



Rapid, colorimetric quantification of lipid from algal cultures

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ABSTRACT

Algae have significant potential as a source of biomass for the production of biofuels, due to their high growth rates and high cellular lipid content. Studies that address the use of algae as biofuels often require the frequent measurement of algal lipid content. Traditional methods for the quantification of lipid are, however, costly if sub-contracted, or involve the use of expensive analytical equipment that is not available in many labs. This study describes a simple, colorimetric method for the quantification of algal lipid from small amounts of culture. The technique is derived from a method for the quantification of fatty acids dissolved in chloroform. Algal lipids are saponified to fatty acids and then mixed with a copper reagent. Chloroform-extractable copper soaps of long-chain fatty acids are then colorimetrically measured by the addition of diethyldithiocarbamate to develop a yellow colored product. Linear responses for fatty acids in the range of C10:0 to C16:0 were observed for a concentration range between 0.025 and 1 μmol of fatty acid per 200 μL of sample. Fatty acids with chain lengths of less than twelve carbons produced significantly reduced signal. Decenoic acid yielded a slightly, but significantly lower signal than decanoic acid indicating that the assay underestimates the presence of unsaturated fatty acids. Lipid contents of *Phaeodactylum tricoratum* and *Chlorella vulgaris* CM2 were monitored for eight days during exponential growth to demonstrate the feasibility of the technique as a monitoring methodology. Overall, the method allowed reliable detection and quantification of fatty acid content from 1 to 2 mL of algal culture. Adaptation of the technique to micro-centrifuge format allows assaying 30 samples in less than 2 h. Considering reagents and time, the total cost per assay was estimated at less than \$5, representing a significant cost savings over traditional lipid quantification procedures.

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1. Introduction

Alternative energy sources in the form of biofuels will be critical to achieving a carbon neutral and sustainable liquid fuel economy. Most current biofuel production efforts, such as oil from agricultural plants or bioethanol, are based on strategies that are highly land and resource intensive and are therefore not sustainable (Chisti, 2008). Approximately 50% of all primary productivity is performed by photosynthetic algae (Chisholm, 2000), suggesting that algae represent an important alternative to agricultural crops for biomass production. The oil content of some algae can exceed 80% (Banerjee et al., 2002; Chisti, 2008), while most agricultural crops used for biodiesel produce yields of less than 5% of biomass (Chisti, 2008). Additionally, doubling times of algae can be on the order of only 4 to 24 h. Such growth rates are unrivaled by any land plant grown for agricultural or biofuel purposes. It has been estimated that even a conversion of a modest proportion (3 to 6%) of total agricultural

cropland for the purpose of producing algal biomass could, in theory, satisfy our demand for transportation fuel (Chisti, 2008).

Despite the enormous potential of algal biofuels, many challenges to making their large-scale production feasible remain. These challenges include problems posed by the design of optimal bioreactors, the isolation, dewatering, and chemical conversion of algal biomass, and the maintenance of physical and chemical environments needed for optimal algal growth.

Another significant component of the design of algal biofuel production processes is the selection and genetic engineering of algal strains. As part of these experiments, it will be necessary to conduct detailed physiological studies that require the frequent quantification of lipid from algal cultures. Traditional approaches for the quantification of lipid from microbial biomass rely on labor- and equipment-intensive methods, such as the GC–MS analysis of fatty acid methyl esters (FAME) or the quantification of phospholipid fatty acid (PFLA) profiles. Commercial laboratories frequently charge between \$50 and \$300 per sample for these analyses, making them cost-prohibitive if analytical equipment is not readily available.

A methodology for the rapid, simple, and colorimetric quantification of lipid from algal cultures is described in this manuscript. The technique was derived from a method for the quantification of fatty acids dissolved in chloroform (Duncombe, 1963). It is based on the

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hydrolysis of algal lipids to fatty acids and subsequent extraction of their copper salts into chloroform. The amount of copper in the chloroform phase is then colorimetrically determined by adding diethyldithiocarbamate to develop a yellow colored product (measured by optical density at 440 nm). In this manner we were able to quantify lipid in micro-centrifuge tube format from 1 mL of log-phase algal culture in less than one hour, and for less than \$5 per sample.

2. Methods

2.1. Strains and isolates

Phaeodactylum tricornutum (CCMP1327) was obtained from the CCMP culture collection (Bigelow Laboratory for Ocean Sciences) and maintained axenically in F/2 medium (Guillard and Ryther, 1962). The *Chlorella vulgaris* CM2 strain used in this study was isolated by extinction dilution from the University of Oklahoma Duck Pond and identified by sequencing its 18S ribosomal RNA gene. The strain was grown and maintained in SN medium (Waterbury et al., 1986).

2.2. Standard curves

All fatty acids used in this study were obtained from Sigma-Aldrich (>99% purity). Sodium salts of decanoic acid, decenoic acid, lauric acid, myristic acid, palmitic acid, and sarkosyl were prepared as 10 mM stock solutions in 1 M NaOH. Longer chain fatty acids had to be solubilized by heating standards to 60 °C. Dilutions were prepared, weight by weight, using an analytical balance in pre-warmed 1 N NaOH. Two hundred microliters of each standard and dilutions thereof were added to micro-centrifuge tubes for quantification.

2.3. Growth curves and cell counts

Phytoplankton cultures were grown on a shaking platform at 20 °C in a light–dark incubator with 12 h light/dark cycles. A 1:10 inoculum was used for 100-mL cultures, which were sampled every 24 h for 10 days. Cell counts were determined by epifluorescence microscopy under blue excitation using a micrometer grid on an Olympus BX-61 microscope. Cells were fixed using 1/10 volume of 37% formaldehyde, and a dilution of this fixed sample was filtered onto 0.22 µm, 25 mm diameter black stained polycarbonate membrane filters (Sterlitech, Kent WA). No staining was necessary due to the auto-fluorescence of phytoplankton pigments. At least 30 fields were counted and averaged for each slide.

2.4. Saponification of cellular lipids

One to two mL of algal culture was spun in 2-mL screw cap tubes at 14,000 ×g for 2 min, and the supernatant was removed. Cell pellets were frozen at –20 °C until extracted. Cell pellets were thawed and 200 µL of saponification reagent (25% methanol in 1 N NaOH) and 50 mg of 0.1 mm muffed glass beads (RPI, Chicago IL) were added to each tube. Cells were disrupted by bead-beating using a Mini Beadbeater-8 (BioSpec Bartlesville, OK) at the maximum setting for 2 min. Complete cell lysis was confirmed by observing wet mounts under light microscopy. Samples were heated in a water bath at 100 °C (boiling) for 30 min and vortexed every 5 min during the process in order to hydrolyze ester bonds of membrane lipids and triglycerides.

2.5. Extraction and colorimetric detection

Two hundred microliters of neutralization reagent (1 N HCl, 100 mM Tris pH 8.0) and 200 µL of copper reagent (9 vol. aq. 1 M triethanolamine, 1 vol. 1 N-acetic acid, 10 vol. 6.45% (w/v) Cu (NO₃)₂·3H₂O) were added to each sample of saponified cells and

standards. Samples were vortexed for 2 min, 250 µL of chloroform was added to each tube and vortexed for an additional 2 min, and then the samples were spun at 14,000 ×g for 1 min. The organic phase was carefully transferred to a separate micro-centrifuge tube. (Note: It is critical at this step that none of the aqueous phase is transferred, since it contains the remaining copper ions which will produce false signal during the detection step). From each tube containing the organic phase, 50 µL was transferred into two separate new tubes. (This two-step pipetting procedure reduces potential carryover of the aqueous phase and increases pipetting accuracy.) One of these tubes received 50 µL of 2-butanol and served as the blank for spectrophotometric detection. The second tube received 50 µL of 1% (w/v) sodium diethyldithiocarbamate in 2-butanol (developing reagent) leading to yellow color development proportional to the amount of fatty acid present. Blanking the instrument by splitting the samples is necessary, because algae contain chlorophylls, which will partially co-extract into the chloroform phase and exhibit significant absorption at 440 nm. Lipid content is therefore significantly overestimated, if no steps are taken to subtract the signal produced by the co-extracted chlorophyll. 1% diethyldithiocarbamate in 2-butanol is optically identical to 2-butanol at 440 nm i.e. no absorption of the developer alone is observed at 440 nm (OD₄₄₀ < 0.004). All tubes were briefly vortexed and absorbances of 4 µL-aliquots were measured at 440 nm using an IMPLEN NanoPhotometer (Implen GmbH, München, Germany). The developing reagent was made every week, stored at 4 °C, and discarded if white precipitate was observed.

2.6. Estimation of the proportion of cellular lipid

The two algal strains used in this study were grown into stationary phase, and 80 mL of culture was centrifuged for each. The cell pellets were washed with 1 × TE buffer (10 mM Tris pH 8.0, 1 mM EDTA pH 8.0) to remove excess salt from the pellets and centrifuged in pre-weighed 1.5-mL micro-centrifuge tubes. Cell pellets were dried for 2 h under vacuum and weighed. Cell counts were obtained as indicated above, and these data were used to calculate average weight per cell. Cell pellets were then suspended in 2 mL of saponification reagent, and replicate 100-µL aliquots were assayed for total lipid content as indicated above. Lipid content per cell was estimated by assuming an average fatty acid chain length of 16 carbons (C16:0 mw = 256.42 g/mol). This assumption is not unreasonable considering observation on *P. tricornutum*, in which C16:0 and C16:1 have been reported to account for between 39.6% and 74.5% of total fatty acids identified (Siron et al., 1989).

3. Results

A simple and fast method for the quantification of lipid from small quantities of algal and bacterial cultures is described. The procedure was designed to enable cheap and rapid monitoring of lipid production during physiological experiments and/or biomass production processes.

All long-chain fatty acids tested as part of this study exhibited linear responses in the assay in the range between 0.025 and 1 µmol of fatty acid per 200 µL of sample (Fig. 1). While generating standard curves, it was observed that the extinction coefficient for lauric acid ($E_{440} = 0.054 \pm 0.004 \times 10^5$ per mole) was significantly greater than the extinction coefficient for decanoic acid ($E_{440} = 0.037 \pm 0.003 \times 10^5$ per mole). This indicated differential behavior of fatty acids of differing chain lengths. Two hundred nanomoles of C10:0, C12:0, C14:0, and C16:0 fatty acids were therefore assayed to compare their behavior (Fig. 2). C12:0, C14:0, and C16:0 fatty acids did not produce significantly different extinction coefficients (pair-wise Student *T*-test *p*-values of 0.27, 0.16, and 0.98 respectively). The extinction coefficient for assayed decanoic acid, however, was significantly lower than the remaining fatty acids that were tested (*T*-test; *p*-

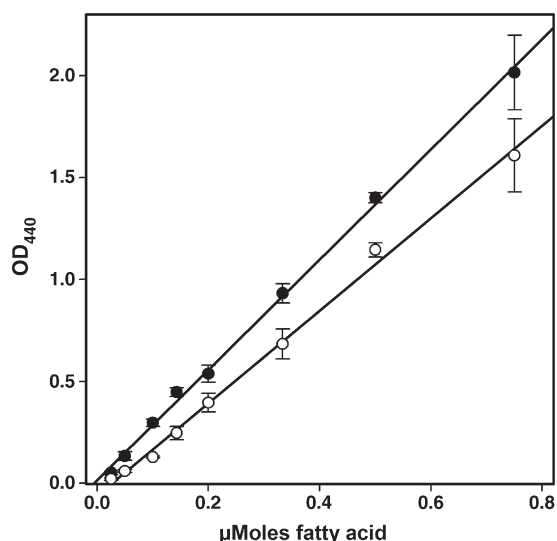


Fig. 1. Optical density at 440 nm as a function of fatty acid concentration. Two standard curves were analyzed. (●) Represents data from five replicate measurements of a dilution series of sodium laurate. (○) Represents data from five replicate measurements of a dilution series of sodium decanoate. Data are shown in μmol of fatty acid per extraction.

values of 0.0008, 0.0003, and 0.0008 for C12:0, C14:0, and C16:0, respectively).

Equal concentrations of decenoic acid and decanoic acid were quantified (Fig. 3A) to determine whether unsaturation of cellular fatty acids would affect their detection. The extinction coefficient for decanoic acid ($E_{440} = 0.034 \pm 0.002 \times 10^5$ per mole), was slightly greater (T -test, $p = 0.057$) than the extinction coefficient for decenoic acid ($E_{440} = 0.031 \pm 0.001 \times 10^5$ per mole), indicating that unsaturation has a small, but significant, effect on the described assay, leading to an underestimation of total cellular lipid content. Similarly, the effect of functional groups was tested. The extinction coefficient for lauric acid ($E_{440} = 0.054 \pm 0.002 \times 10^5$ per mole) was slightly, but significantly (T -test, $p = 0.055$) greater than the extinction coefficient for sarkosyl ($E_{440} = 0.049 \pm 0.004 \times 10^5$ per mole), suggesting that chemical modification of fatty acids (in this case a keto-group and amino acid moiety) has a measurable effect on detection of fatty acids and will also lead to an underestimation of total lipid content.

The described assay was applied to quantify cellular lipid from cultures of *P. tricornutum* and *C. vulgaris* CM2. A 1/10 inoculum was made from stationary phase cultures (grown for eight days) and sampled daily for cell densities and lipid content. We were able to

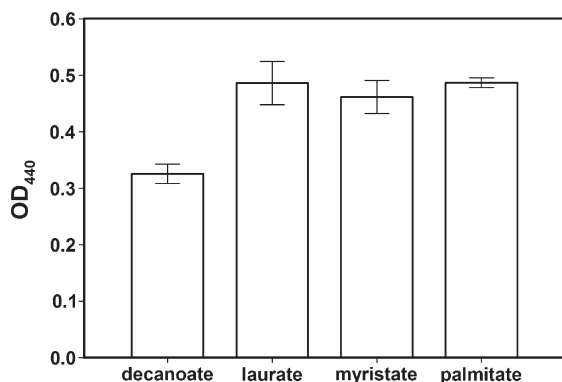


Fig. 2. Average OD_{440} reading observed for 2 mM standard solutions of fatty acids with differing chain lengths. Five replicate extractions were extracted and quantified for each standard. Error bars indicate one standard deviation.

reliably quantify lipid from 1 to 2 mL of algal culture throughout the entire growth curve (Fig. 4). During incubation, cell counts in both cultures increased by approximately one order of magnitude (Fig. 4A), indicating that both cultures were healthy and grew well under the given conditions. Interestingly, it was observed that cellular lipid content decreased by 59% and 81% for *P. tricornutum* and *C. vulgaris* CM2, respectively, during the first 48 h of incubation (Fig. 4B) and only began to recover at the end of growth experiment. Cellular lipid contents for the stationary phase inocula were estimated at 9.4% and 12.0% for *P. tricornutum* and *C. vulgaris* CM2, respectively (Table 1).

4. Discussion

Algae, as a source of biofuel, offer many advantages over traditional biofuel crops including the potential to be grown on marginal land, the use of water sources not suitable for agriculture (e.g. high salt content could be tolerated by halotolerant algae), as well as high growth rates, and high relative lipid content. Approaches to algal biofuel production include the production of hydrogen (Ghirardi et al., 2000) and methane (Spolaore et al., 2006). The largest potential for algal fuel production, however, probably lies with the production of diesel from microalgal oils (Chisti, 2007; Roessler et al., 1994; Sawayama et al., 1995). Primarily, biodiesel is produced by the hydrolysis of cellular lipids (phospholipids and triglycerides) to fatty acids and glycerol. Fatty acids are then esterified with methanol to produce fatty acid methyl esters. Cellular long-chain fatty acid content therefore represents a direct measure of final biofuel yield. The goal of the presented work was to provide a simple and cheap methodology for the quantification of lipid from algal culture to facilitate physiological studies.

Several important caveats of the technique should be mentioned. Most importantly, no structural information is gained from the assay. More traditional approaches provide information about the chemical composition of the fatty acids (e.g. chain length and degree of unsaturation). It might therefore be useful to use a combination of techniques, where FAME and/or PLFA analysis are performed for some samples to provide a baseline of structural information, while routine monitoring of cultures during physiological experiments is achieved with the simpler technique described here. It should also be noted that the described method does not quantify the mass of fatty acids directly. Instead, the molar concentration of chloroform-extractable carboxyl groups, complexed with copper ions, is measured. The total mass of fatty acids has to be inferred via an assumption about their average chain length to convert the detected signal into a quantity of fatty acid. Preliminary FAME analysis on algal strains would therefore be useful to provide a baseline, keeping in mind that fatty acid composition can vary as a function of growth temperature, cell age and media composition (Bezbaruah et al., 1988).

Short chain fatty acids from cellular metabolism are unlikely to affect detection signal, since the signal decreases rapidly for chain lengths of less than twelve carbons. Extinction coefficients for cholesterol, lactic acid, and triglycerides are also very low, indicating that these molecules will not interfere with detection (Guillard and Ryther, 1962). No interference was observed for Tris buffer or EDTA. Detergents frequently used in cell lysis such as sarkosyl (Fig. 3B) and SDS (data not shown), however, produced significant yellow color in the assay. This indicates that detergents are not useful in aiding in the complete cell lysis necessary for saponification. Bead-beating was therefore applied in quantifications from cultures, since the inclusion of glass beads does not affect detection. The extinction coefficient for lauric acid reported here ($E_{440} = 0.0544 \times 10^5$ per mole) was slightly lower than that reported in the original description of the fatty acid detection method (Guillard and Ryther, 1962) ($E_{440} = 0.0776 \times 10^5$ per mole). We attribute this difference to inefficiencies introduced by the extraction step required to solubilize fatty acids from saponified algal biomass into chloroform as well as differences in working and detection volumes.

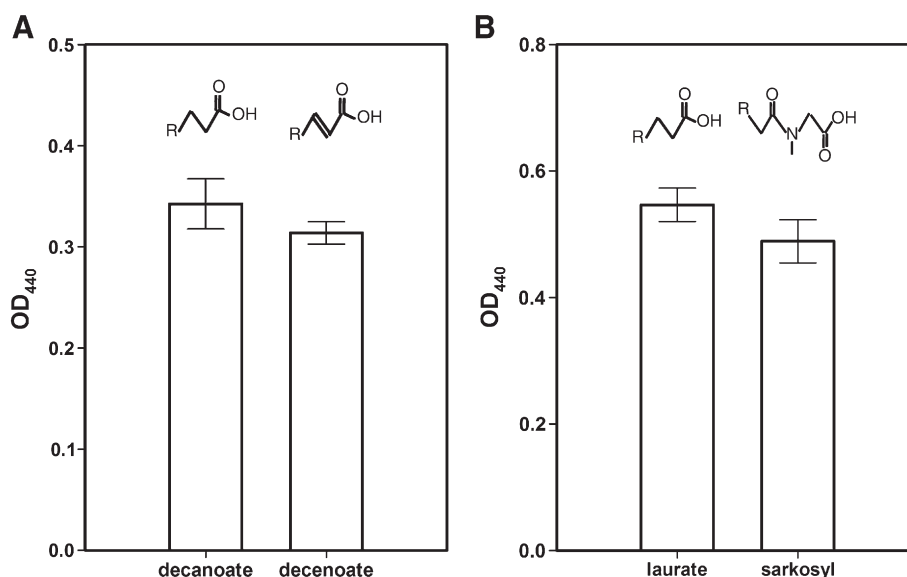


Fig. 3. Average OD₄₄₀ reading observed for 2 μ mol standard solutions of fatty acids with differing chemical structure. (A) Comparison of signals observed for a saturated (decanoic acid) and an unsaturated (deconoic acid) fatty acid. (B) Comparison of signals observed for a saturated (laurate) fatty acid and an amino acid ester of a fatty acid containing a keto-group (sarkosyl).

Measurements of cellular lipid content for *C. vulgaris* CM2 were somewhat lower than prior estimates for *Chlorella* sp., which has been estimated to contain ca. 28–32% dry weight equivalent of oil (Chisti,

2007). This difference might reflect strain-to-strain variation or may, at least in part, be due to a methodological underestimation of lipid here. Lipid content for *P. tricornutum*, however, has been previously estimated between 5.4% and 10.7% (Siron et al., 1989), which is in the same range as estimates obtained for *P. tricornutum* here (9.4% for a stationary phase culture, Table 1). Interestingly, we observed a significant decrease in cellular lipid content when stationary phase cultures were switched to conditions of balanced growth (Fig. 4). The observed decrease in cellular lipid content is consistent with prior observations in *P. tricornutum*, where this parameter has been shown to vary by as much as two-fold, depending on the growth phase of cultures (Siron et al., 1989). The implications for biofuel production are that maintaining algae under optimal, balanced growth conditions may not necessarily lead to the highest production yields. This hypothesis should, however, be more rigorously addressed. Employing the method described here should make answering this and similar questions much easier.

Overall, the described method allowed reliable detection and quantification of fatty acid content from small volumes (1 to 2 mL) of algal culture. This was an important goal of this study, because it allowed the application of the technique in micro-centrifuge format, in which it is possible to run 24 to 30 samples (i.e. the number of tubes that fit into a typical micro-centrifuge rotor) simultaneously. The procedure takes less than 2 h and reagent cost per tube was estimated at less than ten cents per sample. Assuming a technician-hour cost of \$50 and a conservative time estimate of 3 h to run the procedure, the total cost per assay will be less than \$5 per sample, representing a significant cost savings over traditional lipid quantification procedures.

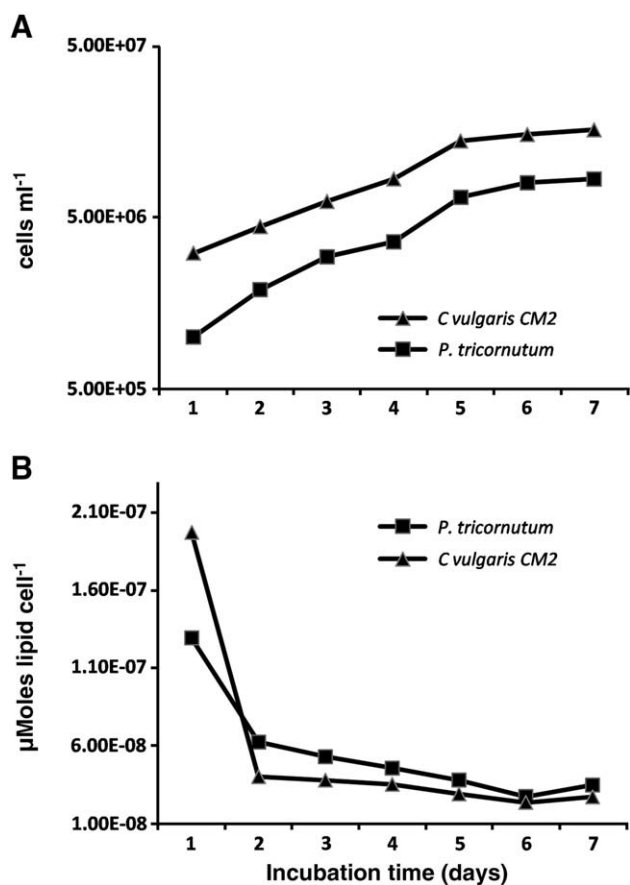


Fig. 4. Detection of lipid from 2 mL of culture sampled from cultures of *Phaeodactylum tricornutum* (■) and *Chlorella vulgaris* (▲). (A) Cell counts per mL for both cultures as a function of time (days of incubation). (B) Cellular lipid content for both cultures as a function of time (days of incubation).

Table 1
Dry weight and lipid content estimates for two algal cultures.

	<i>Phaeodactylum tricornutum</i>	<i>Chlorella vulgaris</i>
Dry biomass per mL of culture (g)	0.025	0.0191
Dry biomass per cell (ng)	71.2 ± 1.7	42.8 ± 2.3
Fatty acid per mL culture (μmol equivalent)	0.115 ± 0.0065	0.111 ± 0.0048
Fatty acid per cell (μmol equivalent)	3.27E-07 ± 0.019	2.50E-07 ± 0.0011
% lipid of dry weight	9.40 ± 1.77	12.0 ± 0.56

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