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A newly described species of unicellular cyanobacterium *Cyanothece* sp BG0011: A potential candidate for biotechnologies

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Abstract

The genome **s**equence of a newly isolated cyanobacterium, *Cyanothece* sp. BG0011 was determined and its phylogenetic relationship to other cyanobacterial taxa described. Growth responses of BG0011 to a range of selected environmental factors were determined, including temperature, salinity, nitrogen sources and natural outdoor culture conditions. The species has high growth rates at salinities from 5 psu to 95 psu, providing the opportunity to use saline or hypersaline water sources for production. BG0011 has high temperature tolerance, with average growth rates of 0.60 d^{-1} at 40°C. High rates of growth in the absence of added nitrogen demonstrate the ability of BG0011 to carry out nitrogen fixation under aerobic conditions, thereby avoiding the need for nitrogen fertilizer in production. Tolerance to high incident solar irradiance and potential ability to adapt to UV exposure indicates less potential for photoinhibition of biomass production in shallow open outdoor production systems. In addition, BG0011 has been shown to produce and excrete large quantities of viscous extracellular polysaccharide polymers, a potentially useful byproduct for a range of applications. The results of this and related studies of BG0011 indicate this species to be a candidate for use in algae-based biotechnologies.

Keywords: algal biomass, nitrogen fixation, hyper salinity, exopolysaccharides

INTRODUCTION

One of the challenges to developing highly productive, sustainable and economically viable algae-based bioproducts and biofuel technologies is finding species capable of meeting the demands and challenges of production systems. Recent research has highlighted the need for low-cost outdoor biomass production systems to be competitive in the market place for many product lines (1, 2). A number of microalgae species have been identified as candidates for such systems because they have high growth rates which exceed those of higher plants (3). However, growth rate is only one of the important factors in the selection process, because outdoor systems are subject to significant variability of a range of environmental factors, such as temperature, salt concentrations, irradiance levels, as well as sources of water and nutrients needed to support growth. Many of these considerations have potentially serious impacts on the sustainability and cost of biomass production, as well as the ultimate quality and quantity of product yields.

The central goal of this study was to describe and examine the ecophysiological characteristics of *Cyanothece* sp. BG0011 with reference to its suitability for algal biomass production systems. The genus *Cyanothece* was first described by Komárek (4). The occurrence of versatile metabolic pathways in *Cyanothece,* including nitrogen fixation, oxygenic photosynthesis and exopolysaccharide production have made some members of the genus potential candidates for bioenergy production (5). The two main objectives of this study were: (1) Sequencing of the genome of BG0011, and identification of its phylogenetic relationship to other cyanobacterial taxa, and (2) Determining the growth responses of the new isolate to a range of selected environmental factors, including temperature, salinity, nitrogen sources and natural outdoor culture conditions, as it relates to its potential use in algae based technologies.

METHODS

Collection and Isolation – Samples used in the isolation of *Cyanothece* sp. BG0011were collected from an interior basin in the north-central region of Florida Bay. Florida Bay is located at the southern tip of the Florida Peninsula west of the Florida Keys (25°00'N x 81°30'W). The north-central region of Florida Bay is characterized by restricted tidal water exchange and long water residence times, resulting in frequent periods of hypersalinity, with values sometimes

exceeding 50 psu (6). The collection area is characterized by an interconnected network of shallow (generally less than 2 meters in depth) basins separated by mud banks and mangrove islands (6). The basin is subject to regular blooms of picoplanktonic cyanobacteria (7).

Both physical and chemical methods were used to achieve unialgal and near axenic cultures of BG0011, including the following procedures: shake-dilution series, plating on enriched agar medium, filtering with millipore filters, an antibiotic protocol, and dilution series following by plating. The antibiotic procedure involved incubating cultures of BG0011 cells in the dark for 24 hours, and then adding antibiotic (i.e. ampicillin), casamino acids, and glucose to the culture in the dark. Every four hours, subsamples were taken of the algae and subsequently centrifuged, rinsed, and plated for a total of 12 total aliquots. Standard sterile protocols were followed in a certified laminar transfer hood. Agar plates were streaked with either a concentrated liquid aliquot and a 1:10 diluted aliquot. After incubation selected colonies were picked, suspended in culture medium, incubated for growth and tested for contamination.

Acridine orange staining, combined with fluorescence microscopy counts at 1000x magnification (8), were used to determine whether the cultures were sufficiently pure to permit genetic analysis.Purifications and culturing of cells were based on Allen's artificial seawater media (9). Sodium chloride was used to adjust the medium to different salinities. Most experiments used media devoid of added inorganic nitrogen since BG0011 is capable of nitrogen fixation under aerobic conditions. The description of BG0011 was based on published information on other *Cyanothece* species (4, 10) and the International Code of Nomenclature of Bacteria (ICBN) guidelines (11, 12).

DNA Extraction, Genomic Sequencing, Genome assembly and Annotation – DNA extraction was accomplished using the modified CTAB protocol and freeze-drying method (13, 14). Approximately 90 micrograms of DNA was submitted for sequencing after a clean-up step using a MoBio Super Clean Kit to remove DNA contaminants. This step removed more than 90-95 % of the sample, 3 micrograms of the sample were left. The DNA submitted behaved anonymously in the fractionation column (G-tube from Covaris); the library was intended to contain approximately 20,000 bp fragments. The SMRT bell library construction protocol removed all but approximately 500 ng of material in the last step, with a size range of approximately 2 to 8 kb with a peak at approximately 3 kb. This material constituted the final SMRT bell PacBio sequencing library. Electrophoretic Lateral Fractionation (SageSciences ELF) system was used to generate a final library with approximately 14 ng of material. The resulting DNA sample concentration was 43.5 ng/μl. Two peaks of size ranges of DNA fragments occurred: the first peak was from 1,012 bp to 5,981 bp with an average size of 3,131 bp representing 47.00 % of the total sample, and the second peak was from 5,981 bp to 37,021 bp with an average size of 12,613 bp representing 45.98 % of the total sample. The resulting library was size-selected for an average size of 6 kb.

DNA was submitted to the University of Florida Interdisciplinary Center for Biotechnology Research (UF-ICBR) for sequencing. The PacBio RS II platform (15, 16) at the UF-ICBR sequenced the genome. The purpose of sequencing the isolate's genome was to generate the longest possible insert libraries for long-read sequencing, to facilitate genome assembly. 14.5 ng of library fragments of 5.8 kb average size (final insert size of the SMRT bell library) was used to set up sequencing reactions in the PacBio RS II for three SMRT cells (Single Molecule Real Time) according to the manufacturer's protocol. *De novo* genome assembly was accomplished using the HGAP.3 (Hierarchical Genome Assembly Process) assembler for PacBio RS II platform. Genome annotation was done using RAST, Rapid Annotation using Subsystem Technology (17) and The SEED (18).

The gene sequence information was submitted to the National Center for Biotechnology Information (NCBI). NCBI registered the Biosample as SAMN08470889 (ID #8470889), in BioProject PRJNA433162.

Phylogenetic Tree Construction - To construct a phylogenetic tree with the newly sequenced BG0011 genome and other cyanobacterial taxa, 20 genomes of other species were collected from the NCBI database GenBank. The genomes were translated to amino acid sequences using RAST. AMORPHA (19) was used to find and select 31protein-coding genes found to be single-copy across all bacteria. The following genes were used: *dnaG*, *frr*, *infC*, *nusA*, *pgk*, *pyrG*, *rplA*, *rplB*, *rplC*, *rplD*, *rplE*, *rplF*, *rplK*, *rplL*, *rplM*, *rplN*, *rplP*, *rplS*, *rplT*, *rpmA*, *rpoB*, *rpsB*, *rpsC*, *rpsE*, *rpsI*, *rpsJ*, *rpsK*, *rpsM*, *rpsS*, *smpB*, and *tsf.* Previous methods using AMORPHA utilized SSU rRNA genes for phylogeny reconstruction. However, organisms with similar SSU rRNA sequences can be incorrectly considered closely related when, in fact, they have distant evolutionary relationships (19). Moreover, it is risky to assume evolutionary relationships from one gene only (19). Many researchers have moved to using multiple protein-coding genes because there is less error associated with amino acids due to the nucleotide compositional bias (19). AMORPHA is a superior pipeline that produces highly reproducible results without manually masking amino acids from the tree-making process (19).

MAFFT (20) was used to create alignments of the amino acid sequences for each of the 31 genes, which were subsequently concatenated using FASconCAT (21). Finally, RAxML was used to reconstruct phylogenetic relationships under maximum likelihood. In addition to a likelihood search for the best tree, 1000 bootstrap replicates were implemented to assess support levels for the relationships recovered.

Laboratory Experimental Design - Laboratory experiments were used to determine the growth response of BG0011 to a range of temperatures, salinities and nitrogen sources. Experiments were carried out in a climate-controlled experimental chambers. Allen's media was used as the base growth medium (9). Modifications to this media, as required by specific experiments, is described below.

In the temperature and salinity experiments, each treatment group was set up in triplicate in 500 ml Erlenmeyer flasks containing 300 mL of medium. Illumination was provided by cool white fluorescent fixtures, yielding incident PAR irradiance of 180-200 µmole photons m^{-2} s^{-1}. The light:dark cycle was 12 hrs. light:12 hrs. dark. At the beginning of each experiment, each flask was inoculated with 10% of the total test volume (depending on the individual experiment) of BG0011 culture in the early exponential phase of growth. Flasks were randomly arranged in the incubation matrix. In temperature experiments Allen's media without nitrogen addition was adjusted to 35 PSU with sodium chloride. Each temperature treatment group was placed in a separate tank filled with water with a circulation heater or chiller. Temperatures were maintained to +/- 1°C of the target treatment temperature, and monitored daily or twice daily (depending on the temperature), along with tank water levels. In salinity experiments treatment groups were adjusted to different salinities using sodium chloride. Flasks were randomly placed in one of two incubation tanks filled with water to the same height and maintained 30°C +/- 1°C.

Illumination for nitrogen source experiments was supplied by flexible LED strips. Each flask was positioned to provide approximately 95 µmol photons $m^{-2}s^{-1}$ of surface illumination. The illumination cycle was 13 hrs. light:11 hrs. dark. At the beginning of each experiment, each flask was inoculated with 10% of the total test volume (depending on the individual experiment) of BG0011 culture in the early exponential phase of growth. Flasks were randomly arranged in the incubation matrix. Dinitrogen gas, air, nitrate, and ammonia were used as nitrogen sources. Erlenmeyer flask cultures inoculated with BG0011 included five treatment groups; no combined nitrogen addition with N_2 gas bubbling (5% CO_2 and 95% N_2), no combined nitrogen addition with air gas bubbling (5% CO_2 and 95% air), two nitrate addition levels (as $NaNO_3$) of 1.5, and 5.0 mM bubbled with inert gas (5% CO_2 and 95% argon) and two ammonium addition levels (as NH_4Cl) of 1.5, and 5.0 mM bubbled with inert gas (5% CO_2 and 95% argon). The assembly was placed in an insulated chamber maintained at 30°C. Each experiment was repeated three times.

Growth in all experiments was determined by removing 5 ml of culture from each flask on a daily basis at mid-day, and chlorophyll *a* fluorescence measured with a Turner Designs benchtop fluorometer.

Outdoor Growth Experiments - Five monthly outdoor mesocosm experiments were conducted from January to June to capture a range of seasonal light and temperature conditions. An experimental outdoor system was designed and constructed. The base of the outdoor system was a 1m x 3m concrete tank filled with water, as a means of damping rapid short-term water temperature variability. At the surface of the base tank, four thirty-liter water filled incubation mesocosms were suspended to provide the opportunity to establish separate light and temperature treatment groups. Three shallow (10 cm) six-liter experimental containers were suspended at the surface of each mesocosms to provide replication in treatment regimes. Light treatments were achieved by using neutral-density plastic panes with different light transmission properties. The basic light treatment groups included full sunlight with ultraviolet radiation (UV), full sunlight minus UV, a reduced percent of full sunlight minus UV, and a seasonally specific treatment regime (e.g. increased temperature in winter). Light conditions were continuously monitored and recorded during the experiment with a Li-cor quantum light meter equipped with a cosine corrected PAR surface probe. Five liters of Allen's medium adjusted to 35 psu salinity was added to each container as the base of the experimental treatments. Each experimental container was aerated to provide circulation and carbon dioxide.

Three percent of a mixed homogenous inoculum of BG0011 in exponential growth phase was used to initiate each experiment. *In vivo* fluorescence was measured daily for 7 days and every-other day for the remaining 14 days.

Growth Rate Determination - Growth rates were determined using the equation of $N_t = N_0e^{rt}$, where N_0 is the population size at time zero, N_t is the final population biomass, r is the intrinsic rate of increase, and t is the time interval (9).

Statistical Analyses - The significance of differences between treatment groups results were tested an ANOVA tests. The significance level alpha was 0.05.

RESULTS AND DISCUSSION

Species Description - Order: Oscillatoriales, Family: Cyanothecaceae, Genus: *Cyanothece.*

Cell Description - *Cyanothece* sp. BG0011 cells are oval or elliptical in shape. Cell lengths range from 5 to 9 µm (average 6.9 µm, *n* =30). Cell widths range 3.5 to 5.5 µm (average 4.4 µm, n=30) (Figure 1). The interior of the cells have a granular appearance under light microscopy (Figure 1). The granular appearance has also been noted for other *Cyanothece* species (10). During reproduction two morphologically identical small semiglobular cells elongate into an oval shape. Cells are solitary and no not form chains.

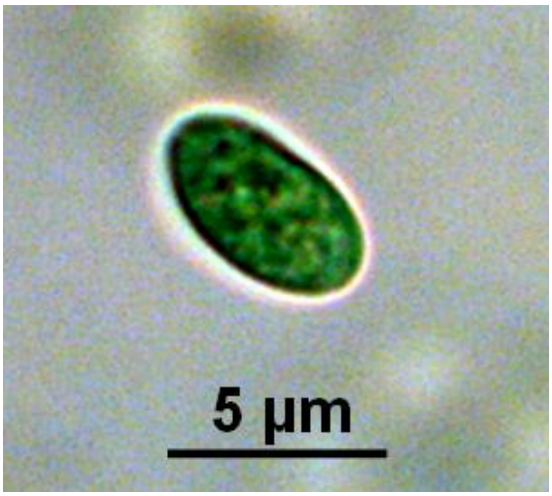

Figure 1. Microscope image of *Cyanothece* sp. BG0011.

Holotype - The *Cyanothece* isolated from Florida Bay, Florida USA, was assigned the strain designation BG0011. Cultures of BG0011 are maintained at the University of Florida in the Phlips Phycology lab, Fisheries and Aquatic Sciences Program of S.F.R.C, 7922 NW 71st Street, Gainesville, FL 32653.

Distribution - *Cyanothece* sp. BG0011 was isolated from samples collected by E. J. Phlips in the tropical marine waters of Florida Bay, located at the southern tip of Florida, USA. At this time, no further information is available on the distribution of the species.

Genetic Analyses - Pacific Biosciences sequencing platform (PacBio) yielded long read lengths (i.e. up to 20kb) and created large overlaps in reads, enhancing the assembly of the genome. Direct comparisons of determining single nucleotide polymorphisms (SNPs) has shown that PacBio has a comparable accuracy rate to shotgun methods (16). The mean read length for BG0011 was 3,538 bp, and the N50 read length was 5,443 bp (Table 1). The number of reads for the run was 84,709, and the number of mapped reads was 72,995. The mean read score was 84%. The number of bases sequenced was 299,731,786, and the depth of coverage was 53.49x. The genome was confirmed to be haploid. The genome size was 5,603,048 bp, and the number of polished contigs was 227.

Table 1. Job metrics of genome assembly using the PacBio H-gap.3 assembler. Metrics include the following: number of polished contigs, percent of adapter dimers, percent of short inserts, number of bases total read by polymerase, number of reads, N50, mean read length, mean read score, mapped reads, mapped read length of insert, average reference length, percent of average bases called, percent of average reference consensus concordance, and average reference coverage.

Job Metric	Value
Polished Contigs	227
Adapter Dimers (0-10 bp)	0.1%
Short Inserts (11-100 bp)	0.01%
Number of Bases	299,731,786
Number of Reads	84,709
N50 Read Length	5,443
Mean Read Length	3,538
Mean Read Score	0.84
Mapped Reads	72,995
Mapped Read Length of Insert	1,824
Average Reference Length	78,177
Average Reference Bases Called	99.99%
Average Reference Consensus Concordance	99.95%
Average Reference Coverage	43.45

RAST (for prokaryotes) genome annotation and Galaxy computing tools at the University of Florida Genetic Institute, revealed that the genome of BG0011 is AT rich; the GC content was 38%. RAST broke the genome down into 382 subsystems. As defined by RAST, "a subsystem is a set of functional roles that together implement a specific biological process of structural complex. Frequently, subsystems represent the collection of functional roles that make up a metabolic pathway, a complex, or a class of proteins." Thirty-three percent of the genome consisted of known subsystems (Fig. 2). In the subsystems, the total coding sequences were 1,837, consisting of 1,774 non-hypothetical coding sequences and only 63 hypothetical coding sequences. For the 67% of the genome not in the subsystem coverage, the total coding sequences for the entire genome is 3,734, consisting of 1,246 non-hypothetical coding sequences and 2,497 hypothetical coding sequences. In the coding sequences of the genome, there are 5,628 features, of which 5,580 are protein coding sequences. 48 of the coding sequences are for RNA.

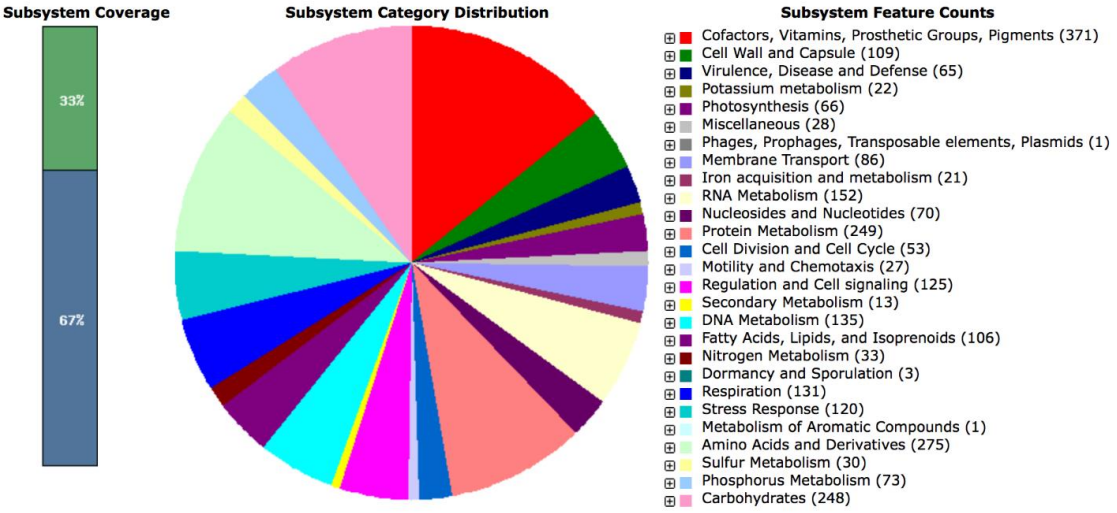

Figure 2. Subsystem categories and their distributions as assigned by RAST annotation software.

Phylogenetic Tree – The availability of a genomic sequence for BG0011 provided an opportunity to compare with sequences of twenty other cyanobacteria obtained from the NCBI Genbank database, including five *Cyanothece* species and four *Synechococcus* species. The 21 genomes were translated to amino acid sequences, and 31 bacterial single-copy genes from the cyanobacteria genomes were selected and used to construct the final phylogenetic tree (Fig. 3).

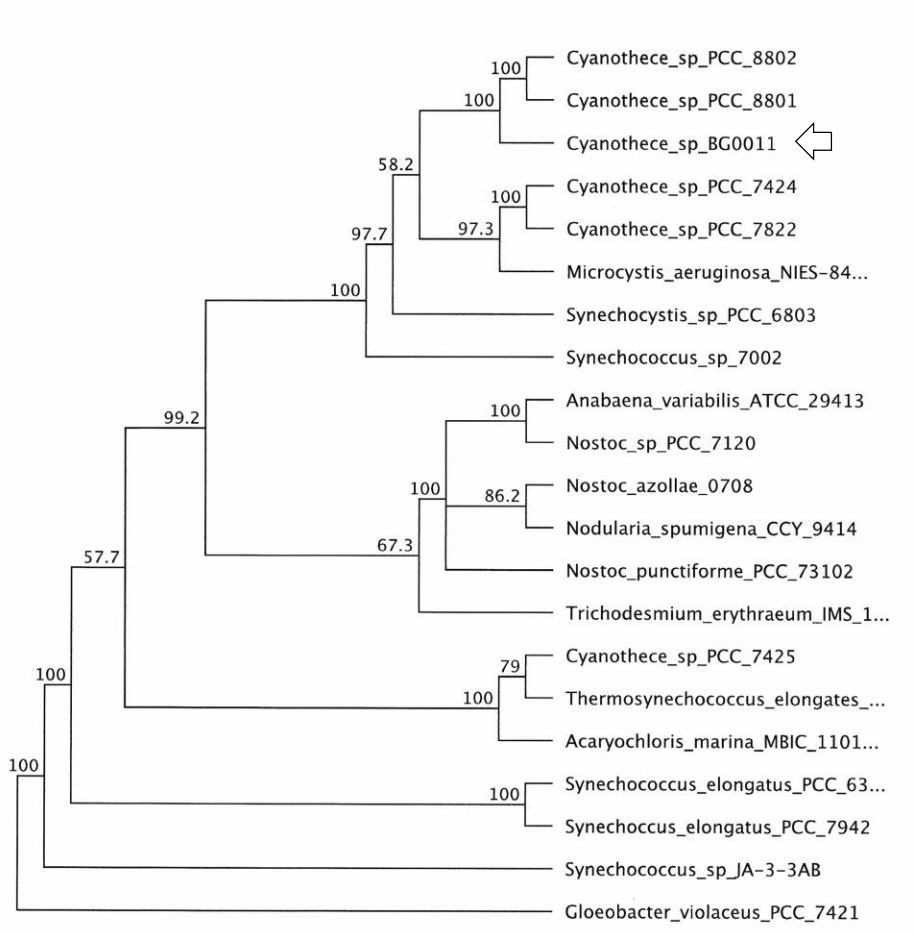

Figure 3. Phylogenetic tree placing *Cyanothece* sp. BG0011 into a broader context with other taxa. Numerical values on nodes represent bootstrap support percentages for the relationships of the organisms.

The results of the phylogenetic tree construction demonstrated that the relationships among the species of *Cyanothece* were well resolved and received strong bootstrap support (ranging from 97 to 100 %). In general, bootstrap support values of 70% or higher show real genetic relationships. This tree confirms that the assignment of the new isolate to *Cyanothece* BG0011 is appropriate. The tree relationships recovered here were similar to those observed by Bandyopadhyay et al. (5), which included many of the same species. Two major groups for the genus *Cyanothece* were recovered by the tree. The placement of BG0011 in the tree is supported by a bootstrap value of 100%. Several other observations support this assignment. The GC content of BG0011 is 38%, similar to other *Cyanothec*e genomes, which have GC contents around 40 % (5). BG0011 and some other *Cyanothece* strains are able to fix nitrogen (23). One unique feature of BG0011 is the large amount of extra-cellular polysaccharides produced by the cells (24).

Ecophysiological Characteristics of Cyanothece sp. BG0011

Temperature Experiments - Microalgae have been grown for biomass production in a range of climatic regimes, although tropical and temperate climates are often best for year around production potential (25). Controlling temperature of large-scale outdoor algal production systems can be challenging from logistic and economic

perspectives, particularly in regions where there are significant seasonal shifts in temperature regimes (26). Even in the sub-tropical climate of Florida water temperatures in shallow aquatic environments can dip below 20ºC in the winter and can rise well above 30ºC during humid summer days when evaporative cooling processes are limited. The upper temperature range is important because temperatures in outdoor production systems can fluctuate more than ambient air temperatures, particularly in high humidity environments and in shallow production pond systems (i.e. <0.5m depth), resulting in temperatures that can reach near 40°C during the day (27). In addition, high algal density in production systems increase the absorption of solar energy, thereby heating the water column. Consequently, it is highly desirable to select algae species which can grow and survive over a broad temperature range (26, 28).

In the controlled laboratory experiments, the average growth rates for BG0011 were similar from 25°C to 40°C, with rates from 0.6-0.7 d^{-1} (Table 2), and upper temperature limit for active growth was between 40ºC and 45 º C (Table 2, Fig. 4). The latter range is similar to another species in this genus, *Cyanothece* sp. ATCC 51142, for which photosynthetic activity drops off above 40 °C (29). For *Cyanothece* sp. ATCC 51142, the reported growth rate is 0.38 d^{-1} at 30°C (29). The high temperature preference of BG0011 is consistent with the broader observation that cyanobacteria are very competitive at high temperatures relative to many other phytoplankton groups (30, 31). Many cyanobacteria species have optimal growth rates at temperatures of 25 °C or higher (31, 32). BG0011 has a temperature preference range between that of mesophiles and thermophiles, which is similar to several other species of cyanobacteria with high temperature tolerances, such as *Nostoc muscorum., Anabaena variabilis, Hapalosiphon fontinalis, Anabaena variabilis, Calothrix elekinii, and Tolypothrix tenuis* (28).

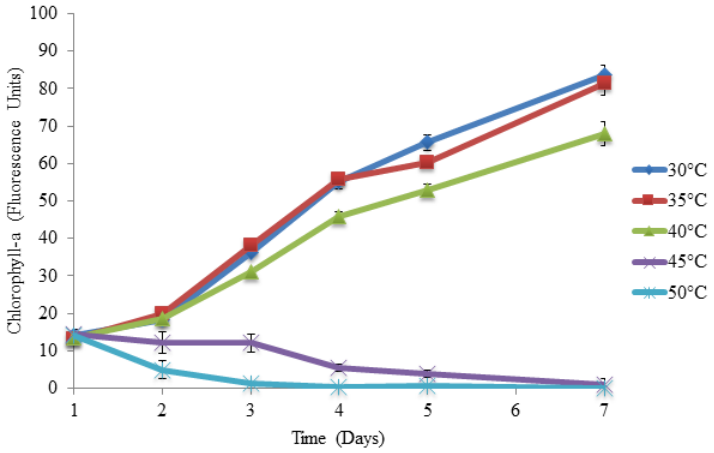

Figure 4. Increase in chlorophyll-a (fluorescence units) over the growth period for five treatment groups in the high temperature experiment: 1) 30 °C, 2) 35 °C, 3) 40 °C, 4) 45 °C, 5) 50 °C. Vertical bars indicate standard deviation of chlorophyll-a expressed as in vivo fluorescence.

The possibility that BG0011 may be able to adapt to temperatures higher than 40°C is a potential target for future research. The chlorophyte *Scenedesmus intermedius* has demonstrated the ability to adapt to higher temperatures over several generations; i.e. from 25°C to 30°C after 15 generations, to 35°C after 30 generations, and to a maximum of 40°C after 135 generations (32). Such plasticity can be helpful in outdoor production systems (32).

The lower threshold for growth of BG0011 lies between 15°C and 20°C (Table 2), which is not surprising considering its tropical origin. In terms of the application of the species to outdoor production systems at cooler ambient temperatures, a heat source may be necessary to maintain high growth rates. In addition to potential for physiological adaptations, structural solutions such as greenhouses offer a potential solution for increasing the temperature of outdoor production systems. Closed cell bioreactors can also maintain higher temperatures in temperate climates due to greenhouse effects (33). Another potential solution to low winter temperatures is utilizing waste heat from cooling waters of power plants, as proposed by Wilde et al. (34).

Table 2. Average growth rates (d^{-1}) at a range of temperature regimes observed in three experiments. NNG refers to no observed net growth.

Treatment Group (˚C)	Experiment	Maximum Specific Growth Rate	Standard Deviation
15	1	NNG	NNG
18	2	NNG	NNG
20	1	0.43	0.07
20	2	0.31	0.13
25	1	0.65	0.03
25	2	0.54	0.24
30	1	0.71	0.04
30	2	0.58	0.26
30	3	0.66	0.07
35	1	0.66	0.02
35	2	0.62	0.27
35	3	0.69	0.02
40	1	0.61	0.06
40	3	0.52	0.06
45	3	NNG	NNG
50	3	NNG	NNG

Salinity Experiments - Open outdoor algal production systems experience evaporative losses of water, which in saline production systems can result in significant fluctuations in salinity (26). High rainfall periods can also result in significant reductions in salinity in saline systems, particularly in the shallow depths used in many production systems because of light requirements to sustain high algal biomass. Both sources of salinity variation highlight the importance of using species capable of adapting to a wide range of salinities (26).

Halotolerant algal strains also have the advantage of growing under hypersaline conditions, which reduces problems of contamination by undesirable invading organisms (26, 35). For example, when cultivating *Dunaliella salina* for β-carotene, hypersaline media is used in the outdoor production systems to prevent contamination (36, 37). In this case, predatory protozoa and the algal competitor *Desmarestia viridis* are the reason for cultivation at higher salinities, rather than optimization of product yield (36, 37).

BG0011 is euryhaline, with similar average growth rates at salinities from 15 PSU to 55 PSU, with rates from 0.64-0.76 d^{-1}, and significant growth down to 5 PSU (Table 3). No growth was observed at 115 PSU (Fig. 5). Cells pre-adapted to high salinities (i.e. 75 PSU or 95 PSU) showed significantly higher average growth rates at elevated salinities versus cells cultured at 35 PSU (Table 3, Fig. 5), demonstrating an ability to adapt to higher salinities. A number of algal species have demonstrated the ability to adapt to changes in salinity over reasonably short periods of time (38). A study by Blumwald et al. (39) found that cells of the cyanobacterium *Synechococcus* sp. 6311 adapted to elevated salinities within 40 hours, and that the primary mechanism for salt adaptation was ionic and compatible solute regulation. Moisander et al. (40) reported that the cyanobacteria *Anabaena aphanizomenoides* and *Anabaenopsis* sp. from the Neuse River Estuary, North Carolina and two strains of *Nodularia* sp. from the Baltic Sea were able to adapt to salt shock within 24 hours. Vonshak et al. (41) also explains that the lag phase in growth, or salt adaptation period, is proportional to the new salinity level to which the organism is exposed. A study by Vonshak et al. (41) found that the cyanobacterium *Spirulina platensis* was able to adapt and grow at higher salinities in 24 hours, and the primary mechanism for salt adaptation in this species was the adjustment of compatible solutes. The salt adapted cells are often characterized by altered internal inorganic ion concentrations (38). Although some of these adapted species show reduced rates of photosynthesis (38), rates of growth of BG0011 did not seem to be negatively impacted by the process of high salinity adaptation, and the adaptation period was relatively short (i.e. within one culture cycle).

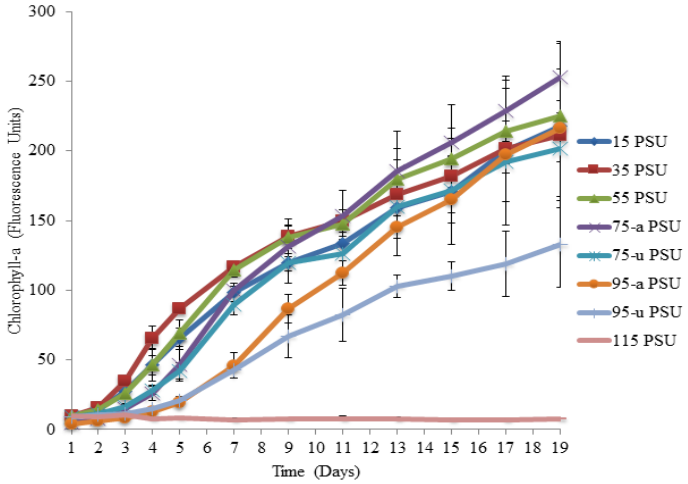

Figure 5. Increase in chlorophyll-a (fluorescence units) over the growth period for eight treatment groups of the second salinity experiment: 1) 15 PSU, 2) 35 PSU, 3) 55 PSU, 4) 75-a PSU, pre-adapted, 5) 75-u PSU, unadapted, 6) 95-a PSU, pre-adapted, 7) 95-u PSU, unadapted, 8) 115 PSU. Vertical bars indicate standard deviation of chlorophyll-a expressed as *in vivo* fluorescence

Table 3. Average growth rates (d^{-1}) at a range of salinity regimes in two experiments. 'A' represents salinity pre-adapted cells and 'U' cells grown at 35 psu. NNG refers to no observed net growth.

Treatment Group (PSU)	Experiment	Maximum Specific Growth Rate	Standard Deviation
5	1	0.42	0.02
15	1	0.55	0.07
15	2	0.65	0.11
25	1	0.50	0.04
35	1	0.51	0.05
35	2	0.76	0.07
45	1	0.54	0.04
55	1	0.48	0.08
55	2	0.64	0.04
65	1	0.39	0.06
75	1	0.26	0.07
75-A	2	0.77	0.06
75-U	2	0.55	0.03
95-A	2	0.57	0.07
95-U	2	0.37	0.02
115	2	NNG	NNG

Nutrient Experiments - Another key issue in the selection of algae species for large scale biomass production systems is nutrient requirements; the supply of which can represent significant logistic and economic considerations. The two primary macronutrients required for growth of all algae is phosphorus and nitrogen, but the focus of this study was nitrogen since some cyanobacteria, can grow in the absence of added reduced nitrogen because of their ability to fix nitrogen (23, 30, 42). The absence of the need for nitrogen fertilizer can be a significant advantage for cyanobacteria if high growth rates can be maintained based exclusively on nitrogen fixation. Average growth rates in the nitrogen experiments were not significantly different between treatment groups (Table 4). For the nitrate treatment group (as $NaNO_3$) average growth rates were 0.51 d^{-1} at 1.5 mM and 0.60 d^{-1} at 5 mM. For the ammonium treatment group (as

NH_4Cl) average growth rates were 0.49 d^{-1} at 1.5 mM and 0.63 d^{-1} at 5 mM. This indicated that the species can readily utilize either ammonia or nitrate as nitrogen source. Average growth rates for the nitrogen fixing treatment groups (i.e. without inorganic nitrogen addition) were 0.54 d^{-1} when air was used and 0.58 d^{-1} when N_2 gas was used for bubbling. The results confirm the nitrogen fixing capability of BG0011.

Table 4. Average rates of growth using different nitrogen enrichments.

Nitrogen Form	Concentration (mM)	Growth Rate (d^{-1})
Nitrate (N as $NaNO_3$)	1.5	0.51
	5.0	0.60
Ammonium (N as NH_4Cl)	1.5	0.50
	5.0	0.63
No addition (with air sparge)	0.0	0.55
No addition (with N_2 sparge)	0.0	0.58

Outdoor Light and Temperature Experiments – Variability in climatic conditions is an important factor to consider when growing algae in outdoor production systems because it is often difficult and costly to control (27). Balancing light availability for growth and high light inhibition of photosynthesis is an important issue in outdoor algal production systems (26). Because algal production systems are generally maintained at high cell densities, high light attenuation coefficients require shallow well-mixed systems, generally around 30 cm (26, 27), resulting in exposure of cells to both high surface irradiances and low bottom light levels. Species that have a high light tolerance and protective mechanisms are advantageous in production systems. Some algae species can produce UV screening pigments and other biochemical processes that provide some protection from harmful effects (30). There is also the issue of seasonal and climate-related variability in temperature. Eurythermal species with broad tolerance to temperature variability are desirable candidates for outdoor applications.

To examine the growth of BG0011 under natural light and temperature conditions a series of monthly outdoor natural experiments were carried out from January through June of 2015 using replicated mesocosms. Each month four treatment groups were included in experiment. All months included three basic treatments, 1) full sunlight, 2) full sunlight minus UV, and 3) 65% (except 50% in June) of full sunlight minus UV. The forth treatment varied by month, i.e. heated treatment (i.e. 30-32^{o}C) in January, February and March, added nitrogen (i.e. ammonium) in April, and 20% of full sunlight minus UV in June. Average mid-day water temperatures in the unheated treatment groups ranged from 18^{o}C in January to near 29^{o}C in April and June (Table 5). Average pre-dawn water temperatures in the unheated treatment groups ranged from 16^{o}C in January to near 26^{o}C in June (Table 5). Average daily irradiance over the incubation periods ranged from 23 mole photons m^{-2} day^{-1} in January to 39 mole photons m^{-2} day^{-1} in April (Table 5).

Table 5. Average temperature and irradiance levels during the five outdoor growth experiments. Temperature means for 'heated' for treatment groups in the January, February and March experiments were 30-32^{o}C.

Month	Average Mid-day Water Temperature in Nine Unheated Tanks	Average Pre-Sunrise Water Temperature of Nine Unheated Tanks	Average Daily Irradiance (mole photons m^{-2} d^{-1})
January	18°C	16°C	23
February	21°C	18°C	24
March	24°C	20°C	29
April	29°C	22°C	39
June	29°C	26°C	32

In the 'full light treatment' group no growth was observed in January, February and March experiments (Table 6). In April and June, the average growth rates were 0.70 and 0.83 d^{-1}, similar to the highest growth rates observed in the other treatment groups in April and June (Table 6). In the 'full sunlight minus UV' treatment group, average growth rates went up from less than 0.01 d^{-1} in January to near 0.80 d^{-1} in April and June. In the '65% full sunlight minus UV' treatment groups, average growth rates went up from 0.15 dbl. day^{-1} in January to near 0.70 d^{-1} in April and June. The average growth rates in the 'heated full sunlight minus UV' treatment group in January, February and March were similar to the growth rates observed in April and June (Table 6). The 'added nitrogen' treatment group in April had similar average growth rates as the other treatment groups, in which no combined nitrogen was added (Table 6). The '20% full sunlight minus UV' treatment group in June had somewhat lower average growth rate than the other treatment groups (Table 6).

Table 6. Average maximum growth rates for different treatment groups in the five outdoor growth experiments. NNG refers to no observed net growth.

Month	Treatment Group	Maximum Specific Growth Rate	Standard Deviation
January	UV, Vis	NNG	NNG
	No UV, Vis	0.01	0.02
	No UV, 65% Vis	0.31	0.03
	No UV, Vis, Heat	0.85	0.03
February	UV, Vis	NNG	NNG
	No UV, Vis	0.24	0.07
	No UV, 65% Vis	0.25	0.07
	No UV, Vis, Heat	0.86	0.08
March	UV, Vis	NNG	NNG
	No UV, Vis	0.70	0.09
	No UV, 65% Vis	0.72	0.07
	No UV, Vis, Heat	0.93	0.03
April	UV, Vis	0.70	0.02
	No UV, Vis	0.76	0.08
	No UV, 65% Vis	0.73	0.07
	No UV, Vis, N	0.79	0.05
June	UV, Vis	0.83	0.12
	No UV, Vis	0.79	0.10
	No UV, 50% Vis	0.68	0.06
	No UV, 20 % Vis	0.55	0.02

Average growth rates were similar in all seasons of the year in treatment groups with similar temperature regimes (i.e. heated groups in January – March and unheated in April and June) (e.g. Fig. 6), indicating that incident irradiance was not a major limiting factor for BG0011 growth rates even in the winter. However, the sufficiency of irradiance in the winter for large-scale high cell density continuous culture systems was not tested in these experiments and would be an important next step.

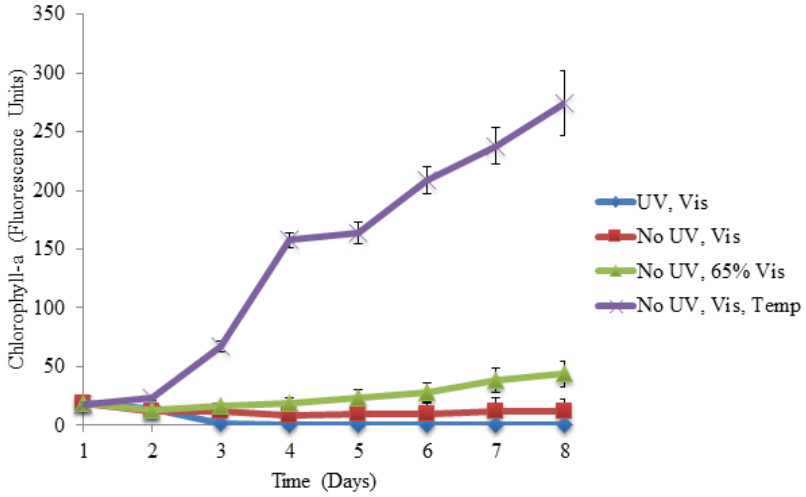

Figure 6. Increase in chlorophyll-a (fluorescence units) over the growth period for four treatment groups of the January light experiment: 1) full sunlight (UV, Vis), 2) full sunlight minus UV radiation (No UV, Vis), 3) 65% of full sunlight minus UV radiation (No UV, 65% Vis), 4) full sunlight minus UV radiation at an elevated temperature (approximately 32 °C, No UV, Vis, Temp). Vertical bars indicate standard deviation of chlorophyll-a expressed as in vivo fluorescence.

In terms of UV radiation, negative effects on the growth of BG0011 were observed in the winter months. In the April and June experiments the inhibitory effects of UV light appeared to dissipate after an extended lag phase (e.g. Fig. 7). The extended lag phase for initiation of rapid growth suggests a period of adaptation to UV exposure. It is well established that many algae can produce UV screening pigments and other biochemical processes that provide some protection from harmful effects (30). For example, the production of carotenoids, such as β-carotene, and aromatic amino acids absorb UV radiation and keep the UV radiation from damaging the photosynthetic apparatus. Moreover, oxygenated carotenoids, such as xanthophylls, undergo light-dependent reactions that convert the xanthophyll compounds eventually to zeaxanthin and heat (30). Potential adaptation of BG0011 to UV exposure in the warmer months suggest that higher ambient temperatures facilitate the adaptation process.

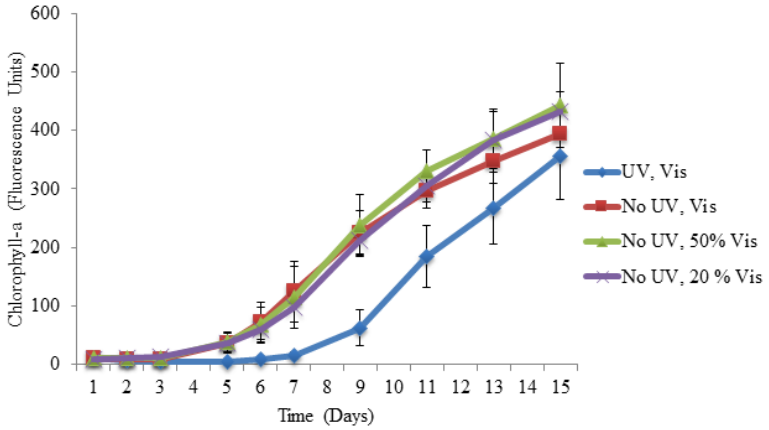

Figure 7. Increase in chlorophyll-a (fluorescence units) over the growth period for four treatment groups of the June light experiment: 1) full sunlight, 2) full sunlight minus UV radiation, 3) 50% of full sunlight minus UV radiation, 4) 20% of full sunlight minus UV radiation. Vertical bars indicate standard deviation of chlorophyll-a expressed as *in vivo* fluorescence.

In terms of water temperature in the unheated outdoor bioreactors, there were significant increases in growth rates from the winter to spring. The responses of BG0011 to low temperature in the controlled laboratory experiments support the observations from the outdoor bioreactors, namely that temperature less than 20ºC appear to have negative effects on growth. The results indicate that to maintain high long-term growth rates in the winter may require a source

of heating. The possibility that BG0011 may adapt to lower temperatures was not tested in these experiments, but is another important question for future research.

Another result from the outdoor bioreactors supported by the results from controlled laboratory experiments is the similarity of growth rates for the nitrogen added and nitrogen–free groups. The extended lag phase in the no added nitrogen treatment group compared to the nitrogen-added group before the onset of exponential growth suggests that the development of strong nitrogen fixation potential is related to the development of sufficient nitrogenase activity (i.e. the catalyst for nitrogen fixation). This should be looked at further in the future, because growing BG0011 in nitrogen fixing conditions contribute to reduction contaminating organisms in outdoor production systems, as well as lowering the costs associated with the need for nitrogen fertilizer.

SUMMARY

The growth rates and responses of *Cyanothece* sp. BG0011 to a range of key environmental factors suggest that it is a potential candidate for outdoor production of algal biomass. The maximum observed growth rate of BG0011 (i.e. 0.94 day^{-1}) falls within the range of maximum rates for other cyanobacteria species that have been identified as candidates for biotechnological applications, i.e. 0.81-1.15 day^{-1} (23, 43). Several characteristics observed in this study represent strengths of BG0011 as a potential biomass producer in outdoor production systems. The species has high growth rates at salinities from 5 psu to 95 psu, providing the opportunity to use saline or hypersaline water sources for production. The species has high temperature tolerance (i.e. between 40-45^{o}C), which increases the sustainability of production at high summer water temperatures encountered in some geographical regions. High rates of growth in the absence of added nitrogen, because of nitrogen fixation, eliminates the need for nitrogen fertilizer in production. Tolerance to high incident solar irradiance and potential ability to adapt to UV exposure indicates less potential for photoinhibition of biomass production in open systems. In addition, BG0011 has been shown to not only yield high biomass levels, but also produces and excretes large quantities of viscous extracellular polysaccharide polymers, which have potential utility for (24). The EPS represents the potential for the production of a range of other valuable products, such as a range of food and cosmetic applications, contributing to the economic viability of new technologies using the species.

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