EFFECTS OF ENHANCED MIXING AND MINIMAL CO_2 SUPPLEMENTATION ON BIOMASS AND NITROGEN CONCENTRATION IN A NITROGEN-FIXING *ANABAENA* SP. CYANOBACTERIA BIOFERTILIZER PRODUCTION CULTURE

Joshua Wenz, Heather N. Storteboom, Jessica G. Davis
Department of Soil and Crop Sciences, Colorado State University

ABSTRACT

Nitrogen-fixing cyanobacteria are attractive as a nitrogen fertilizer because they are ubiquitous in nature and have minimal nutrient requirements. Our lab is scaling up production of a local strain of the nitrogen-fixing cyanobacterium *Anabaena* sp. in on-farm open raceways to determine its economic potential as a nitrogen fertilizer for horticultural crops. Our goal is to increase productivity in an organically certifiable growth medium above the current two week batch production levels of 30 mg L^{-1} total Kjeldahl nitrogen. To improve production, we tested delta wing arrays to improve mixing and mass transfer of nutrients. We also supplemented production raceways with CO_2 to maintain a pH of 9.5 or lower. We determined that improved mixing produces no significant differences in biomass or nitrogen production in CO_2 limited cultures. Supplementation of CO_2 to limit pH at 9.5 produced small improvements in biomass but no difference in nitrogen concentration. Increasing the levels of CO_2 supplemented and coupling the delta wings with CO_2 supplementation still has strong potential to improve nutrient transfer, and production of biomass and total nitrogen.

INTRODUCTION

Nitrogen fixing cyanobacteria have the potential for use as a nitrogen fertilizer. The Colorado State University (CSU) Biofertilizer Lab has been conducting trials to scale up production of an *Anabaena* sp. nitrogen (N) fixing cyanobacterium in open raceways. An organically certifiable growth medium (RB medium) has been successfully developed for field use (Barminski, 2014). Healthy raceway cultures regularly produce 30 mg L^{-1} of total N during a 14-d batch production cycle. In 2014, we explored two methods for increasing production above baseline levels. The first incorporated delta wings to improve mixing and mass transfer in the raceways. The second introduced carbon dioxide (CO_2) into raceways as a supplemental source of carbon (C).

Mixing

One of the key factors limiting scale up of microalgae cultures in open raceways is mixing (Grobbelaar, 2012). The primary benefit of increased mixing is the improvement of the mass transfer of nutrients, including CO_2, to the cyanobacteria, thereby increasing photosynthetic efficiency (Grobbelaar, 1994). The Utah State University (USU) raceway hydraulics group designed delta wings that improve vertical mixing (vs. the horizontal mixing provided by the paddlewheel) (Voleti, 2012; Lance, 2012; Vaughan, 2013). The USU group determined that delta wings at a 40-degree angle, spanning the width of the raceway channel, and placed every 5.4’ could achieve sustained vortices throughout the raceway. When these delta wings were added to

USU model raceways in a greenhouse, the biomass of *Chlorella vulgaris* (a green algae) increased by 27.1% compared with controls (Vaughan, 2013). We tested the USU delta wing configuration in an effort to achieve a 25% increase in *Anabaena* sp. biomass and total N.

CO_2 Supplementation

N-fixing cyanobacteria require CO_2 for photosynthesis. Because of the difficulty in mass transfer between the production culture (liquid) and CO_2 in ambient air (gas), CO_2 in the production raceways will not be replenished naturally (Borowitzka, 2005). The fixation of dissolved inorganic carbon in the forms of CO_2 or H_2CO_3 also increases pH. *Anabaena* sp. continues to grow at high pH, and high pH can aid in the suppression of biological contaminants. However, it is likely that CO_2 is limited (Olaizola et al, 1991). Literature shows up to a four-fold increase in biomass with the introduction of low levels (.5-3%) of CO_2 (Olaizola et al., 1991, Arudchelvam and Nirmalakhandan, 2012). Our use of a N-fixing microorganism and a N-free growth medium likely reduces the upper limits of production that can be expected. Despite this, supplementation of CO_2 appears to have the best potential for increasing biomass and total N in our production cultures. We tested the supplementation of CO_2 in an *Anabaena* sp. N-fixing bacterial culture to determine if it could cost effectively increase biomass and total N during 14-d batch production.

METHODS

Field production experiments were conducted in the summer of 2014, at the CSU Horticultural Research Center in Fort Collins, CO. Six 107-ft^2 open raceways with 10in depth were installed in 20ft x 50ft high tunnels. The RB growth medium was added to the raceway and allowed to mix prior to inoculation with *Anabaena* sp. cultures. To monitor the growth and health of the production culture, pH, temperature, and dissolved oxygen (DO) were measured daily between 2-4PM at three points within each raceway using an ORION 5 STAR portable meter (Thermo Scientific). Samples (15 mL) were collected at the same three data points within each raceway for measurement of optical density (OD). Every three to four days, additional samples were taken from the same sampling points for determination of Total Kjeldahl Nitrogen (TKN). A Hach DR 3900 Benchtop Spectrophotometer (Hach Company, Loveland, CO. USA) was used to measure OD (at 550 nm) and TKN. Statistical analysis was performed in R with $\alpha = .05$.

For the delta-wing trials, delta wings were designed using information from Vaughan (2013). A total of five two-delta arrays were placed 5.4 ft apart along the channels of three raceways, except where the paddlewheel was attached. Three raceways served as controls. Two trials were conducted for mixing with delta wings. Due to high levels of algal contamination during the initial delta wings trial, conducted 6/25 to 7/9/2014, a second mixing trial was conducted 8/21 to 9/8/2014 using a healthier inoculum. For the CO_2 treatment, three raceways were fitted with PINPOINT pH Controller (American Marine, Inc.) to continuously monitor pH. A brass AC110V solenoid (Duda Energy, Alabama, U.S. Model 2W-200-20N) was attached to the pressure regulators on a 20-lb compressed CO_2 tank and plugged into the pH controller. CO_2 was bubbled into the raceway using 1.5 in x .5 in Sweetwater ceramic diffusers attached to ¼” ID x 3/8” OD ATP Vinyl-Flex clear plastic tubing. The pH controller for each raceway was set to open the solenoid when the pH reached 9.6 and bubble CO_2 until the pH reached 9.5.

RESULTS AND DISCUSSION

Delta Wings Mixing

In delta wings production trials, no significant differences were found at the end of production between raceways with delta wings and control for either OD or TKN (Table 1, Figure 1).

Table 1. Total Kjeldahl Nitrogen and Optical Density levels at the end of two batch experiments comparing raceways fitted with delta wings with control raceways. Data are means of 3 raceways (n=9).

	Deltas	Control	p-value (t test)
Trial 1 End of Batch (Day 14)			
Total Kjeldahl Nitrogen (mg L^{-1})	13.9	14.1	0.8928
Optical Density 550 nm (Abs)	0.293	0.314	0.3917
Trial 2 End of Batch (Day 18)			
Total Kjeldahl Nitrogen (mg L^{-1})	28.8	24.5	0.0807
Optical Density 550 nm (Abs)	0.333	0.312	0.0808

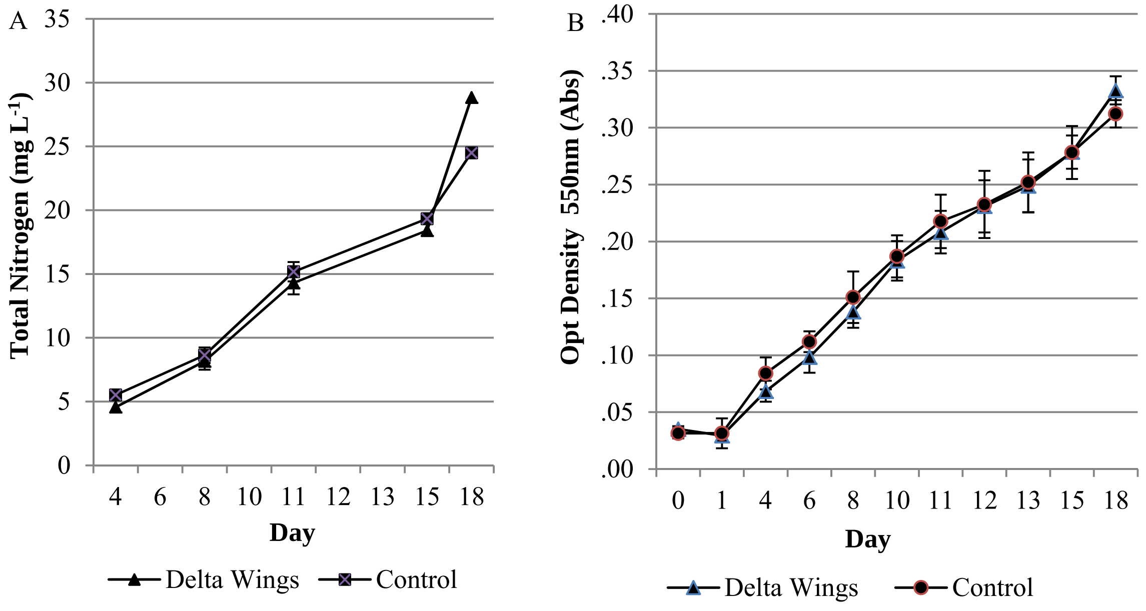

Figure 1. Total Kjeldahl Nitrogen (mg L^{-1}) (A) and Optical Density 550 nm (Abs) (B) as a function of time in a batch experiment comparing delta wings with control in 10 m^2 raceways (Trial 2).

CO_2 Supplementation

One control raceway (WSE) was excluded from statistical analysis as it was taken over by a *Scenedesmus* sp. alga shortly after inoculation. There was no significant difference in TKN between CO_2 supplemented and control raceways (Fig. 2). Raceways receiving CO_2 supplementation to maintain pH at a maximum of 9.5 began showing significant differences in OD vs. control on day 11 (Fig. 3). The pH was also significantly different in raceways receiving CO_2 most days after day 6, but not on the final day (day 18) of the production batch.

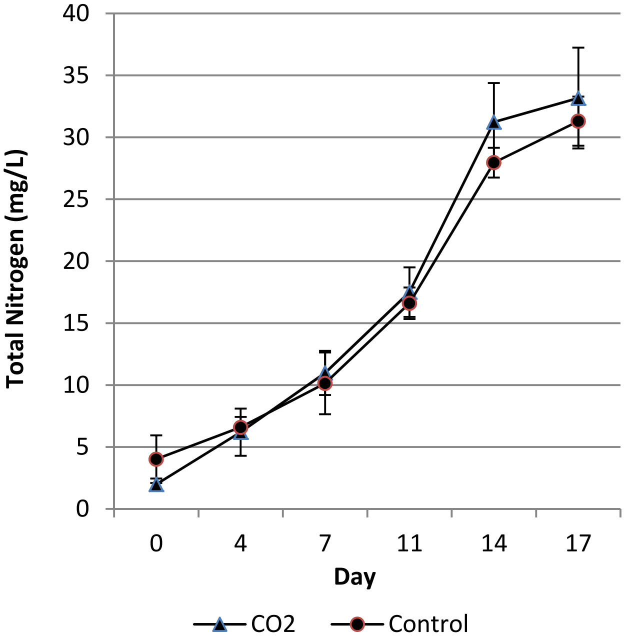

Figure 2. Total Kjeldahl Nitrogen (mg L^{-1}) as a function of time in a batch experiment comparing CO_2 supplementation to maintain a maximum pH of 9.5 with control in 10 m^2 raceways.

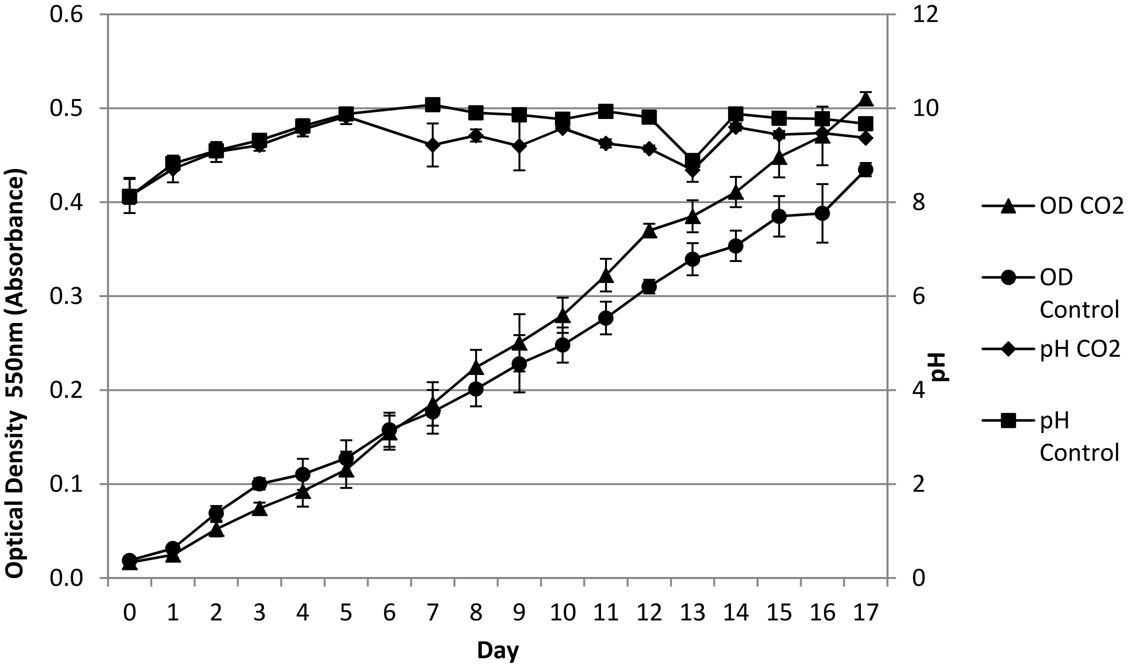

Figure 3. Optical Density 550 nm (Abs) and pH as a function of time in a batch experiment comparing CO_2 supplementation to maintain a maximum pH of 9.5 with control in 10 m^2 raceways.

Table 2. Effect of CO_2 Supplementation to Anabaena sp. Grown in 10 m^2 Raceways on Total Kjeldahl Nitrogen, Optical Density, and pH at the end of an 18-d Batch Experiment. Data are means of 3 raceways for treatment (n=9), and 2 for control (n=6)

	CO_2 to maintain max pH 9.5	Control	p-value (two sample t test)
Mean Total Kjeldahl Nitrogen (mg L^{-1})	31.2	31.3	.2473
Mean Optical Density 550nm (Abs)	.510	.435	<.001
Mean pH	9.37	9.67	<.001

In both delta wing trials, there was no significant difference in biomass or total N production between raceways with delta wings and the controls. It is possible that the spacing between delta wings within an array was too small, resulting in early deterioration of vortices. Another possibility is that the cultures have an overall nutrient limitation that cannot be addressed by improved mixing of the culture. Once the nutrient limitation (e.g., CO_2) is resolved, the delta wings could lead to enhanced growth, either by increasing overall biomass and total N or by increased growth rates and shorter production cycles.

During the CO_2 trial, beginning day 7 until the end of the batch, most days showed significant differences in pH between treatment and control raceways (Table 2). Overcast weather (notably day 13) reduced photosynthesis and pH in both control and CO_2 supplemented raceways. Beginning on day 11, there were statistically significant differences in OD. However, there was no difference between total N in control raceways compared with pH controlled raceways. The increase in OD in supplemented raceways holds little practical significance, and it would be difficult to justify the costs of bubbling CO_2 to maintain a pH of 9.5.

Other nutrients may be limiting growth and N fixation. Increasing pH above 9.0 can render some nutrients, such as potassium and iron, unavailable to cyanobacteria (Evan and Prepas, 1997). Using Moreno et al. (2003) raceway production ranges of 9 g m^{-2} d^{-1} to 20 g m^{-2} d^{-1} as a reference for a marine N-fixing *Anabaena* sp., it could be reasonable to expect a 50% to 200% increase over our current baseline production. Moreno et al. (2003) maintained a pH between 8.5 and 9.0 to achieve those production levels, so it is possible that increasing CO_2 supplementation and reducing pH further to at least 9.0 could lead to cost effective increases in biomass and total N.

SUMMARY

Improving mechanical mixing and supplementing CO_2 remain the best candidates for economical increases in biomass and total N of nitrogen-fixing *Anabaena* sp. cultures. The differences in biomass late in the CO_2 supplementation experiment suggest that a pH ceiling of 9.5 may be the upper boundary for altering growth of our *Anabaena* sp. It could prove worthwhile to run the CO_2 supplementation experiments at levels that maintain incrementally lower pH values to 1) determine the efficacy of supplementation on cyanobacterial nitrogen fixation and 2) to find the most economical level of CO_2 supplementation for production of a N-fixing cyanobacteria-based fertilizer.

This study has shown that improved mixing may not be effective in a CO_2-limited culture. However, coupling the delta wings with CO_2 supplementation may yet result in improved nutrient transfer, photosynthetic efficiency, and shorter fertilizer production times.

REFERENCES

Arudchelvam, Y., and N. Nirmalakhandan. 2012. Energetic optimization of algal lipid production in bubble columns. International Conference on Lignocellulosic Ethanol. 46: 757-772.

Barminski, Rosalyn. 2014. Development of an Organically Certifiable Growth Medium for Nitrogen-Fixing Cyanobacteria in a Raceway Biofertilizer Production System. Master's thesis, Soil and Crop Sciences, Colorado State University, Fort Collins, CO.

Borowitzka, M. 2005. Culturing Microalgae in Outdoor Ponds In: Andersen (ed) Algal culturing techniques. Elsevier Academic Press, San Diego, pp 205-218.

Evan, J.C., and E.E. Prepas. 1997. Relative importance of iron and molybdenum in restricting phytoplankton biomass in high phosphorous saline lakes. Limnology and Oceanography 42(3):461-472.

Grobbelaar, Johan U. 1994. Turbulence in mass algal cultures and the role of light/dark fluctuations, Journal of Applied Phycology 6: 331-335.

Grobbelaar, Johan U. 2012. Microalgae mass culture: the constraints of scaling-up. J Appl Phycol 24:315–318.

Lance, B. 2012. Using Stereo Particle Image Velocimetry to Quantify and Optimize Mixing in an Algae Raceway using Delta Wings, Master's thesis, Mechanical and Aerospace Engineering, Utah State University, Logan, UT.

Moreno, J., M.A. Vargas, H. Rodríguez, J. Rivas, and M.G. Guerrero. 2003. Outdoor cultivation of a nitrogen-fixing marine cyanobacterium, *Anabaena* sp. ATCC 33047. Biomol Eng. 20(4-6):191-7.

Olaizola, Miguel, Eirik O. Duerr, and Donald W. Freeman. 1991. Effect of CO_2 enhancement in an outdoor algal production system using *Tetraselimis.* Journal of Applied Phycology 3: 363-366.

Vaughan, Garret F. 2013. Experimental Studies of Vertical Mixing Patterns in Open Channel Flow Generated by Two Delta Wings Side-by-side. Master's thesis, Mechanical and Aerospace Engineering, Utah State University, Logan, UT.

Voleti, R. 2012. Experimental Studies of Vertical Mixing in an Open Channel Raceway for Algae Biofuel Production. Master's thesis, Mechanical and Aerospace Engineering, Utah State University, Logan, UT.

PROCEEDINGS
OF THE
WESTERN NUTRIENT
MANAGEMENT CONFERENCE

Volume 11

MARCH 5-6, 2015
RENO, NEVADA

Executive Committee
Galen Mooso, Simplot, 2014-15 Chairman
Joan Davenport, Washington State University, 2014-15 Vice Chairman
Jim Walworth, University of Arizona, 2014-2015 Secretary

Program Chair
David Tarkalson
USDA - ARS
3793 N 3600 E
Kimberly, ID 83341
(208) 423-6503
David.tarkalson@ars.usda.gov

Coordinator
Phyllis Pates
International Plant Nutrition Institute
2301 Research Park Way, Suite 126
Brookings, SD 57006
(605) 692-6280
ppates@ipni.net