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Review of Methods Used for Microalgal Lipid-Content Analysis

Yuchi Han, Qinxue Wen, Zhiqiang Chen, Pengfei Li*

State Key Laboratory of Urban Water Resource and Environment, Harbin Institute of Technology, Harbin, China

Abstract

This paper provides a brief overview of most recent strategies that used to analyze microalgal lipid content, including NIR spectroscopy and TD-NMR methods etc. Common methods like gravimetric quantification and staining quantification are also introduced in this report. The physiology background of microalgal lipid accumulation is stated in order to clarify the purpose of each individual analytical method. After all, online lipid content measurement method that has good accuracy has the best chance to be generalized for all the lipid analyzing researches.

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Keywords: microalgae; lipid content; analytical methods

1. Introduction

Due to the limited fossil fuels and the increasing emission of greenhouse gas into the atmosphere from combustion of fossil fuel, research has begun to focus on alternative biomass-derived energy [1-5]. As we all known, microalgae have long been recognized as the most potential source of biofuels alternative to the fossil fuels, because of the good biomass productivity and the high-lipid content of large scale algal cultures[6,7]. The critical point for the whole microalgal biofuel production process is to select the optimal strain with the suitable lipid yield [8]. Therefore, to identify the best strain and culture conditions will require a lipid screening technique which suits for the lipid analysis for the microalgae.

The current techniques for screening the lipid content are mainly the old techniques we mastered for non-algal lipid analysis. The problem is that for the lab scale, not all the techniques fit for the small amount algal-lipid testing. Moreover, for different strains of microalgaes, the results from same technique may vary. The lipid content analysis originated from early 30's. After several decades' improvement and evolution, the processes had become handy and the results had been more accurate than the old times.

* Corresponding author. Tel.: +86-451-82339268.
E-mail address: lipengfei_19881018@126.com

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Therefore, this paper will give an overview of the most recent techniques as well as the most common ones used for algal-lipid content analysis.

2. Microalgea Physiology and Algal Lipids

Microalgae have been known to be rich in lipids. It produces many different kinds of lipids, triglycerides and diglycerides, phospholipids and glycolipids, hydrocarbons and others [6, 9-10]. The renewed interest in the use of algal-lipid fuels has refocused research on algal lipids and lipid metabolism. The distinction (polar and neutral) in the main lipid classes is important for the subsequent conversion of microalgal oils to biofuels especially when the composition of the feedstock affects the efficiency of fuel conversion by catalytic routes. The relative composition of algal lipids depends greatly on the species used and the nutrient, environmental and developmental conditions in which the cells are cultured and harvested [11-14].

More specifically, the lipid synthesis includes the following steps:

1). ATP and acetyl-CoA to form malonyl-CoA.

2). Tran-acylation step involving ACP and two acyl-CoA.

3). Acetyl-CoA/ACP reacts with low-molecular-weight acyl-CoA to give rise to branching fatty acids [15, 16].

The fatty acids produced are incorporated into lipid components in chloroplast or the endoplasmic reticulum [17]. In fast growing cells, lipid metabolism is focused around membrane lipids biosynthesis to support the growing. Therefore most lipids in these cells are synthesized under stress conditions [15]. Overall triglyceride metabolism is complex and is regulated at several levels, based on signals reflecting nutritional and environmental signals.

Lipid synthesis is important for the algal cells and the physiological background of microalgae do give us inspiration when we dealing with lipid-content analysis problems as well as developing new analytical methods.

3. Methods Used for Lipid Content Analysis

Usually, GC-MS will be applied for most of the methods below for accurate composition analysis. For instance, the Gravimetric Methods, the biomass will experience centrifugation, washing, grinding, extraction, evaporation, weighting and GC-MS sampling. Therefore, the methods we described in the paper are beneficial to the analysis if coupled with other tests.

3.1. Gravimetric quantification methods

The most common approach is the macro-gravimetric method in which lipids are extracted from a sample, the extraction solvent is evaporated and the retained material is measured as the lipid contend[18,19]. This traditional gravimetrical methods requires a relatively large quantity of sample and is time consuming and labor-intensive when analysis of many samples is needed. The mechanism of the gravimetric methods will be introduced below with detail, and it will provide a basis for all the other methods that differentiated from the method.

Microalgal cultures were harvested and then dewatered by centrifugation. The centrate was discarded and the microalgal paste was obtained. In the experiments, the dried microalgae was used, the paste was dried at 80℃ in oven for 10 h. Then the dried paste was grind into powder. The powder could be stored at

5℃for 45 days before they used for lipid extraction. For hexane extraction for example, 150mL of n-hexane was used for 2g of microalgal powder. Addition of alcohol on hexane extraction worked better. A 3/2 v/v mixture of n-hexane and isopropanol could work for 2g of powder. The conical flasks were sealed and agitated at 800 rpm at ambient condition for 8 h. then the Whatman CF/C paper was used for residue filtering. The filtrate was transferred into a funnel and sufficient water and hexane were added to induce biphasic layers. After settling, the mixture partitioned into two phases, a top dark-green hexane layer containing most of the extracted lipid, and the bottom layer contained all the co-extracted non-lipid contaminants. The hexane phase was transferred to a pre-weighted flask and heated to dryness in the oven to enable gravimetric quantification of the extract. The lipid was dissolved in hexane and sealed for storage.

There are various extraction strategies for gravimetric quantification: Straight hexane extraction, Alcohol combined co-solvent extraction, Soxhlet apparatus extraction and SCCO2 extraction [20]. One recent report indicates that the SCCO2 extraction is much better than soxhlet and solvent extraction. But the results may vary due to different extraction situations.

3.2. Staining methods

Another common methods used for lipid quantification, the advantage is the handling time is much shorter than the traditional ones. Fluorospectrophotometer is usually combined with staining methods. The two results reading from both before staining and after staining will be compared and the differences will be used to estimate the lipid content.

1) Nile Red/BODIPY Staining

Fluorescent lipophilic dyes, such as Nile Red and BODIPY are used for lipid quantification and strain screening because of their selective affinity for neutral lipid droplets inside the cells. However, the disadvantage of the assays is that they are affected by uneven dye uptake due to the variability of different algal strains and cell wall composition that affected by the growth conditions[21]. Also, the Nile Red seems not working well with died algal cells [22].

2) Nile Red modifications

There are lots of modifications on the Nile Red Staining Methods. Most of them focused on overcome the obstacles like cell wall and organelle composition. For instance, calibration modification, microwave assist, lyophilized alga powder preparation and 96-well plate technology etc. [23, 24]. These modifications provide rapid, easily manipulated and reliable methods for in vivo quantification of neutral lipids in various algal taxa.

3.3. Colorimetric SPV method

The colorimetric sulfo-phospho-vanillin (SPV) method developed by Chabrol et al. [25, 26] is an attractive alternative for lipid measurement because of its fast response and relative ease in sample handling. The SPV method has been modified for diverse applications [27-32] and the newly developed assay method possesses many advantages. For instance, it requires a small amount sample and it requires less time and less labor when large number of samples is analyzed. The coloration developed during the reaction can be read and compared very easy. Although it's possible to analysis the lipid content in tested oils, the preparation is still inconvenient.

3.4. TD-NMR method

TD-NMR is commonly used to determine lipid content in foods and seeds. The results of recent study

revealed that the method is also applicable to quantitate lipid in microalgae quickly. TD-NMR is based on the different relaxation times of hydrogen nuclei in different phases of the sample analyzed [22]. The solids such as protein exhibit the shortest relaxations times, whereas the relaxation time for lipid is about few hundred times slower. TD-NMR method can achieve higher accuracy and reproducibility. And the method has less restriction on the lipid concentration of samples. The sample tested can be reused for other analysis since the method is not invasive.

3.5. TLC/HPLC method

Similarly, HPLC (high-performance liquid chromatography), TLC (Thin-layer chromatography) and MS are also powerful techniques for lipid analysis [21]. However, these methods rely on a considerable sample preparation step to isolate the lipid fraction prior to analysis. HPLC is a chromatographic technique that can separate a mixture of compounds and is used in biochemistry and analytical chemistry to identify, quantify and purify the individual components of the mixture. HPLC typically utilizes different types of stationary phases, a pump that moves the mobile phase and analyte through the column, and a detector to provide a characteristic retention time for the analyte. The detector may also provide additional information related to the analyte. Analyte retention time varies depending on the strength of its interactions with the stationary phase, the ratio/composition of solvent(s) used, and the flow rate of the mobile phase. It is a form of liquid chromatography that utilizes smaller column size, smaller media inside the column, and higher mobile phase pressures. With HPLC, a pump (rather than gravity) provides the higher pressure required to move the mobile phase and analyte through the densely packed column. The increased density arises from smaller particle sizes. This allows for a better separation on columns of shorter length when compared to ordinary column chromatography.

3.6. NIR and FTIR spectra

At NREL's National Bioenergy Center, researches are working towards developing a new method for estimating the oil or lipid content and chemical composition in a wide range of algal strains using near infrared (NIR) spectroscopy. NIR spectroscopy measures the absorption of energy in the IR region of the spectrum by chemical bonds in molecules. The advantage of NIR is its tolerance to a certain level of variation in the samples and minimal sample preparation requirement. Overall, it can be applied as a fast, accurate and non-destructive method that only requires very small amounts of homogenized biomass (about 10 mg) using a 96-well plate set up. Future work will include testing and developing models for different algal strains and also models on growing algal culture. Calibration models are also needed for unknown samples in order to apply on a routine basis.

4. Comparison for Different Analytical Methods

From all the methods we described above, we can simply find out which one is more suitable for specific microalgal strains and which ones are more favoring to certain experimental environment. Each method has its own advantages, for better results for the lipid content analysis, combined techniques are used. Table at the end gives a summary of rankings for all the methods describes in this paper. Further estimations for each aspect are still needed for the methods that will be modified later.

The most time consuming method, which is the Gravimetrical quantification, is still the most accurate way to monitor the lipid content. It can be used as a standard model when developing new screening strategies. The newly innovated NIR spectroscopy is still under development, but we can count on that for further online monitoring for lipid content for microalgal cultures. The staining methods introduced in the

paper have the most modifications. Especially the Nile-Red Staining, it can be used to estimate more and more algal strains due to the contribution of the modifications. Therefore, Staining is on average, the most suitable method over all. Other methods like SPV and TD-NMR have very limited strain coverage. For further development on these methods, modifications have to be applied to enhance the ability to test unknown samples. TLC/HPLC methods will cost a lot during sampling, therefore, is not suitable for large scale sampling in many situations.

Table 1: Comparison of introduced methods

Aspect	Quantitative relations from High to Low
Preparation	GRA. TLC. SPV. STA. TD-NMR. NIR
Time/Labor	GRA. TLC. SPV. STA. TD-NMR. NIR
Cost per trail	TLC. GRA. STA. SPV. TD-NMR. NIR
Modification	STA. GRA. (SPV. TLC. NIR.)**
Strain Coverage	GRA. STA. TLC. TD-NMR. NIR. SPV
Response Rate	(TD-NMR. NIR.) SPV. (STA. TLC.) GRA.
*Accuracy	GRA. (NIR.TLC) STA. TD-NMR. SPV
Reproducibility	(SPV. TD-NMR. NIR.) (GRA. STA)
Destructiveness	GRA. (STA. TLC) (SPV. TD-NMR. NIR.)

*Gravimetric accuracy was used as the standard in the Accuracy column. ** The content in the bracket had similar quantitative relations. GRA is short for gravimetric, STA is short for Staining.

From all the results and analysis above, we can conclude that there is no single method that fit for every aspect, but we can always find one that fit for our research. For instance, laboratory condition, research objectives, research fund etc. will influent the choice of methods picking. The common methods that used in the microalgal research field are still the gravimetric and staining method. A combined method that contains two or more strategies may work fast and give accurate results; but will cost more than traditional methods for sure. Also, the online measuring techniques will facilitate the researches about the microalgal biodiesel. It provides a quick monitoring for cultures and the data can be collected more easily. With the development speed of the online measuring methods, it will replace the traditional methods eventually. By for now, the most suitable method is still the staining method that has modification superiority and great value for application.

5. Future Prospective

Microalgae, as promising biofuel production energy source that alternative to the fossil fuel, draws more and more attentions nowadays. High lipid content with high productivity makes it suitable for future development. The lipid content analysis, as a key analytical step for biofuel production, will affect the research progresses in many ways. Newly innovated methods will facilitate the process and make more benefits to human beings. Therefore, both modifications on exist methods and new ideas are critical to the biofuel industry. Methods that can provide accurate result with short preparation time and low trial cost are the ones that will contribute to the field.

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