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Article

Optimizing *Arthrospira platensis* growth for biofuel production via symbiosis between cyanobacteria strains

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SUMMARY

The exacerbation of climate change due to the release of greenhouse gases is leading researchers to explore renewable energy sources such as biofuels, which are near carbon neutral. Producing fuel derived from cyanobacteria, which exhibit rapid growth and minimal growth requirements, circumvents issues caused by conventional biofuel crops. To increase the economic viability of cyanobacterial fuel, we hypothesized that co-culturing *Arthrospira platensis*, a valuable cyanobacteria genus, with other symbiotic cyanobacteria genera *Nostoc* sp. and *Anabaena* sp. would increase culture resilience and *A. platensis* biomass compared to mono-culturing. We selected *Nostoc* sp. and *Anabaena* sp. as secondary species for their unique characteristics, including colony formation and nitrogen-fixing abilities, which enable them to provide nutrients and structural support for other organisms in nature. We used bright-field microscopy, spectrophotometry cell density measurements, and general observations (e.g., color, visual indicators of culture health) to compare mono-cultured A. platensis with its co-cultures containing either *Nostoc* sp. or *Anabaena* sp. This study confirms that *A. platensis* takes advantage of mutualistic relationships and forms a more robust culture alongside *Anabaena* sp. Specifically, *Nostoc* sp. and *Anabaena* sp. reduced invasive species growth and harmful algal blooms. However, *Nostoc* sp. did not effectively support *A. platensis* growth, and there were signs of antagonism. Compared to the monocultures, co-cultures with *Anabaena* sp. demonstrated enhanced resistance to environmental stressors, prolonged culture health even in low-nutrient environments, and increased biomass. These findings suggest that commercial co-culturing of *A. platensis* with *Anabaena* sp. could improve the feasibility of cyanobacteria-derived biofuel.

INTRODUCTION

Anthropogenic activities, especially the immense burning of fossil fuels for energy generation, are a leading cause of climate change (1). As annual worldwide carbon dioxide emissions surpass 36 billion tons, the need for bioenergy—renewable energy derived from biomass—is rising, encouraging bioenergy production and exploration into newer production methods (2). In the United States, bioenergy makes up 60% of renewable energy, with biofuels constituting 32% of total renewable energy used (3). The most prevalent types of biofuels are ethanol and biodiesel, which are both used in transportation, and they are mostly used in blends with traditional fuels (4). Typically, fuel blends containing ethanol are 10% ethanol combined with 90% gasoline, and fuel blends containing biodiesel are 5–20% biodiesel, with the rest being petroleum diesel (4, 5). Not only are biofuels renewable, whereas fossil fuels and conventional fuels are not, but they also represent a near-carbon-neutral fuel. They are carbon neutral since the production of biofuel, specifically the growth of plant biomass, sequesters carbon dioxide. This removal of carbon from the atmosphere offsets the greenhouse gases that will be released when the biofuel is burned, meaning they greatly reduce greenhouse gas emissions compared to traditional fuels (6, 7). Specifically for biodiesel, the percentage of biodiesel that makes up the blend of fuel is approximately the percentage of reduction of greenhouse gases compared to using petroleum diesel only (8). However, biofuels do have a few drawbacks. For instance, while typical engines can run on the aforementioned blends of biofuels, not all engines are optimized to use biofuels (5). Moreover, another drawback of biofuels is that they contain slightly less energy per gallon than traditional fuels (5). Per gallon, biofuel blends with 98% ethanol provide 30% less energy than gasoline (5). However, there is no noticeable difference in performance when using many blends, such as a fuel with 20% biodiesel content (4,5). Ultimately, the benefits of biofuels far outweigh any drawbacks, especially in the face of the urgency of climate change.

While biofuels are promising, one issue that must be addressed is that most biofuels used today are currently derived from edible energy crops. This is problematic as edible energy crops, such as corn and sugarcane, threaten biodiversity and food security due to the immense use of land and resources needed for growth (9). In contrast, biofuels derived from microorganisms such as cyanobacteria offer a more sustainable alternative as they circumvent land-use conflicts and have a myriad of other benefits. Some of these benefits include their higher growth rates compared to crops and their ability to directly utilize concentrated carbon dioxide emissions from industrial waste sources during cultivation (10).

Cyanobacteria can be used for biofuel production as they contain several molecules that are precursors to biofuel, including hydrocarbons, diacylglycerol, and triacylglycerol (11). Besides their biochemical makeup, they are especially favorable as they have a rapid rate of lipid synthesis and

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growth. Compared to traditional methods, they can be more than 100 times more efficient at lipid production than conventional crops (measured in yield per unit area) (11, 12). Moreover, cyanobacteria are adaptable, easy to cultivate, and can grow in wastewater to aid purification since they uptake excess nutrients present in wastewater (13).

One of the most widely available and promising cyanobacteria species for biofuel production is *Arthrospira platensis*, the species of focus in this study (14). However, cultivating *A. platensis*, and other species of cyanobacteria, currently faces challenges in economic viability due to the high costs of technology and methods implemented in cultivation and harvesting (15). To overcome these challenges, scientists are actively endeavoring to inexpensively optimize biomass, in turn increasing economic viability and expanding the market (15). Therefore, our study regarding bioenergy production presents a novel procedure where we optimize *A. platensis'* growth through the formation of synergistic relationships with other cyanobacteria strains. Currently, researchers have extensively studied the symbiotic relationships between cyanobacteria and plants to enhance crop growth; however, studies regarding associations between cyanobacteria strains are very limited (16). In our study, the secondary cyanobacteria species that we paired with the primary species *A. platensis* were *Nostoc* sp. and *Anabaena* sp., which are also suitable for biofuel production but have a lower growth rate due to their periphytic nature—their tendency to adhere to underwater surfaces (**Figure 1**) (17, 18). We specifically selected these species because they are known to form symbiotic relationships in their natural habitat due to their unique characteristics, namely their abilities to fix nitrogen and form colonies that support other organisms (19).

Generally, Nostocales cyanobacteria, which include *Nostoc* sp. and *Anabaena* sp., naturally form symbiotic relationships with plants in terrestrial and aquatic environments. *Nostoc* sp. are known for their relationships with liverworts, ferns, and the Angiosperm *Gunnera*, while *Anabaena* sp. are known for their symbiosis with the water fern *Azolla* (20, 21). Both *Anabaena* sp. and *Nostoc* sp. have specialized cells, heterocysts, which contain nitrogenase enzymes that convert atmospheric nitrogen (N_2) into ammonia (NH3), allowing them to survive unfavorable conditions and develop these associations, specifically commensalism or mutualism (22). Their cell membranes allow the nitrogenase enzyme-generated ammonia to diffuse into their surroundings, thereby increasing bioavailable nitrogen and benefiting nearby nutrient-dependent species (23). Moreover, the structure of these species also aids in their ability to form symbiotic relationships; *Nostoc* sp. cells specifically develop mucilaginous envelopes, which aid in their development of symbiotic relationships since the gelatinous matrix provides a protective space where other organisms may live (**Figure 1C**) (24, 25). Although researchers have not extensively studied this phenomenon, some cases report symbiotic microorganisms living within a *Nostoc* sp. colony and depending on the colony's metabolism to survive (25).

Based on the evidence for various symbiotic relationships that *Nostoc* sp. and *Anabaena* sp. develop, this study explores developing consortia to foster a synergistic association between *A. platensis* and each of these two species. *A. platensis*, the target species of our study, grows optimally in high-nutrient environments, further highlighting the possible benefits of a synergistic relationship as *Nostoc* sp. and *Anabaena* sp. both fix and secrete nitrogen (26). We hypothesized that developing these consortia would foster symbiotic relationships and allow for increased culture resilience and biomass of *A. platensis*, as opposed to mono-culturing. This includes culture resilience to stressful conditions, namely the depletion of provided nutrients from media. Our findings support this hypothesis, revealing that *A. platensis* and *Anabaena* sp.'s co-cultivation fosters commensalism; co-cultivation improves *A. platensis* growth in terms of both health and biomass, decreases the growth of invasive species *Chlorella vulgaris*, and prolongs culture life. While *Nostoc* sp. lengthened the overall culture life as well as decreased *C. vulgaris* growth, *Nostoc* sp. did not support as much *A. platensis* (while there was a significant amount of *Nostoc* cells in the co-culture, there were few *A. platensis* cells). This indicates a possible antagonism effect due to competition. This study suggests that producers can grow *Anabaena* sp. alongside *A. platensis* cultures on the commercial level to strengthen the resilience of the culture, possibly cutting costs by reducing the need for additional nutrients or materials for culture resiliency.

RESULTS

To increase the economic viability of cyanobacteria-derived biofuel, we explored fostering synergistic associations between the target species *A. platensis* and secondary species *Anabaena* sp. and *Nostoc* sp. to improve culture health and increase biomass. If successful, cyanobacteria cultivation facilities can apply this methodology of building a consortium between cyanobacteria strains to improve growth and decrease costs. We compared the growth of a monoculture of *A. platensis* (control group) to two co-cultures. The first co-culture contained *A. platensis* (the primary species) and *Nostoc* sp., and the other co-culture contained *A. platensis* and *Anabaena* sp. We conducted two experimental trials, each spanning a total of 28 days consisting of an initial 14-day growth period and then a 14-day period for additional observations. We collected brightfield microscopy and spectrophotometry data at the beginning and end of

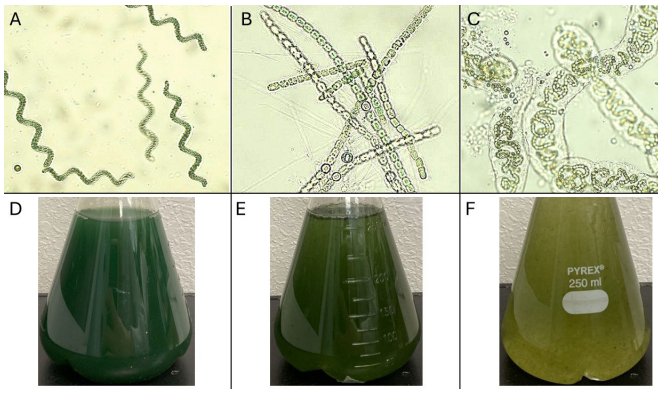

Figure 1: Starting cultures of *A. platensis*, *Anabaena* sp., and *Nostoc* sp. A-C) Microscopy photos of our cultures of *A. platensis* **(A),** *Anabaena* sp. **(B)**, and *Nostoc* sp. **(C)**, under 40X magnification before we set up the experiment. **(D-F)** *A. platensis* **(D)**, *Anabaena* sp. **(E)**, and *Nostoc* sp. **(F)** starting cultures. A-C were taken with the Zeiss Model Axiovert 100m microscope, with a Sony Alpha A5000 digital camera, under 40X magnification. D-F are the cultures we homogenized before setting up and starting experimentation.

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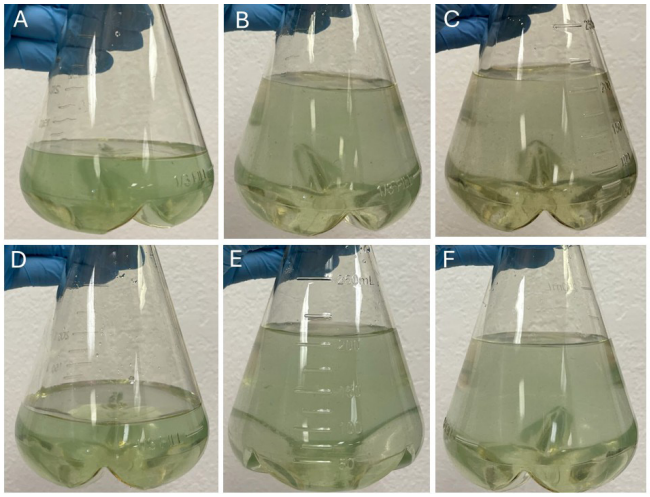

Figure 2: Day 1 of cultures in experimentation during the central growth phase. A-C) Cultures from the first trial: *A. platensis* **(A)**, *A. platensis* and *Anabaena* sp. **(B)**, and *A. platensis* and *Nostoc* sp. **(C). D-F)** Cultures from the second trial, in that order, respectively. The species were homogenized and used to set up these starting cultures.

the initial 14-day growth period to observe the microscopic interactions between species. We collected qualitative data from microscopy and gained insight into the physical arrangements between the primary species, *A. platensis*, and secondary species, *Nostoc* sp. or *Anabaena* sp. We used spectrophotometry to measure light absorbance of the cultures, directly assessing optical density and biomass.

General Observations of the Cultures

At the beginning of the experiment, all cultures resembled a light green hue. As experimentation progressed, clear distinctions in color, consistency, and texture emerged (**Figure 2**). As cultivation progressed, the co-cultures developed a drastically darker shade than the control groups (**Figure 3**). While all cultures exhibited cell sedimentation at the bottom by the end of each day, the control groups experienced the most increased clumping. This increased clumping made it more difficult to maintain homogeneity in the control cultures. The co-cultures all formed a distinct overall shape where most cells appeared connected, suggesting a symbiotic relationship or a possible communal formation (**Figure 3**). However, despite this, they were easy to aerate and allowed us to make the culture homogeneous. Notably, the co-cultures with *A. platensis* and *Anabaena* sp. had pronounced amounts of oxygen bubbles (**Figure 3B, 3E**).

Nanodrop Spectrophotometer Data

All cultures were homogeneous at the start of the experiment, and the optical densities across the cultures were lower than each of their measurements at the end. While initial monoculture readings were close in similarity (0.007 and 0.001 nm), initial readings of the *A. platensis* and *Nostoc* sp. co-cultures were slightly lower (0.002 and 0.001 nm), whereas the initial readings of the *A. platensis* and *Anabaena* sp. co-cultures were marginally higher (0.012 and 0.040 nm) (**Table 1**). After the 14-day period, co-cultures with *Nostoc* sp. reached 0.163 nm and 0.126 nm, and the optical density of the co-cultures with *Anabaena* sp. similarly increased greatly, reaching 0.75 nm and 0.175 nm, for the first and second trials respectively for each (**Table 1**). In comparison, the end readings for both control groups were relatively low, reflecting lower cell density.

The cultures with a consortium experienced the most prominent increase in biomass, as reflected by the larger differences between beginning and ending optical density readings. While the *A. platensis* and *Nostoc* sp. cultures increased by an average of 0.143 nm and the *A. platensis* and *Anabaena sp.* cultures increased by 0.099 nm (average), the controls merely increased by 0.031 nm (average) (**Table 1**). Reflecting this, in the first trial, the *A. platensis* and *Nostoc* sp. co-culture experienced an increase in cell concentration that was six times greater than the cell concentration increase of the control. Similarly in the first trial, the *A. platensis* and *Anabaena* sp. co-culture experienced an increase in cell concentration that was 2.5 times greater than the control. In the second trial, the *A. platensis* and *Nostoc* sp. co-culture experienced a cell concentration increase that was over three times greater than the cell concentration increase of the control, while the *A. platensis* and *Anabaena* sp. co-culture was 3.5 times more. The control groups' minor increase in optical density reflects less biomass increase compared to the other cultures, further confirming the more robust growth of both consortia. It is important to note that these co-culture optical measurements reflect the growth of the consortia as a whole, not *A. platensis* only.

Microscopy

Using the microscope, we observed little to no free-floating cells in the control cultures; rather, they were strongly conglomerated in masses, reflecting the observation of clumping (**Figure 4**). The cause of this conglomeration was the growth of an invasive species, which we identified as *C. vulgaris* based on cell shape and size. The growth of *C. vulgaris* most likely harmed *A. platensis* growth as conglomeration harmed the *A. platensis* cells (27). On the other hand, the consortium with *Anabaena* sp. visibly

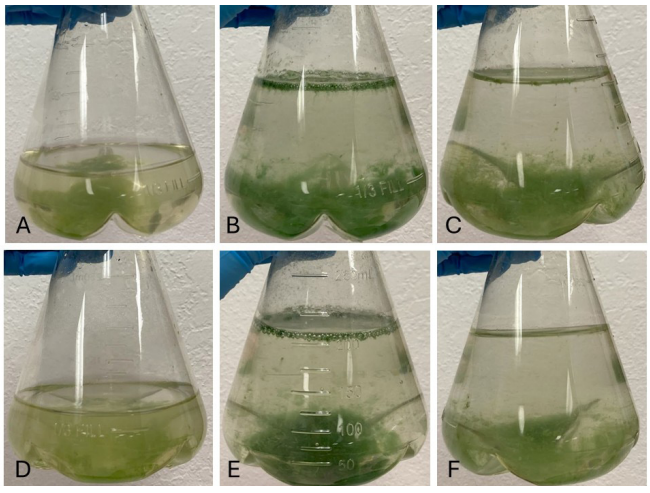

Figure 3: Day 14 of cultures in experimentation of the central growth phase. A-C) Cultures from the first trial, *A. platensis* **(A)**, *A. platensis* and *Anabaena* sp. **(B)**, and *A. platensis* and *Nostoc* sp. **(C). D-F)** Cultures from the second trial, in that order, respectively. The species were homogenized and used to set up these starting cultures.

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Sample name	Trial	Day 1 600 nm (a.u.) absorbance	Day 14 600 nm (a.u.) absorbance	Difference in absorbance (a.u.)
A. platensis monoculture	1	0.007	0.033	0.025
	2	0.001	0.039	0.038
A. platensis and *Nostoc* sp.	1	0.002	0.163	0.161
	2	0.001	0.126	0.125
A. platensis and *Anabaena* sp.	1	0.012	0.075	0.063
	2	0.040	0.175	0.135

Table 1: Optical density readings of cultures on day 1 and day 14 of experimentation. Absorbance values of the cultures in the beginning and the end of the central growth period, with the last column as the difference between the beginning and ending absorbance values. These readings were measured with a NanoDrop ND-1000 UV-Vis Spectrophotometer with wavelength of 600 nm.

supported an abundance of *A. platensis* (**Figure 4**). Notably, we detected little to no *C. vulgaris* presence in all co-cultures. However, the consortia with *Nostoc* sp. contained little to no *A. platensis* cells, which indicates that the cell concentration increases in the *Nostoc* sp. co-culture mainly reflect the growth of *Nostoc* sp., not the growth of the target species, *A. platensis* (**Figure 4**). One factor we also noted was that while most *Nostoc* sp. cells were in mucilaginous envelopes, others were filamentous (**Figure 4**).

Additional 14-day Observation Period

We also analyzed the growth of the cultures in the additional 14-day period, after our main experimental growth period, with microscopy. The consortium in trials one and two with *Anabaena* sp. supported *A. platensis* to the 24th or 25th day. On the other hand, the invasive species completely outcompeted *A. platensis* in the monocultures around the 19th day and the consortia with *Nostoc* sp. had a very minimal amount of *A. platensis* up to the 27th and 28th day. However, although the co-cultures continued to prolong the culture, the health of all cultures visibly declined after the 14-day central growth period.

DISCUSSION

This study found that co-culturing *A. platensis* with *Anabaena sp.* improved culture health and produced longer-lasting cultures, possibly by decreasing invasive species contamination. Oxygen bubbles from photosynthesis and qualitative observations of the color of the cultures indicated culture health. A vibrant green indicated high health while a dull color indicated a decline in health. Optical density measurements from the spectrophotometer showed the increase in growth and cell concentration, which further provided insight into the health of the culture. *Anabaena* sp. supported the healthiest culture, lasting 24 to 25 days with the most prominent amount of *A. platensis* observed by microscopy. This study reveals that co-culturing *A. platensis* with *Anabaena* sp. resulted in commensalism, whereas *Nostoc* sp. failed to support much *A. platensis* despite decreasing contamination, improving overall culture health, and prolonging culture lifespan to around 27 days. Thus, the effect of *Nostoc* sp. on *A. platensis* should be explored further. The results of our study confirm the benefits of co-culturing and suggest that supplementing *A. platensis* cultivation with *Anabaena* sp. specifically can improve culture growth and health, possibly cutting costs and increasing the competitiveness of cyanobacteria-derived biofuels in the market.

Our general observations, specifically noting the color, formation, and signs of photosynthesis, provided insight into how *Anabaena* sp. and *Nostoc* sp. both improved culture health. The deeper green hues, the oxygen bubbles, and the connected appearance of the co-cultures with *Anabaena* sp. indicate robust health and the formation of a beneficial synergistic association. We used the presence of oxygen bubbles as a qualitative indication of photosynthesis. We ensured that the observed bubbles were not a result of or influenced by the mixing of cultures, which was done daily, by allowing the cultures to remain undisturbed for 24 hours prior to any observations on bubbles or color. The buoyancy of the cells due to the bubbles further suggested possible improved aeration achieved in co-culturing with *Anabaena* sp. Conversely, the control groups, one in each trial, had difficulty being aerated due to prominent cell clumping.

Further, we used optical density data, which was measured by the spectrophotometer and reflects the concentration of cells in the culture, to observe differences in growth. Reflecting healthy growth, the optical density data of the first trial's co-cultures depicted increases in cell concentration that were six times and 2.5 times greater than the control (*Nostoc* sp. co-culture and *Anabaena* sp. co-culture, respectively). This is notable as an increase in cell concentration indicates growth and an increase in biomass. Similarly, the second trial co-culture cell concentration increases were over three times and 3.5 times greater than the control (*Nostoc* sp. co-

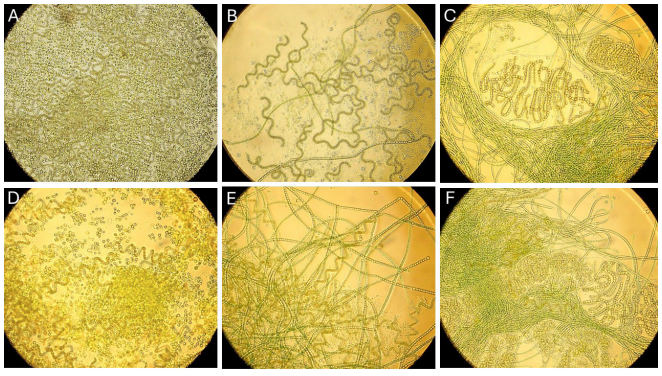

Figure 4: Bright-field microscopy after the 14-day central growth period. A-C) Microscopy photos of cultures from the first trial. The cultures are the *A. platensis* monoculture **(A)**, *A. platensis* and *Anabaena* sp. co-culture **(B)**, and *A. platensis* and *Nostoc* sp. co-culture **(C). D-F)** Microscopy photos of cultures from the second trial: *A. platensis* monoculture **(D)**, *A. platensis* and *Anabaena* sp. co-culture **(E)**, and *A. platensis* and *Nostoc* sp. co-culture **(F).**

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culture and *Anabaena* sp. co-culture, respectively). However, while all the co-cultures with *Anabaena* sp. and *Nostoc* sp. displayed more growth than the control, microscopy revealed that the consortia with *Anabaena* sp. supported more *A. platensis* cells. The consortia containing *Nostoc* sp. had very little *A. platensis* cells. This indicates that the increase in cell concentration, as shown by the spectrophotometry for the *Nostoc* sp. co-culture, mainly reflects the growth of the *Nostoc* sp. rather than that of the target species *A. platensis*. Microscopy also enabled us to monitor the cultures in the extended observation period, where growth lessened, possibly due to the lower nutrient levels as we did not add additional media. The extended observation period highlighted the abilities of *Anabaena* sp. and *Nostoc* sp. to prolong the survival of a culture. However, *Nostoc* sp. supported very little *A. platensis* in both the central growth period and extended observation period, thus the prolonging of the culture is less significant.

Our microscopy data revealed the key finding that only the control groups experienced the growth of unwanted species, which we identified as *C. vulgaris*, which caused the aforementioned conglomeration of cells. The growth of *C. vulgaris* caused undesirable clumping in the control groups, which inhibited *A. platensis* growth (27). While *C. vulgaris* is a microalgae that is also used for biofuel production, this growth is nonetheless problematic as it inhibits the production of cyanobacteria- specific biofuel by inhibiting cyanobacteria growth, the focus of this research. The contamination of any unauthorized species may complicate the process and impair the quality of the culture (28). Noticeably, we observed no unwanted growth in any of the co-cultures, and we predict that this results from *Anabaena* sp. and *Nostoc* sp.'s ability to release toxins, specifically microcystin-LR (29). While these toxins are innocuous to *A. platensis*, microcystin-LR inhibits the growth of *C. vulgaris*. When in contact with microcystin-LR, *C. vulgaris* secretes excessive glycolipids due to oxidative stress that the toxin causes, which damages the cell structure. Moreover, microcystin-LR harms *C. vulgaris'* cell membrane and mitochondria by disturbing metabolic pathways (30). Based on this line of reasoning, we recommend co-culturing *A. platensis* with *Anabaena* sp., and possibly *Nostoc* sp., in environmental conditions favorable to other unwanted species such as *C. vulgaris*. This further emphasizes the beneficial relationship and symbiosis between *A. platensis* and *Nostoc* sp. or *Anabaena* sp., where *Nostoc* sp. or *Anabaena* sp. are not impacted but *A. platensis* benefits. Whereas these findings align with existing studies on the benefits of symbiotic connections between nitrogen-fixing cyanobacteria and other species, the nascent nature of studying beneficial relationships between cyanobacteria restricted this study (16). The strategy of fostering symbiotic relationships in the biofuel production field is relatively new, with extremely limited earlier research available on any interactions between *A. platensis*, *Nostoc* sp., and *Anabaena* sp. This research gap made predicting the exact cause behind our results somewhat difficult.

Limited time and resource availability constituted a major limitation of this study, which restricted us to two experimental trials. Thus, we suggest further research and replication of these trials to confirm these results. Another limitation is the difficulty in analyzing the biomass of *A. platensis* alone in the co-cultures since *A. platensis* biomass mixed with the secondary species biomass. To address this, future directions can include identifying methods to replicate the commensalism while keeping cells of different species physically separate. This would allow us to use a hemocytometer and dry weight, in addition to a spectrophotometer, to obtain a more accurate measurement of cell count and biomass.

Future research should investigate measuring the amount of nitrogen in these cultures, confirm the presence of microcystin-LR, and explore the applicability of co-culturing with *Anabaena* sp. and *Nostoc* sp. with other cyanobacteria strains. Repeating the experiment without the presence of *C. vulgaris* will help researchers observe the effects of co-culturing without the presence of a third, invasive species. Moreover, measuring the amount of nitrogen in these cultures can aid in exploring the role of nitrogen fixation in this symbiosis, as *Anabaena* sp. and *Nostoc* sp. are both nitrogen-fixing species. Similarly, confirming the presence of cyanotoxins with liquid chromatography and mass spectrometry will aid in confirming the accuracy of our predictions that cyanotoxins were present in the co-cultures (31).

The findings of this study highlight the practicality of co-culturing procedures to address the challenges of large-scale cyanobacteria cultivation for bioenergy. This approach offers an effective pathway to improve the feasibility of cyanobacteria-based biofuels, making them a more appealing renewable energy alternative. The *A. platensis* and *Anabaena* sp. co-culture's improved health, growth, prolonged survival, and resilience to lower nutrient environments and invasive species underscore the vigor of this method. The decrease in unwanted species contamination, enabled by *Anabaena* sp. and *Nostoc* sp., is especially critical in cyanobacteria growing facilities that need to anticipate contamination, such as open-pond systems, but still require biofuel purity. This study advocates for the usage of *Anabaena* sp. in *A. platensis* growing facilities and the increased exploration of co-culturing methods with *Nostoc* sp. Further research into fostering synergistic associations to optimize cyanobacteria growth will enable the biofuel industry to better harness the potential of cyanobacteria.

MATERIALS AND METHODS

This study was conducted in the controlled environment of an indoor greenhouse to establish optimal cultivation conditions for *A. platensis* and the secondary species *Nostoc* sp. and *Anabaena* sp. The experiment was conducted under controlled Fecida LED growth lights and adhered to a photoperiod regime of 16 hours of light followed by 8 hours of darkness. A temperature of 25°C (±2°C), suitable for cyanobacteria growth, was maintained throughout. This environment ensured uniform light distribution and temperature conditions across all cultures, thereby promoting optimal growth and minimizing external variability. All trials were prepared in 250 mL sterilized flasks, with the control containing 100 mL of Alga-Gro Freshwater Medium (Carolina Biological Supply) and 5 mL of *A. platensis* stock culture (Carolina Biological Supply). The experimental groups each consisted of 200 mL of media, 5 mL of *A. platensis*, and 5 mL of either *Nostoc* sp. (Carolina Biological Supply) or *Anabaena* sp. (Carolina Biological Supply), adhering to recommended starter culture ratios published by Carolina Biological Supply (32). Before beginning the experimental period, cultures were homogenized to ensure even distribution (**Figure 1**).

Sterilization involved subjecting all tools used to high-

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pressure saturated steam at 120°C for 20 minutes, eliminating the possibility of parasite infections. To maintain homogeneous distribution, prevent conglomeration, and ensure the growth of *A. platensis*, which cannot live in stagnant waters, each culture was stirred daily for ten minutes (27). The main experimental period lasted 14 days per trial, without the addition of new media, and trials were cultivated for 14 extra days to observe the effects of low-nutrient conditions.

Throughout the experiment, daily photographs were captured to facilitate visual tracking of growth and morphological changes in the cultures over time. Concurrently, daily observations were documented, noting changes in pigmentation and overall health of the cultures. At the end of the experiment (day 14), data was collected via the Zeiss Model Axiovert 100m microscope under 40x magnification, with a Sony Alpha A5000 digital camera, and the NanoDrop ND-1000 UV-Vis Spectrophotometer. All spectrophotometer readings were measured with a 600 nm wavelength, as this wavelength corresponds to the absorption peak of phycocyanin—a pigment in cyanobacteria—and allowed us to monitor cell density (33). To analyze the impact of the consortium on biomass, we utilized the spectrophotometer to obtain the difference between the optical density between the beginning and end of the experiment. Both measuring optical density and performing microscopy were done on all cultures at the beginning of the experiment (to collect baseline data) and at the end.

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