Comparison of biomass and lipid production under ambient carbon dioxide vigorous aeration and 3 % carbon dioxide condition among the lead candidate *Chlorella* strains screened by various photobioreactor scales

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19 **Abstract**

20 *Chlorella* species from the UTEX collection, classified by rDNA-based phylogenetic analysis,
21 were screened based on biomass and lipid production in different scales and modes of culture.
22 The lead candidate strains of *C. sorokiniana* UTEX 1230 and *C. vulgaris* UTEX 395 and 259
23 were compared between conditions of vigorous aeration with filtered atmospheric air and 3 %
24 CO₂ shake-flask cultivation. The biomass of UTEX 1230 produced 2 times higher at 652 mg L⁻¹
25 dry weight under both ambient CO₂ vigorous aeration and 3 % CO₂ conditions, while UTEX 395
26 and 259 under 3 % CO₂ increased to 3 times higher at 863 mg L⁻¹ dry weight than ambient CO₂
27 vigorous aeration. The triacylglycerol contents of UTEX 395 and 259 increased more than 30
28 times to 30 % dry weight with 3 % CO₂, indicating that additional CO₂ is essential for both
29 biomass and lipid accumulation in UTEX 395 and 259.

1. Introduction

Some microalgal species can produce biomass that is useful as a biofuel feedstock. The unicellular green microalgae of the *Chlorella* genus are potential candidates for biofuel (*e.g.*, biodiesel) production due to inherently high rates of biomass production and oil accumulation with minimal nutrient requirements (Bumbak et al., 2011). Most conventional fuels, including petroleum and diesel, contain aliphatic hydrocarbons that are chemically similar to the fatty acid components of triacylglycerol (TAG) (Hu et al., 2008). TAGs are neutral lipids consisting of a glycerol backbone esterified to three fatty acids. The fatty acid composition of *Chlorella* TAGs is similar to that observed in higher plants, with predominantly 16- and 18-carbon fatty acids (Durrett et al., 2008; Hu et al., 2008). Biodiesel refers to fatty acid components derived from trans-esterification of TAGs from renewable sources, such as soybean oil or animal fats. Algal oil is an attractive alternative to vegetable oils as a feedstock for biofuel production because its cultivation can be maintained on land that is unsuitable for conventional agriculture while yielding much higher amounts of oil per acre compared to conventional oilseed crops such as soybean (Dismukes et al., 2008; Greenwell et al., 2009). *Chlorella* cultivation will also be readily performed in industrial processes (Rodolfi et al., 2009; Schenk, 2008).

Chlorella produces substantial biomass under minimum nutrients while requiring nitrogen, phosphorus and other micronutrients in the presence of light and carbon dioxide (CO₂). Nutrient uptake from different manure-based anaerobic digested products and industrial effluents has been evaluated for production of biomass, lipids, carbohydrates and proteins in *Chlorella* species (Kobayashi et al., 2013; Singh et al., 2011). Production of biomass and lipid has been studied broadly under different media in the *Chlorella* species: *Chlorella vulgaris*, *C. protothecoides*, *C. sorokiniana*, *C. zofingiensis*, *C. pyrenoidosa*, *C. minutissima* and other

species. For example, the biomass and lipid contents in *C. vulgaris* UTEX 259 were reported as 250 mg L⁻¹ and 38 % dry weight (DW), respectively, in modified Bold's Basal Medium (BBM) (Liang et al., 2009) and the biomass and productivity of *C. protothecoides* UTEX 255 were 11.7 g L⁻¹ and 654 mg L⁻¹day⁻¹, respectively, under the 2.4 g L⁻¹ KNO₃ medium (Shen et al., 2010) which contains ten times higher nitrogen concentration than BBM. The biomass and lipid contents in *C. sorokiniana* were between 165-261 × 10⁶ cells ml⁻¹ (0.82 g L⁻¹) and 13 % DW respectively (Zheng et al., 2012) and those in *C. zofingiensis* (ATCC 30412) were 1.9 g L⁻¹ and 15 % DW, respectively, in Kuhl medium (Liu et al., 2011). In *C. pyrenoidosa* SJTU the contents of biomass and lipid were 1.6 g L⁻¹ and 24 % respectively in BG11 medium with 10 % CO₂ (Tang et al., 2011) and in *C. minutissima* UTEX 2219 they were at 21-140 × 10⁶ cells ml⁻¹ (61-726 mg L⁻¹) and 4-4.8 % DW, respectively, in Bold 3 N medium (Tang et al., 2012). Although much information regarding biomass and lipid production in different *Chlorella* species has been collected, comparative analyses are limited by lack of uniformity in growth conditions.

Many *Chlorella* strains have previously been compared by the rDNA-based phylogenetic analysis (Blanc et al., 2010; Rosenberg et al., 2013). In this study, fourteen *Chlorella* strains from *C. vulgaris*, *C. sorokiniana*, *C. kessleri*, *C. protothecoides* and *C. zofingiensis* species were selected by ribosomal DNA (rDNA)-based phylogeny comparing the distance with *C. variabilis* NC64A before biomass and lipid comparisons were pursued using a standard photoautotrophic growth medium (BBM). Considering the potential biofuel applications using genetically engineered *Chlorella* strains in the future, the candidate *Chlorella* strains have been compared by rDNA-based phylogenetic analysis (Rosenberg et al., 2013) to *C. variabilis* NC64A, whose genome has been elucidated (Blanc et al., 2010). In cases for related cultivars comparison of the

rDNA segment including internal transcribed spacers (ITS) regions among species and phylogenetic analysis are appropriate for understanding evolutionary proximity.

The production of biomass and lipids by these strains in BBM were compared using different types and volumes of photobioreactors (sterilized 1 or 3 L bioreactors, 80 L aquarium tanks and 80 L hanging-bags). BBM is a minimal nutrient media, which is appropriate to maintain the large-scale cultures like aquarium tanks and hanging-bags. In preliminary studies at the National Renewable Energy Laboratory (NREL), *C. vulgaris* UTEX 395 was found to have more biomass production than *C. sorokiniana* UTEX 1230 in BBM with 2 % CO₂ in 250 ml shake flask at 150 rpm and 1 L Roux bottle at 500 rpm under illumination at 200 μmol m⁻² s⁻¹ (unpublished data). Interestingly, UTEX 1230 at the University of Nebraska-Lincoln (UNL) has produced more biomass than UTEX 395 in BBM (pH 7.0) with vigorous aeration at a flow rate of 30 L min⁻¹ (0.38 vvm) containing atmospheric CO₂ (0.04 %) under fluorescence illumination at 200 μmol m⁻² s⁻¹. Furthermore, the transcriptome of *C. vulgaris* has been analyzed in nitrogen-replete and deplete conditions and high production of biomass and fatty acid methyl ester (FAME) lipid contents were reported at 8 × 10⁸ cells ml⁻¹ and 10 % DW, respectively, under the nitrogen-replete while at 5 × 10⁸ cells ml⁻¹ and 60 % DW under the nitrogen-deplete condition (Guarnieri et al., 2011). In an analogous study, the differential biomass production and lipid profiles of *C. sorokiniana* UTEX 1230 have been characterized during photoautotrophy and heterotrophy, yielding from 40 to 115 × 10⁶ cells ml⁻¹ and from 12 to 24 % DW of FAME TAG (Rosenberg et al., 2013). To compare the growth and lipid production between these and other *C. sorokiniana* and *C. vulgaris* strains, the rDNA sequences of each strain obtained from NREL and UNL were confirmed to be identical and the biomass and lipid production of the lead *Chlorella* candidates UTEX 1230, 395 and 259 were compared under the ambient CO₂ vigorous aeration

and 3 % CO₂ conditions at the same corresponding conditions at 25 ± 2 °C under the light intensity 200 μmol m⁻² s⁻¹ on the shaker at 150 rpm in BBM (pH 7.0). After screening based on biomass production and lipid content and composition, the most promising *Chlorella* strains and optimal carbon sources for biofuel application are discussed.

2. Materials and Methods

2.1. Algae strains and growth conditions

Samples of *C. vulgaris*, *C. sorokiniana*, *C. kessleri*, *C. protothecoides*, *C. fusca* var *vacuolata*, *C. minutissima* and *C. zofingiensis* strains were obtained from the Culture Collection of Algae at the University of Texas at Austin. *C. variabilis* NC64A and *C. sorokiniana* CS-01 were provided by Dr. James Van Etten at the University of Nebraska-Lincoln and Dr. Minxi Wan at Johns Hopkins University, respectively. All algae strains at UNL were transferred to Bold's Basal Media (BBM) (Bold, 1949) sterile agar plates containing 100 μg ml⁻¹ tetracycline and 10 μg ml⁻¹ ampicillin and grown at 25 °C under continuous illumination at 160 μmol m⁻² s⁻¹. The algae at NREL were maintained in BBM sterile agar plates containing 100 μg ml⁻¹ ampicillin. Liquid culture was initiated by inoculation of a single isolated colony into 5 ml of sterile BBM. The 5 ml cultures were shaken at 250 rpm for 7 days under continuous illumination (160 μmol m⁻² s⁻¹) at 25 °C.

2.2. rDNA-based phylogenetic analysis

Nucleic acids were extracted from clonal algal populations using a 5 % Chelex-100 solution, as described previously (Rosenberg et al., 2013; Wan et al., 2011). After boiling at 100 °C for 15 min, samples were centrifuged at $16,000 \times g$ for 2 min and DNA recovery was quantified using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Delaware, USA). The primer pair designed for this study (5'-ACTCCGCCGGCACCTTATGAG-3'; forward, 5'-CCGGTTCGCTCGCCGTTACTA-3'; reverse) was used to amplify the ITS region with the Top Taq Master Mix Kit (Qiagen, California, USA) according to the manufacturer's protocol employing thermocycler conditions with an initial melting at 95 °C for 2 min, followed by 35 cycles of [94 °C for 30 sec, 60 °C for 30 sec, 72 °C for 2 min]; and a final elongation at 72 °C for 10 min. Amplified fragments were separated by electrophoresis on a acrylamide (1 % w:v) tris-borate-EDTA gel. Confirmation of molecular weights was determined with a GeneRuler 1 kb Plus DNA Ladder (Fermentas, Delaware, USA) and ultimately purified using the GenCatchTM PCR Extraction Kit (Epoch).

The ITS region nucleotide sequences (Eurofins MWG Operon, Ebersberg, Germany) were assembled manually and poor quality ends were removed. Sequences were saved in multifasta format, and aligned using Multiple Alignment and Fast Fourier Transform (MAFFT) v7.029b. The alignment was annotated to include a partial 18S, ITS1, 5.8S, and ITS2. The final alignment contains 1226 bp to 1720 bp, while some species contain a ~400 bp intron in the 18S (*C. saccharophila* SAG 211/1d and *C. minutissima* UTEX 2219). The phylogenetic tree was generated using Phylogeny by PhyML, a software package using maximum likelihood to generate the tree. 1000 bootstrap replicates were used in the construction of the tree.

2.3. Algae cultivation in bioreactors, aquarium tanks and hanging-bags

All cultures at stationary phase were inoculated at 4×10^6 cells ml^{-1} and cells growth was measured by hemocytometer. Algae growth was monitored during the algal growth cycle and the algae were collected at the stationary phase (Table 1) by centrifugation at $5,000 \times g$ for 5 min. The pellet was stored at -80°C and lyophilized overnight and used for lipid analyses.

For the preparation of bioreactors, 15 ml cultures were grown for 14 days and then transferred to 300 ml BBM in the aerated photo-bioreactors. *Chlorella* liquid cultures in 250 ml, 500 ml, 1 L and 3 L were grown in photobioreactors under continuous fluorescence illumination at $200 \mu\text{mol m}^{-2} \text{s}^{-1}$ in a greenhouse environment at $25 \pm 2^\circ\text{C}$. Exponential-phase cells were transferred after 5 days to 3 L BBM in the 3 L aerated photobioreactors and grown to the stationary phase. Aeration flow for the 300 ml and 3 L photo-bioreactors were maintained by an air-pump (Whitewater, MTL66) with a flow rate of 30 L min^{-1} (0.38 vvm) (in addition to agitation by spinning turbine impellers (180 rpm)). For the aquarium tanks, the 3 L cultures were again scaled-up and transferred to 80 L BBM in the 170 L open aquarium tanks with two Power Head water pumps (1500 L h^{-1} ; PETCO King 225) arranged in hanging bag preparation, the 3 L cultures were transferred to 80 L BBM in the polyethylene hanging bag. Air stones with tubing were inserted in adjacent columns providing vigorous and ascending aeration supplied from an external compressed air source (30 L min^{-1} , 0.38 vvm). Both aquarium tanks and hanging bags were grown in a greenhouse environment at $25 \pm 2^\circ\text{C}$. The contamination of culture was monitored and checked by both microscope and the rDNA sequences.

2.4. *Maintenance and collection of UTEX 1230, CS-01 UTEX 395 and UTEX 259 culture under the ambient CO₂ vigorous aeration and 3 % CO₂ conditions*

The cells (1.68×10^5 cells ml⁻¹) were inoculated both to 500 ml BBM in the 1 L bioreactors for the air treatment and 150 ml BBM in the 250 ml flasks under the CO₂ condition. Aeration flow was maintained by an air-pump (Whitewater, MTL66) with a flow rate of 30 L min⁻¹ (0.38 vvm) in addition to agitation by spinning turbine impellers (120 rpm). 3 % CO₂ was saturated at 23 °C in the CO₂ chamber (Forma Environmental Chamber, Thermo Electron Corporation, USA) and the cultures were maintained on the shaker at 150 rpm. The growth was monitored during the algal growth cycle and the algae were collected at the stationary phase (day-12) by centrifugation at $5,000 \times g$ for 5 min. The pellet was stored at -80 °C and lyophilized overnight and used for lipid analyses.

2.5. *Lipid extraction*

Lipid extraction followed the procedure provided by (Kobayashi et al., 2013). The dried algae pellets (100 mg) were homogenized using a mortar and a pestle with liquid nitrogen. Lipid extraction was performed by a modification of the method described by (Bligh, 1959). Two ml of chloroform:methanol (1:2, v:v) containing 0.01 % butyl hydroxyl toluene was added to the ground cells and pooled in a glass tube. This step was repeated 3 times. 500 µg of tripentadecanoin (15:0 TAG, Nu-Check Prep, MN, USA) was added as an internal standard. Two ml of zirconia beads (0.7 mm, BioSpec Products, OK, USA) were added and the mixture was shaken by a vortex at room temperature for 30 min. Two ml of chloroform and 4 ml of water were added and vortexed. After centrifugation at $1,500 \times g$ for 5 min, the chloroform phase was

collected and the aqueous phase was re-extracted with 5 ml of chloroform. This step was repeated 5 times. The pooled chloroform was evaporated to dryness under a stream of nitrogen. Three biological replicates of total lipid content were determined gravimetrically.

2.6. *Chromatographic methods*

Thin layer chromatography (TLC) for neutral lipid separation and trans-methylation to FAME were performed as previously described (Kobayashi et al., 2013). FAMES were analyzed using an Agilent 6890 Series gas-chromatography (GC) System with Agilent 5973 Network Mass Selective Detector (Agilent Technologies, Delaware USA) and chromatography was carried out using a 200 m x 250 μ m x 0.25 μ m Varian GC Capillary column (Varian Inc., CA, USA). GC inlet was held at 270 °C and 1 μ L of the sample was injected with splitless or 20:1 split. The oven temperature was programmed from 130 °C (10 min hold) to 160 °C (7 min hold), from 160 °C to 190 °C (7 min hold), from 190 °C to 220 °C (22 min hold) and from 220 °C to 250 °C (17 min hold) at a rate of 10 °C min⁻¹ for each step with helium as the carrier gas. The total analysis time was 75 min. The GC/MS was carried out using 70 eV EI and the data was evaluated with total ion count (TIC). The total lipid content was calculated based on the 15:0 TAG internal standard and the ration of peak area in total FAME composition sum and 15:0 FAME was applied to obtain the lipid content percentages in dry weight.

3. Results and discussion

3.1. Strain collection and phylogenetic characterization in *Chlorella* strains

The *Chlorella* strains used in this study were collected mainly from UTEX collection (<http://web.biosci.utexas.edu/utex/>) and maintained in BBM, which is a minimal nutrient medium for photoautotrophic algal cultivation. In order to determine the phylogenetic relationships between *Chlorella* strains, DNA was extracted from the selected *Chlorella* strains and analyzed by ribosomal DNA (rDNA)-based phylogenetic analysis (Fig.1). Coding regions of rDNA are highly conserved among species but ITS regions are variable due to insertions, deletions, and point mutations. In cases for sibling species, comparison of the rDNA segment including ITS regions is appropriate for phylogenetic analysis (Ahmad et al., 2013; Cha et al., 2011). In comparing the genomic DNA-based *Chlorella* identities, the three strains were identified newly by rDNA based-phylogenetic analysis with alignment by the MAFFT platform, which is able to focus on only *Chlorella* strains, not including other genera, and supports the tree with bootstrap values from 1000 replicates (Kato, 2014). The ITS sequences of all strains listed in the phylogenetic tree is released into the GenBank sequence database (Rosenberg et al., 2013). While our previous rDNA-based phylogenetic analysis of *Chlorella* and *Scenedesmus* strains employed the MUSCLE alignment algorithm (Rosenberg et al., 2013), this study focused on UTEX *Chlorella* strains (e.g., *C. vulgaris*, *C. sorokiniana*, *C. kessleri*, *C. protothecoides*, *C. fusca* var *vacuolata*, *C. minutissima* and *C. zofingiensis*) based on MAFFT alignments (Fig. 1). The relation of the closer distance with *C. variabilis* NC64A by rDNA-based phylogenetic analysis with MAFFT was the same order as the previous study with MUSCLE (Rosenberg et al., 2013), which was from *C. sorokiniana*, *C. vulgaris*, *C. kessleri*, *C. protothecoides* and others. The correct identity of UTEX 29 was found to be *C. vulgaris* rather than *C. protothecoides* in the UTEX collection and UTEX 2714 was *C. sorokiniana* instead of *C. vulgaris* in the UTEX

collection, which agrees with previous results by MUSCLE platform (Rosenberg et al., 2013). Interestingly, UTEX 265 was identified as *C. sorokiniana* by MAFFT alignment and *C. vulgaris* by MUSCLE, which may require further investigation for its definitive identification.

The physiology of *Chlorella variabilis* NC64A has been extensively studied and its genome well-characterized; however, the maintenance of NC64A requires additional nutrients such as peptone and its biomass production is lower than other *Chlorella* strains (Blanc et al., 2010). Therefore, it is critical to search for alternative strains in the *Chlorella* genus as platforms for biomass production and species closely related to NC64A were selected: *C. sorokiniana*, *C. vulgaris*, *C. kessleri*, *C. protothecoides* by rDNA-based phylogenetic analysis (Fig. 1). Considering bioenergy applications, the levels of biomass and lipid production were compared among the selected strains from *C. sorokiniana*, *C. vulgaris*, *C. kessleri*, *C. protothecoides*, *C. fusca* var. *vacuolata* and *C. zofingiensis*.

3.2. Growth curves of the selected *Chlorella* strains

The selected *Chlorella* strains were maintained under BBM in 1 or 3 L bioreactors, 80 L aquarium tanks or 80 L hanging-bags. UTEX 259, 29 and 395 were selected as representative *C. vulgaris* strains; UTEX 265, CS-01, UTEX 2805, 1230, 2714 and 246 for *C. sorokiniana*; UTEX 2229 and 263 for *C. kessleri*; *C. protothecoides* UTEX B25; *C. fusca* var. *vacuolate* UTEX 252; and *C. zofingiensis* UTEX 32 (Table 1). *C. vulgaris* UTEX 259, 29 and 395 and *C. sorokiniana* UTEX 265, CS-01, 2805 1230 and 2714 exhibited high biomass production of greater than 296 mg L⁻¹ DW at the maximal cell density in the different cultures, while the biomass of the strains from *C. kessleri*, *C. protothecoides*, *C. fusca* var. *vacuolate* and *C. zofingiensis* was less than 262

250 mg L⁻¹ DW and was susceptible to rapid population decline during the growth cycle. The
251 maximal cell densities of *C. vulgaris* UTEX 29 and 395 were 467 and 400 mg L⁻¹ DW under the
252 vigorous aeration (0.38 vvm) containing 0.0 4% CO₂ from the atmosphere, respectively, and *C.*
253 *sorokiniana* UTEX 265, CS-01, UTEX 2805, 1230 and 2714 cell densities were 405, 491, 436,
254 571 and 296 mg L⁻¹ DW, respectively. The cell densities biomass production of *C. vulgaris*
255 UTEX 29, *C. sorokiniana* UTEX 265 and CS-01 reached maximal in 1 or 3 L bioreactors, while
256 *C. vulgaris* UTEX 259 and 395 and *C. sorokiniana* UTEX 2805, 1230 and 2714 reached
257 maximal in 80 L hanging-bags. The growth of *C. sorokinian* and *C. vulgaris* were lower or not
258 measurable in the aquarium tanks in comparison of bioreactors and hanging bags, which
259 indicates that the type and size of the culture vessel most likely affect biomass production due to
260 different light intensities and circulation patterns. Chinnasamy *et al.* also showed the higher
261 biomass productivities of the mixture culture (*Chlamydomonas globasa*, *Chlorella minutissima*
262 and *Scenedesmus bijuga*) at 21.1 g m⁻² d⁻¹ in the polyethylene hanging-bags rather than at 5.9 g
263 m⁻² d⁻¹ in the horizontal raceways by using the wastewater, which was due to the higher
264 photosynthetic efficiency (based on full solar spectrum) of 8.1% in the hanging-bags compared
265 to 1.0 % in the raceways. This mixed culture study also demonstrated the requirement of more
266 air flow in raceways (10 L min⁻¹) than in the hanging-bags (0.4-0.8 L min⁻¹) (Chinnasamy et al.,
267 2010). This indicates the optimal light intensity and aeration play important roles in algal
268 biomass productivity. Furthermore, the biomass production of *C. kessleri*, *C. protothecoides*, *C.*
269 *fusca* var. *vacuolata* and *C. zofingiensis* in BBM were lower than 263 mg L⁻¹ DW or
270 unmeasurable due to the requirement of higher nutrients in the media such as TAP, B5, KNO₃
271 rich- and Kuhl media (Liu et al., 2011; Shen et al., 2010; Shiratake et al., 2013). The large

standard deviations of the biomass and dry weight were caused by the annual temperature difference in the greenhouse although it is set at 25 °C.

The growth curves of *C. sorokiniana* CS-01, UTEX 1230, 2714, 265 and 2805, *C. vulgaris* UTEX 395 and 259, *C. kessleri* UTEX 2229 and *C. zofingiensis* UTEX 32 were compared in different type and size of culture; 1 or 3 L bioreactors, 80 L aquarium tanks and 80 L hanging-bags (Fig. 2). As the cell size of *Chlorella* is about 2 µm in diameter and dry weight is similar among the strains (*e.g.*, the ratio of dry weight vs. cell density; UTEX 1230 = 4.6; UTEX 2714 = 4.9; CS-01 = 4.8), the unit was expressed as cell density (cells ml⁻¹). The growth curves of *C. sorokiniana* UTEX 1230, 2714 and 2805, *C. vulgaris* UTEX 395 and 259 showed the range at cell density 74-131 × 10⁶ cells ml⁻¹ in 80 L hanging-bags and 43-66 × 10⁶ cells ml⁻¹ in 1 or 3 L bioreactors under the vigorous aeration with 0.04 % atmospheric CO₂. In contrast, *C. sorokiniana* CS-01 and UTEX 265 showed the cell density at 92-112 × 10⁶ cells ml⁻¹ in the 1 or 3 L bioreactors but 73 × 10⁶ cells ml⁻¹ or were not measurable in 80 L hanging-bags since the algae did not grow at the very early log phase (3-5 days after inoculation). *C. sorokiniana* CS-01 and UTEX 1230 in the 80 L aquarium tanks presented maximal cell densities of 73 and 87 × 10⁶ cells ml⁻¹, respectively, however aquarium tanks did not produce as much as biomass as cultures grown in bioreactors and hanging-bags. The growth differences in the different type and size culture might be due to the difference of the light penetration and circulation of the *Chlorella* strains in the culture. Other strains like *C. kessleri* UTEX 2229 and *C. zofingiensis* UTEX 32 either grew less than 60 × 10⁶ cells ml⁻¹ or crashed easily during the growth cycle in all types and sizes of cultures.

To address the question of whether *C. sorokiniana* or *C. vulgaris* produces more biomass during autotrophic cultivation under vigorous aeration with 0.04 % CO₂, *C. sorokiniana* UTEX

1230, CS-01 and UTEX 2805 and *C. vulgaris* UTEX 395 and 259 were selected and the growth curve data from Fig. 2 were combined to obtain the general growth curve patterns of *C. sorokiniana* vs. *C. vulgaris* (Fig. 3 a). All cell densities of *C. sorokiniana* UTEX 1230, CS-01 and UTEX 2805 were in the range of $70\text{-}90 \times 10^6$ cells ml^{-1} , while *C. vulgaris* UTEX 395 and 259 were 27-35 % lower at $45\text{-}66 \times 10^6$ cells ml^{-1} , which demonstrates higher biomass production of *C. sorokiniana* relative to *C. vulgaris* under the vigorous aeration with atmospheric CO_2 levels at UNL. However, *C. vulgaris* UTEX 395 from NREL reached the cell density more than 200×10^6 cells ml^{-1} under 2 % CO_2 condition in 1 L Roux bottle (Fig. 3 b), which suggests that supplemental CO_2 might be required for the higher photoautotrophic biomass production in *C. vulgaris*.

3.3. Lipid contents of the selected *Chlorella* strains grown under ambient CO_2 vigorous aeration

The lipid contents of total lipid, FAME total lipid and TAG as the corresponding samples of Fig. 2 were analyzed and quantified at the stationary phase in *C. sorokiniana* UTEX 1230, CS-01, UTEX 265 and 2714, *C. vulgaris* UTEX 395 and UTEX 259, *C. kessleri* UTEX 2229 and *C. zofingiensis* UTEX 32 (Fig. 4). TAG is the neutral lipid which is chemically similar to most conventional fuel and was obtained by removing the charged membrane lipids from FAME total lipid on thin layer chromatography, while the other lipids such as total lipid from the extraction or bio-crude from hydrothermal liquefaction (Chapman and Ohlrogge, 2012; Elliott, 2013) contains a lot of charged moieties in the lipid chemical structures which may cause the degradation of the oil. To access the quality and quantity of the accurate neutral lipid production in algae, TAG analysis is critical. The contents of total lipid, FAME total lipid and TAG of the

selected strains were measured at highest contents of 24, 19, 8 % DW, respectively, in *C. sorokiniana* UTEX 1230, 18, 13, 6 % DW, respectively, in CS-01 and 17, 8, 7 % DW in UTEX 265 (Fig. 4 a), which indicates the high production of both membrane and storage lipid. The contents of total lipid and FAME total lipid in *C. sorokiniana* UTEX 2714 were at the same range of 22 and 14 % DW, respectively, but TAG contents were less than 1 % DW, which is lower than UTEX 1230, 265 and CS-01. In *C. vulgaris*, the contents of total lipid, FAME total lipid and FAME TAG were measured as 19, 16, 1 % DW respectively in UTEX 395 and 16, 4, <1 % DW, respectively, in UTEX 259 in which FAME TAG was hardly detected (Fig. 4 b). The higher FAME total lipid contents but lower TAG contents may indicate more membrane lipid synthesis than storage lipid accumulation. The difference between total lipid and FAME total lipid in *C. sorokiniana* UTEX 265 and *C. vulgaris* UTEX 395 and 259 most likely include the higher production of non-fatty acids such as chlorophylls, pigments, sterols, vitamins than fatty acid. The lowest lipid contents were measured in *C. kessleri* UTEX 2229 and *C. zofingiensis* UTEX 32. The contents of total lipid, FAME total lipid and FAME TAG were quantified at 16, 7, <1 % DW, respectively, in UTEX 2229 and 5, 3, <1 % DW, respectively, in UTEX 32 where FAME TAG was also hardly detected (Fig. 4 c, d), which might indicate that lipids did not manifest as both membrane and storage lipids due to the low growth rates. The results showed the highest TAG accumulation as 6-8 % DW in *C. sorokiniana* strains CS-01, UTEX 1230 and 265.

3.4. Comparison of biomass and lipid production between *C. sorokiniana* UTEX 1230 vs. *C. vulgaris* UTEX 395 and 295 under the ambient CO₂ vigorous aeration and 3 % CO₂ conditions

339 *C. vulgaris* is one of the strongest candidates to produce high levels of biomass and lipids
340 in *Chlorella* strains according to the National Renewable Energy Laboratory (NREL) (Guarnieri
341 et al., 2011). Furthermore *C. vulgaris* UTEX 395 from NREL yielded higher cell density $200 \times$
342 10^6 cells ml^{-1} under 2 % CO_2 condition (Fig. 3 b) than 130×10^6 cells ml^{-1} of UTEX 1230 from
343 UNL under the vigorous aeration with ambient CO_2 (Fig. 2, Table 1). The rDNA sequences of *C.*
344 *sorokiniana* UTEX 1230 and *C. vulgaris* UTEX 395 and 295 from NREL and University of
345 Nebraska-Lincoln (UNL) were confirmed identically by the MAFFT alignment and the biomass
346 and lipid contents were compared between *C. sorokiniana* UTEX 1230 vs. *C. vulgaris* UTEX
347 395 and 295; 1) in BBM under the ambient CO_2 vigorous aeration at flow rate 30 ml min^{-1} (0.38
348 vvm) and 2) in BBM with 3 % CO_2 maintained in the CO_2 saturated chamber (Fig. 5A). Since
349 the preliminary characterizations of *C. sorokiniana* and *C. vulgaris* growth varied between
350 NREL and UNL studies due to different growth conditions, such as CO_2 concentration and
351 culture vessels (e.g., 1 L Roux bottles vs. large-scale photobioreactors), these strains were
352 compared under the same conditions to assess biomass and lipid production between *C.*
353 *sorokiniana* and *C. vulgaris* based on CO_2 requirement. *C. sorokiniana* UTEX 1230 and CS-01
354 increased about 2 times from 123-144 to 483-652 mg L^{-1} DW (cell density $110\text{-}150 \times 10^6$ cells
355 ml^{-1}) with ambient CO_2 vigorous aeration (Fig. 5A, b), while the biomass of *C. vulgaris* UTEX
356 395 and 295 were less than 271 mg L^{-1} (cell density 60×10^6 cells ml^{-1}) under the ambient CO_2
357 vigorous aeration, which agrees with the maximal cells densities reported in Table 1 and
358 indicates that the aeration is essential for the growth of *C. sorokiniana* UTEX 1230. The biomass
359 yields of *C. sorokiniana* UTEX 1230 and CS-01 under the 3 % CO_2 condition also reached the
360 same levels of 483-652 mg L^{-1} DW, however the biomass of *C. vulgaris* UTEX 395 and 295
361 produced 652-863 mg L^{-1} DW (cell density $150\text{-}200 \times 10^6$ cells ml^{-1}), which is approximately 3

times higher than those of the ambient CO₂ aeration condition (Fig. 5A, c) and imply that the carbon concentrating mechanisms may be different between *C. sorokiniana* and *C. vulgaris*, which the pH was constant at the same pH range (7.0-7.5) conditions during the growth cycle of strains. The higher yield of biomass production under different CO₂ concentration was reported at the broad ranges of CO₂ concentrations; 10.0 g L⁻¹ dry weight in *C. vulgaris* under both 6.5 % CO₂ and 0.4 vvm aeration (Anjos et al., 2013), 821-1303 mg L⁻¹ dry weight of *C. vulgaris* grown in wastewater with addition of 5 % CO₂ in air (Arbib et al., 2014) and 1.55g L⁻¹ in *C. pyrenoidosa* under 10 % CO₂ which demonstrated the best biomass production in comparison of 0.03-50 % different CO₂ application (Tang et al., 2011). The biomass of all strains without both air and CO₂ were under 261 mg L⁻¹ DW (60×10^6 cells ml⁻¹ DW) (Fig. 5A, a), which is the same range as UTEX 395 and 259 under the ambient CO₂ vigorous aeration (Fig. 5A, b). These results indicate that *C. sorokiniana* may be highly productive with minimal CO₂ and vigorous aeration, while CO₂ supplementation is required for *C. vulgaris* to reach similar biomass yields. It is important to determine the optimal CO₂ and aeration level in various *Chlorella* strains.

The lipid contents of the corresponding samples were also quantified and compared between *C. sorokiniana* UTEX 1230 and *C. vulgaris* UTEX 395 and 259 under ambient CO₂ aeration and 3 % CO₂ conditions (Fig. 5B). The contents of total lipid, FAME total lipid, and TAG of *C. sorokiniana* UTEX 1230 and CS-01 were measured at 23-26, 15-19, 8 % DW, respectively, under ambient CO₂ vigorous aeration, while the contents of *C. vulgaris* UTEX 395 and 295 were at 18-19, 7-18, <2 % DW respectively which detection of FAME TAG was very low under the ambient CO₂ vigorous aeration (Fig. 5B, a). In contrast, total lipid, FAME total lipid and TAG accumulation of *C. vulgaris* UTEX 395 under the 3 % CO₂ condition increased to 44, 42 and 31 % DW respectively which correspond to 2, 2, and 28 times higher accumulation

under ambient CO₂ vigorous aeration. The contents of total lipid, FAME total lipid, and TAG of *C. vulgaris* UTEX 259 were 39, 33, and 30 % DW, respectively, which are roughly 2, 5, and 15 times higher accumulation respectively in comparison to ambient CO₂ vigorous aeration. The levels of FAME total lipid and TAG were same in *C. vulgaris* under the 3 % CO₂ condition, which may indicate the high storage lipid accumulation. Total lipid, FAME total lipid and TAG of *C. sorokiniana* CS-01 increased to 33, 25, and 20 % DW, respectively, which are 1.4, 1.6 and 2.5 times higher than under the ambient CO₂ vigorous aeration, however all lipid contents of *C. sorokiniana* UTEX 1230 under the 3 % CO₂ condition were at the same ranges as those under the ambient CO₂ vigorous aeration. A dramatic difference was observed between *C. sorokiniana* and *C. vulgaris* under the ambient CO₂ vigorous aeration and 3 % CO₂ condition. The results indicate that the CO₂ treatment includes both strains of *C. vulgaris* and *C. sorokiniana* CS-01 to produce higher biomass and lipid contents, especially high levels of TAG accumulation, while it does not affect *C. sorokiniana* UTEX 1230. In a previous study performed with *C. vulgaris* UTEX 395, the FAME lipid content was measured as more than 10 % in nitrogen replete conditions and 60 % in nitrogen deplete conditions with 2 % CO₂ supplement (Guarnieri et al., 2011). TAG contents of *C. sorokiniana* UTEX 1230 under heterotrophic condition with glucose and without light accumulated to 24-39% DW also at the same level as the FAME total lipid contents (Rosenberg et al., 2013). This indicates that CO₂ is required for both biomass and lipid accumulation for *C. vulgaris*, while *C. sorokiniana* UTEX 1230 can achieve comparable biomass densities with the continuous air, but its lipid and biomass production can be enhanced by other carbon sources, such as glucose. This might suggest that *C. vulgaris* and *C. sorokiniana* have different HCO₃⁻ transporters (Duanmu et al., 2009).

The main fatty acid compositions of all these strains under both ambient CO₂ vigorous aeration and 3 % CO₂ conditions in FAME total lipid and TAG were detected as 16:0, 18:1, 18:2 and 18:3 (carbon : double bond number), which are the main FAME in *Chlorella* (Fig. 5B, c-f) (Hu et al., 2008). The FAME total lipid and TAG levels of 16:0, 18:1, 18:2 and 18:3 in *C. sorokiniana* UTEX 1230 and CS-01 remained within the same ranges both under the ambient and 3 % CO₂ conditions, however the level of 18:1 of *C. vulgaris* UTEX 395 and 259 increased from 1.6-2.5 % to 14-19 % DW in FAME total lipid (Fig. 5B, c, d) and 0.4-0.8 % to 13-14 % DW in FAME TAG (Fig. 5B, e, f). The main FAMES, 16:0, 18:2 and 18:3 in FAME total lipid were the same range at 2-8, 0.8-4.5, and 1.8-5.8 % DW respectively in *C. vulgaris* UTEX 395 and 259 both under the ambient CO₂ vigorous aeration and 3 % CO₂ conditions (Fig. 5B, c, d), while 16:0, 18:2 and 18: 3 in FAME TAG increased from 0.007-0.011, 0.02-0.3 and 0.12-0.37 % under ambient CO₂ vigorous aeration to 5.6-5.8, 2.7-2.8 and 3.6-5.0 % DW with 3 % CO₂ condition, respectively (Fig. 5B, e, f), which also demonstrated higher TAG accumulation with supplemental CO₂. The lipid composition (18:1) of FAME total lipid and (16:0, 18:1, 18:2 and 18:3) in TAG increased in *C. vulgaris* UTEX 395 and 259 under the 3 % CO₂ condition. In particular, 18:1 was 16-35 times higher under the 3 % CO₂ condition compared to ambient CO₂ vigorous aeration. In contrast, the lipid composition (18:2) of *C. sorokiniana* UTEX 1230 under heterotrophic condition increased dominantly in the lipid composition (Rosenberg et al., 2013), which may indicate the involvement of desaturase in plastid or endoplasmic reticulum observed in plants (Chapman and Ohlrogge, 2012; Wallis and Browse, 2002).

4. Conclusion

After selecting the *Chlorella* species by rDNA based-phylogenetic analysis and screening their biomass and lipid production, *C. sorokiniana* and *C. vulgaris* demonstrated the highest biomass and lipid production among other species. Comparison of different levels of CO₂ during the growth of *C. sorokiniana* and *C. vulgaris* showed higher biomass and lipid production in *C. sorokiniana* under conditions of ambient CO₂ vigorous aeration, while *C. vulgaris* prospers more with 3% CO₂. For biofuels applications, the *Chlorella* strains examined in this study represent that carbon can be uniquely paired based on the abundant natural resources sources (*i.e.*, industrial CO₂ or organic wastes).

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Figure Legends

Fig. 1 Proposed phylogenetic tree of rDNA gene sequences from *Chlorella* species. Selected sequences from the NCBI database were included. Grey arrows; the strains used in this study. Black arrow; the characterized genome of *C. variabilis* NC64A (Blanc et al., 2010).

Fig. 2 Growth curves of *Chlorella* species in different types and sizes of culture. A, 80 L hanging-bag; B, 80 L aquarium tank; C, 1 or 3 L bioreactors. Black lines and symbols, *Chlorella sorokiniana* CS-01, UTEX 1230, UTEX 2714, UTEX 265 and UTEX 2805; Grey lines and symbols, *C. vulgaris* UTEX 395 and UTEX 259; White grey lines and symbols, *C. kessleri* UTEX 2229 and *C. zofingiensis* UTEX 32. Error bar: standard deviation of three biological replicates.

Fig. 3 Comparison of the growth patterns between *C. sorokiniana* and *C. vulgaris* under vigorous aeration with ambient atmospheric CO₂ (UNL). Each growth curve was combined from the results of hanging-bags and bioreactors (a). Growth curve of *C. vulgaris* UTEX 395 grown in 1 L Roux Bottle under 2 % CO₂ condition at NREL (b). The graph was converted from OD (750 nm) to cell count based on the equation ($y=00398x-0.0245$) of the relationship between cell count and OD (750 nm) in *Chlorella*.

Fig. 4 Lipid contents of *C. sorokiniana*, *C. vulgaris*, *C. kessleri* and *C. zofingiensis*. a, *Chlorella sorokiniana* UTEX 1230, CS-01, UTEX 2714 and UTEX 265; b, *C. vulgaris* UTEX 395 and UTEX 259; c, *C. kessleri* UTEX 2229 and d, *C. zofingiensis* UTEX 32. Black bar; total lipid, grey bar; FAME total lipid; white grey bar; FAME TAG. Error bars: standard deviation of three technical replicates.

Fig. 5 Comparison of growth curves (A) and lipid analyses (B) of *C. sorokiniana* UTEX 1230 and *C. vulgaris* UTEX 395 and 259 under ambient and 3 % CO₂ conditions. A. Growth curves of

UTEX 1230 and CS-01 (black), UTEX 395 and UTEX 259 (grey). a; no air and CO₂ application, b; ambient CO₂ vigorous aeration, c; 3 % CO₂ application. B. Lipid contents under ambient (a) and 3 % CO₂ (b) conditions. Black column; Total lipid, grey column; FAME total lipid and white grey column; FAME TAG. Fatty acid analysis of FAME total lipid under ambient (c) and 3 % CO₂ (d) conditions. Fatty acid analysis of FAME TAG under the ambient (e) and 3 % CO₂ (f). Black column, UTEX 1230 and dark grey, CS-01, *Chlorella sorokiniana*; light grey column, UTEX 395 and white grey, UTEX 259, *Chlorella vulgaris*. Error bar: standard deviation of six biological replicates for panel A graph b and c and three biological replicates for panel A graph a.

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Table 1 Biomass and stationary days of Chlorella species in different container size of cultivation.

Chlorella strains	Bioreactors			Aquarium tanks			Hanging-bags		
	Stationary phase (days)	Cell density (x10 ⁶ cell ml ⁻¹)	Dry weight (mg L ⁻¹)	Stationary phase (days)	Cell density (x10 ⁶ cell ml ⁻¹)	Dry weight (mg L ⁻¹)	Stationary phase (days)	Cell density (x10 ⁶ cell ml ⁻¹)	Dry weight (mg L ⁻¹)
<i>Chlorella vulgaris</i>									
UTEX 259	14	44.93±8.11 ^a	207.46±34.29 ^a	-	-	-	23	85.6	379.48
UTEX 29	13	106.20±2.40 ^a	466.62±10.15 ^a	15	62.8	283.03	-	-	-
UTEX 395	9	60.20±30.21 ^a	272.04±127.80 ^a	15	52.6	239.89	20	90.60±33.95 ^a	400.63±143.60 ^a
<i>C. sorokiniana</i>									
UTEX 265	14	91.80±29.42 ^a	405.70±124.43 ^a	7	34.4	162.90	-	-	-
CS-01	9	112.00±21.38 ^a	491.15±66.91 ^a	17	87.30±6.47 ^a	386.67±27.14 ^a	15	72.67±26.35 ^a	324.77±111.46 ^a
UTEX 2805	13	58.9	266.54	-	-	-	15	99.0	436.16
UTEX 1230	8	42.90±9.38 ^a	198.85±39.67 ^a	14	73.15±2.57 ^a	326.80±10.86 ^a	22	130.93±15.40 ^a	571.24±65.29 ^a
UTEX 2714	13	65.8	295.72	-	-	-	20	74.15±12.94 ^a	296.26±54.74 ^a
UTEX 246	14	56.0	254.27	-	-	-	-	-	-
<i>C. kessleri</i>									
UTEX 2229	15	31.58±12.22 ^a	150.99±51.69 ^a	7	29.5	142.18	16	23.0	114.68
UTEX 263	-	-	-	14	38.5	180.25	-	-	-
<i>C. protothecoide</i>									
UTEX B25	-	-	-	14	58.0	262.73	-	-	-
<i>C. fusca</i> var. <i>vaculata</i>									
UTEX 252	-	-	-	16	25.3	124.41	-	-	-
<i>C. zofingiensis</i>									
UTEX 32	14	42.8	198.43	7	54.1	246.23	-	-	-

^aStandard deviation from three biological replicates, -Not measured.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 4

Figure 5

A

B