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A high throughput Nile red method for quantitative measurement of neutral lipids in microalgae

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ABSTRACT

Isolation of high neutral lipid-containing microalgae is key to the commercial success of microalgae-based biofuel production. The Nile red fluorescence method has been successfully applied to the determination of lipids in certain microalgae, but has been unsuccessful in many others, particularly those with thick, rigid cell walls that prevent the penetration of the fluorescence dye. The conventional "one sample at a time" method was also time-consuming. In this study, the solvent dimethyl sulfoxide (DMSO) was introduced to microalgal samples as the stain carrier at an elevated temperature. The cellular neutral lipids were determined and quantified using a 96-well plate on a fluorescence spectrophotometer with an excitation wavelength of 530 nm and an emission wavelength of 575 nm. An optimized procedure yielded a high correlation coefficient ($R^2 = 0.998$) with the lipid standard triolein and repeated measurements of replicates. Application of the improved method to several green algal strains gave very reproducible results with relative standard errors of 8.5%, 3.9% and 8.6%, 4.5% for repeatability and reproducibility at two concentration levels (2.0 μg/mL and 20 μg/mL), respectively. Moreover, the detection and quantification limits of the improved Nile red staining method were 0.8 μg/mL and 2.0 μg/mL for the neutral lipid standard triolein, respectively. The modified method and a conventional gravimetric determination method provided similar results on replicate samples. The 96-well plate-based Nile red method can be used as a high throughput technique for rapid screening of a broader spectrum of naturally-occurring and genetically-modified algal strains and mutants for high neutral lipid/oil production.

1. Introduction

Due to the limited stocks of fossil fuels and the increasing emission of greenhouse gas carbon dioxide into the atmosphere from the combustion of fossil fuels, research has begun to focus on alternative biomass-derived fuels (Scurlock et al., 1993; Kosaric and Velikonja, 1995). One promising source of biomass for alternative fuel production is microalgae that have the ability to grow rapidly, and synthesize and accumulate large amounts (20–50% of dry weight) of neutral lipid (mainly in the form of triacylglycerol, TAG) stored in cytosolic lipid bodies (Day et al., 1999; Hu et al., 2008). Some species of diatoms (e.g., *Chaetoceros muelleri*) and green algae (e.g., *Chlorella vulgaris*) have been considered to be ideal sources of neutral lipids suitable for biodiesel production (McGinnis et al., 1997; Illman et al., 2000). However, there is still much to be done to move the effort from laboratory research to commercial production of biofuel feedstock. The critical starting point for this process is identification of suitable algal strains that possess high constituent amounts of total lipids in general, and neutral lipids, in particular, and are capable of rapid accumulation of large quantities of neutral lipids under various culture conditions. Identification of desirable algal species/strains will require screening of the lipid contents in both naturally-occurring and genetically-modified algae.

The conventional method used for lipid determination involves solvent extraction and gravimetric determination (Bligh and Dyer, 1959). Further quantification of neutral lipids requires the separation of the crude extractions and quantification of the lipid fractions by thin-layer chromatography (TLC), HPLC or gas chromatography (GC) (Eltgroth et al., 2005). The procedure used for lipid analysis must ensure complete extraction and at the same time avoid decomposition and/or oxidation of the lipid constituents. A major drawback of the conventional method is that it is time- and labor-intensive, making it difficult to screen large numbers of algae. As a result, increasing attention has focused on *in situ* measurements of the lipid contents (Cooksey et al., 1987; Izard and Limberger, 2003).

Nile red, a lipid-soluble fluorescent dye, has been frequently employed to evaluate the lipid content of animal cells and microorganisms, such as mammalian cells (Genicot et al., 2005), bacteria (Izard and Limberger, 2003), yeasts (Evans et al., 1985; Kimura et al., 2004), zooplankton (Kamisaka et al., 1999), and microalgae (McGinnis et al., 1997; Eltgroth et al., 2005; Elsey et al., 2007). However, most of the studies have provided only a qualitative or semi-quantitative analysis of

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the lipids. An *in situ* quantitative determination of cellular neutral lipid content using a Nile red method has yet to be established. Furthermore, the Nile red assay was usually carried out in individual cuvettes, using a UV spectrophotometer or flow cytometer one sample at a time, resulting in a time-consuming process.

Of the algal species and strains analyzed by the Nile red method, most have belonged to the Bacillariophyceae (Cooksey et al., 1987; McGinnis et al., 1997), Xanthophyceae (Eltgroth et al., 2005), Chrysophyceae (de la Jara et al., 2003) Phaeophyceae (Brzezinski et al., 1993) with only a few species from the Chlorophyceae (Lee et al., 1998; Elsey et al., 2007). On the other hand, over the past several decades, numerous algal species and strains belonging to the Chlorophyceae have been reported to contain, based on the conventional solvent extraction and gravimetric determination analysis, high levels (25–50% of dry weight) of neutral lipid, suggesting that this class of algae may represents a large pool of organisms from which desirable candidates could be obtained for lipid/oil feedstock production (Hu et al., 2006, 2008). During our preliminary screening of algae for high neutral lipid producers, we realized that the high neutral lipid content measured in a number of green algal species resulted from the solvent extraction and gravimetric method (Bligh and Dyer, 1959), but failed to be detected by the commonly used Nile red method (Cooksey et al., 1987). We speculated that the failure of the Nile red method to determine the neutral lipid content in the green algae in our studies and perhaps also in previous efforts was due to the composition and structure of the thick and rigid cell walls common in many green algae which may prevent the Nile red dye from penetrating the cell wall and cytoplasmic membrane and subsequently binding with the intracellular neutral lipid and polar lipids to give the desired fluorescence.

In this study, we re-examined the Nile red method with nine algal species of various classes. Various physical and chemical treatments were applied to the existing Nile red method to improve the effectiveness and efficiency. A modified, improved Nile red method was developed primarily using the green alga *C. vulgaris* as a model system. An optimal protocol was successfully applied to six other green algal species and strains varying in cell size and morphology. The modified, improved Nile red method can be used as a sensitive, quantitative, and high throughput method for screening of cellular neutral lipid content in green algae as well as other classes of algae.

2. Materials and methods

2.1. Organisms and culture conditions

Species of algae were obtained from the Culture Collection of Algae at the University of Texas at Austin (*Ankistrodesmus pseudobraunii* UTEX LB1380, *Nannochloris* sp. UTEX LB2291 the Provasoli-Guillard National Center for Culture of Marine Phytoplankton, U.S.A..(*Chaetoceros lauderi CCMP 193*; *Emiliania huxleyi CCMP 372*; *Crypthecodinium cohnii CCMP 316*) the CSIRO Marine Microalgae Research Center, Australia. (*Rhodomonas salina CS 24*; *Nannochloropsis* sp. CS 246; *Pavlova pinguis CS 375*), and from our laboratory isolations (*Chlorella zofingiensis. C. vulgaris LARB#2*, *Palmelococcus miniatus*, *Desmodesmus quadricauda*, *Pseudochlorococcum* sp.). *C. vulgaris*; and *C. zofingiensis,* and *Pseudochlorococcum* sp. were maintained in BG-11 growth medium, *Nannochloropsis* sp, *R. salina*, *P. pinguis*, and *E. huxleyi* were grown in f/2 growth medium, and *C. cohnii* was grown in f/2+NPM culture medium. *C. lauderi* was grown in f/2 + Si medium. All the algal strains were maintained in a glass column photobioreactor at 25 °C, and exposed to a continuous illumination at a light intensity of 300 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Culture mixing was provided by bubbling air containing 1.5% CO_2 (v/v).

2.2. Chemicals and reagents

Nile red and lipid standards were purchased from Sigma (USA). Silicon gel plates used for separation of neutral lipids was from Merck (Germany). All other chemicals and solvents of analytical grade were purchased from Sigma or other commercial suppliers.

2.3. Lipid analysis

2.3.1. Gravimetric determination of neutral lipids

Lipids were extracted in a chloroform–methanol–water system according to Bligh and Dyer (1959). The extract was evaporated in a water bath (30 °C) using a rotary evaporator (Büchi, Switzerland) to remove solvents. Crude lipids were further separated by column chromatography using silicon gel (60–200 mesh) (Merck Corp., Germany) according to Alonzo and Mayzaud (1999) using the following solvent sequence: 6 volumes of chloroform to collect the neutral lipid class and 6 volumes of methanol to collect the polar lipids. Each lipid fraction was transferred into a pre-weighed vial, initially evaporated in a water bath (30 °C) using a rotary evaporator and then dried under high vacuum. The dried residuals were placed under nitrogen and then weighed.

2.3.2. Nile red fluorescence determination of neutral lipid

After the algal cell suspensions were stained with Nile red, fluorescence was measured on a Varian 96-well plate spectrofluorometer using medium scan control and high PMT detector voltage mode. According to the pre-scan of excitation and emission characteristics of neutral lipid standards, the excitation and emission wavelengths of 530 nm and 575 nm were selected.

2.4. Optimization of the modified Nile red fluorescence method

2.4.1. DMSO concentration

5 μL algal samples of known cell concentration were introduced into individual wells of a 96-microplate containing 3 μL of a 50 μg Ml^{-1} Nile red solution. Then, 292 μL DMSO aqueous solutions with the concentrations ranging from 1% (v/v) to 40% were added. The 96-well plate was vortexed (120 rpm) and incubated at 40 °C for 10 min. After algal cells were stained, fluorescence emissions were recorded with a Varian spectrophotometer equipped with a 96-well plate reading mode. Unless stated otherwise, six replicates of each treatment were analyzed.

2.4.2. Nile red dye concentration

Nile red dye, at different concentrations, ranging from 0.1 to 10 μg/mL was used following the experimental procedure detailed above to optimize the dye concentration. In this experiment, 25% DMSO (v/v) was used in the staining solutions.

2.4.3. Staining time

For the optimization of staining time, algal suspensions of defined cell concentrations were stained with Nile red in 25% DMSO aqueous solutions. Staining times of 5 min, 10 min, 20 min, 30 min, 60 min and 100 min were evaluated.

2.4.4. Staining temperature

Staining temperatures, ranging from 20 °C to 80 °C were investigated following the staining procedure described above.

2.4.5. Algal cell concentration

To optimize cell concentrations for determination of cellular neutral lipid, several cell concentrations, ranging from 10 to 8×10^5 cells mL^{-1} were evaluated using the above procedures for cell staining.

2.5. Comparison of lipid content by Nile red fluorescence method and conventional gravimetric method

To verify that the modified Nile red fluorescence method was effective in determining neutral lipid content, the modified method and conventional gravimetric method were compared for the green alga *C. vulgaris*. The cells used were from a 12-day liquid culture. For