1 Characterization and Classification of Highly Productive Microalgae Strains Discovered
2 for Biofuel and Bioproduct Generation

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30 ABSTRACT

31 This paper describes the characteristics of microalgal strains that originated out of an
32 isolation and screening project included within the National Alliance for Advanced
33 Biofuels and Bioproducts (NAABB). The project's goal was to identify new potential
34 platform strains with high growth rates and/or lipid productivities. To classify the best
35 performing strains, we conducted a combined microscopic and phylogenetic analysis.
36 Among the best performing strains were many coccoid green algae. Several strains
37 belong to the species *Acutodesmus (Scenedesmus) obliquus* and to the species *Chlorella*
38 *sorokiniana*, thus expanding on existing germplasm. Identified at the genus level were
39 some *Desmodesmus* strains and one *Ankistrodesmus* strain. Several strains were classified
40 as belonging to the genus *Coelastrella*, a taxon reported for the first time for North
41 America. Multiple additional strains had ambiguous identities, with some strains possibly
42 representing novel species. Reporting on the above strains, some of which have been
43 tested successfully in outdoor ponds and most of which are deposited at the University of
44 Texas Culture Collection of Algae, is a step forward in expanding the biological
45 resources available for algae biofuel production.

52 *Key index words:* biofuel, bioprospecting, green algae, *Scenedesmus*, *Coelastrella*,
53 *Chlorella*
54
55 *Abbreviations:* UHPLC, Ultra high performance liquid chromatography; PDA, Photo
56 diode array; MS, Mass Spectrometry; APCI, Atmospheric Pressure Chemical Ionization;
57 ITS, internal transcribed spacer; SSU, small-subunit; LSU, large-subunit; TAG,
58 triacylglycerols

1. Introduction

Algae are seen as a more appealing biofuel feedstock than land plants because of their faster biomass doubling cycles, more accessible forms of stored carbon, and their ability to thrive on waste or salt water sources [1, 2, 3]. But the best strains for such production are unlikely to be known or utilized at large scales [4]. Therefore, a need exists for “phyco-prospecting” [4] – the identification of novel algal “platform strains” – which sustain high growth rates and accumulate lipids [5, 6, 7, 8, 9]. In addition, strains that produce significant amounts of higher-value products, such as carotenoids, are also desired [1, 2, 10, 11, 12, 13]. Such novel “platform strains” could, in turn, be developed further via artificial selection, genetic engineering, or other “crop improvement” methods [3, 14, 15] and used to generate biofuel feedstocks at lower costs.

Commercial scale algae ponds have been operated for more than a decade [3, 16, 17, 18], mainly to harvest pigments and metabolites that are desired as nutritional supplements [10]. The algae cultivated in these ponds include *Spirulina* (*Arthrospira*), for high protein powder [19, 20], *Haematococcus*, for the antioxidant astaxanthin [21, 22], and *Dunaliella salina*, for pro-vitamin A production [23, 24]. Due to the success demonstrated with these used species [17], the as of yet untapped diversity of microalgae appears to offer possibilities in the field of biofuels [25, 26, 27], as well as the production of high-value products [1], such as pharmaceuticals [10, 13, 28].

It is not clear how many microalgae species there are, with estimates running from 70,000 to one million [29, 30, 31]. Only about 44,000 have been described [29, 32]. New species and genera are consistently being discovered; this consistent rate of discovery indicates that a large proportion of undocumented species exists [29].

Based on the known utility of already characterized species and the known absence of knowledge regarding the large amount of undocumented strains, it is hypothesized that previously uncharacterized strains of microalgae exist which exhibit high growth rates and lipid productivities. These novel “strains” may be new species or varieties of species with more favorable growth characteristics for biofuel production. Some of these species or strains may also produce high value products. To test this hypothesis, a large-scale effort was conducted to isolate, screen, identify, and characterize microalgae strains that could be used as platform strains for biofuel and/or high-value product generation. For a number of the most promising strains to come out of the screen, a classification was conducted. In deciding on an appropriate barcode, the nuclear rDNA Internal Transcribed Spacer 2 Region (ITS2) was chosen because, unlike the 18S – which often does not vary enough to distinguish algal species [33] – the ITS2 has proved to be a helpful tool for discrimination at the genus [33, 34] and species level [35, 36, 37, 38, 39, 40, 41, 42]. At the same time, its two dimensional structure is highly conserved throughout the eukaryotes [43, 44, 45]. Combining the fast evolving sequences with its slowly evolving structure has allowed for it to be used in high level classifications while still discriminating at the low species level, within the same phylogenic tree [37].

The overall goal of our research was to discover new microalgal strains that could enhance the genomic/biological resources available for algae biomass production. This biomass would then be used as feedstock for biofuels and/or for bioproducts generation. The focus of this report is on freshwater algae originating mainly from the NAABB phyco-prospecting project [46]. The objective of this report is to document some of these strains and begin to have a cladistic understanding of where they fall in the algal tree of

life. It is hoped that this work may provide some clues for targeted phyco-prospecting approaches in the future.

2. Materials and methods

2.1. Sampling, isolation, and screening.

Cells were sampled and screened as described previously in detail [47]. Briefly, environmental samples were collected over the three years of the NAABB project (2010-2012) from a variety of habitats (e.g. freshwater lakes and soils) across the continental U.S. and during all seasons. As described in detail in [47], for strain isolation an approach was taken that combined the traditional plating method [48] with high-throughput flow cytometry using fluorescence aided cell sorting [49, 50].

For the first-level screen, in summary, batch cultures of strains were grown in Erlenmeyer flasks in defined minimal media suitable for this selection: C Medium, Bold Basal Medium, and BG11 Medium [51], with the only CO₂ enrichment being the sodium bicarbonate which was added to BG11. Flasks were grown under 50 $\mu\text{E m}^{-2} \text{s}^{-1}$ continuous light provided by daylight fluorescent lamps (See the SN-1 for more details on screening methods) at room temperature.

Strains that passed the first-level screen were deemed to be potential producers and were selected for further characterization (Figure 1). In a second-level screen, the strains were cultivated under identical conditions as in the first screen in multiple different

media (C Medium, Bold Basal Medium, BG11) to determine if another growth medium was better suited for use in continued strain characterization (Figure 1).

In a third-level screen for analysis of actual biomass and lipid production potential, strains were grown at 28 Degree Celsius in glass columns (3 cm width with 500 mL volume) into which CO₂ enriched air was bubbled through the cultures from the bottom. Twenty-eight degrees Celsius was used at this point in the screen, because it was deemed a good intermediate temperature to find strains that grow well both in the spring and fall as well as in the summer. Cultures were illuminated by daylight fluorescent lamps from one side at an intensity of 200 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$.

Biomass was determined daily by measuring the Ash-Free-Dry-Weight (AFDW, Supplemental Notes 1(SN-1)). The daily AFDW was graphed using the ggplot package, within the R software environment for statistical computing and graphics (<http://cran.r-project.org/>), to create weighted polynomial curves, which represented algal growth curves. The doubling time was also calculated in R for the exponential growth phase with the formula $\log(2)/k$, with k being the slope of the growth curve.

When the algal cultures reached their stationary phase, biomass also was taken and analyzed via a gravimetric method to determine the total lipid content [52].

2.2. Metabolite analysis.

Pigments and lipids were extracted using a modified version of an established protocol [53] (See SN-2). Pigments and lipids were analyzed using a Ultra High-Performance Liquid Chromatography (UHPLC) C18 reverse-phase column, connected to

a Photo Diode Array detector (Flexar FX-15 UHPLC system from Perkin Elmer), and then passed through an Atmospheric-Pressure Chemical Ionization (APCI) probe connected to a Time-of-Flight Mass Spectrometer (Perkin Elmer, Massachusetts) (UHPLC-PDA-APCI-TOF-MS). The samples were run through the LC with solvent A consisting of 39% water, 59% Acetonitrile, and 2% ammonium acetate and solvent B consisting of 88.2% 2 propanol, 9.8% acetonitrile, and 1.9% ammonium acetate. The column was first equilibrated for 15 minutes with 60% A and 40% B and a flow of .4 ml/min. A 10 minute gradient transition then brought solvent B to 100% — and it was held for 6 minutes. For one minute, then, solvent B was brought down to 40% and solvent A was brought to 60%. Following, the column was washed for 5 minutes. For the PDA, the differential absorbance was set at 450nm/550nm to detect the carotenoids. For the MS, the following settings were used – Corona 5uA, Endplate -2000 Volts, Cap Entrance -3000 Volts, Drying Gas Flow 5.0, Drying Gas Heater 300°C, Auxilary Gas 25, APCI Heater 200°C, Capillary Ext 100 Volts, Skimmer 25.

2.3. *Benchmark for comparison.*

Our ‘gold-standard’, or benchmark, for biomass and lipid productivity comparison of newly isolated strains was the species *Nannochloropsis salina* strain CCMP1776. This strain was used by the National Alliance for Advanced Biofuels and Bioproducts [46, 54] in outdoor cultivation trials in raceway ponds [46]. It had also previously been evaluated in outdoor photobioreactors [55].

173 2.4. *DNA Extraction, polymerase chain reaction (PCR), and sequencing.*

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175 A 50 ml culture in the light-limited growth phase was concentrated by centrifugation,
176 and then the DNA of 1 ml of the concentrated liquid sample was extracted using a MO-
177 BIO DNA Isolation Kit (USA). The ITS2 rDNA region was amplified using the universal
178 primers ITS3 (GCATCGATGAAGAACGCAGC) and ITS4
179 (TCCTCCGCTTATTGATATGC) [56]. GoTaq® (Promega) master mix was used as the
180 PCR reaction mixture. We performed 30 cycles (1 minute 95°C, 1 minute 48°C, and 1
181 minute at 72°C – with an initial denaturing step of 5 minutes at 95°C). In cases where,
182 even with the ITS2 barcode, the identity of the species was still not clear, the entire 18S,
183 ITS1, 5.8S, and ITS2 regions were also amplified using universal primers AL1500af
184 (GCGCGCTACACTGATGC) and LR 1850 (CCTCACGGTACTTGTTC). In these
185 instances, 30 cycles (30 seconds 95°C, 30 seconds 48°C, and 1.5 minutes at 72°C – with
186 an initial denaturing step of 2 minutes at 95°C) were performed. PCR products were then
187 purified and sequenced by Genewiz (www.genewiz.com). For one of these organisms,
188 DOE0101, the DNA was also extracted using a DNeasy Plant Maxi Kit (Qiagen), and the
189 genome was then sequenced using Illumina technology at Los Alamos National
190 Laboratory, NM.

191
192 2.5. *Alignment and phylogenetic analysis.*

193
194 All 28 ITS2 sequences obtained from our lab samples were delimited and cropped
195 with the hidden Markov model (HMM)-based annotation tool present at the ITS2

database [57] (E-Value <0.001, Viridiplantae HMMs). In addition to the 28 ITS2 sequences from our top performing strains, we also downloaded ~50 algae ITS2 sequences from the ITS2 database (<http://its2.bioapps.biozentrum.uni-wuerzburg.de/>). As the sequences were to be used to help determine the taxonomic position of our strains, sequences from algae branches were chosen that NCBI blast analyses suggested were close to our specimens – within the Chlorophyceae and Trebouxiophyceae clades. From the Trebouxiophyceae, the Chlorellales order seemed particularly well represented. Within the Chlorophyceae, the Sphaeropleales and Chlamydomonadales orders were well represented. Since one of our strains appeared to be an *Ankistrodesmus*, yet no sequences of that genus were available in the ITS2 database, an *Ankistrodesmus* sequence was also downloaded from NCBI and cropped using the aforementioned model. As outgroups, two species from the prasinophytes (*Mantoniella squamata* and *Micromonas pusilla*) were chosen. *Mantoniella squamata* has been used previously to root phylogenetic trees showing the evolution relationship of chlorophytes based on SSU rRNA sequence data [58]. *Micromonas pusilla* is a well characterized prasinophyte whose complete genome had been sequenced [59].

The cropped sequences and structures were then automatically aligned with 4SALE 1.7 using an ITS2-specific scoring matrix for sequences and structures [60, 61]. To find simultaneously the evolutionary distance between organisms according to sequence and secondary structures, profile neighbor joining (PNJ) was applied using ProfDistS 0.9.9 (Qt-Version) [62, 63]. An ITS2-specific general time-reversible substitution model was applied [60]. The resulting tree was displayed with FigTree (<http://tree.bio.ed.ac.uk/>) and further processed in R (<http://cran.r-project.org/>) using Ape [64].

The 18S, ITS1, 5.8S, ITS2, and 28S sequences were aligned with ClustalW and reconstructed using Maximum Likelihood with a Tamura-Nei Model and Nearest-Neighbor-Interchange (NNI) heuristic method, with 500 bootstrap replications performed. Significant bootstrap values are based on 500 replicates and mapped to the appropriate internode. Branch lengths were drawn proportional to inferred changes.

Most of the strains described in this study were deposited at the UTEX Culture Collection of Algae (<http://web.biosci.utexas.edu/>), and all sequences generated were also deposited at the National Center for Biotechnology Information (NCBI) [65].

2.6. Secondary Structure Prediction.

The structures of most of the sequences downloaded from the ITS2 database were determined either by direct-fold or homology modeling within the ITS2 database [41, 66, 67]. The secondary structures of most of the sequences from our strains were also determined by the same homology model. For four of our sequences, that of DOE0101, EN1423, DOE0259, and the *Ankistrodesmus* sequences from NCBI – because no related organisms were found in the ITS2 database – RNAstructure 5.6 was used to predict the ITS2 structure [68].

3. Results and discussion

The focus of our manuscript is on the phylogeny and characterization of some of the most promising strains that were isolated in the course of our phyco-prospecting work for

the NAABB consortium [46]. Nevertheless, the first part of this section provides an overview of the phyco-prospecting project.

3.1. Summary of the NAABB phyco-prospecting project.

The multi-year strain isolation and screening process, which yielded a variety of novel strains, consisted of multiple stages [47]. For a better understanding of the results, the overall approach that resulted in potential future platform strains is discussed below:

1. A dual spatial and temporal sampling strategy was employed. Sampling sites represented very diverse, mostly aqueous but also several terrestrial environments, with an emphasis on the southwestern United States, because the southwestern states were viewed as the most promising potential sites for algal mass cultivation [9]. Many shallow and temporal water bodies were sampled, because it was anticipated that future use of strains would include raceway-type ponds.
2. Aliquots of environmental samples were plated onto different agar-solidified media in Petri dishes. The rationale for this first step was that different types of algae could be easily distinguished by colony color and size. Often microscopic examination of colonies and its cells allowed a broad taxonomic categorization of potential isolates. Furthermore, introduction of this first step allowed for the determination of isolates that could grow on agar-solidified media. This was viewed as important for future strain development, which might be done in high-throughput applications [69].

3. Transfer of colonies onto index plates was done with the rationale that more biomass could be used for inoculation of liquid medium, which reduced the lag-phase of the liquid cultures, thus speeding up the isolation process.
4. Transfer into liquid medium was done because the cell sorter could only process algal cells provided in liquid. This had the additional benefit of demonstrating that the cells did indeed grow in liquid culture, which was important as later applications were anticipated to involve cultivation in open ponds or photobioreactors.
5. FACS cell sorting onto Petri dishes was applied with the rationale to provide a high-throughput method to obtain a large number of unialgal strains rapidly. Sometimes new strains were also already axenic following cell sorting onto plates. Sorting into liquid wells did not prove as efficient, because determination of potentially unialgal strains was more difficult in liquid medium.
6. Unialgal strains were then transferred into storage tubes consisting of agar-solidified medium overlaid with liquid medium. This storage method was chosen because all strains could be maintained in this manner. Also, we found that strains in agar/liquid-overlay cultures under a 12 h light/dark regime could survive without transfer for up to three years.

Figure 1 summarizes our phyco-prospecting efforts for the NAABB program. Within the isolation and screening project, several bottlenecks were encountered at different levels due to the labor-intensive steps involved. Out of our total of 2,465 isolated NAABB strains, only 1,575 strains could be subjected to a first-level screen, of which 334 passed as candidates for potential use as future biofuel/nutraceutical platform species. Subsequently, from the 334 candidate strains only 200 strains could be tested in a

second-level screen to determine the optimal growth medium. In the following third-level screen only 110 strains could be examined for actual biomass and lipid productivities in bubbling columns. Of the 110 strains tested in bubbling columns, more than 30 freshwater strains outperformed our ‘gold’ standard, *Nannochloropsis salina*, in terms of growth rate and final biomass yield. In addition to having high growth rates, several of our best performing strains also accumulated carotenoids in cytosolic oil bodies.

Figure 1: Schematic summarizing the overall phyco-prospecting process performed in our laboratory as part of the NAABB Consortium to identify the best performing microalgae strains for biofuel production.

Overall, 30 strains were deposited with the UTEX culture collection. Currently, these strains are not in the main UTEX collection, but they are in a special collection. Out of these 30 deposited strains, general information on 25 strains is contained in Table 1. Most strains were isolated from samples collected in summer. Three strains came out of fall samples (DOE0686, DOE0717, DOE1357) and strain DOE1135 originated from a February sample. Additionally, Table 1 includes information on three strains from a previous phyco-prospecting effort.

Strain Number	Taxon	State	Latitude	Longitude	Habitat	NCBI Accession
DOE0013	<i>Scenedesmus</i>	CA	37.97	-122.56	Fountain	KJ434964
DOE0043	<i>Desmodesmus</i>	CA	37.9	-122.25	Lake	KJ434974
DOE0088	<i>Coelastrrella</i>	CA	37.96	-122.34	Birdbath	KJ43972
DOE0101*	Chlamydomonadales	TX	29.92	-95.82	Roadside Mud	KJ434990
DOE0111	<i>Scenedesmus obliquus</i>	CA	37.96	-122.34	Water from a Garden Pot	KJ439665
DOE0152*	<i>Scenedesmus obliquus</i>	NY	40.65	-74.01	Water from a Turtle Tank	KJ434966
DOE0155	<i>Coelastrrella</i>	TX	29.84	-95.87	Bayou	KJ434970
DOE0202*	<i>Coelastrrella</i>	CA	37.96	-122.34	Water from a Garden Pot	KJ434971
DOE0259	<i>Ankistrodesmus</i>	TX	29.87	-95.81	Roadside Ditch	KJ434991
DOE0314	<i>Dichloster-related</i>	TX	29.87	-95.81	Roadside Ditch	KJ434977
DOE0369	<i>Coelastrrella</i>	CA	38.89	-121.97	Fountain	KJ43967
DOE0623	Chlorellaceae	TX	29.77	-95.62	Bayou	KJ434983
DOE0686	<i>Chlorella sorokiniana</i>	CT	41.6	-72.7	Greenhouse Hydroponics	KJ434981
DOE0700	Chlorellaceae	NM	32.31	-106.78	Wastewater Pond	KJ434979
DOE0717	Chlorellaceae	CT	41.6	-72.7	Greenhouse Hydroponics	KJ434986
DOE1041	Chlorellaceae	TX	30.29	-97.84	Roadside Ditch	KJ434978
DOE1044	Chlorellaceae	TX	30.78	-95.95	Unknown	KJ434987
DOE1051	<i>Desmodesmus</i>	TX	30.04	-101.2	Ditch	KJ434975
DOE1070	Chlorellaceae	TX	27.71	-97.18	Bay	KJ434985
DOE1095	Chlorellaceae	TX	29.01	-95.22	Estuary	KJ434984
DOE1116	<i>Chlorella sorokiniana</i>	TX	29.28	-94.83	Estuary	KJ434982

DOE1135	Chlorellaceae	TX	27.71	-97.18	Bay	KJ434988
DOE1357	<i>Desmodesmus</i>	TX	30.36	40.63	Lake	KJ43976
DOE1412*	<i>Chlorella sorokiniana</i>	NY	40.63	-73.95	Culture Contaminant	KJ434980
DOE1418	<i>Desmodesmus</i>	IL	39.5	-88.18	Lagoon	KJ34973
EN1234	<i>Coelastrella</i>	CA	32.77	-117.19	Bird Bath	KJ434969
EN1235	<i>Coelastrella</i>	NY	Unknown		Bird Bath	KJ43968
EN1423	<i>Borodinellopsis texensis</i>	NM	34.34	-105.57	Roadside Saline Soil	KJ434989

Table 1: Listed are the top performing strains that were classified in the work reported here. Included in the table are the strain numbers, taxon, and location along with the habitat of collection. BG11 was the best growth media for all strains in the list. Strains with the ID DOE##### are from the NAABB phycoprospecting project. All these strains have been deposited at the University of Texas Culture Collection of Algae and are available to researchers. Strains with the ID EN##### resulted from an Airforce Office of Science funded phyco-prospecting effort. The asterisk next to Strain ID numbers indicates that the strain was tested successfully in outdoor ponds at NAABB testbed sites [46].

3.2. Growth evaluation of strains.

Following sampling and strain isolation a multi-level screen was applied to identify potential lipid producers. The first-level screen evaluated growth by optical density changes and lipid productivity using Nile Red fluorescence [47]. The second-level screen evaluated performance of strains in different growth media [47]. In the third-level screen strains were cultivated in bubbling columns [47] and biomass productivities and lipid levels were determined gravimetrically.

For most strains the third-level screen only consisted of one culture having been tested. Only for a few selected groups of strains was more than one culture grown. Figure 2 shows growth curves of selected groups of the best performing microalgal strains identified: what our phylogenetic analysis would classify as *Chlamydomonadales* strain

DOE0101, *Chlorella sorokiniana* strain DOE1412, three novel *Coleastrella* sp., and two strains of the species *Scenedesmus obliquus* - also known as *Acutodesmus obliquus* [34]. Because all bubbling column cultures were inoculated from pre-cultures that were in the light-limited growth phase, the lag-phase for all the bubbling column cultures were usually very short. For all strains shown in Figure 2 the exponential growth phase was followed, between about 50 to 100 hours, by the light-limited growth phase. During the light-limited growth phase the biomass productivity of the best strains identified was between 0.6 to 0.9 g AFDW l⁻¹ d⁻¹ (Figure 2). In comparison, *N. salina*, our ‘standard’, only produced about 0.6 g AFDW l⁻¹ d⁻¹. A recent literature review discussed the difficulties of making rigorous comparisons between different algae growth experiments because of variations in culture conditions [70]. Nevertheless, the biomass productivities of our strains were also within the range of the reviewed most promising species of algae for lipid productivity [70].

Figure 2: Shown are growth curves based on the AFDW (grams/liter) of some of the most productive strains. Biomass productivities are given for the light-limited growth phase between 50-100 hours. At least two biological replicates were included for each strain. Shading represents the 95% confidence region for the regression fit of the biological replicates.

For the data shown in Figure 2, for *S. obliquus* the growth curve shown represents

two strains DOE0111 and DOE0152. The growth curve of *Coelastrella* sp. represents the summary results of three independently isolated strains DOE0088, DOE0202, and DOE0369. Most of our tested strains reached the stationary growth phase with total biomass yields of sometimes more than 4.0 g AFDW L⁻¹. Noteworthy is that at about 200 hours, when the batch cultures were terminated, the strains of the *Coelastrella* had still not reached the stationary growth phase. At that time, the biomass yield was about 4.5 g AFDW L⁻¹. When grown for a longer time period, the biomass yields were sometimes upwards of 6.0 g AFDW L⁻¹ and the cultures had still not reached the stationary phase.

When in the stationary growth phase at 180 hours the strains had accumulated the following amounts of total lipids as part of their biomass: *Chlamydomonadales* sp. strain DOE0101 (25%); *Chlorella sorokiniana* DOE1412 (25%); *Coelastrella* sp. strain DOE0088 (17%) and strain DOE0202 (23%); *S. obliquus* strain DOE0111 (29%) and strain DOE0152 (28%). It should be noted that due to their thick cell walls it did not appear that all lipids could be extracted from cells of the *Coelastrella* strains, as the remaining biomass in the extraction was often still green. Thus, total lipid contents was likely underestimated. Overall, the total lipid productivities of all strains shown in Figure 2 were similar at about 0.2 g lipids L⁻¹ d⁻¹.

Although growing slower, the lipid content of *N. salina* could reach over 30% lipids within the five days in batch culture, thus ultimately also reaching about 0.2 g total lipid L⁻¹ day⁻¹. However, compared to *N. salina*, our top strains had shorter lag-phases and higher daily biomass productivities. This is important, as the latest biofuels applications aim at use of hydrothermal liquefaction technologies (HLT), which convert biomass into crude oils rather than extracting lipids only [71, 72, 73, 74]. Consequently, rather than

technologies that are dependent on the lipid content of algal cells, Hydrothermal liquefaction technologies can rely on fast growing, biomass-accumulating strains to increase higher overall crude oil productivities.

3.3. Characterization of carotenogenic capabilities of selected strains.

When cultivated in shaker flasks during the first-level screening process, it was noticed that a variety of strains over-accumulated secondary carotenoids in cytosolic lipid bodies [47] similar to *Haematococcus pluvialis* [75, 76]. All *Coelastrella* strains listed in Table 1, the strain DOE0101 belonging to the Chlamydomonadales, and *Borodinellopsis texensis* strain EN1423 displayed such carotenogenic capability. This over-accumulation of secondary carotenoids, including Astaxanthin, may be of value for future biotechnological application in carotenoid production. This is especially the case for DOE0101 and DOE0202, as both strains were successfully cultivated in outdoor ponds at the NAABB testbed site in Pecos, TX [46]. Therefore, these two strains were further characterized. As the secondary carotenoids accumulate in cytosolic oil bodies, the carotenoid and triacylglycerol (TAG) profiles were compared for both strains for actively growing (green) cultures and carotenogenic (orange) cultures (Figure 3). In the center, Figure 3 displays exemplary UHPLC chromatogram traces of extracted pigments. Peaks shown in the UHPLC chromatogram traces are either carotenoids or chlorophylls. Identification of carotenoids was done based on their absorbance spectra and based on their molecular mass (<http://metlin.scripps.edu/index.php>). Nevertheless, for some of the peaks, identifying the molecules as one carotenoid rather than another could not be done,

as they were either stereoisomers or different ions with the same mass. However, carotenoids of pharmaceutical relevance are present in these algae. As indicated by the orange color of cells and cultures, the three strains investigated contained different carotenoids such as Peak 1 with Adonirubin or Astaxanthin, Peak 2 with Lutein or Zeaxanthin, Peak 3 with Canthaxanthin or Adonixanthin, Peak 4 with Echinenone or Cryptoxanthin. and minor amounts of Astaxanthin diesters (Peaks at later retention times). In brief, strain DOE0101 seemed to have a distinctly different profile with a particularly high accumulation of either canthaxanthin or adinoxanthin when compared to the *Coelastrella* species strain DOE0202 (Figure 3).

Growing Cultures

Stressed Cultures

Figure 3: Compared are cells and metabolites of the *Chlamydomonadales* sp. strain DOE0101 and the *Coelastrella* sp. strain DOE0202. The upper panel shows EIC traces obtained by Mass Spectrometry representing Triacylglycerol (TAG) profiles. The lower panel displays PDA profiles (absorbance traces at 450 nm) of extracts from cells of actively growing cultures and of carotenogenic (orange) cultures. Photos for representative cells from these cultures are shown for both strains. The black bars underneath the cells of strain DOE0101 indicate 10µm. The same scale was used for photos of cells from strain DOE0202. Extractions were conducted from the same volume of cells for cultures of the same age (SN-3).

Figure 3 also displays profiles of overlaid Extracted Ion Counts (EIC) traces for a variety of triacylglycerol molecules identified for both strains. The individual EIC traces were obtained by UHPLC separation followed by APCI-TOF-MS detection of TAG molecules from lipids extracted from algal strains. Each EIC peak represents a different specific TAG molecule [77]. All strains investigated contained dozens of different molecular classes of TAGs (SN-4). Superficially, under the light microscope the cells look somewhat similar (Figure 3). Nevertheless, stressed cells of DOE0101 have comparatively very little C₁₆-C₁₆-C₁₈ TAGs, and have mostly saturated C₁₆-C₁₈-C₁₈ molecules. In contrast, cells of the *Coelastrella* species have more C₁₆-C₁₆-C₁₈ TAGs. Also, its C₁₆-C₁₈-C₁₈ s are weighted, if anything, toward the unsaturated molecules (Figure 3). These differences among the cells of the two species may be due to the different amounts of the various secondary carotenoids, in the two strains, that are sequestered in the oil bodies. Investigating this further is of pharmaceutical/cosmetic relevance, but is beyond the scope of this work.

3.4. Strain classification.

For the phylogenetic analysis, rDNA ITS2 was used as the molecular marker because both its sequence and secondary structure can be used for classification, and the presences of many other ITS2 sequences in the ITS2 database (<http://its2.bioapps.biozentrum.uni-wuerzburg.de/>) [33, 78, 79, 80]. Sequences of 49 previously identified strains of Chlorophytes and three strains from a previous phycoprospecting project were compared with sequences of 25 of our most promising strains (Figure 4).

Figure 4: ITS2 structure-aligned sequence tree of novel strains for biofuel production and also known strains of the Chlorophyta – rooted with two prasinophyceae. Significant bootstrap values (>50%) are based on 500 replicates and mapped to the appropriate internode. Unlabeled internal branches are not significant (<50% bootstrap support). Branch lengths are drawn proportional to the number of nucleotide substitutions. Arrows point to strains discussed in this paper in more detail.

Within the ITS2 phylogenetic tree - including 25 newly isolated strains from the NAABB project, 3 previously isolated novel strains, and the 49 known strains – some clades were not resolved, because of low support in some internal branches (Figure 4). Nevertheless, the major branches were well supported in their monophyly, and for the known sequences from the ITS2 database, our phylogeny is consistent with the established relationship among the Chlorophyta [81]. Similarly, in the systematics of the major clade within our study, the Scenedesmaceae, there is low bootstrap support for the bases of the trees and only good support for the groups of species within genera [33, 34, 82]. Therefore, despite the presence of some low bootstrap values in our tree, that the known taxa fall in accordance to other published ITS1, ITS2, and 18S trees yields some confidence for the phylogenetic information revealed in the branches. Our investigation corroborates conclusions of other studies [83], which showed that sequence-structure analysis of ITS2 provides a taxon-rich means of testing phylogenetic hypothesis at high taxonomic levels.

In general, our best performing strains were coccoid green algae that either fell within the green algal order of the Sphaeropleales (Chlorophyceae), the Chlamydomonadales (Chlorophyceae), or the Chlorellales (Trebouxiophyceae) (Figure 4). Among the thousands of strains that were isolated preliminary microscopic investigation of many strains during the isolation and screening process demonstrated presence of flagellate,

coccoid, and even some filamentous strains of the green algae. In addition, many cyanobacteria, diatoms, and other algae (e.g. Eustigmatophytes) were identified based on microscopic examination of cells. Therefore, our overall isolation protocol did not per se discriminate against non-green algal species. This report focuses on the green algae, because among the best performing strains emerging from our multi-level screen the majority were coccoid green algal species. This may be because non-motile coccoid cells do not ‘waste’ resources on active movement, a process that consumes large amounts of energy. Moreover, coccoid cells cannot determine their location and may have a need to store high-energy containing molecules as reserve substances. In consequence, many coccoid species could have evolved to store energy in oil bodies, specifically under environmentally unfavorable conditions.

3.4.1. *Sphaeropleales* (Class of the *Chlorophyceae*)

With regard to the newly isolated strains that are predicted to be within the class of the *Chlorophyceae*, many strains belong to the order of the *Sphaeropleales* and the family of the *Scenedesmaceae*. Several of our top performing strains (DOE0013, DOE0111, DOE0152) are similar to strains identified in the ITS2 database as *S. obliquus*. Although the species *S. obliquus* is synonymous to *A. obliquus* [34], in this paper, we refer to it as *S. obliquus* to keep consistent with the papers we are comparing our results to. Cells of our newly isolated strains have the characteristic oblong shape and coenobia. As an example, we show cells of *S. obliquus* strain DOE0152 in Figure SM-1.

That several top performers independently isolated were strains of *S. obliquus* was not surprising. Strains of *S. obliquus* have been known to grow well in mass cultures –

even demonstrating potential in outdoor raceway ponds in the developing world [84, 85]. Strains of the genus *Scenedesmus* have been found to be able to accumulate lipids [86] even under colder temperatures such as 10°C [87], which is important as winter strains are needed for biofuel production. Other studies have found that *S. obliquus* has a high tolerance and growth rates under elevated levels of CO₂, suggesting that the species has potential to reduce CO₂ in the flue gases emitted by thermoelectric power plants [88].

In bubbling columns with 3% CO₂ at 25°C, biomass productivity for *S. obliquus* strains was about 1.0 g L⁻¹ d⁻¹ [89], and another report used bubbling column with 0.5% CO₂ at 25°C resulting in about 0.6 g L⁻¹ d⁻¹ [90]. Both reported productivities for bubbling columns were in our range of productivity results (Figure 2). Further, a comparison can be found in a study looking at eight microalgae from a total of 33 isolated cultures of water samples from freshwater rivers and livestock wastewater treatment plants at Wunju, South Korea [91]. One of the highest performers was a strain of *S. obliquus*. In that study, among multiple isolated *S. obliquus* strains, which were the same species according to LSU rDNAD1-D2 regions, significant differences in lipid productivity existed. Such different productivities within strains of a species from the same geographical region demonstrate that surveying culture collections may not be sufficient, as different strains of the same species may have different biomass or lipid productivities. Similarly, showing that different strains of the same species can have widely varied physiological parameters, some *S. obliquus* isolates have been found to have 3-4 times higher carbon fixation efficiencies than other *S. obliquus* isolates [92]. In agreement with these studies, our work also resulted in several independent strains of *S. obliquus*, that not

only grew well under laboratory conditions, but also outdoors even when compared to *N. salina* [46].

Another group of strains (DOE0088, DOE0155, DOE0202, DOE0369, EN1234, EN1235) nested closely together in a clade with *Coelastrella* sp. strain SAG217-5 [33, 93] being the sister group (Figure 5). To gain a better taxonomic overview, a second and specific phylogenetic analysis was performed including all publically available ITS2 sequences of *Coelastrella* strains from the NCBI database. Our results showed that our six novel strains fell into one clade together with other *Coelastrella* strains SAG2123 and SAG217-5 (Figure 5).

Figure 5: Un-rooted ITS2 sequence tree including our novel strains DOE0088, DOE0155, DOE0202, DOE0369, EN1234, EN1235, and also known strains of the Scenedesmaceae. Bootstrap values are based on 500 replicates and mapped to the internal branches. Branch lengths are drawn proportional to inferred nucleotide substitutions.

Figure 6: Light microscopic photos of cells of *Coelastrella* sp. strain DOE0202 (A-H). A) Several autosporangia containing daughter cells. B) Side view of vegetative, oval, coccoid cells with one cup-shaped chloroplast, which contains one pyrenoid. C) Cells stained with iodine show a ring of starch indicated by black color on the outside of the pyrenoid. Note the Scenedesmus-like coenobia of smaller cells that were released from the autosporangia. D) Larger sized vegetative cells with the hyaline cell wall. E) Daughter cells just released from an autosporangium. The hyaline cell wall of the mother cell is visible in the background. F) A variety of vegetative cells in different growth stages. G) An autosporangium is visible on the lower left side with cells that are in the carotenogenic process. H) Center view of two vegetative carotenogenic cells where protruberances are visible on the cell wall. I) Shown are two SEM images where striated cells ridges are visible along the oval cells. In addition, some connections are visible between ridges. The black bar represents 10 μ m for the light microscopic and the electron microscopic images. The SEM images were taken by Dr. Cooke at New Mexico State University.

Based on microscopic investigation, we found that the new *Coelastrella* strains had a broad range of coccoid cell morphologies. As one example, images of the strain

DOE0202 are presented in Figure 6. Flagellate cells were never observed regardless of growth conditions. Reproduction occurs through division of autospores, which release two to eight daughter cells by rupture of the parental cell wall. Depending on the growth conditions, cells were either solitary or found in coenobia. Only sometimes, during the exponential growth phase in batch cultures, coenobia were found that looked similar to coenobia of *Scenedesmus* species (Figure 6C). Solitary cells were round to ellipsoid and 3-10 μm long. When ellipsoid, cells often had polar thickenings. Cells contained one chloroplast with one pyrenoid. In growing cultures, vegetative cells were green. Resting cells from cultures in the stationary phase became first brown and then orange due to accumulation of orange colored oil bodies in the cytosol (Figure 6). Regardless of growth conditions, cell walls were hyaline. By light microscopy only sometimes cell walls had ridges appearing as protruberances (Figure 6H), but additional electron microscopy confirmed that all cells had ridges on their cell walls (Figure 6I). Nevertheless, there was variability in the appearance of the ridges. Such variability in the cell wall appearance and ridges is in agreement with the description of *Coelastrella* sp. strain SAG 217-5 [33], which according to our recovered ITS2 phylogenetic trees (Figures 4 & 5) is a close relative to our novel strains. Our novel strains cluster not only very closely together in the phylogenetic analysis, but they also have identical cell morphologies and physiological characteristics. Therefore, we assume that all our strains (DOE0088, DOE0155, DOE0202, DOE0369, EN1234, and EN1235) belong to the same species. Currently, not all species within the genus of *Coelastrella* are well defined and our strains fall into the *sensu lato* group [93]. Consequently, we refrain from providing species names for our novel strains, which will have to wait until the *Coelastrella* genus undergoes a closer

taxonomic reinvestigation.

Our newly isolated *Coelastrella* strains originated from different freshwater habitats from a variety of locations within the United States (Table 1), indicating that this *Coelastrella* species has a broad distribution within the United States. In their original habitats such as birdbaths, fountains, and temporary waterbodies (roadside ditches), the *Coelastrella* strains were often found co-existing with strains of the green alga *Haematococcus pluvialis*. Similar to *H. pluvialis*, our new isolates were also carotenogenic and formed spores that survived desiccation. To the best of our knowledge, this is the first description of *Coelastrella* strains isolated from the United States. Thus, all these *Coelastrella* isolates represent novel strains, possibly of a new species. Of note is that all of these novel *Coelastrella* strains performed not only well in the laboratory, but strain DOE0202 also tested positively in small raceway-type ponds [46], indicating that – similar to *H. pluvialis* – the new *Coelastrella* strains might be employed as new platform strains for biofuels and/or bioproducts generation.

Several of the well performing strains (DOE1418, DOE1357, DOE1051) appear to be of the *Desmodesmus* genus. This was confirmed by the strains cell morphology (Figure SM-2) as oval cells also exhibit the characteristic spines at the ends of their coenobia [80]. For the genus of *Desmodesmus*, detailed species level studies comparing morphology and ITS2 rDNA phylogenies are lacking to date [80], except for an account on four closely related species bearing lateral spines [39]. Based on us finding multiple well performing strains belonging to the *Desmodesmus* genus, we hypothesize that species of this genus may be excellent platforms for biofuel production. However, proving this hypothesis would require further large-scale testing in mass culture, which is

beyond the scope of this work.

The strain DOE0259 falls into one clade with *Ankistrodesmus* sp. SP2-15 (Figure 7), although quite some evolutionary distance exists between the two. Currently, only three other *Ankistrodesmus* strains besides DOE0259 have their ITS2 region sequences in the NCBI database. All three strains showed similar far distances from our newly isolated strain, so only one was included in the tree shown in Figure 4. Extending a search in the NCBI database with the 3' end of the 18S, ITS1, 5.8S, and ITS2 sequences from DOE0259 (Genebank Accession Number KT274017) also identified this strain as an *Ankistrodesmus*, with it sharing the highest percent identity (99%) with *Ankistrodesmus* RS-2012, although with low query coverage (59%). Lack of exact matches for the 18S gene, the ITS1 spacer, the 5.8S gene, or the ITS1 spacer for strain DOE0259 suggests that it may represent a new species. Unfortunately, the genus of *Ankistrodesmus* is polyphyletic [94, 95, 96] as there has been difficulty discerning the different crescent-shaped morphological features of species, which are highly variable and challenging to identify by light microscopy. Figure 7 shows images of cells of the strain DOE0259. The crescent-shaped cells were always solitary. The basic cell morphology is coccoid with cells being elongated sickle-shaped having rounded ends (Figure 7). Due to the known uncertainty regarding the taxonomic position of species within the genus *Ankistrodesmus* and the family of the Selenastraceae in general [94, 95], any further classification of strain DOE0259 was beyond the scope of this manuscript.

Figure 7: Shown are images of cells of the novel *Ankistrodesmus* strain DOE0259. Some cells have oil bodies that are visible as blue roundish structures in the cytosol. The black bar represents 10 μm . A) Two cells accumulating numerous oil bodies in the cytosol. B) Images of several cells illustrating the plasticity in cell morphology. C) Single cell. D) Two cells in the early division process. E) Two cells released from an autosporangium. F) Four daughter cells still in the autosporangium. G) Four cells released from the autosporangium.

3.4.2. *Chlamydomonadales* (Class of the *Chlorophyceae*)

Two taxa, strain DOE0101 and strain EN1423, fell into the *Chlamydomonadales* order (Figure 4). Strain EN1423 was isolated from hyper-saline soil from a roadside ditch close to the Laguna Del Perro in New Mexico, USA (Table 1). Originally the strain was maintained in a 0.5 M NaCl containing artificial *Dunaliella* medium [97] and also tested for biomass productivity in bubbling columns. However, in the saline medium large cell clumps developed that stuck to the glass vessel, thus preventing accurate productivity

determination. Later the strain was transferred to BG11 freshwater medium [51] and found to grow well in that medium.

As the ITS2 sequence alone could not provide an accurate species determination for EN1423, the rDNA sequence containing the 3' end of the 18S gene, ITS1, 5.8S gene, ITS2, and the 5' end of the 28S gene (Accession Number KT274016) was used for a BLASTn search into the NCBI database. This search resulted in a hit with 100% identity for our partial 18S gene sequence to the species *Borodinellopsis texensis* (NCBI Accession number KM020129). In addition to the molecular marker sequence, the cell morphology and physiology of strain EN1423 was investigated and found to match the description of *B. texensis* (Figure SM-3) [98]. To our knowledge, as based on its lipid over-accumulation characteristics, this is the first report of this species having potential as a platform strain for biofuel generation.

While strain DOE0101 may not have been the very best biomass producer in the laboratory (see section 3.2. above), this strain was highly carotenogenic (see section 3.3. above) and it was successfully tested in small raceway-type ponds [46]. Classification of strain DOE0101 to the species or genus level remained challenging. From our genome sequences (unpublished) of the strain DOE0101, we located the 18S gene (NCBI Accession number AJ249515), the 28S gene (NCBI Accession number KC145458), the *psaB* gene (NCBI Accession number JN63055), and the *rbcl* sequence (NCBI Accession number KC145509). For each of these four molecular markers, searches within the NCBI database showed the best hits to be *Dysmorphococcus globosus* (SM 1), which is in the Chlamydomonadales order, but it has no ITS2 sequence available. Although the sequence identity was high, for DOE0101's *psaB* and *rbcl* genes, the query coverage with *D.*

globosus was only 85 and 86 %, respectively, indicating that strain DOE0101 is not a close relative. Also, in contrast to the flagellate *D. globosus*, cells of strain DOE0101 were always coccoid (Figure 8). Therefore, though the ITS2 sequence-structure phylogeny places DOE0101 within the Chlamydomonadales, even use of further molecular marker sequence could not improve classification, because not enough sequences are available from public databases.

Figure 8: Shown are images of cells of the strain DOE0101 indicating the variety of cell types. The bar indicates 10 μm . A) Vegetative, coccoid cell from an actively growing culture in the beginning stages of division of the pyrenoid. B) Several cells each containing one cup-shaped chloroplast and one pyrenoid per chloroplast. C) One large cell in the beginning phase of carotenogenesis and one autosporangium. D) Single cells with orange oil bodies. E) Orange cells with large oil bodies and unknown white appearing structures. F) Two autosporangia originating from orange cells.

In summary, while *Borodinellopsis texensis* strain EN1423 and the Chlamydomonadales strain DOE0101 are in the same order as the species *H. pluvialis*, in contrast to flagellate green cells of *H. pluvialis*, non-stressed green cells of the novel strains were always coccoid under our growth conditions. Also in contrast to *H. pluvialis*, which is well-known for application in mass production of the brick-red carotenoid astaxanthin, cells of DOE0101 appear orange under stress (Figure 3 & 8) with the orange pigments in DOE0101 having been identified as precursors of astaxanthin (Figure 3). As the strains EN1423 and DOE0101 accumulate secondary carotenoids, both strains may find further use in future applications for carotenoid production.

3.4.3. *Chlorellales* (Class of the *Trebouxiophyceae*)

Green algae with the spherical morphotype named *Chlorella* belonging to the class of the Trebouxiophyceae have traditionally been used as model organisms for studies of photosynthesis and biotechnological applications for decades [99]. As shown in Figure 4, many of our top performing strains clustered within the class of the Trebouxiophyceae. However, the taxonomic status of many of the new strains within the order of the Chlorellales was not too well resolved when the rDNA ITS2 marker was used (Figure 4). The best support for identification was obtained for strain DOE1135, which appears to be closely related to *Chlorella vulgaris*. DOE0314 falls with *Dicloster acutus*, though a significant distance exists between the two. The most internal branches of the other new strains were also not well supported, but in the terminal parts of the phylogenetic tree they clearly nested into highly supported clades. As the phylogeny based only on the rDNA ITS 2 spacer region was not providing sufficient information regarding the

taxonomic position at the genus or even species level, an additional molecular comparison was performed using the rDNA region including the 3' end of the 18s gene, the ITS1 region, the 5.8s gene, the ITS2 region, and 5' end of the 28s gene [100]. The resulting phylogenetic tree (Figure 9) allowed classification of the strain DOE1412 as *C. sorokiniana*.

Figure 9: Phylogenetic tree based on the rDNA region including the 3' end of the 18s gene, the ITS1 region, the 5.8s gene, the ITS2 region, and 5' end of the 28s gene. The sequences for the strains *Chlorella sorokiniana* UTEX 1230 and *Chlorella sorokiniana* LANL 1228 were kindly provided by Dr. Starkenburg.

In the past few years, the classification of coccoid taxa within the Chlorellaceae including the genus of *Chlorella* has received a lot of attention and many species were reclassified [96, 99, 100, 101, 102]. Molecular and morphological characters together are used for delineation of coccoid species [96, 100]. *C. sorokiniana* strain DOE1412 is of unique interest, because of its high productivity, and is the subject of research in several major laboratories. As a reference, we included images of cells of in Figure 10 below.

Figure 10: Shown are photos of cells of *Chlorella sorokiniana* strain DOE1412. The black bar represents 10 μm . A) Autosporangia. B) Some autosporangia and several cells in the early stages of cell division. C) Several larger cells with the pyrenoid clearly visible. D) Several autosporangia and one larger cell. One autosporangium and an opened autosporangium with released daughter cells. E) Larger cells with the pyrenoids clearly visible within the chloroplast. F) Larger cells with the pyrenoids clearly visible within the chloroplast.

Although strain DOE1412 is not a representative of a new species and concern about redundancies regarding already existing strains in culture collections may exist, the first analysis of a draft genome of strain DOE1412 revealed that its genome contains significant differences to two other *C. sorokiniana* strains UTEX1230 and LANL1228 (Starkenburger, personal communication). Therefore, it can be concluded that the new isolate strain DOE1412 greatly expands the germ plasma of the species *C. sorokiniana*. Comparative genomic work is ongoing and the analysis of the results will be published separately.

In addition to determining its biomass productivity, the fatty acid profile for *C.*

sorokiniana DOE1412 was obtained and analyzed for a culture grown in a photobioreactor (Figure SM-6). Cells of *C. sorokiniana* had C16:0 and C18:1 fatty acids as the main constituents, which is in line with a previous report about *C. sorokiniana* strains having long-straight chain alkanes and fatty alcohols being major compounds of extracts [103].

Within the Chlorellales clade, *C. sorokiniana* DOE01412 proved to be one of the best growers of the first strains isolated during the NAABB project. It is of note that *C. sorokiniana* strain DOE1412 was found to grow well in LED-lighted 800L indoor raceway ponds [104]. When tested outdoors, it grew very well, significantly better than even gold standard *N. salina*. In 23,000 L NAABB outdoor raceway ponds with paddle wheels, it had a maximum productivity of 30g/m²/day, tolerating temperatures from 40°-110°F, withstanding a range of salinities, and accumulating about 25% lipids. The strain also fared well in economical models, and oil from it was successfully converted to jet fuel. Consequently, *C. sorokiniana* strain DOE1412 was brought forward as a top producer, and its discovery was cited as a major deliverable for the NAABB project [46].

Like *Scenedesmus*, *Chlorella* strains have also been of interest as biofuel feedstocks. For example, *C. sorokiniana* is regarded as one of the most promising algae feedstocks, because the algae can grow in autotrophic, heterotrophic, and mixotrophic conditions [105]. Our new strains offer an expansion of the genetic resources available to create even more productive algae from this clade.

4. Conclusions

This work characterized and classified the top algal strains to come out of a multi-year screening effort to find algal strains suitable for further development as biofuel feedstocks. Thirty of the best performing strains were deposited with the UTEX algal culture collection. The project successfully isolated a variety of strains, which showed potential as future platform strains. Several novel strains belonging to the *Coelastrella* genus were found, which are reported on for the first time for the United States. In addition, one new strain designated as DOE0101 was from the Chlamydomonadales. Both, the Chlamydomonadales strain DOE0101 and all the *Coelastrella* strains, accumulate carotenoids and may become platform strains for future carotenoid production. That these novel strains appear previously uncharacterized in a biofuel context despite their high growth rates and carotenoid-rich profiles demonstrates the untapped diversity of the green microalgae. In addition, several new strains of species already known to be fast growers were isolated, thus validating our overall phyco-prospecting approach. These strains of known species such as *S. obliquus*, now offer an expansion of the genomic resources available for these clades. Lastly, the discovery of *C. sorokiniana* strain DOE1412 is particularly noteworthy as it is currently one of the most promising algal species being developed for biofuels applications. Understanding the evolution of the highly productive strains isolated in this study and characterizing their metabolic and growth traits is a key step in bringing forth cultivars of microalgae for biofuel, and high-value compound, production.

Contributions

J.P. designed and oversaw the entire isolation and screening project. P.N., J.P., and A.H. conceived and wrote the paper. A.H., W.C, and F.J. conducted the screening, P.N., J.P., S.T., and F.J., conducted the lipid analysis, and P.N., J.P., K.S., and Q.W. conducted the phylogenetic analysis. Light microscopy for many strains was performed by A.G. and the Transmission Electron Micrograph for *Coelastrella* strain DOE0202 was provided by O.H. The fatty acid profile for *C. sorokiniana* strain DOE1412 was provided by S.T. Together, J.P. (jpolle@brooklyn.cuny.edu) and P.N. (pneofotis@gc.cuny.edu) declare the integrity of this work as a whole.

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