Chapter 7

Extraction and Characterization of Lipids from Macroalgae

David R. Nobles Jr. and Schonna R. Manning

Abstract

Although most algal biofuel research has focused on microalgae, macroalgae are also potential sources of lipid for the production of biodiesel and other liquid fuels. Reliable, accurate methods for assessing the lipid composition of biomass are essential for the development of macroalgae in this area. The conventional methods most commonly used to evaluate lipid composition, such as those of Bligh and Dyer and Folch, do not provide complete extraction of lipids in photosynthetic cells/tissues and therefore do not provide an accurate accounting of lipid production. Here we present a 2-EE lipid extraction protocol, a method which has been demonstrated to be superior to conventional lipid extraction methods for microalgae, adapted for use with macroalgae.

Key words Algae, Macroalgae, Lipid extraction, 2-Ethoxyethanol, Lipid profiling by thin-layer chromatography, Lipid analysis

1 Introduction

Algal biomass is a promising resource for the production of renewable fuels. Algae offer the advantages of high aerial productivity, growth in saline and brackish water, use of marginal land for cultivation, and the coproduction of high value products [1, 2]. The production of biodiesel from triacylglycerols (TAGs) has been central to the efforts to commercialize algae at a very large scale. Microalgae are known to produce TAGs at up to 50% of their biomass dry weight [3] and thus have been the primary focus of most algal biofuels development and research. Macroalgae have generated much less interest as potential biofuel feedstocks because they have generally been characterized as having low-lipid productivity, often producing less than 5% lipids by dry weight. However, there are multiple examples of macroalgae with lipid production exceeding 10% of the dry biomass. These higher values for observed lipid accumulation, combined with the relative ease of cultivation and harvest of macroalgae as compared to microalgae,

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make them viable candidates for biofuel production and worthy of additional study in this area [4].

Accurate and repeatable methods for the extraction and quantification of lipids are key to the meaningful evaluation of biomass from diverse algae and their suitability as biodiesel feedstocks. To date, the most commonly employed protocols for lipid extraction from microalgal biomass have been the methods of Folch and of Bligh and Dyer [4-7]. However, it has been demonstrated that the use of 2-ethoxyethanol (2-EE) provides superior lipid recovery (150-200%) in microalgae when compared to these methods [8]. Here we describe methods for biomass preparation and related metrics, total lipid extraction using 2-EE, and the identification of lipid classes by thin layer chromatography (TLC). The standardized 2-EE protocol is widely used for microalgae and is adapted here for the extraction of lipids from macroalgae.

2 Materials

Prepare all solutions using ultrapure or double-distilled water and analytical grade reagents. Adhere to all waste disposal regulations when disposing of waste materials.

2.1 Fresh Weight (FW), Dry Weight (DW), and Ash-Free Dry Weight (AFDW) Determination

1. Porcelain crucibles.
2. Scalpel, scissors, or razor blade for harvesting biomass.
3. Flat-tipped forceps.
4. Filter paper or Kimwipes.
5. Weigh boat or weighing paper.
6. Drying oven (80 C).
7. Crucible tongs.
8. Desiccator with desiccant (e.g., calcium sulfate, Drierite) (see Note 1).
9. Muffle furnace (550 C).
10. Analytical balance.
11. 15-mL conical centrifuge tubes.
12. 5-mL serological pipettes with pipette pump.
13. 0.5 M NaCl solution (filter sterilized and stored at 4 C).
14. 0.5 M Ammonium Bicarbonate (filter sterilized and stored at 4 C).
15. Fresh macroalgal tissue (generally material from blades), 100-200 mg per sample/replicate.

2.2 Lipid Extraction

1. 100-mL round-bottom flask (RBF).
2. Vortex mixer.

3. Water bath (60 C).
4. 40-mL glass vials with PTFE-lined screw caps.
5. 5-mL glass vials with PTFE-lined screw caps.
6. P1000 micropipettor and 1 mL micropipette tips. 7. 10-mL serological pipettes and pipette bulb.
8. Methanol in squeeze bottle.
9. Acetone in squeeze bottle.
10. Rotary evaporator.
11. Resuspension solvent—toluene-hexane-acetone-methanol 2:2:1:1 (v/v/v/v).
12. 2-Ethoxyethanol (2-EE).
13. Macroalgal tissue, 100-200 mg per sample/replicate.
14. Glass Pasteur pipettes and bulb.
15. Glass fiber membrane, 1.2 μm porosity (see Note 2).
16. Parafilm (see Note 3).

2.3 Analysis of Biomass Lipids by Thin-Layer Chromatography (TLC)

1. TLC plates—silica gel 60 aluminum sheets with 254 nm fluorescent indicator, F254 (EMD-Millipore 1055670001). 2. 5-μL semiquantitative capillary micropipettor.
3. Chromatography tank.
4. Iodine crystals (Fisher Scientific 7553-56-2) and silica gel mesh 60-200 (Fisher Scientific 7631-86-9) mixed 1:1, sealed in plastic bin, and stored in a dedicated fume hood.
5. Lipid class reference standard mixture with 2 mg/mL final concentration of each analyte, including TAG, DAG, MAG, FFA, PC, PE, FAME, and BC (see Note 4).
6. Polar solvent system—chloroform-methanol-acetone-acetic acid-water 65:10:20:10:3 (v/v/v/v/v; see Note 5).
7. Nonpolar solvent system—hexane-diethyl ether-acetic acid 80:20:1 (v/v/v; see Note 5).
8. Ultraviolet (UV) light handwand (254 nm and 365 nm).

3 Methods

If dried tissue will be used for the lipid extraction, prepare two sets of tissue samples: one set of samples to be reserved for lipid extraction after drying and one set to determine ash-free dry weight (see Fig. 1; see Note 6).

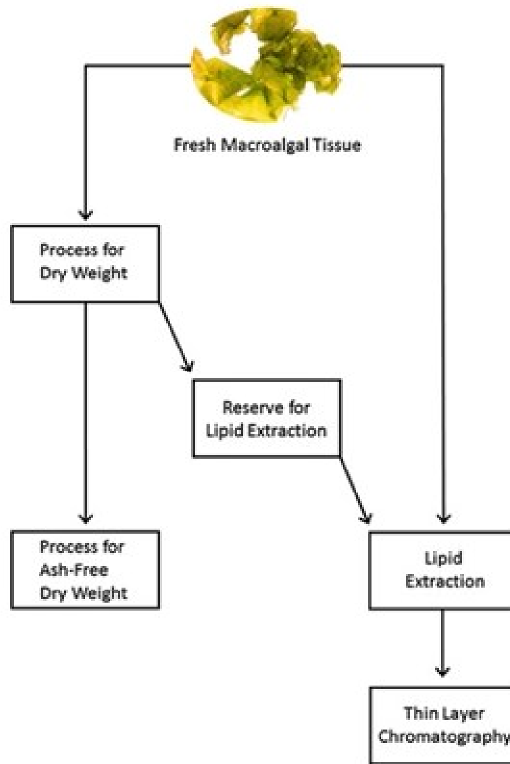

Fig. 1 This flow diagram illustrates the workflow streams for lipid extraction from fresh and dried biomass. Lipid extractions can be performed directly using fresh biomass or alternatively, on dried biomass after determination of dry weight. For the latter option, samples are split with half utilized to determine ash-free dry weight and half utilized for lipid extraction

3.1 Fresh Weight (FW), Dry Weight (DW), and Ash-Free Dry Weight (AFDW) Determination

1. Weigh a labeled crucible for each sample to be processed. Record the values.
2. Using a scalpel, scissors, or razor blade, harvest macroalgal tissue (typically from the blades) and place in a 15-mL conical tube.
3. Use a serological pipette to wash the tissue with 5 mL of sterile 0.5 M NaCl; remove epiphytes and any other foreign material by gently inverting the sealed tube 10-15 times.
4. Decant the NaCl solution. Use a serological pipette to rinse the tissue with 5 mL of 0.5 M ammonium bicarbonate and gently invert 10-15 times.
5. Remove tissue with flat-tipped forceps and gently pat dry with filter paper or Kimwipes.
6. Weigh the washed tissue in a tared weigh boat or on weighing paper to determine the FW. Record the value.

7. Transfer tissue to a preweighed crucible and place in a drying oven set to 80 C. Check the weight of the crucible every 2 h until a constant weight is obtained.
8. Store the sample in the desiccator overnight to remove the last traces of water.
9. Transfer the crucible to the analytical balance and weigh. Record the value.
10. If using dried tissue for lipid extraction, one set of samples should be returned to the desiccator and reserved for lipid extraction. The other set will proceed to step 11 for ashing (see Fig. 1).
11. Place the crucible in a muffle furnace set to 550 C.
12. Combust the tissue sample for 2 h then allow the furnace to cool for 1 h.
13. Remove the crucible using crucible tongs and place on an insulated surface—items will be very hot. Allow the crucible and contents to cool to room temperature, place in a desiccator, and weigh once an hour until a constant weight is achieved.
14. Transfer the crucible to the analytical balance and weigh. Record the value. Subtract the weight obtained from step 1 to determine the weight of the ash.
15. Calculate the tissue DW, AFDW, % ash, and % moisture for sample Duplicate 1 (DWD1, AFDWD1, etc.) as follows:

$$\mathrm{DWD1} = (\mathrm{crucible} + \mathrm{dried\ tissue}) - \mathrm{crucible}$$
$$\mathrm{AFDWD1} = (\mathrm{crucible} + \mathrm{dried\ tissue}) - (\mathrm{crucible} + \mathrm{ashed\ tissue})$$
$$\%\mathrm{Ash} = (\mathrm{crucible} + \mathrm{ash}) - \mathrm{crucible}()/\mathrm{DWD1} \times 100\%$$
$$\%\mathrm{Moisture} = (\mathrm{FWD1} - \mathrm{DWD1})/\mathrm{FWD1} \times 100\%$$

where

FWD1 = fresh weight of duplicate #1 in grams (see Subheading 3.1, step 4).

DWD1 = dry weight of duplicate #1 in grams.

AFDW1 = ash-free dry weight of duplicate #1 in grams.

Repeat calculations for each sample Duplicate 2 (DWD2, AFDWD2, etc.).

3.2 Lipid Extraction

1. Weigh 25-50 mg of dried tissue reserved in step 8 from Subheading 3.1 and transfer to a 40-mL glass vial. Alternatively, fresh biomass can be used. In which case, an amount of fresh tissue proportional to that of the dried material, based on the ratio of fresh weight to dry weight can be used (see Subheading 3.1, step 13 for dry weight calculation method).
2. Use a serological pipette to transfer 5 mL of MeOH to the vial containing harvested tissue. Cap the vial tightly and extract the tissue by heating in a 60 C water bath for 30 min.

3. Use a serological pipette to transfer 5 mL 2-EE to the vial, cap tightly, and extract lipids by heating in a 60 C water bath for 60 min. Mix by gentle vortexing for a few seconds every 5 min.
4. Transfer the liquid extract to a preweighed 100-mL RBF using a glass Pasteur pipette. To maximize recovery, wash the vial containing the residual solid biomass with acetone and methanol (1:1, v/v), to recover all of the lipids, using chlorophyll as the indicator pigment (see Note 2). Pool the washes for each sample in the 100-mL RBF containing the lipid extract.
5. Remove the solvents using the rotary evaporator at 60 C at a moderate speed. Weigh the flask periodically. The flask with residue will ultimately achieve a constant dry weight with no appreciable reduction due to loss of solvent(s). Record the weight of the RBF with lipid residue.
6. Using a micropipettor, resuspend the lipid extract in 2 mL 2:2:1:1 (hexane-toluene-acetone-methanol, v/v/v/v) with gentle swirling and transfer to a 5-mL glass vial; rinse the RBF with another 2 mL of 2:2:1:1 and pool with the previous (see Note 3).
7. Dry the RBF using the rotary evaporator at 60 C until a constant weight is achieved. Record the value.
8. Subtract the weight of the RBF minus extract from the weight of the RBF with extract to obtain the grams of TLE. Divide the grams of total lipids extracted by the resuspension volume (4 mL) to obtain the concentration of lipids in grams per liter for subsequent TLC analyses.

   If beginning with dried material, calculate the % biomass extracted as total lipid as follows for sample Duplicate 1 (D1):

%TLED1 of dry weight ¼ TLED1=DWD1 100%
%TLED1 of ash-free dry weight ¼ TLED1=AFDWD1 100%

where

TLED1 ¼ total lipid extract of duplicate #1 in grams.

DWD1 ¼ dry weight of duplicate #1 in grams.

AFDW1 ¼ ash-free dry weight of duplicate #1 in grams.

   Repeat calculations with sample Duplicate 2 (D2).

   Perform statistical averages, %RSD, and SD of replicate samples, D1 and D2.

9. If beginning with fresh material, calculate the moisture correction (mc) for duplicate samples D1 and D2 as follows to estimate the sample DW and AFDW:

$$\%\text{Moisture of D1} = (\text{FWD1} - \text{DWD1})/\text{FWD1} \times 100\%$$
$$\%\text{Moisture of D2} = (\text{FWD2} - \text{DWD2})/\text{FWD2} \times 100\%$$
$$\text{Average}\%\text{moisture of D1\&D2} = (\%\text{moisture D1} + \%\text{moisture D2})/2$$
$$\text{DWD1}_{mc} = \text{FWD1} \times (100 - \text{average}\%\text{moisture of D1\&D2})/100$$
$$\text{AFDWD1}_{mc} = \text{DWD1}_{mc} - \text{average ash weight for D1\&D2}$$

Perform statistical averages, %RSD, and SD of replicate samples, D1 and D2.

FWD1 = fresh weight of duplicate #1 in grams.

DWD1_{mc} = moisture-corrected dry weight of duplicate #1 in grams.

DWD2_{mc} = moisture-corrected dry weight of duplicate #2 in grams.

AFDWD1_{mc} = moisture-corrected ash-free dry weight of duplicate #1 in grams.

3.3 Analysis of Biomass Lipids by Thin-Layer Chromatography (TLC)

1. Prepare a 10 cm × 10 cm TLC plate; using a pencil, label the origin, solvent front, mid-way point, and sample spots as shown in Fig. 2 (see Note 7).
2. Spot 5 and 10 μL of each extract for TLC with capillary micropipettors along with 5 μL of lipid reference standards (see Fig. 2; see Note 4).
3. Add polar solvent system 65:10:20:10:3 chloroform-methanol-acetone-acetic acid-water (v/v/v/v/v) to the chromatography tank and cover (see Notes 5 and 8).
4. Carefully insert the TLC plate straight down and lean behind the ridge with the face of the plate toward the front, and then cover the tank. Develop the plate to the mid-way point, then remove the plate with flat-tipped forceps, lean against the front of the tank, and allow to dry completely (front and back should be dry).
5. Next, develop the plate with the nonpolar solvent system 80:20:1 hexane-diethyl ether-acetic acid (v/v/v) up to the solvent front, then remove the plate from tank (see Notes 5 and 8).
6. Permit to dry, then expose the TLC plate to UV light using the handwand at each wavelength to examine absorption and fluorescence properties. Take images of the plate in visible light and with UV.
7. Place the TLC plate in the chamber containing iodine-silica 1:1 (w/w) and seal. Expose for 2-3 min; lipids will stain darker with increased exposure time.
8. Diagram the TLC plate as shown in Fig. 3—photograph and indicate where lipids are observed (plates can be reexposed to iodine if needed).

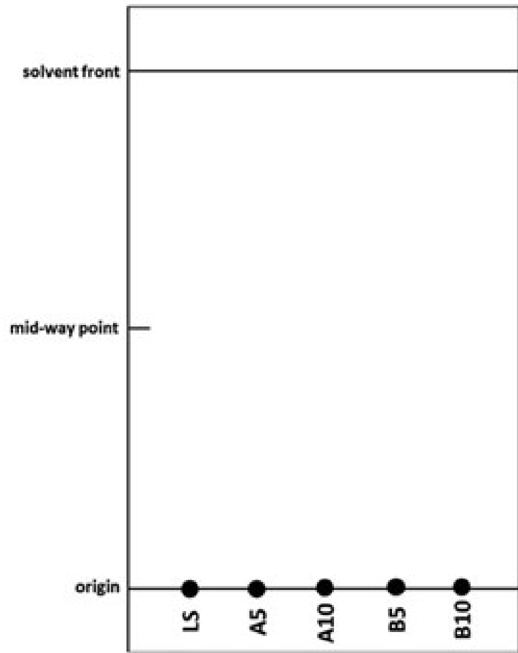

Fig. 2 This diagram illustrates the setup and labeling of a TLC plate for analysis of TLE. LS ¼ Lipid Standards. Lanes A5 and B5 represent 5 μL of TLE from samples A and B, Lanes A10 and B10 represent 10 μL of TLE from samples A and B

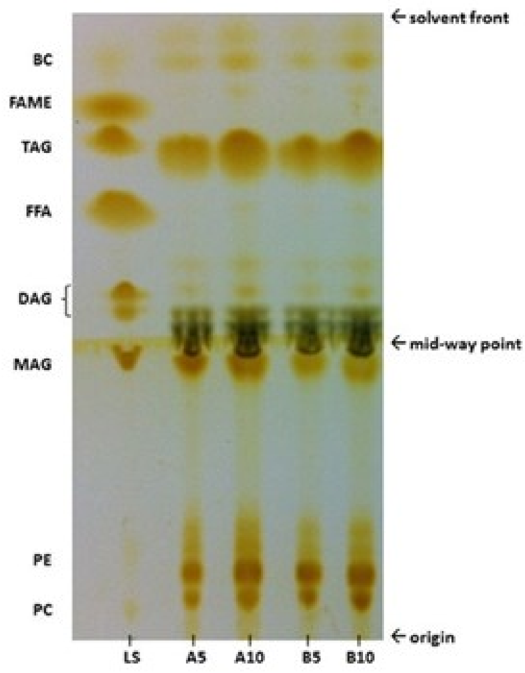

Fig. 3 The TLC shows the development of TLE samples A and B, applied at two different concentrations. Lane 1 contains the lipid standards (LS), lane 2 contains 50 μg of TLE A (A5), lane 3 contains 100 μg of TLE A (A10), lane 4 contains 50 μg of TLE B (B5), and lane 5 contains 100 μg of TLE B (B10)

4 Notes

1. Exercise caution if using color-changing desiccant containing cobalt(II) chloride, a known carcinogen, and follow local guidelines regarding the handling and disposal of hazardous waste.
2. If the tissue is not completely decolorized, rinse the biomass with methanol and acetone and homogenize with 5 mL of 2-EE. Transfer the homogenate to the RBF, add another 5 mL 2-EE and extract for 30 min at 60 C or until the tissue is decolorized. Filter extracted solids over a 1.2 μm glass-fiber membrane and pool the filtrates.
3. Do not collect any solids. Cap the vial tightly, wrap with Parafilm, and store at 4 C.
4. TAG ¼ Triacylglycerol (triolein, Sigma T7140-500MG), DAG ¼ Diacylglycerol (1,2- and 1,3-diolein, Sigma D8894-50MG), MAG ¼ monoacylglycerol (monoolein, Sigma M7765-50MG), FFA ¼ free fatty acid (oleic acid, Sigma O1008-5G), PC ¼ phosphatidylcholine (dioleoyl phosphatidylcholine, Sigma P6354-25MG), PE ¼ phosphatidylethanolamine (dioleoyl phosphatidylethanolamine, Sigma P1223-25MG), FAME ¼ fatty acid methyl ester (methyl oleate, Sigma 311111-5G), and BC ¼ beta-carotene (Sigma C4582-10MG). Resuspend standards in toluene-hexane-acetone--methanol 2:2:1:1 (v/v/v/v) to a prepare 20 mg/mL stock solutions; prepare in glass vials with screw caps, wrap in Parafilm, and store at 20 C. Combine 100 μL of each stock solution plus 200 μL of the 2:2:1:1 solvent in a screw-cap glass vial to prepare 1 mL of the Lipid Standard (LS) mixture containing 2 mg/mL of each analyte (1,2- and 1,3-DAG are counted together as total DAG). Wrap in Parafilm and store at 20 C.
5. Use glacial acetic acid.
6. Two replicates, at a minimum, are recommended for each measurement.
7. Use a dull #2 pencil to lightly draw a line at the origin, a line at the solvent front, a dash at the mid-way point, small dots where spots are to be applied, and sample labels. Take care not to break the surface of the silica gel. A 1-cm rule should be applied—the origin and solvent front are 1-cm from the bottom and top of the plate, respectively, and sample spots should be 1 cm from the edges and 1 cm between samples.
8. Do not exceed 0.8 cm in depth (usually just below the ridge in standard tanks) to avoid loss of sample. Equilibrate the chromatography tank with developing solvent for at least 15 min prior to developing the plate.

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References

1. Hu Q, Sommerfeld M, Jarvis E, Ghirardi M, Posewitz M, Seibert M, Darzins A (2008) Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances. Plant J 54:621-639
2. Quinn JC, Davis R (2015) The potentials and challenges of algae based biofuels: a review of the techno-economic, life cycle, and resource assessment modeling. Bioresour Technol 184:444-452
3. Scott SA, Davey MP, Dennis JS, Horst I, Howe CJ, Lea-Smith DJ, Smith AG (2010) Biodiesel from algae: challenges and prospects. Curr Opin Biotechnol 21(3):277-286
4. Magnusson M, Mata L, de Nys R, Paul NA (2014) Biomass, lipid and fatty acid production in large-scale cultures of the marine macroalga Derbesia tenuissima (Chlorophyta). Mar Biotechnol 16:456-464
5. Xu X, Kim JY, Oh YR, Park JM (2014) Production of biodiesel from carbon sources of macroalgae, Laminaria japonica. Bioresour Technol 169:455-461
6. Folch J, Lees M, Stanley GHS (1957) A simple method for the isolation and purification of total lipids from animal tissues. J Biol Chem 226:497-509
7. Bligh E, Dyer WJ (1959) A rapid method of total lipid extraction and purification. Can J Biochem Physiol 37:911-917
8. Jones J, Manning S, Montoya M, Keller K, Poenie M (2012) Extraction of algal lipids and their analysis by HPLC and mass spectrometry. J Am Oil Chem Soc 89:1371-1381