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An efficient and scalable extraction and quantification method for algal derived biofuel

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ABSTRACT

Microalgae are capable of synthesizing a multitude of compounds including biofuel precursors and other high value products such as omega-3-fatty acids. However, accurate analysis of the specific compounds produced by microalgae is important since slight variations in saturation and carbon chain length can affect the quality, and thus the value, of the end product. We present a method that allows for fast and reliable extraction of lipids and similar compounds from a range of algae, followed by their characterization using gas chromatographic analysis with a focus on biodiesel-relevant compounds. This method determines which range of biologically synthesized compounds is likely responsible for each fatty acid methyl ester (FAME) produced; information that is fundamental for identifying preferred microalgae candidates as a biodiesel source. Traditional methods of analyzing these precursor molecules are time intensive and prone to high degrees of variation between species and experimental conditions. Here we detail a new method which uses microwave energy as a reliable, single-step cell disruption technique to extract lipids from live cultures of microalgae. After extractable lipid characterization (including lipid type (free fatty acids, mono-, di- or tri-acylglycerides) and carbon chain length determination) by GC–FID, the same lipid extracts are transesterified into FAMEs and directly compared to total biodiesel potential by GC–MS. This approach provides insight into the fraction of total FAMEs derived from extractable lipids compared to FAMEs derived from the residual fraction (*i.e.* membrane bound phospholipids, sterols, *etc.*). This approach can also indicate which extractable lipid compound, based on chain length and relative abundance, is responsible for each FAME. This method was tested on three species of microalgae; the marine diatom *Phaeodactylum tricornutum*, the model Chlorophyte *Chlamydomonas reinhardtii*, and the freshwater green alga *Chlorella vulgaris*. The method is shown to be robust, highly reproducible, and fast, allowing for multiple samples to be analyzed throughout the time course of culturing, thus providing time-resolved information regarding lipid quantity and quality. Total time from harvesting to obtaining analytical results is less than 2 h.

1. Introduction

Consumption rates of traditional fossil fuels in the United States currently exceed 18 million barrels per day with a significant percentage being imported from foreign sources (EIA, 2012). The political, economic and environmental controversies over these resources have resulted in increased interest in advanced alternative fuels (Dismukes et al., 2008; Greenwell et al., 2010; Groom et al., 2008; Hill et al., 2006; Sheehan, 1998). Although algae-derived biofuels command a relatively small percentage of this emerging market, they hold great potential for truly displacing traditional sources due to their high biomass productivity, relatively small land mass requirements and high lipid yield (Chisti, 2007; Hu et al., 2008). Further, algal fuels do not directly contend in the "fuel *vs.* food" debate because they can grow in non-arable environments (Ferrell and Sarisky-Reed, 2010).

Biodiesel is the end result of a transesterification reaction in which lipids such as phospholipids, free fatty acids (FFA), and mono, di, and tri-acylglycerides (MAG, DAG, TAG), derived from plant or animal sources, react with methanol in the presence of heat and base or acid, to produce fatty acid methyl esters (FAMEs) (Laurens et al., 2012a). Biodiesel is considered a "drop-in" fuel since no modifications have to be made to the current distribution infrastructure or diesel combustion engines. Biodiesel has similar combustion properties to petroleum diesel and a higher combustion efficiency than gasoline (Demirbas, 2007).

However, if biodiesel is to replace a large part of petroleum hydrocarbon based fuels, the compositional makeup of biodiesel will become highly important since biodiesel can have poorer performance than petroleum-based diesel (Hunter-Cevera et al., 2012). For example, (i) the viscosity of fully saturated hydrocarbons increases significantly at low temperatures and can lead to operational issues and (ii) short chain fatty acid methyl esters tend to be more susceptible to oxidation which can lead to corrosion, resulting in reduced engine durability (Xue et al., 2011). Therefore a comprehensive and diverse

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feedstock of lipid precursors along with the derived fatty acid methyl esters will be necessary to achieve the desired properties of the biodiesel fuel (Knothe, 2005).

Traditionally, microalgae species have been screened for strains that produce a high abundance of TAG using fluorometric techniques such as the Nile Red assay (Cooksey et al., 1987) and total lipid extracts have been quantified gravimetrically (Bligh and Dyer, 1959) or by gas chromatography after transesterification (*e.g.* Guckert and Cooksey, 1990). However, these methods are limited by their inherent inability to identify all, or only those, compounds which may be utilized for fuel. Nile Red, for instance, roughly correlates with the amount of TAG precursors per cell (Chen et al., 2009; Cooksey et al., 1987; Elsey et al., 2007; Gardner et al., 2011, 2013). Hence, Nile Red-based measurements may underestimate the total biofuel potential of algal species that produce high fractions of FFA, MAG, DAG or membrane lipids. Additionally, the Nile Red assay is qualitative (possibly semi-quantitative) and cannot be compared between species. In contrast, gravimetric analysis can overestimate total biofuel potential by including non-fuel components (*e.g.* chlorophyll) in the total weight (Laurens et al., 2012b). Gas chromatographic analysis of FAMEs has proven to be a reliable method for quantifying total biodiesel potential (Eustance et al., in press; Laurens et al., 2012b), but fails to identify the biological precursor molecules.

Here, a new method is described which allows for high-throughput harvesting and extraction of live cultures by utilizing microwave energy for a one-step cell disruption-and-extraction of lipid precursors (FFA, MAG, DAG, TAG). Microwave energy has recently been shown to be an effective cell disruption technique, generating comparative or better lipid yields than more traditional methods such as sonication or bead beating (Lee et al., 2010; Patil et al., 2012; Prabakaran and Ravindran, 2011). Additionally, extraction of lipids from live culture significantly reduces process time and decreases the potential for degradation of intracellular lipid compounds. A portion of the lipid extract is analyzed by GC coupled with flame ionization detection (GC–FID) to identify the lipid class and carbon chain length of the fatty acid(s). In parallel, a portion of the lipid extract is transesterified and analyzed by GC–MS to identify FAMEs derived from extractable lipid. Additionally, total biodiesel potential is determined by direct *in situ* transesterification of live cultures. Comparisons can be made between extractable lipid precursors, FAMEs derived from extractable lipids and total biodiesel potential by contrasting the carbon chain length and saturation of each molecule respectively. This approach was demonstrated for three different frequently used microalgae species; the model Chlorophyte *Chlamydomonas reinhardtii*, the marine diatom *Phaeodactylum tricornutum* and the freshwater green alga *Chlorella vulgaris*. These three organisms have been extensively studied (*e.g.* Gardner et al., 2012; Merchant et al., 2007; Stephenson et al., 2010; Valenzuela et al., 2012) and represent a diverse set of microalgae which are commonly used for biofuel investigations (Gardner et al., 2013; Liu et al., 2011; Spiekermann et al., 2003). The method described herein is relatively simple, fast, utilizes fairly standard equipment, and results in a comprehensive lipid profile within 2 h of harvesting.

2. Materials and methods

2.1. Microalgae strains

2.1.1. Phaeodactylum tricornutum

P. tricornutum strain Pt-1 (CCMP 2561) (Pt-1) was acquired from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (East Boothbay, Maine, USA) and accessions of *P. tricornutum* have previously been described (Martino et al., 2007). Pt-1 was cultured on ASP_2 medium (Provasoli et al., 1957) amended with 50 mM Tris buffer (Sigma-Aldrich, USA), pKa 7.8, to maintain a stable pH throughout culturing. Cultures were continuously sparged with ambient air and amended with 25 mM of sodium bicarbonate (final concentration) just prior to nitrate depletion in the medium to induce TAG accumulation as demonstrated by Gardner et al. (2012).

2.1.2. Chlorella vulgaris

C. vulgaris, UTEX 395 (University of Texas at Austin) was cultured on Bold's basal medium (Nichols and Bold, 1965) with pH adjusted to 8.7 prior to autoclaving. Cultures were sparged with 5% CO_2 in air during the light hours.

2.1.3. Chlamydomonas reinhardtii

C. reinhardtii CC124, obtained from the Chlamydomonas Center (Minnesota State University, Minneapolis MN), was kindly provided by John Peters, Department of Chemistry and Biochemistry at Montana State University, and was cultured on Sager's minimal medium (Harris, 1989). Cultures were sparged with 5% CO_2 (v/v) until just prior to ammonium depletion, at which time the gas sparge was switched to ambient concentrations of CO_2 (0.04%; v/v) and 50 mM of sodium bicarbonate (final concentration) were added to induce TAG accumulation (Gardner et al., 2013).

2.2. Culturing conditions

All organisms were checked for bacterial contamination by inoculation into respective medium supplemented with 0.05% yeast extract and 0.05% glucose and incubation in the dark. Experiments were conducted in triplicate batch cultures using 70 × 500 mm glass tubes containing 1.2 L medium submersed in a water bath to control temperature. Rubber stoppers, containing ports for aeration and sampling, were used to seal the tubes. Temperature was maintained at 24 °C ± 1 °C. Light (400 μmol photons m^{-2} s^{-1}) was maintained on a 14:10 light–dark cycle using a light bank containing T5 tubes. Aeration (400 mL min^{-1}) was supplied by humidified compressed air (supplemented with 5% CO_2 (v/v) for *C. reinhardtii* and *C. vulgaris*) and controlled using individual rotameters for each bioreactor (Cole-Parmer, USA). ACS grade sodium bicarbonate was used in all experiments involving bicarbonate addition (Sigma-Aldrich, St. Louis, MO).

2.3. Culture analysis

Cell concentrations were determined using an optical hemacytometer with a minimum of 400 cells counted per sample for statistical reliability. Cell dry weights (CDWs) for *C. reinhardtii* and *C. vulgaris* were determined by harvesting 30 mL of culture into a tared 50 mL Falcon tube (Fisher Scientific, Palatine, IL) followed by centrifugation at 4800 ×*g* and 4 °C for 10 min (Thermo Scientific, Sorvall Legend XTR, Waltham, MA). The concentrated biomass was rinsed with deionized H_2O (diH_2O), 18 MΩ, to remove media salts and excess bicarbonate, before centrifuging again. Remaining algae pellets were frozen and lyophilized (Labconco lyophilizer, Kansas City, MO) for 48 h. CDWs were calculated by subtracting the weight of the biomass-free Falcon tube from the weight of the Falcon tube with freeze-dried biomass.

CDWs for *P. tricornutum* were determined by filtering 10 mL of culture using 1 μm pore size glass fiber filters (Fisher Scientific) to collect the biomass. The biomass was washed with diH_2O to remove media salts and excess bicarbonate. Algal cells were dried at 70 °C until the weight of the filter (plus biomass) remained constant. CDWs were calculated by subtracting the dry weight of the clean filter from the oven-dried weight of the filter with biomass. This method was employed for the diatom due to the concern that the fragility of the organism's silicon-based frustules may result in cell disruption during the centrifugation and lyophilization steps utilized for the Chlorophytes, thereby underestimating overall CDWs. This technique was not employed for the Chlorophytes because it has been shown by