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Isolation, characterization, and validation of oleaginous, multi-trophic, and haloalkaline-tolerant microalgae for two-stage cultivation

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ABSTRACT

Mass outdoor cultivation of microalgae for biofuels and co-products faces challenges of low lipid productivity, contamination, inefficient CO_2 supply, and difficulties in harvesting. A two-stage cultivation process was developed to address these challenges. This involves culturing microalgae in a fermentor heterotrophically or photobioreactor mixotrophically as first-stage to rapidly obtain high cell densities for inoculating a phototrophic open-pond culture featuring high levels of $NaHCO_3$, pH, and salinity as second-stage. A microalgae that is tolerant of these phototrophic conditions, can use organic carbon, and can prolifically produce oil is key to the success of such a two-stage process. Two oleaginous, haloalkaline-tolerant, and multi-trophic green microalgae from soda lakes were isolated, identified, and compared in this study using a multi-instrument approach as candidates for such process. A model triacylglyceride (TAG) was developed for rapid, non-destructive lipid quantitation using liquid-state ^1H NMR. A two-stage cultivation system and a high pH-mediated auto-flocculation method were tested on the selected strain ALP2 with a 1 L fermentor and 40 L open-tank. In unoptimized conditions, the stain achieved a final biomass concentration of 0.978 g DCW/L, lipid content of 39.78% DCW, and auto-flocculation harvesting efficiency of 64.1%.

1. Introduction

Lipids from biomass are precursors for renewable fuels like biologically-derived synthetic paraffin kerosene (Bio-SPK) for aviation [1] and biodiesel for electrical power generation [2] and ground transportation [3]. The National Renewable Energy Laboratory's (NREL) Aquatic Species Program long ago identified oleaginous photosynthetic microalgae as a biomass feedstock for such lipid-based fuels [4]. These algae accumulate neutral lipid as triacylglycerides [TAGs] in lipid bodies, typically under nitrogen or phosphate depletion stress conditions [4]. However, commercialization of microalgal biofuel involving large-scale, outdoor open-ponds is still constrained by low lipid productivity [5], contamination by invasive species [6,7], sustainable supply of nutrients (e.g.; nitrogen and phosphorous [8,9]) and inorganic carbon [10], limited available acreage for inoculum and primary cultures [7,11], and inefficient harvesting [4,12].

The use of a microalgae that can be efficiently cultured under heterotrophic or mixotrophic conditions for inoculum and phototrophically for biomass growth and lipid accumulation was proposed as a two-stage cultivation strategy to address the challenges associated with long growth times and contamination [11] in large-scale open-pond operations. Heterotrophic or mixotrophic culture conditions in a closed reactor in the first stage enable the use of organic carbon substrates [11] to rapidly achieve high densities as inoculum for the outdoor phototropic open-pond culture that harnesses sunlight for lipid accumulation in the second stage. With a larger inoculum and reduced growth time, the microalgae in the second-stage phototrophic open-pond culture could more likely outcompete or withstand undesirable environmental contaminants [11]. Low-maintenance, low surface area–volume ratio industrial fermentors currently used for commercial production of medicines, beverages, food additives, and energy [13,14] could be used for such first-stage heterotrophic cultivation without light-limitation. Additionally, the organic carbon used in the first-stage heterotrophic cultivation could be derived from various waste sources, including cellulosic sugars from agricultural crop residues [15], acetate from pyrolyzed forestry residues [16], and crude glycerol from trans-esterification processes [17].

Challenges associated with outdoor contamination [18], low volumetric lipid productivity [19], harvesting [12,20], and supply of

Abbreviations: TAG, triacylglyceride; GC-FAME, gas chromatographic fatty acid methyl ester; PBR, photobioreactor; DCW, dry cell weight; NMR, nuclear magnetic resonance; ITS, internal transcribed spacer; 2NBH, double-nitrogen Bristol medium with Hutner's trace elements; BLAST, basic local alignment search tool.

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inorganic carbon to second stage open-pond phototrophic cultures [10] could also be overcome by using high levels of bicarbonate salts that maintain a moderately high pH via bicarbonate buffering. For example, the high pH and high alkaline salt conditions employed in this scheme can serve to limit contaminants and competing organisms [18]. In this study, a cyanobacterium was used as a model contaminant, demonstrating the ability of a haloalkaline-tolerant microalgae to outcompete and thrive relatively unchallenged. Alkaline conditions in phototrophic cultures also support $Mg(OH)_2$-mediated auto-flocculation of cells and facilitate harvesting [12,20]. Furthermore, the addition of bicarbonate has reportedly enhanced microalgal growth and lipid accumulation [19]. For example, carbon-limited *Synechococcus leopoliensis* grown in chemostats exhibited a very high photosynthetic affinity for HCO_3^- over CO_2 during the growth phase [21]. Supplementation of HCO_3^- to nitrogen-starved, CO_2-sparged *Chlamydomonas reinhardtii* microalgal cultures reportedly triggered a photosynthetic increase in their neutral TAG–lipid accumulation [19]. Using bicarbonate salts that are more soluble and storable at night when least needed compared to CO_2 gas has been additionally advocated. This represents a way to avoid the inefficient gas–liquid mass transfer [10], outgassing losses, medium acidification, and low one-pass 25–30% capture [22] associated with supplying concentrated CO_2 from flue-gas emissions to large, highly evaporative, second-stage open-pond phototrophic cultures [10].

Such a two-stage mass-cultivation process, however, requires identification of a microalgal strain that is suitable for these special conditions. Specifically, the ideal algae strain will grow well under heterotrophic, mixotrophic, and phototrophic conditions, tolerate strongly haloalkaline conditions, use bicarbonate salts as a carbon source during both growth and lipid accumulation, and efficiently produce significant quantities of lipid. There have been no previous efforts to identify and propagate an algal species meeting these criteria. The purpose of this study was to fill this technical gap. Two oleaginous, multi-trophic, halo-alkaline-tolerant microalgae from soda lakes, Alkali Lake and Soap Lake, in the State of Washington, U.S.A., were isolated and identified as candidates for this application. A two-stage cultivation process, featuring both a fermentor and an open-tank photobioreactor (PBR), and an auto-flocculation strategy adapted to the process, were then demonstrated using one of the strains.

2. Material and methods

2.1. Microalgal and cyanobacterial strain isolation

Water and rock samples were collected with transparent screw-cap 0.5 L plastic bottles during spring (April 15, 2010) and summer (June 21, 2010), respectively, from the shallow shores of Alkali Lake (Latitude = 47.5165° N and Longitude = −119.5009° W) and the meromictic and terminal Soap Lake (Latitude = 47.4050° N and Longitude = −119.4975° W, elevation = 330.71 m, and total depth = 4.27 m) in the lower Grand Coulee of Grant County, Washington, U.S.A. Soap Lake phytoplankton population density is reportedly highest in the spring when it thermally stratifies via a metalimnion layer and when grazing zooplankton population density is lower [23,24]. Clear lake water or visibly phototrophic green mats that were scraped with wire-brush from rocks were used within 1 day to inoculate 0.25 L flasks containing a previously described 2NBH medium [25] supplemented with 12 mM $NaHCO_3$ and adjusted to pH 9.60 to mimic enriched soda lake water. The high pH cultures were grown for 20 days at 130 rpm agitation on an orbital shaker under 70 μmol m^{-2} s^{-1} of poly-chromatic light intensity from overhead fluorescent bulbs set on a 16 h-light/8 h-dark diurnal cycle with a timer. As visualized under an optical light microscope, two green coccoidal phototrophs, ALP2 from Alkaline Lake, and SLP2 from Soap Lake, were sorted from the spring lake water-inoculated cultures containing debris using a FACS Vantage SE fluorescence activated cell sorter (BD Immunocytometry Systems, San Jose, CA) equipped with FACSDiva v.2008 software. These two isolates were then streaked and grown on 2% (w/v) agar plates containing modified 2NBH medium. Single ALP2 and SLP2 phototrophic colonies were then cloned and loop-inoculated into 10 mL glass tubes containing modified 2NBH for phototrophic axenic cultivation. A freshwater cyanobacterial strain, *Synechococcus PCC7942* (Targeted Growth, Inc., Seattle, WA) [26], was later used to co-inoculate an ALP2 flask culture for flow-cytometrically monitoring contamination experiments. Additional characterization work, subsequent two-stage cultivation, and ^1H NMR model development was conducted using the more promising ALP2 strain that yielded higher biomass than SLP2 during organic carbon screening experiments.

2.2. Microalgal strain identification

Genomic DNA was isolated from SLP2 and ALP2 cultures grown in BG-11 medium [27] as previously described [25]. A TD-700 Nanodrop fluorometer (Turner Designs, Inc., Sunnyvale, CA) was used to measure DNA concentration and $OD_{260/280}$ purity ratio. Full segments of internal transcribed spacer (ITS) regions 1 and 2 and 5.8S rDNA with fragment segments of 18S rDNA and 28S rDNA were PCR amplified using ITS1 forward and ITS4 reverse primers as previously described using a DNA Engine Peltier Thermal-Cycler (Bio-Rad, Hercules, CA) with the exception that 20 μg/mL of template DNA was used [25]. PCR reactions were run alongside a 2-log DNA ladder on a 1% (w/v) agarose gel at 95 V electrophoresis. Single-band PCR products were visualized by ethidium bromide staining and with a Universal Hood II UV-light exposure (Bio-Rad, Hercules, CA) and cleaned with a PCR clean-up kit (Qiagen, Venlo, The Netherlands). The products were then subjected to a dideoxy big-dye incorporation thermal-cycler reaction and then cleaned by centrifugation through a size-exclusion chromatography elution column, vacuum evaporated for 30 min, and then directly distinguished and sequenced with capillary electrophoresis at the WSU Sequencing Center. Sequences were cleaned and edited using Geneious software version 5.0 bioinformatic software (Biomatters, Ltd., Auckland, New Zealand). A contig assembly was generated, from which nucleotide BLAST searches were performed with NCBI GenBank. BLAST results in the form of GenBank accession numbers were ranked according to e-value and query match percentage.

2.3. Microalgal carbon and trophic mode screening experiments

To initially test the effects of carbon source and light intensity conditions during heterotrophic, mixotrophic, and phototrophic conditions, SLP2 and ALP2 from maintenance cultures were washed 4× in PBS and inoculated at an OD_{680} = 0.035 into 0.25 L flasks. These flasks contained 0.10 L of BG-11 medium [27] adjusted to pH 7.0 and were supplemented with 330 mM carbon from various candidate carbon sources (glucose (55.5 mM), xylose (66.6 mM), glucose + xylose (28 mM + 33 mM), glycerol (119 mM), potassium acetate (137 mM), arabinose (33 mM), mannose (28 mM), sodium bicarbonate (333 mM)). Cultures were agitated at 150 rpm on an orbital shaker in foam-capped flasks for 10 days at 21 °C, either side by side or at different times under phototrophic/mixotrophic (70 μmol m^{-2} s^{-1} of continuous polychromatic light intensity from overhead fluorescent bulbs) or heterotrophic (flasks covered with aluminum foil except for the top for gas exchange) conditions. Stock solutions of $NaNO_3$ were autoclaved separately from glucose. Samples of 1 mL were aseptically removed for measurements of OD_{680}, OD_{750}, and monomer sugar content. On the final culture days, the remaining biomass was sampled for measurement of dry cell weight (DCW). The minimal Na_2CO_3 in BG-11 medium was not included in calculations of total $NaHCO_3$ supplemented to phototrophic cultures.

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