Algal Research 13 (2016) 126–133

Contents lists available at ScienceDirect

Algal Research

journal homepage: www.elsevier.com/locate/algal

Stabilizing continuous mixed cultures of microalgae

Lucie Novoveská [1], Dylan T. Franks, Tristan A. Wulfers, William J. Henley *

Department of Botany, Oklahoma State University, Stillwater, OK 74075, USA

ARTICLE INFO

Article history:
Received 23 April 2015
Received in revised form 4 November 2015
Accepted 25 November 2015
Available online xxxx

Keywords:
Turbidostat
Continuous culture
Mixed species culture
Competitive exclusion
Dunaliella
Aphanothece

ABSTRACT

Microalgae are a promising source of renewable biofuels because they are highly productive, can utilize low quality water, and do not compete with cropland. Recent studies have shown that monocultures are difficult to maintain, but mixed cultures of two or more strains, species or higher taxa may increase and stabilize productivity. Continuous cultures have the potential to support higher long-term average productivity and yields than batch cultures. However, methods are lacking to preclude competitive exclusion and maintain desired mixture composition in long term continuous cultures. First, we grew mixed batch cultures of *Dunaliella* sp. GSP-980625-1E and the diatom *Phaeodactylum tricornutum* (CCMP 1327) and found no difference in growth rates or lipid productivity compared to monocultures. Next, we determined the responses (growth rate and photosynthetic efficiency) of those species plus the cyanobacterium *Aphanothece* sp. CCMP 2529 to four salinity × three nutrient full factorial conditions. Salinity responses were confirmed in single species cycloturbidostat continuous cultures. Finally, we used that information to stabilize mixed continuous cultures of *Dunaliella* and *Aphanothece* by alternating salinity to successively favor one or the other species.

1. Introduction

Various biofuel feedstocks are being developed to meet societal targets for carbon neutral renewable energy. Among these, algae are widely regarded as a potentially transformative feedstock, pending solutions to many techno-economic and environmental challenges [1–5].

To date, the majority of nascent commercial facilities focus on mass cultivation of single microalgal species with high lipid yields or other desirable traits. Except for alkali- or halophiles such as *Arthrospira* and *Dunaliella* [6,7] or other extremophiles, monocultures will succumb to contamination with competing algae, grazers and pathogens [2,8], particularly in open pond systems that tend to be more economical than photobioreactors [9]. Contaminant monitoring, application of biocides and other pest-reduction efforts presuppose known and well-understood pests, are expensive and pose environmental risks [2,10]. Alternatively, polycultures of functionally complementary species potentially offer several advantages for algal mass culture, including greater resource use efficiency, stability under variable abiotic conditions, and resistance to contaminating algae, grazers and pathogens [11–14]. Polycultures also exhibit increased C:P (and presumably C:N) ratios consistent with accumulation of high energy biofuel precursor compounds such as lipids [15–17]. These topics were recently reviewed [18].

Although overyielding (productivity of multi-species mixtures exceeds that of monocultures) may be quite nuanced [19], biomass and lipid overyielding occurred in fed-batch polycultures of natural phytoplankton communities and mixed laboratory strains, which the authors attributed to complementarity rather than selection effect [15,16]. This dictates further exploration of the potential for improving algal biomass and feedstock productivity.

Despite these potential advantages, uncontrolled polycultures offer little opportunity for consistent species and biochemical composition, which complicates harvesting and post-harvest processing into end products. It may be possible to design assemblages of two or more algae that optimize yields of desired products, whether total biomass, lipids, carbohydrates, animal feed, specialized bioproducts, or a combination thereof. Especially in long-term continuous culture, one species or strain is likely to competitively exclude or dominate the other(s), and the species balance, hence biomass quality, will change seasonally and with weather conditions (light and temperature), which cannot be controlled economically. Thus, we propose manipulation of other environmental factors, such as CO_2, pH, nutrients and salinity, to maintain an optimal species/strain balance.

Another factor in algal productivity is the mode of cultivation: batch, fed batch, semicontinuous or continuous. Most work to date has featured batch cultures, which result in high terminal cellular lipid content upon depletion of the limiting nutrient [20,21], but this may not represent the maximum time-averaged lipid or biomass yield due to the inherent intermittent nature of batch cultivation. We recently demonstrated that even continuously growing *Dunaliella* cells accumulate some neutral lipids [22]. This observation, combined with the encouraging studies of mixed

* Corresponding author.
E-mail address: bill.henley@okstate.edu (W.J. Henley).
[1] Current address: 186 Rolling Hill Dr., Daphne, AL 36526 USA.

http://dx.doi.org/10.1016/j.algal.2015.11.021
2211-9264/

culture stability and overyielding, led us to test whether two species can be maintained in continuous cultures by manipulating conditions to prevent competitive exclusion. If successful, this would combine the potential advantages of mixed species and continuous culture. Salinity is one of several possible manipulable variables having a large effect on algal competitive abilities, with the long-term goal being control of assemblage composition to optimize biomass yield. We present proof-of-concept bench scale experimental results with halotolerant algae manipulated by salinity changes that will facilitate future scale-up and application to other (multiple) species and other means of potentially automated polyculture stabilization and optimization of biomass.

2. Materials and methods

2.1. Biomass and lipid production in single and mixed species batch cultures

Monocultures and mixtures of the chlorophyte *Dunaliella* sp. GSP-980625-1E [23], isolated from hypersaline soil of the Salt Plains National Wildlife Refuge in Oklahoma, USA, and the marine diatom *Phaeodactylum tricornutum* CCMP 1327 were grown in 150 ml batch cultures at 24 °C for 15 days in 250 ml flasks on an orbital shaker at 100 rpm. Triplicate flasks per monoculture and mixture were cultured under 130 μmol photons m^{-2} s^{-1} 400-W metal halide illumination on a 14:10 h light:dark cycle. We used UTEX AS medium [23] modified to contain 35 g NaCl L^{-1}, 600 μM nitrate, and 120 μM phosphate, and was pH buffered only by 2 mM initial $NaHCO_3$. All cultures were inoculated with the same initial biovolume of each species (11 nl cells ml^{-1}), therefore mixtures had 22 cells ml^{-1} total initial biomass, which allowed a direct comparison of growth response between mono- and mixed cultures. Biovolumes were calculated based on the cell number and dimensions of each species [24]. Cell densities were counted on alternate days using a hemocytometer.

2.2. Monoculture semi-continuous experiments to determine species salinity response

The cyanobacterium *Aphanothece* sp. CCMP 2529 and chlorophyte *Dunaliella* sp. 980625-1E, both isolated from the Great Salt Plains in Oklahoma, USA, and the marine diatom *P. tricornutum* CCMP 1327 were grown in unbuffered UTEX AS medium [23] in 125 ml flasks at 24 °C and 90 μmol photons m^{-2} s^{-1} 400-W metal halide light on a 14:10 h light:dark cycle for 14 days. Biweekly-transferred batch stock cultures were maintained under similar conditions except at lower irradiance. Triplicate semi-continuous monocultures were grown in factorial combinations of four salinities (10, 50, 100 and 150 g NaCl L^{-1}) × three nutrient regimes (12,200:370, 600:120 and 40:8 N:P μM), with the nitrogen comprising a molar proportion of 96% nitrate and 4% ammonium. Cultures were diluted daily at photoperiod hour 6 to the same A_{750} (absorbance at 750 nm) as a proxy for cell density, and the fractional dilution equals the specific growth rate μ (d^{-1}). Thus, cultures were semi-continuous turbidostats rather than chemostats. After dilution, cultures were bubbled for one minute with ~1.5% CO_2 (controlled by Omega FMA A2400 electronic mass flow meter/controller and measured with a CO_2 meter.com K33-BLG 0-30% sampling CO_2 data logger). pH was within the 6–8 range immediately after bubbling.

2.3. Stabilization of two-species mixed cycloturbidostat culture

We developed a custom four-unit cycloturbidostat continuous culture system on a light–dark cycle and with fresh culture medium supplied on-demand, based on turbidity of the suspension [25]. Turbidity, a function of cell size and density [22], was continuously monitored at high temporal resolution and pumps triggered in each culture unit when the 5-min mean signal exceeded an arbitrary set point. Each 2 or 3 min pump cycle delivered ~4 or 6 ml medium and removed an equal culture volume per cycle, as described previously.

Growth rate response to salinity of monocultures of the three species was assessed by starting the turbidostat units (one per species) at 10 g NaCl L^{-1} and incrementing to 50, 100 and 150 g NaCl L^{-1}. Within species, growth (= dilution) rates were averaged for 2–5 days; although days are pseudoreplicates, this was merely a preliminary experiment to determine salinity response in support of subsequent experiments, and the results were not statistically analyzed. *P. tricornutum* did not grow well in the turbidostats, thus we selected *Dunaliella* and *Aphanothece* for mixed turbidostat cultures based on high growth rates and differential salinity response between 50 and 100 g NaCl L^{-1}.

In the monoculture and first mixed species experiments, duplicate 500-ml Pyrex culture bottles contained 220–235 ml of culture in full strength unbuffered AS medium (11.75 mM NO_3^-, 0.5 mM NH_4^+, 0.37 mM PO_4, 2 mM HCO_3^-) at ~80 μmol photons m^{-2} s^{-1} (measured adjacent to the bottles) of 400-W metal halide illumination on a 14:10 L:D cycle. In the second mixed species experiment, we used 250 ml culture in four 1-L translucent FEP Teflon bottles (same diameter as the glass bottles) to reduce cell adhesion; nutrients were reduced to 200 μM NH_4^+ (no NO_3^-) and 40 μM PO_4, initial HCO_3^- was increased to 10 mM, and light was 250–300 μmol photons m^{-2} s^{-1} of 1000-W metal halide illumination on a 14:10 L:D cycle. The suspension was continuously aerated (via 0.2 μm filters in the air line) at 25–50 ml min^{-1} per culture, equivalent to a headspace turnover rate of approximately 2–4 h^{-1} and mixed with a Teflon-coated magnetic stir bar. The entire apparatus was autoclaved prior to each experiment. Culture temperature was 25 ± 3 °C (diel fluctuation in room temperature) for all turbidostat experiments.

Cell densities were monitored at the same time daily using a hemocytometer. Daily dilution rates (DR, d^{-1}) were calculated as effluent volume divided by nominal culture volume. In monocultures, the DR equals the specific growth rate (μ). In the second mixed culture experiment, μ of each species in each culture unit was calculated as $DR + \ln(N_{t+1} / N_t)$, where N_t and N_{t+1} are cell densities for that species on successive days. For example, if DR was 1.5 d^{-1} and the cell density of *Dunaliella* decreased by 15% and *Aphanothece* increased by 25% on that day, $\mu_D = 1.5 + \ln(0.85 / 1) = 1.34$ d^{-1}, and $\mu_A = 1.5 + \ln(1.25 / 1) = 1.72$ d^{-1}.

2.4. Flow cytometric relative quantitation of neutral lipids (TAG) with Nile Red

On days 4, 6, 8, 11, 13, and 15 during the batch culture experiment, we measured relative content of neutral lipids (triacylglycerides or TAG) using the lipophilic fluorescent dye Nile Red (NR; 9-diethylamino-5-benzo [α]phenoxazine; Sigma-Aldrich) using a modified protocol [26,27]. Briefly, we added 16 μl of 250 μg ml^{-1} NR in acetone to 4 ml cell suspension to give a final concentration of 1 μg ml^{-1}, vortexed for 10 s and incubated in darkness for 5–20 min. We measured NR fluorescence of individual cells using a BD Biosciences FACSCalibur® flow cytometer, excited by a 488 nm argon laser, and yellow fluorescence of neutral lipids was measured in the 560–640 nm FL2 channel [27]. A total of 10,000 cells were analyzed and compared to the autofluorescence of unstained cells. The flow cytometer settings were the same for all samples and the data are expressed in arbitrary units. NR fluorescence is directly proportional to TAG content in a given species, both in bulk and by flow cytometry [27,28]. However, it is only a relative measure of lipids and the relationship between NR fluorescence and neutral lipid content may vary among species, thus it is not trivial to compare NR signals in mixtures and monocultures. Moreover, *Dunaliella* sp. and *P. tricornutum* were not fully differentiated by gating in any of the detector channels, thus flow cytometry could not be used to resolve the lipid content of the species. Therefore, we determined the fractional species composition of each mixed culture sample by light microscopy, and combined that with NR fluorescence of corresponding samples of monocultures to predict NR fluorescence of the mixed cultures on sampling days. Predicted values were compared to measured values in mixed cultures to infer whether cellular TAG content $cell^{-1}$ differed between mono- and mixed cultures.