Figure**
478 **5C and 5D**). In all of these experiments, canola oil was used as a standard and resulted in a linear
479 correlation (R^2 of 0.98 for the first experiment and 0.99 for the second) between fluorescence
480 and canola oil mass in the well. Note that if all samples have low lipid content, then the highest
481 point or two on the standard may be dropped. This correlation can be used to calculate the
482 quantity of neutral lipid (μg) in each of the algae sample wells. The oil mass can then be converted
483 to a concentration in the lipid extract applied to the microplate well. This value is multiplied by
484 the dilution factor used (*e.g.,* 3x) to obtain the neutral lipid content of the original Folch extracts.

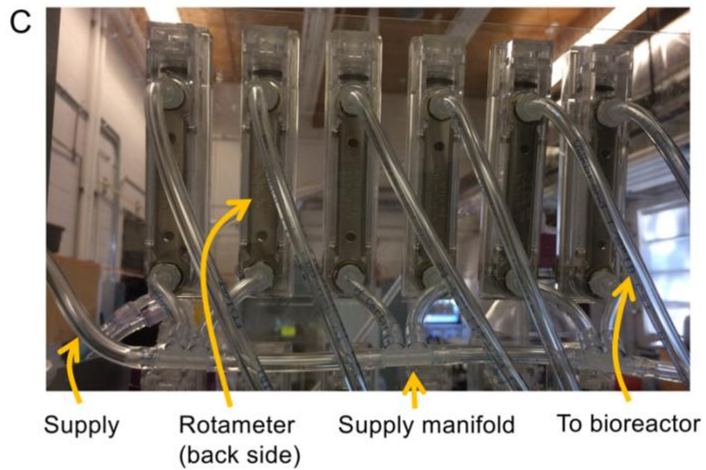
485 This concentration is then multiplied by the volume of the extract (should be close to 4 mL) and
486 then divided by the total mass of algae biomass used for lipid extraction (should be close to 20
487 mg). The result is the neutral lipid content of the microalgae.
488
489 [Place **Figure 5** here]
490
491 Also, it is good practice to calculate the coefficient of variation (standard deviation divided by the
492 mean) of the raw fluorescence readings across all technical replicates in the neutral lipid assay.
493 Assuming technical replicates were carried out in the microplate in quadruplicate as noted in the
494 procedure, the coefficient of variation typically should not exceed 10%. High coefficients of
495 variation are usually the result of poor mixing (particularly during the addition of isopropyl
496 alcohol) and potentially inaccurate use of the multichannel pipette.
497
498 **FIGURE AND TABLE LEGENDS:**
499



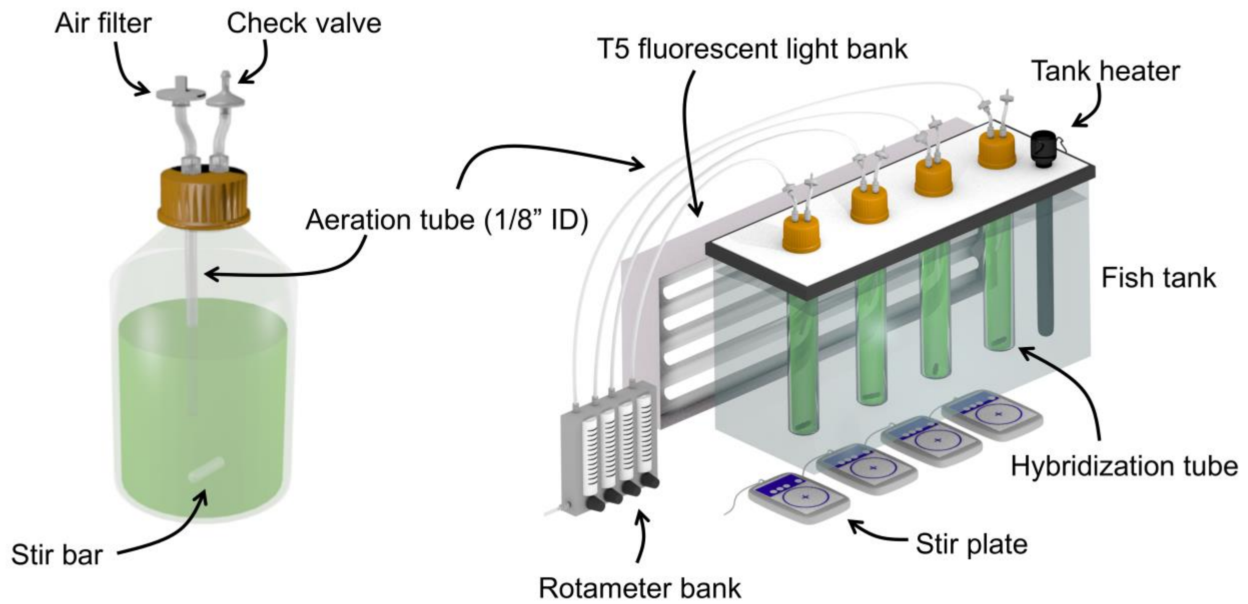
500
501 **Figure 1. Schematic and photos for constructing bioreactors.** (**A**) Schematic for construction of
502 the bioreactor lids (**B**) photo of the assembled bioreactor lid, and (**C**) photo of the assembled lid
503 used for the humidifier. Note that the humidifier fittings should be coated in water-proof silicone
504 to ensure an airtight seal with the lid.





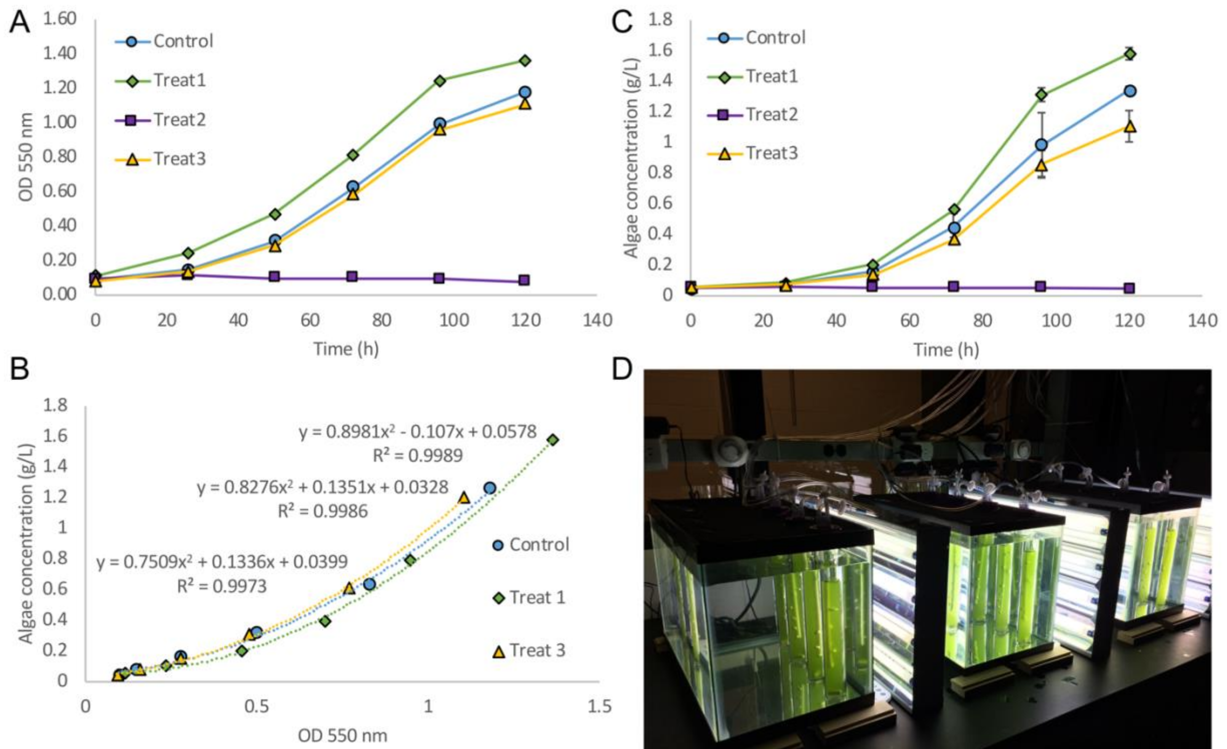


505
506 **Figure 2. Schematic and photos for assembling bubble column system.** (**A**) Schematic of the
507 aeration system (**B**) photo of the humidifier, mixing trap, and rotameter bank, and (**C**) photo of
508 the manifolds used to connect the rotameter banks together.



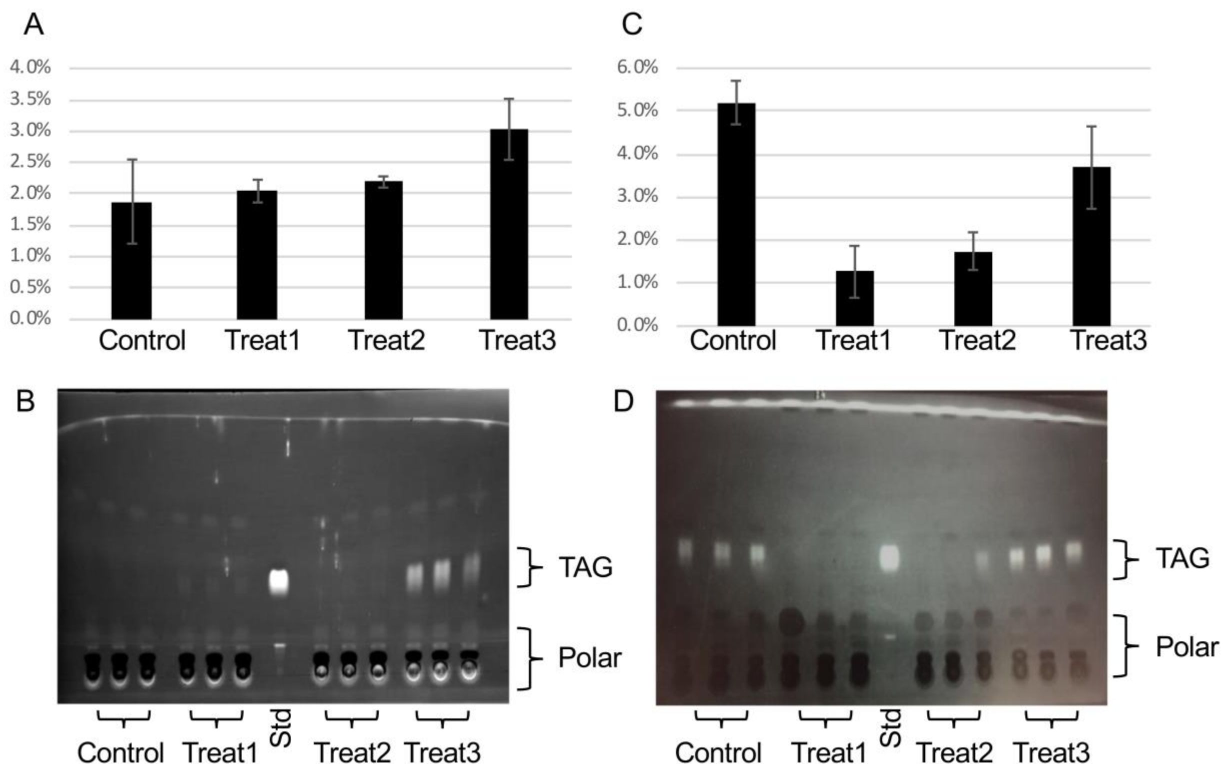
509

510 **Figure 3. System schematic for the bottle bioreactors (left) and the bubble column**
511 **photobioreactors (right)**. This figure has been modified from Higgins *et al.*[17].



512
513 **Figure 4. Algae growth results in bubble column photobioreactors.** (**A**) The optical density (550
514 nm) growth curve of *Auxenochlorella protothecoides* (UTEX 2341) shows cultures entering late
515 logarithmic growth at 120 hours. Control cultures were grown on fresh N8-NH_4 medium,
516 treatment 1 is co-cultures of *A. protothecoides* and *Azospirillum brasilense* grown on fresh N8-

517 NH_4 medium, treatment 2 is axenic *A. protothecoides* grown on N8-NH_4 medium supplemented
518 with 50 mg/L indole-3-acetic acid (IAA), and treatment 3 is axenic *A. protothecoides* grown on
519 spent medium from *A. brasilense*. The spent medium was prepared by culturing *A. brasilense* for
520 96 hours on N8-NH_4 medium supplemented with 2 g/L malic acid. Cells were removed, and the
521 medium was re-supplemented with ammonium to restore its initial level, pH was adjusted, and
522 the medium was sterile filtered (0.2 μm). Note that the 50 mg/L IAA treatment completely
523 inhibited algae growth. (**B**) Correlation curves between OD 550 nm and final dry weight
524 concentration using a second order polynomial fit. No correlation is shown for treatment 2
525 because no algae could be harvested at the end of the culture period. (**C**) Application of the
526 polynomial correlation to the optical density data yields a growth curve with dry weight
527 concentration on the y-axis. The control culture correlation was applied to the OD data for
528 treatment 2. Error bars are standard deviations based on 3 biological replicates. (**D**) Photo of the
529 bubble column photobioreactors shortly after culture inoculation.



530
531 **Figure 5. Neutral lipid data obtained from cultures of *Chlorella sorokiniana* (UTEX 2714).** (**A**)
532 Neutral lipid content (% dry weight) for algae in experiment 1 in which algae were grown for 120
533 hours. The control culture was axenic and cultured on fresh N8 medium[23], treatment 1 was a co-
534 culture of *C. sorokiniana* and *A. brasilense* on fresh N8 medium, treatment 2 was fresh N8
535 medium supplemented with 50 mg/L IAA, and treatment 3 was spent medium from *A. brasilense*.
536 The spent medium was prepared by culturing *A. brasilense* for 96 hours in N8 medium
537 supplemented with 2 g/L malic acid, removing cells, supplementing lost nitrate, adjusting pH, and
538 sterile filtering the medium (0.2 μm). Unlike *A. protothecoides*, 50 mg/L of IAA did not inhibit *C.*
539 *sorokiniana* growth. (**B**) TLC plate image for experiment 1 showing relative TAG abundance. (**C**)
540 Neutral lipid content (% dry weight) for algae in experiment 2 which had the same treatments as

541 experiment 1 but the cells were harvested after 72 hours of growth. (**D**) TLC plate image for
542 experiment 2 showing relative TAG abundance. Error bars are standard deviations based on 3
543 biological replicates.
544
545 **DISCUSSION:**
546 The most important consideration when culturing algae is an understanding of the specific needs
547 of the organism or group of organisms. The algae cultivation system described here can be used
548 to culture a wide range of algae but the specific abiotic factors (temperature, media, pH, light
549 intensity, CO_2 level, aeration rate) need to be adjusted to the needs of the organism. Note the
550 parameters described here were used for the cultivation of *Chlorella* and *Auxenochlorella*. These
551 organisms are of industrial interest because they are tolerant to high nutrient, light, and
552 temperature levels[27]. However, light levels can be reduced through removal of fluorescent bulbs
553 and the day/night cycle can be adjusted to represent seasonality. Likewise, the water heaters can
554 be turned up or down to control the water bath temperature on the system. While the system
555 described here does not employ such a feature, it is possible to chill the fish tank water baths
556 below room temperature through a low-cost cooling system. Place a bucket of water in a small
557 refrigerator and use a variable speed pump to pump water from the cold-water bucket to the
558 fish tank and back. The faster the pumping rate, the colder the fish tank reservoir will become.
559
560 Although the algae cultivation system generally provides consistent results, there are a few
561 important caveats that should be considered. The first is water siphoning that occurs in the event
562 that the aeration system experiences a sudden loss of pressure (*e.g.,* a blown fitting or an air
563 compressor failure). Pressure that is in the humidifiers will push humidifier water backward
564 through the tubing, including through the rotameter. Installing an upstream trap or backflow
565 preventer can help. Note that siphoning will not impact the bioreactors themselves because they
566 operate at atmospheric pressure. Routine inspection of the piping and fittings can help alleviate
567 system failures. Another important consideration is to routinely inspect and replace air filters and
568 check valves on the bioreactors. Be sure to follow the manufacturer's recommendation for the
569 number of autoclave cycles allowable. This is particularly important if maintenance of axenic
570 cultures is critical to the experiment. Finally, it is recommended to purchase or construct an
571 autoclavable rack for safe transport of the bioreactors between the water baths, autoclave, and
572 biosafety cabinet. A stainless-steel wire rack can serve this purpose.
573
574 The bioreactor system described here has several limitations. A key limitation is the need to
575 handle the reactors in a biosafety cabinet using sterile technique. The reactors lack an anti-
576 siphoning sampling port, requiring the user to move the reactor to a biosafety cabinet to sample
577 without contaminating the culture. The reactors also require manual pH reading and adjustment
578 which introduces potential for human error.
579
580 The bioreactor system described here fills a niche between simple flask cultivation and fully-
581 controlled bioreactors. The bioreactor system was developed in response to a need for more
582 consistent results than were achievable using bottles. The data shows that this system generates
583 consistent growth results when operated appropriately. Note that aerated bottles were
584 employed in the procedure for cultivation of algae stock, but this was done only to produce

585 inoculum for the experiment. This is acceptable because stock bottles were pooled for
586 inoculation and hence variability among reactors is not an issue.
587
588 As many algae researchers are interested in monitoring both growth and neutral lipid content,
589 we have included our approach to measuring both of these parameters here. Use of optical
590 density to measure growth is standard practice and is unparalleled in its simplicity. However,
591 correlations between dry weight and optical density change over time and depend on culture
592 conditions. It is recommended to produce a correlation equation for each experimental
593 treatment within every experimental batch. This is possible in the proposed procedure because
594 freeze dried algae will be obtained after every culture experiment. A critical assumption of the
595 optical density approach is that the correlation between optical density and dry weight holds
596 constant over the course of batch growth. As long as deviations from this assumption are small,
597 the result will be reasonably accurate. The relative accuracy can be assessed by comparing the
598 calculated algae dry weight concentration at time zero. Assuming cultures were well mixed, and
599 pipetting was accurate, all of the cultures should have the same initial inoculation density. The
600 optical density approach can also be challenged when background absorbance of the medium is
601 high (*i.e.,* when working with certain wastewaters). Subtraction of the medium absorbance (prior
602 to inoculation) from each OD reading can help with this issue.
603
604 Extraction of lipids from the dry algae follows the well-established Folch approach[21,24]; however,
605 there are important considerations. Different algae species have different cell walls with varying
606 degrees of toughness. The zirconia/silica beads used here are sharp and are designed to pierce
607 strong, polysaccharide cell walls. A softer bead type (*e.g.,* glass) or fewer bead disruption cycles
608 may be used on algae with weaker cell walls. However, a rule of thumb is that the resulting cell
609 pellet after extraction should be free of pigment, indicating that all chlorophyll was extracted.
610 One of the most common sources of failure during the lipid extraction step occurs due to
611 improper freeze drying. If the sample melts in the freeze dryer before it is fully dry, the result will
612 be a very hard, dark, waxy pellet. This is the result of cells that lysed under the vacuum conditions
613 of the freeze dryer. The pellet may be weighed to obtain a dry weight, but it cannot be used for
614 lipid extraction as the waxy particles do not break down in the Folch solvent. To ensure that
615 freeze drying always yields soft, powdery samples, it is essential to freeze all samples to -80 °C
616 and promptly transfer them to the freeze dryer. Moreover, using parafilm (with a hole poked in
617 it) rather than loosened tube lids will ensure that moisture can be continuously removed from
618 the sample before it thaws.
619
620 The neutral lipid assay described in this procedure has been published previously and includes a
621 discussion of alternative lipid assays[22]. However, some important improvements have been made
622 to that procedure since publication. Most notably, the Nile red solution concentration was
623 increased from 0.5 µg/mL to 1 µg/mL. The effect of this change was higher signal intensity,
624 improved repeatability, and an elimination of signal decline over time during the incubation
625 period. The results show that the assay compares well to results from qualitative thin layer
626 chromatography. This assay was developed and validated using various species of *Chlorella* and
627 *Auxenochlorella* so its applicability to species of significantly different composition has not been
628 determined. All green pigment should be completely removed from the assay during the bleach

629 incubation, leading to samples that are clear or very pale yellow. Also note that lipid extracts that
630 are degraded (as indicated by a change from green to brown color) typically fail to deliver
631 accurate results in this assay. It is thus imperative to store lipid samples at no higher than -20 °C
632 in the dark.
633
634 The methods presented here for culturing algae, measuring growth, and quantifying neutral lipids
635 are useful for a variety of engineering applications of algae, but are particularly suitable for
636 research on biofuel production. These methods are also being used to study algal growth
637 inhibition on wastewaters[28] as well as impacts of organism interaction on the growth and
638 composition of microalgae.
639

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645

646 **DISCLOSURES:**
647 The authors have nothing to disclose.
648

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