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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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HIGHLIGHTS

- *Chlorella* species were classified by rDNA-based phylogenetic analysis.
- Selected *Chlorella* species were screened based on biomass and lipid production.
- The candidate strains were compared under vigorous aeration with air and 3% CO_2.
- *Chlorella* species of UTEX 395, 259 and 1230 yielded the most biomass and lipids.
- Depending on the carbon source the strains may be suited for biofuel applications.

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ABSTRACT

Chlorella species from the UTEX collection, classified by rDNA-based phylogenetic analysis, were screened based on biomass and lipid production in different scales and modes of culture. The lead candidate strains of *C. sorokiniana* UTEX 1230 and *C. vulgaris* UTEX 395 and 259 were compared between conditions of vigorous aeration with filtered atmospheric air and 3% CO_2 shake-flask cultivation. The biomass of UTEX 1230 produced 2 times higher at 652 mg L^{-1} dry weight under both ambient CO_2 vigorous aeration and 3% CO_2 conditions, while UTEX 395 and 259 under 3% CO_2 increased to 3 times higher at 863 mg L^{-1} dry weight than ambient CO_2 vigorous aeration. The triacylglycerol contents of UTEX 395 and 259 increased more than 30 times to 30% dry weight with 3% CO_2, indicating that additional CO_2 is essential for both 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

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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 et al., 2008).

Chlorella produces substantial biomass under minimum nutrients while requiring nitrogen, phosphorus and other micronutrients in the presence of light and carbon dioxide (CO_2). 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., 2013a,b; Singh et al., 2011). Production of biomass and lipid has been studied broadly under different media in the *Chlorella* species: *Chlorella vulgaris*, *C. prototheocoides*, *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. prototheocoides* UTEX 255 were 11.7 g L^{-1} and 654 mg L^{-1} day^{-1}, respectively, under the 2.4 g L^{-1} KNO_3 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 $\times$ 10^6 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% CO_2 (Tang et al., 2011) and in *C. minutissima* UTEX 2219 they were at 21–140 $\times$ 10^6 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. prototheocides* 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% CO_2 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 CO_2 (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 8×10^8 cells ml^{-1} and 10% DW, respectively, under the nitrogen-replete while at 5×10^8 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 $\times$ 10^6 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 CO_2 vigorous aeration and 3% CO_2 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. Methods

2.1. Algae strains and growth conditions

Samples of *C. vulgaris*, *C. sorokiniana*, *C. kessleri*, *C. prototheocoides*, *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*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 s, 60 °C for 30 s, 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 GenCatch™ 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