VOL. 22, NO. 3, 2023, 1-8
elektrika.utm.my
ISSN 0128-4428

ELEKTRIKA
Journal of Electrical Engineering

Microalgae Lipids Detection and Quantification Method Towards Online Real-Time Monitoring: A Review

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Abstract: Bioprospecting toward biofuel has appeared as a sustainable and alternative energy option as fossil fuels are finite and causing a large number of environmental problems such as global warming by the greenhouse effect, leading the development of alternative substantial energy has attracted more and more attention. Microalgae are potential candidates as third generation biofuel producers to tackle this problem as they can produce an abundance of value-added products such as triacylglycerols which is an energy-rich fatty acid under stress-condition. However, the productivity of such biofuels is still low as the lipid accumulation phase of microalgae varies from time to time. In order to improve productivity, microalgae lipids must be harvested at the most suitable time when microalgae accumulated the highest amount lipids. Hence, this review highlights various quantification and detection methods of microalgae lipids, ranging from conventional methods like gravimetric and chromatographic techniques to non-conventional methods like fluorescent staining, Raman scattering, and dielectric insulation. While conventional methods require a series of procedures, including biomass dehydration and lipid extraction, non-conventional methods provide advantages such as rapidity, low cost, and smaller sample sizes needed. Future prospects revealed that real-time monitoring and online measurement of lipid content in microalgae is required to quantify the intracellular lipid level of microalgae in controlling and rapidly adjusting the growing conditions for cultivation. These real-time results, combined with the Internet of Things (IoT) technology, can be further processed using machine learning to maximize the productivity of microalgae as an alternative biofuel producer.

Keywords: Lipid quantification method, microalgal lipids, third generation biofuel.

Article History: received 7 May 2023; accepted 6 December 2023; published 26 December 2023.

1. INTRODUCTION

Fossil fuels are finite and cause a large number of environmental problems [1] such as global warming by the greenhouse effect, leading the development of alternative substantial energy has attracted more and more attention [2], [3]. In the search for alternatives to fossil-derived oils, bioprospecting toward biofuel has appeared as a sustainable and alternative energy option. Biofuel is well-known as a substitution to replace petroleum-based fuels or fossil fuels for their renewable feature that burns cleaner, resulting in fewer greenhouse gas emissions. Microalgae, yeasts, fungi, and bacteria, can accumulate high amounts of lipids (> 20% of cell dry weight) in their cellular compartments and are considered oleaginous feedstocks for biofuel production [4]. Hence, microalgae are potential candidates as third generation biofuel producers to tackle this problem.

Unlike other generation biofuel production, third generation biofuel producers are able to overcome competitive factors faced by first- and second-generation biofuel producers such as land factor, availability, and price. Microalgae are able to live within and present in all the existing ecosystems including lakes, springs, ponds, wetlands, and rivers as well as wastewater. Moreover, its high biofuel production aligns with the high growth rate enabling it to yield high productivity. Under stress-condition, microalgae can produce an abundance of value-added products such as lipids [5]. For example, numerous studies have been efforts in nutrient-limiting conditions for microalgae to accumulate significant amounts of lipids known as single-cell oils [6]–[9]. Neutral triacylglycerols (TAGs) which are energy-rich fatty acids are mainly found in these lipids produced and can be used for biodiesel production.

Although this is an attractive and renewable feedstock, microalgae lipid production still possesses limitations and challenges that must be overcome to upgrade the technology from the lab to the industrial level [10]. At larger scale production, the cost and time must be kept low and productivity must be kept high to fully leverage microalgae capabilities. Hence, it is imperative to maintain continuous monitoring of microalgae lipids throughout the accumulation phase and harvest when it accumulates the highest amount of lipids to achieve the highest productivity. This involves the identification and detection

of lipid content especially during the lipid accumulation period of microalgae. Thus, it is important to review various current methods in the quantification and detection of microalgae lipids to achieve real-time monitoring with online measurement generated substantial interest, particularly with the emergence of IoT technology and machine learning. This work reviewed the conventional as well as non-conventional methods to quantify and detect microalgae lipids. The conventional method for lipid quantification relies on extraction either with gravimetric or chromatographic method not only time-consuming and involves multi-stage, but also put microalgae to death and this process is unreversible. Hence, it is inadequate for high-throughput analysis that allows for rapid screening of oil accumulation under varied conditions. Several techniques have emerged as high-throughput and reliable lipid quantification such as fluorescent staining, Raman scattering, and dielectric insulation.

2. CONVENTIONAL METHODS

Conventional lipid quantification methods in microalgae included gravimetric and chromatographic methods. These methods involve a series of procedures from collecting microalgae, and biomass dehydration to cell membrane disruption, and finally lipid extraction. While gravimetric can only be used to estimate the total amount of lipids but is unable to know its composition weightage, the chromatographic method is able to overcome this problem.

2.1 Gravimetric method

The gravimetric method is based on the estimation of the mass percentage of a substance to a known quantity of mass and it is the most common technique used as a reference standard to quantify microalgae lipids [11]. However, it must involve the step of lipid extraction according to their polarity such as polar lipids of phospholipids and non-polar lipids of TAGs [12], [13]. Different extraction approach has been developed to extract lipids from microalgae samples such as traditional organic solvent extraction, supercritical fluid extraction, and switchable solvent extraction.

In the case of non-polar lipid which is more soluble in non-polar solvents, non-polar solvents are used so that it is able to react and produce any observable changes for measurement. However, nonpolar organic solvents, such as hexane, benzene, toluene, diethyl ether, ethyl acetate, and chloroform, are usually combined with the polar organic solvents to maximize the extraction efficiency of non-polar lipids [14]. Chloroform is commonly mixed with methanol in different aspects to extract lipids and determine its total lipid weight gravimetrically after solvent extraction [15]–[19].

Supercritical fluid extraction (SFE) has turned out to be a promising green technology that is possible to replace the traditional lipid extraction solvent. As a consequence of the short extraction time without the use of harmful organic solvents, it has been implemented in microalgae lipid oil extraction [20]–[22]. Even though a relatively high cost associated with this type of extraction, scCO2 has been frequently adopted in SFEs because it offers the advantages of negligible environmental impact, high diffusivity, no toxicity, no oxidation or thermal degradation of extracts, and easy separation of desired bioproducts [23].

Compared to those traditional extraction methods, switchable solvent extraction known as “reversible” solvents is a relatively new approach for the extraction of lipids from microalgae. This kind of extraction empowers the solvent to change its properties upon the addition or removal of an external stimulus such as a temperature change or the addition or removal of a gas. The advantages of SSE include its relatively low toxicity, low energy consumption, and potential for high selectivity and efficiency in lipid extraction which provide a more practical application for researchers to extract and quantify lipids [24]–[26].

An ideal solvent for lipid extraction should be free of toxicity, easy to remove, and more selective towards target products Among all, the organic solvent-based extraction methods are extensively used because of their lower cost and easy availability even though they require a relatively large quantity of biomass and have few environmental impacts. Nevertheless, each method has its own advantages and disadvantages, and the choice of extraction method may depend on factors such as the desired yield, quality, and composition of the extracted lipids, as well as the availability of equipment and resources.

2.2 Chromatographic method

The chromatographic method operates on the fundamental objective of differentiating molecules in a mixture and quantitatively determining the components of a mixture. It separates lipids based on their size and their elution time with standard reference. Various chromatography methods have been developed and the most common chromatographic methods for lipid analysis are thin-layer chromatography (TLC), gas chromatography (GC), and high-performance liquid chromatography (HPLC),

Thin-layer chromatography (TLC) is a simple and inexpensive method used to separate and analyze lipids based on their polarity [27]. It is often used to separate and analyze different classes of lipids, such as glycolipids, phospholipids, and neutral lipids like triglycerides. Hence, quantitative analysis can be performed based on the lipid composition that reacts with various types of reagents such as acidic ferric chloride [28], and Bial’s and Dittmer reagents [29].

On the other hand, gas chromatography (GC) is a chromatographic technique used to separate and analyze volatile and semi-volatile organic compounds, including fatty acids in lipid samples. Flame Ionization Detector (FID) which measures the concentration of the individual components is a common detector that is often coupled with GC for the identification of lipids from microalgae [30]–[32]. Microalgae are identified as feedstock production of fatty acid methyl ester (FAME), a type of biodegradable biodiesel to replace mineral diesel and gas oil in the engine which is more volatile and hence suitable for GC analysis [33], [34].

In HPLC, a liquid mobile phase is pumped through a column packed with a stationary phase to increase its efficiency. The individual lipid classes can be separated

using specific stationary phases and mobile phase compositions and can be detected using a variety of detectors. Quadrupole time-of-flight mass spectrometry coupled with HPLC was employed to allow the selection and isolation of precursor ions as well as provide high efficiency, sensitivity, and mass accuracy [35]. Besides, HPLC combined with an evaporative light-scattering detector was developed to quantify lipids from microalgae with a shorter time and higher accuracy [36].

GC and HPLC are highly sensitive and precise methods for lipid analysis, but it requires specialized equipment and expertise to perform. On the other hand, TLC is a relatively simple and inexpensive method for lipid analysis, but it has limitations in terms of resolution and sensitivity compared to other chromatographic techniques such as HPLC and GC. Nonetheless, GC has a limitation which is not suitable to use in some lipid classes that are not easily converted to volatile derivatives.

3. NON-CONVENTIONAL METHODS

As the conventional method is involved in lipid extraction, a large sample, time-consuming, non-conventional lipid quantification method in microalgae enables rapid and low cost as well a smaller sample is needed. These methods included fluorescent staining, Raman scattering, and dielectric insulation.

3.1 Fluorescent staining

Microalgae lipid fluorescent staining is a technique used to visualize and quantify lipids in microalgae. A fluorescent dye that specifically binds to lipids in the microalgae is pre-treated and then only can be detected using fluorescence microscopy or flow cytometry. The most commonly used fluorescent dyes for microalgae lipid staining are Nile Red and BODIPY [37]. Once the dyes bind to lipids, they will emit fluorescence with the emission spectrum depending on the polarity of the lipid environment.

Nile Red is a lipophilic dye that stains neutral lipids such as triglycerides and has been used to determine neutral lipids in microalgae cells [38]. Nile Red can interact differently with neutral lipids which result in shorter emission wavelength than polar lipids permitting the differentiation of lipid [39]. However, it does have some limitations such as uneven dye uptake, interference with the autofluorescence of chlorophyll, and non-specific fluorescence. Hence, optimization has to be done for each species [40]. Stain carrier such as solvent dimethyl sulfoxide (DMSO) was introduced into microalgae samples and gave very reproducible results [41].

BODIPY stains are able to tackle the defection of Nile Red by being able to bind to both neutral and polar lipids, making it a useful tool for studying lipid accumulation in a variety of cell types, especially in microalgae [42]. Besides, it can stain a variety of algae irrespective of cell wall properties [43]. However, it has been reported that BODIPY straining produced a high fluorescence background [44]. BODIPY strain improvements have been done involving single-cell methodologies on single-cell lipid screening procedures to increase the accuracy and eliminate uncertainties caused by cellular physiology and different staining conditions [45].

The high throughput method has been integrated with the staining method which is flow cytometry able to concurrently measure fluorescence and sort individual stained cells [46]. In vivo flow cytometry quantification of lipids with staining either using Nile Red or BODIPY has been applied in individual microalgae [47], [48]. With technology becoming more and more advanced, this laboratory technique is the potential to be used in large-scale detection as it is able to rapidly quantify the amount of lipid yet it still needs the usage of microscopic imaging techniques.

3.2 Raman scattering

Raman scattering is based on the inelastic scattering of monochromatic light produced by a laser in the range of visible light, NIR, or UV [49]. It is a rapid and non-destructive method to quantify based on the interaction of light with the molecular vibrations of the sample, resulting in a unique spectral fingerprint. Among all the biomolecules presented in the microalgae cellular environment, lipids have been widely investigated as one of the major biomolecules due to large Raman scattering cross-sections [50].

Studies claimed that the use of Coherent Anti-Stokes Raman Scattering (CARS) in Raman Spectrometry allowed accurate determination of harvesting time for algae [51]. Moreover, it successfully allows the separation of lipid-specific signals from the excited chlorophyll fluorescence and is able to analyze the formation of lipid droplets. Besides, with a combination of optical confocal microscopy, it can be used to measure the volume, lipid weight, and dry cell weight of individual cells [52]–[54].

Overall, Raman spectroscopy-based strategies offer immense potential for non-invasive analysis of biochemical composition and imaging of microalgae cells, particularly for analyzing microalgae lipids. Nevertheless, Raman spectrometry requires the use of costly equipment, and employment of such technique in photosynthetic organisms is limited by the intense autofluorescence of pigments, which hinders its potential for precise and accurate measurements [50].

3.3 Dielectric insulation

Microalgae, like any living organism, exhibit complex dielectric properties. The dielectric properties of microalgae are defined as their ability to store electrical energy in an electric field. Hence, it will alter the permittivity and conductivity of a medium depending on their cell components where lipid plays an important role in varying the capacitance of the cell. Therefore, dielectric insulation can be referred to as the dielectric property of microalgae which is able to block the flow of current, and this property is exploited as one of the quantification methods of microalgae lipid [55], [56].

Dielectrophoresis (DEP) uses the principles of polarization and the motion of cell components which was induced by non-uniform electric fields for the separation of cells with different lipid contents [57]. Briefly, there are two types of dielectrophoresis response known as negative dielectrophoresis (nDEP) and positive dielectrophoresis

(pDEP). Lipids produced by microalgae are not stored in dedicated reserve organelles but formed vesicles in the cytosol and hence the dielectric constant and conductivities of microalgae depend significantly on the lipid content [58]. Based on the intracellular lipid content together with the help of hydrodynamic forces, microalgae can be digitally quantified and separated by hydrodynamic force into different outlets [59].

Electrorotation used an electric field to rotate or spin the microalgae is another method to detect and quantify the lipid content of microalgae [60], [61]. During electrorotation, the microalgae are subjected to an alternating rotating electric field normally with a 90-degree phase difference. The electric field causes the microalgae to rotate as a result of Maxwell-Wanger polarization, and the frequency and amplitude of the field can be adjusted to control the rotation speed and direction. The rotation of the microalgae is affected by their size, shape, surface charge, and lipid content [60]. As a result, this technique can be used to determine the lipid content of microalgae which directly affects their electrical properties such as their dielectric constant and conductivity by measuring the rotational speed of microalgae in response to the electric field.

Impedance spectrometry is another technique that leverages the dielectric properties of microalgae. This technique works by measuring the impedance of microalgae cells at different frequencies of electrical current and relating it with lipid content through developed mathematical models. However, studies have reported that the changes in impedance at lower frequencies (10-500KHz) due to lipid accumulation were ignorable [62], [63] while impedance at higher frequencies (1–15 MHz) is observable in response to lipid accumulation [64]. Since lipid is non-conducting molecules, which means that as the lipid content of microalgae cell increase, the conductivity of cells decreases. This led to an increase in the impedance of the cells [62], which is also defined as the resistance to the flow of electrical current through the cells. Besides, the dielectric constant of the inner cell constituents would decrease with lipid accumulation [56], [61], and indirectly decrease its capacitance.

Dielectric insulation is a notable property to investigate the lipid content of microalgae, however, more studies and developments are needed to fully utilize its potential. DEP is a rapid and straightforward technique to access the lipid content of microalgae but it is only able to separate microalgae in the range of lipid content but not accurately detect the amount of lipid accumulated. Electrorotation on the other hand has been reported as the most accurate technique to quantify lipid content in microalgae but it needs additional resources such as microscopy or other optical setups and imaging technique to detect the rotational speed of microalgae. Lastly, impedance spectrometry suffered from the usage of bulky equipment such as an Alternative Current (AC) generator, impedance analyzers, and complex interpretation of data. Besides, it remained a major challenge where fitting of appropriate circuit model and determining of frequency for the analysis is crucial for accurate analysis of lipid content in microalgae.

4. FUTURE PROSPECTS FOR REAL-TIME MONITORING AND ONLINE MEASUREMENTS

Throughout the year, there have been numerous technological advancements that have enhanced the competitiveness of the microalgae industry. However, most of the monitoring and measurement rely on offline analysis of samples taken from the biorefinery process and cultivation. Cultivation systems no matter in the open or closed system would require the real-time monitoring of microalgae growth status and continuous measure of the lipid produced to ensure maximum productivity is achieved [60] in making sure the commercial viability of microalgae as an alternative biofuel producer.

To accomplish continuous monitoring and immediate measurement in real-time, a rapid, simple procedure, non-invasive, high throughput as well as accurate output method is required. The conventional method can give a high-accuracy result and frequently being used as a reference when developing lipid detection methods but it afflicted by a series of tedious procedures. On the contrary, the non-conventional method was able to rapidly measure lipid accumulated in microalgae as a non-invasive method and required simple procedure, but it often demanded the use of additional high-cost equipment and expertise.

The usage of pretreated samples and the specific solution presents another challenge in achieving real-time and online monitoring. Even though the non-conventional method is known to have simpler procedures but methods such as fluorescent staining require the dye to penetrate into the sample before being able to complete the analysis which is time-consuming. Besides, DEP and electrorotation required the usage of a low conductivity medium [58], [60] to minimize the effect of polarization. However, impedance spectrometry can be done at either high conductivity medium such as phosphate-buffered saline (PBS) solution [62] or a low conductivity medium of deionized water.

When evaluating the present status of the optimal method for real-time monitoring and online measurements, dielectric insulation can be regarded as the most suitable method for achieving real-time monitoring and online measurement, as it can produce results quickly and requires low-cost, lightweight equipment. While Raman scattering is a feasible approach, its usage for commercial purposes may not be advisable due to the heavy and costly equipment involved. Table 1 summarizes the currently available methods for detecting and quantifying microalgae lipids

Table 1. Summary of lipid detection and quantification method

Method	Category	Advantages	Disadvantages	References
Gravimetric		Well-developed and mature to be used as a reference standard	Requires the step of lipid extraction based on specific lipid polarity only	[11]–[13]
	Organic solvent extraction	Extensively used, lower cost, easy availability	Requires a relatively large quantity of biomass, which can have environmental impacts	[14]–[19]
	Supercritical fluid extraction	Promising green technology, short extraction time, negligible environmental impact, high diffusivity	Relatively high-cost equipment	[20]–[23]
	Switchable solvents extraction	Low toxicity, low energy consumption, the potential for high selectivity and efficiency	A relatively new approach, may require specialized equipment	[24]–[26]
chromatographic		Able to differentiate molecules in a mixture and quantitative determine the components of a mixture	Requires the step of lipid extraction, needed to wait for elution time	
	Thin-Layer Chromatography (TLC)	Simple, inexpensive, and suitable for qualitative and quantitative analysis of lipids	Limited resolution and sensitivity, not suitable for volatile compound	[27]–[29]
	Gas Chromatography (GC)	Highly sensitive and precise for volatile and semi-volatile organic compounds	Not suitable for some lipid classes that are not easily converted to volatile derivatives	[30]–[34]
	High-Performance Liquid Chromatography (HPLC)	Highly sensitive and precise with high resolution	Requires specialized equipment and expertise to perform	[35], [36]
Fluorescent Staining	Nile Red dye	A shorter emission wavelength is needed which interacts differently with neutral lipids	Only bind to neutral lipids, optimization has to be done for each species	[38]–[41], [46]
	BODIPY	Able to bind to different types of lipids, applicable to a variety of cell types, high-throughput potential	produces a high fluorescence background, hard to quantify neutral lipids.	[42]–[45], [47]
Raman Scattering	Coherent Anti-Stokes Raman Scattering (CARS)	Rapid and non-destructive method, lipid-specific signals separation, analysis of lipid droplets, volume and weight measurement of individual cells	High equipment cost, limited application in photosynthetic organisms due to intense autofluorescence of pigments	[51]–[53]
Dielectric insulation		A non-destructive and non-invasive method	Fitting of appropriate circuit model and determining of frequency is crucial	
	Dielectrophoresis	Rapid and straightforward, able to continuously detect and sort microalgae based on the amount of lipid	Only able to separate microalgae in the range of lipid content but not accurately detect the amount of lipid accumulated	[57]–[59], [61]
	Electrorotation	The most accurate method to quantify lipid content in microalgae	Needs additional resources such as microscopy or other optical setups and imaging techniques to detect the rotational speed of microalgae	[60], [61]
	Impedance	Rapid, Non-invasive, and	Usage of bulky equipment,	[55], [56],

	spectrometry	capable of real-time analysis	complex interpretation of data	[62]–[64]

5. CONCLUSION

In conclusion, the conventional method although time-consuming and results in low throughput, these methods have been well developed and mature enough to be used as a standard reference in the experiment. Non-conventional methods have the upper hand of high throughput but it still has limitation such as required heavy equipment. Hence, a portable and high-accuracy device to quantify the microalgae lipid is the future breakthrough in order to boost the effectiveness of biofuel production using microalgae. In spite of that, real-time monitoring and online measurement of microalgae lipids still remain challenges to be overcome. Thus, it is necessary to have a method for quantifying microalgae lipids that can detect changes in less than a minute such as dielectric insulation technique, which would facilitate control and rapid adjustment of growing conditions to achieve specific growth objectives and highest productivity of lipids. The outcomes should also be accessible online for seamless integration with the Internet of Things (IoT), and to allow for further analysis using machine learning to counteract unusual cultivation environments. Therefore, this review holds significance in selecting the optimal approach for maximizing harvest efficiency by optimizing the time of harvest time else low productivity might happen if harvest time is not optimized especially for the rapidly growing microalgae.

ACKNOWLEDGMENT

This work has been supported by Universiti Teknologi Malaysia via Hi-Tech (F4) grant Q.J130000.4623.00Q15.

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