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Qualitative and quantitative estimation of algal lipids for biofuel production

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Abstract
The current rising demand for energy, global warming, and depletion of fossil fuels have led to increased consideration of alternate renewable biofuel sources. Microalgae serve as an excellent feedstock for biofuel production. Many qualitative and quantitative methods are available for lipid estimation from microalgae. In this review, we describe the various lipid estimation methods. Among the various methods discussed *viz.,* conventional, and non-conventional methods, Nile red method offers a high throughput technique for rapid screening of high neutral lipid producing microalgae.

Keywords: Biodiesel, spectroscopy, neutral lipids, staining, fatty acid

Introduction

The best alternative to meet the existing and future demand for fuel is to replace it with renewable biofuels. Biofuels not only serves as an alternative to fossil fuels but also prevents the emission of harmful gaseous pollutants (Chen *et al.,* 2018) [15] . Guo *et al.* (2015) [23] have reported that biofuels exist in various forms like solids (charcoal, sawdust, etc.), liquid (bioethanol, biodiesel, etc), and gaseous (biogas, bio-syngas, etc). The major concerning issue with the commercialization of biofuel is its high cost of downstream processing (Yan *et al.,* 2014) [73].

Based on the feedstocks used, biofuels are classified into a) primary and b) secondary biofuels. Primary biofuel is referred to as natural biofuels. This is obtained from materials like firewood, wood chips, animal waste, etc. secondary biofuels are further classified into a) First-generation - The feedstocks used are food crops like wheat, barley, corn, coconut, etc. which are grown on arable lands. The use of arable lands for biofuel production resulted in less available land for human and animal food production. b) Second-generation, The feedstocks used are non-food crops such as jatropha, cassava etc and non-edible parts of food crops like corn, sugarcane, etc. unlike crop plants, it grows on marginal lands. The feedstocks include lignocellulosic woody biomass which will not interfere with the food source and chain of the human (Alam *et al.,* 2012) [2]. Waste frying oil can also be used as a feedstock but it results in high viscosity due to its high free fatty acid content (Menard *et al.,* 2018) [47] and c) the third generation biofuels- Algae with high lipid content is used as a source for fuel extraction. Algae are photoautotrophic organisms that will accumulate a certain amount of lipids and carbohydrates during their metabolic processes which can be used in extracting the fuel. Microalgae can produce different renewable biofuels such as methane, biodiesel, biohydrogen, etc., (Rajkumar *et al.,* 2014) [56]. Microalgae, similar to higher plants produce both neutral and polar lipids. Polar lipids like glycolipids and phospholipids are produced under favorable conditions. These enrich the cellular and chloroplast membrane compositions. But, under unfavorable conditions like nutrient stress mainly nitrogen starvation, these algae alter their biochemical pathways to produce non- polar (or) neutral lipids such as triacylglycerols which accumulate in the cytoplasm as lipid droplets and are involved in energy storage (Held and Raymon, 2011; Aratboni *et al.,* 2019) [32, 3]. Microalgae have potential as feedstocks for biodiesel (Singh and Dhar, 2011; Suchitra *et al.,* 2014; Suchitra *et al.,* 2015) [63, 65-66] besides their value as a source of bioactive molecules, nutraceuticals, and pigments (Gupta *et al.,* 2013) [29]. Figure 1 has shown a flowchart for various methods of algal lipid estimation.

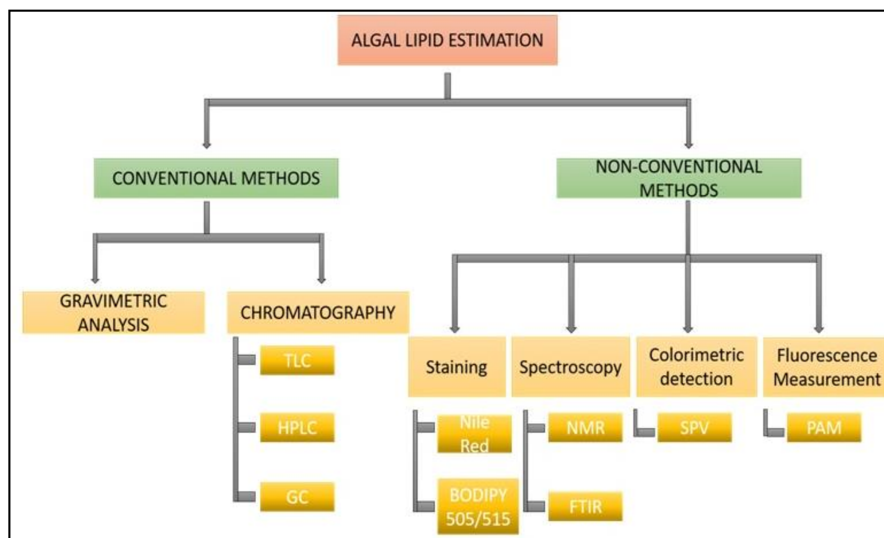

Fig 1: Flowchart for algal lipid estimation by various methods

Algal lipids

Algae comprise most of the major classes of lipids like glycolipids, phospholipids, triacylglycerols, hydrocarbons, sterols, etc (Beisson and Harwood, 2019) [6]. Microalgae comprise unsaturated fatty acids like palmitoleic, oleic, linoleic, linolenic acid, and a small amount of saturated fatty acids like palmitic and stearic acid. One of the disadvantages of using highly unsaturated sources includes complications related to contamination due to sediment formation as a result of oxidation. So, the composition is very much important concerning feedstock for biofuel production (Parveen *et al.,* 2015) [54]. Polyunsaturated fatty acids (PUFAs) are derived from polar lipids which consist of long-chain fatty acids. PUFAs include docosahexaenoic acid, docosapentaenoic acid, eicosapentaenoic acid, hexadecadienoic acid, hexadecatrienoic acid, linoleic acid, etc as shown in Table 1. These can be used in biofuel production and can also be used in the field of medicine to treat diseases like atherosclerosis, Parkinson, and Alzheimer (Aratboni *et al.,* 2019) [3].

Table 1: Fatty acid composition (% of total lipids) of the various microalgae strains

Organism	16:0	16:1	16:2	16:3	18:0	18:1	18:2	18:3	18:4	20:4	20:5	References
Chlorella vulgaris	11.3	15.6	-	9.7	-	2.9	24.9	29.5	-	-	-	Beisson *et al.,* 2019
Nannochloropsis gaditana	15.2	30.2	-	-	-	5.3	-	9.2	-	3.9	35.1	
Tetracystis intermedia	21.3	-	5.9	-	22.4	10.6	21.6	7.6	-	-	-	
Scenedesmus obliquus	21.78	5.95	3.96	0.68	0.45	17.93	21.74	3.76	0.21	-	-	Gouveia and oliveira, 2008
Neochloris oleabundans	19.35	1.85	1.74	0.96	0.98	20.29	12.99	17.43	2.1	-	-	
Botryococcus braunii	21	2	6.5	15.2	2.9	3.2	13.6	33	-	-	-	Schlagermann *et al.,* 2012
Nannochloropsis salina	37.5	23.3	-	-	0.9	11.9	1.5	-	-	3.3	15.3	
Nostoc commune	43.5	11.3	0.4	-	1.5	6.9	19.3	16.3	-	-	-	Lang *et al.,* 2011
Parietochloris incisa	10	2	1	1	3	16	17	3	-	46	1	
Lauderia borealis	12	21	-	-	-	3	1	-	-	1	3	Harwood, 2019
Phaeodactylum tricornutum	19	25	-	-	-	8	6	1	-	1	18	

Suchitra *et al.* (2019) [67] confirmed the presence of octadecadienoic acid, octadecenoic acid, and hexadecanoic acid methyl ester in *Botryococcus* sp. and *Tetradesmus wisconsinensis*, which are responsible for biodiesel production. The presence of phosphate usually hinders the transesterification process and leads to sulfur emissions from vehicles. So, due to the presence of high fatty acid content and lack of phosphorus and sulfur, TGAs are preferred over polar lipids for biofuel production (MacDougall *et al.,* 2011) [43]. Algal species with rich TAG or oil content are referred to as oleaginous algae (Hu *et al.,* 2008) [35]. The structure of the TAG molecule comprises a glycerol backbone and three fatty acids attached to it. Depending on the degree of unsaturation present in these attached fatty acid chains, they can be further classified as saturated fatty acid (SFA) and unsaturated fatty acid as Monounsaturated fatty acid (MUFA), and Polyunsaturated fatty acid (PUFA) (Sharma *et al.,* 2018) [62]. TAG accumulation is linked with photosynthesis and increases with stress conditions like high salt concentration, heat stress, nutrient starvation, etc. Certain chemicals like Brefeldin A, lipase inhibitors, etc. can also be used to induce TAG accumulation. Specific acyltransferases are involved in the biosynthesis pathway of TAG. The acyl chains are

sequentially added to the Glycerol-3- phosphate (Wase *et al.,* 2018) [70].

Nile red staining

Unlike traditional methods of analysis of lipids which are time-consuming, Nile red staining is relatively simple and easy to perform the technique. Staining with Nile red will not involve any extraction methods rather the whole algal cells are directly used for lipid analysis. Neutral lipids, due to its lower unsaturation have much importance in biofuel production. These are normally found as lipid droplets in the cytoplasm and are produced under unfavorable or stress conditions and at the end of the growth stage (Han *et al.,* 2011) [31]. Nile red is a lipophilic dye with a heterocyclic structure. The fluorescence of the stain is highly dependent on the hydrophobicity of the environment (Malapascua *et al.,* 2012) [44]. When Nile Red interacts with the lipid bodies in a hydrophobic environment, it emits intense fluorescence. But, the fluorescence is fully quenched in an aqueous environment (Halim and Webley, 2015) [30]. Due to its ease in use, it is used widely in the estimation of lipids in bacteria, yeasts, microalgae, etc. The earlier studies on the Nile Red staining failed and this may be due to the thick and rigid cell wall composition of the algae. This would have perhaps restricted the entry of the dye into the cells. To overcome such issues and to make use of Nile red as an ideal stain for lipid analysis, certain modifications are done to the process which showed the rise in the graph of the results in a positive way. This includes the use of dimethyl sulfoxide (DMSO) as a carrier that allows the entry of the stain at elevated temperatures (Chen *et al.,* 2009) [12].

The advantage of Nile Red over BODIPY 505/515 is that the latter has a strong background fluorescence which distorts the actual lipid fluorescence (Morschett *et al.,* 2016) [49]. The guidelines for the standardization of Nile red staining for microalgae strains were formulated by Halim and Webley (2015) [30]. They further concluded that Nile red staining can be used as a quick diagnostic tool for screening hyper-lipid producing microalgae cells. Gerde *et al.* (2012) [24] reported that Nile red staining can be utilized to quantify the cell disruption efficiency of heterotrophic microalgae *via* ultrasonication. They also found the Nile red fluorescence method as an instrumental for the rapid determination of the best extraction technique to maximize oil extraction. Suchitra *et al.* (2019) [67] confirmed the presence of neutral lipid in microalgal cells *via* Nile red staining.

Bodipy 505/515 staining

BODIPY is a lipophilic fluorescent dye, which can be used as an alternative to Nile red dye. Algal cells stained with BODIPY shows stained green lipid bodies with red chloroplasts (Cooper *et al.,* 2010) [16]. It belongs to a class of strong UV absorbing molecules with a sharp emission peak. Small modification to the structure of the dye results in different fluorescent and excitation properties (Rumin *et al.,* 2015) [57]. The high lipid yielding cells can be identified and isolated microscopically *via* micromanipulator, flow cytometry, or fluorescence-activated cell sorter. The main advantage of BODIPY 505/515 is that it does not bind to cytoplasmic compartments other than lipid and chloroplast. Due to the high oil/water coefficient, it allows the stain to the lipid components of the live cells. The lipid probe is blue which can be distinguished from the green chloroplasts (Brennan *et al.,* 2012) [10]. Encarnacao *et al.* (2018) [19] proposed a new fluorescent probe BODIPY BD-C12 to determine the lipid content in *Nannochloropsis*. It showed multicolor fluorescence and showed increased lipid content with nitrogen stress conditions. The advantages of this dye include high molar extinction coefficient, high fluorescent quantum yields, sharp emission bands, relative insensitivity to changes in pH and polarity, and good photochemical stability (Govender *et al.,* 2012) [27].

Sudan black B staining

Sudan Black B is a dark-colored non-fluorescent thermostable fat-soluble diazo dye that was first used for staining of neutral triglycerides and lipids by Lison (1934) [41]. Ru-rong *et al.* (2011) [58] observed microalgal lipids by Sudan Black B staining and recorded maximum absorbance at a wavelength of 645 nm. Kitcha and Cheirsilp (2011) [37] Screened 23 lipid producing yeast strains by Sudan Black B test. The eight freshwater blooming green algae of keral were screened preliminarily for lipid richness by the Sudan Black B staining method (Santhosh *et al.,* 2016) [59].

FTIR spectroscopy

FT-IR (Fourier Transform Infrared Spectroscopy) can be used for lipid content determinations in algal as lipid, carbohydrate and protein have their absorbance at specific frequency regions in the mid-infrared zone (Lv *et al.,* 2010) [42]. Dean *et al.* (2010) [17] have demonstrated the use of Fourier transform infrared micro-spectroscopy for lipid and carbohydrate estimation from freshwater microalgae. It is a technique that provides information regarding changes taking place *in-situ* and *in-vivo* inside living cells. The strength and frequency of absorption are estimated with factors like the mass of atoms, bond type, symmetry, etc (Vidyadharani and Dhandapani, 2013) [68]. Ansari *et al.* (2019) [1] performed FT-IR analysis of *Scenedesmus obliquus* which showed different absorption bands of various functional groups. The variations in the peak intensities correspond to changes in biochemical composition due to different nutrient conditions. It is reported that FT-IR is an efficient and rapid tool for monitoring algal lipid accumulation. Highly significant correlations were noticed between FT-IR and the Nile red based lipid measurement (Dean *et al.,* 2010) [17]. Other than lipid estimation, FT-IR can also be used to analyze growth prediction, changes in elemental carbon allocation, and physiological responses to nutrient stress (Mayers and Shields, 2013) [46]. The advantages of this method over conventional chemical analyses include high reliability, sensitivity, and speed of measurement procedure (Wagner *et al.,* 2010) [69]. Feng *et al.* (2013) [21] compared FT-IR and the Nile red with gravimetry for microalgal lipid determination. They further confirmed that Nile red is more appropriate for small samples when high-frequency screening is required.

PAM fluorometry

Pulse Amplitude Modulation (PAM) fluorescence gives information about the photosynthetic yield. The instrument (fluorometer) used is capable of measuring the intensity changes of fluorescence without disturbing the state of the sample (Schreiber *et al.,* 2004) [61]. PAM can be used for qualitative estimation of the physiological characteristics of oxygenic photosynthesis e.g. linear and cyclic electron transfer (Schuurmans *et al.,* 2015) [64]. It is not only a rapid and non-intrusive method but also can be used to determine properties like photoprotection and photoinhibition (Jebali *et al.,* 2019) [36]. Ranglova *et al.* (2019) [55] used the PAM fluorometry to analyze the rate of photosynthesis and its

variables. They related it to the growth of the culture and used it for the estimation of suitable temperature for optimum growth of a particular strain.
The screening of microalgae bioprospecting has to be comprehensive in assessing the lipid producing potential as well as the kinetics of growth and tolerance. The success of downstream processing is dependent on reliable biochemical and physiological screening tools such as the Nile red staining, BODIPY lipid stain, FTIR spectroscopy, and PAM fluorometry.

Sulfo-phospho-vanillin (SPV) method
The colorimetric (SPV) method is known for its fast response and it is relatively easy in handling the samples. It was first introduced by Chabrol and Charonnat (1937) [11]. This method was widely used as standard protocol human cerebrospinal fluid estimation for the detection of total lipid content. Because of the reactivity of SPV with the human serum, it has a prominent role in the medical field. Mishra *et al.* (2014) [48] used the SPV method for lipid estimation in microalgae like *Chlorella* sp., *Nannochloropsis* sp., etc. and was successfully employed as a direct quantitative measurement of algal lipids. It is a microplate assay where the reaction mixture is maintained in a 96-well plate throughout the assay. SPV when reacting with lipids, produces pink color, and the absorbance measured at 530 nm using spectrophotometric methods refer to the lipid concentration. The main reagents used include concentrated sulphuric acid, methanol, chloroform, o-phosphoric acid. Phoshovanillin is prepared by mixing vanillin with phosphoric acid at standard proportions (Park *et al.,* 2016) [51].
Certain advantages are using this method which includes 1) absorbance reading is comparatively easy 2) less risk of handling the samples containing concentrated sulphuric acid and 3) color development and its intensity refer to the concentration of lipids present. So, this method can be used for the quantitative measurement of lipids (Cheng *et al.,* 2010) [13]. SPV assay is sensitive to the degree of unsaturation and shows high intensities with high degrees of unsaturation present. One of the main disadvantages is that it may lead to false results if the unsaturation degree changes during the process or chemical treatment of the samples (Higgins *et al.,* 2014) [33].

Mass spectrometry
Mass spectrometry is a powerful analytical technique used to determine the molecular weight, chemical composition, the position of branching, and nature of substituents in the fatty acid structures of microalgal lipids. Many new strategies for Mass Spectrometry based analysis of lipids have been developed. Using the Matrix-Assisted Laser Desorption and Ionization Time-of-Flight (MALDI-TOF) Mass Spectrometry (MS) in combination with Thin-Layer Chromatography (TLC) technique, glycerol-trimethylhomoserine and phosphatidylethanolamine are exclusively detected in the green algae *Chlamydomonas reinhardtii* extracts, and phosphatidylcholine, a characteristic lipid of *Cyclotella meneghiniana* (Diatom) have been detected (Astrid *et al.,* 2007) [4]. The Liquid Chromatography – Mass Spectrometry (LC-MS) is used to profile the total Glycolipids and Phospholipids of microalgal species of *Chlamydomonas reinhardtii, Chlorella vulgaria, Nannochloropsis sp., Scenedesmus sp.,* and *Schizochytrium limacinum* (Linxing *et al.,* 2015) [40].

Raman spectroscopy
Raman spectroscopy is a non-destructive method used to identify the components, determine the chemical composition and various properties of biomolecules in the individual algal cells. It is based on the principle of Raman scattering that takes place due to inelastic collision between photons and electrons. The difference in energy between incident photon and emitted photon generates Raman lines. Depending on the emitted frequency of the photons the lines generated are called Stokes lines, if their frequency of the emitted photon is less than the incident frequency of photons and anti stokes line if the frequency of emitted photons is greater than the incident frequency of photons. Depending on the number of scattered photons, the raman spectrum shows various peaks that change with changes in the characteristics of biomolecules in the sample. These characteristic peaks can be used to identify the structural components, or chemical composition of the sample (Niranjan and Vikas, 2012) [50]. Laser Tweezers Raman Spectroscopy (LTRS) is a combination of a near-IR optical trap and a Raman spectroscopy, used to obtain the Raman spectral library of lipid extract from microalgal cells (Huawen *et al.,* 2011) [34] (Figure 2).

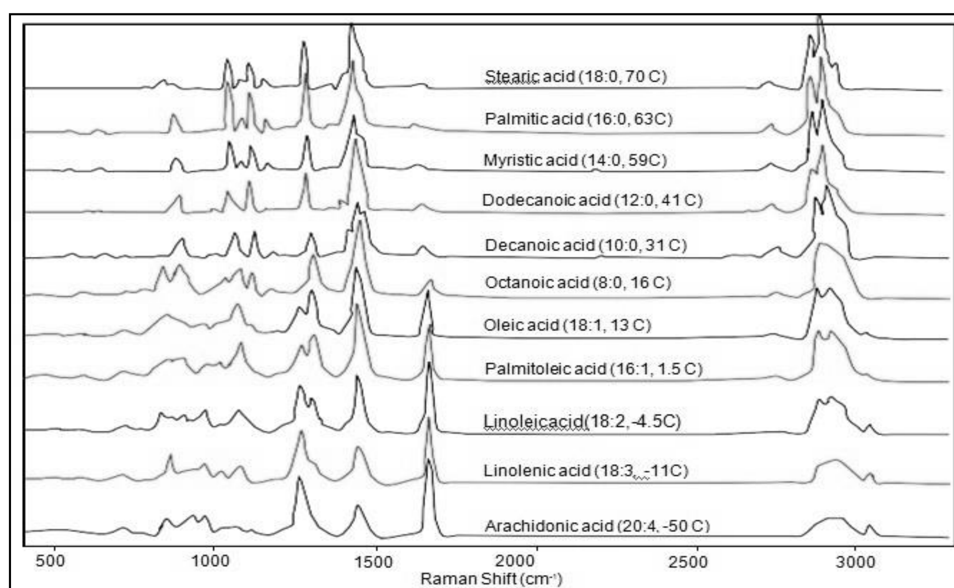

Fig 2: Raman spectral library of several single fatty acids typically found in algal lipid extracts

The Raman spectral peaks obtained from the lipid bodies of single microalgal cells determine the ratio of unsaturated-to-saturated carbon-carbon bonds in fatty acids (Table 2). Advances in Raman Spectroscopy techniques such as Surface Enhanced Raman Spectroscopy (SERS), Tip Enhanced Raman Spectroscopy (TERS), Coherent Anti-Stokes Raman Spectroscopy (CARS) and Laser Tweezers Raman Spectroscopy (LTRS) enable us to identify the real-time changes in the microalgal cells and to detect the biomolecule changes in the algal cells in different environments (Niranjan and Vikas, 2012) [50].

Table 2: Degree of lipid unsaturation in microalgae using Raman spectroscopy

S. No	Microalgal Strains	Raman Spectral Peaks	Characteristics	References
1	*Botryococcus braunii*	1650-1670 cm^{-1} and 2800-3000 cm^{-1} peaks	Double bond stretching peak in long chain unsaturated hydrocarbons	Largeau *et al.,* 1980
2	*Chlorella sorokiniana Neochlorisoleo abundans*	1650 cm^{-1} peak 2800-3000 cm^{-1} peak	C=C Stretching peak C=C-H vibration peak	Huang *et al.,* 2010
3	*Botryococcus sedeticus Chlamydomonas sp. Trachydiscus minutes*	1656 cm^{-1} peak 1445 cm^{-1} peak	Cis C=C stretching mode proportional to no. of unsaturated bonds. CH_2 scissoring mode proportional to no. of saturated bonds. *Trachydiscus minutes* have significantly higher content of unsaturated fatty acids.	Samek *et al.,* 2010

Near infrared reflectance spectroscopy

Near Infrared Reflectance Spectroscopy (NIRS) is an analytical method in which the sample is irradiated with the near-infrared region of the electromagnetic spectrum (800-2500 nm) and the reflected wavelength of light is recorded in the form of a spectrum. The spectrum contains information about the composition of the sample as different molecules absorb and reflect near-infrared light in a different wavelength. The NIRS technique is used to determine the TFA across three Chlorella species of *Chlorella vulgaris*, *Chlorella protothecoides*, and *Chlorella zofingiensis* (Bin *et al.,* 2015) [7]; using FAME as a proxy measurement the concentration of biomass and lipid was estimated in microalgal cultures of *Kirchneriella sp*. and *Nannochloropsis sp*. (Malcolm *et al.,* 2014) [45]. The NIR combined with FTIR (Fourier Transform Infrared) is used for predicting the levels of spiked neutral and polar lipids in microalgae species of *Nannochloropsis sp., Chlorococcum sp., Spirulina sp.,* and an unknown Diatom (Lieve and Edward, 2010) [39]. NIRS technique can bypass the involvement of cell disruption, oil extraction, and transesterification processes. Thus, it is a fast and non-destructive method that has great potential for screening purposes, in particular the high throughput screening of oleaginous microalgal fatty acids for biodiesel production (Bin *et al.,* 2015) [7].

Hyper spectral imaging

The traditional way of the gravimetric method to determine the lipid concentration of microalgae involves a set of chemical treatments which is a tedious and time-consuming process. Although the chemical analysis based on gravimetric method is accurate, it is difficult to apply in large scale industrial applications and the use of chemical treatment also increases the potential of environmental pollution. To address this problem many potential detection techniques have been proposed. Among them, hyperspectral imaging is an ecofriendly, nondestructive, fast, and real-time in situ method that is capable of visualizing the microalgal lipid concentration in live microalgal cultures. It helps in understanding the dynamic effect of nitrogen stress on the lipid content accumulation in microalgae *Scenedesmus obliquus* (Xiaoli *et al.,* 2020) [71]. Hyperspectral imaging is an extension of near-infrared spectroscopy, obtained by recording the reflections of hundreds of different bands of the electromagnetic spectrum from the biological samples (Akin and Kemal, 2020) [5]. The hyperspectral imaging of microalgae revealed a continuous process of lipid accumulation from live algal cells under different conditions, which to the best of our knowledge has not been investigated in the literature. The hyperspectral image method provides an intuitive visual way to display the lipid accumulation of algae, which has enormous potential in both microalgae-based industrial and biofuel research (Xiaoli *et al.,* 2020) [71].

NMR spectroscopy

High resolution nuclear magnetic resonance spectroscopic technique offers many advantages over existing analytical methods. Sarpal *et al.* (2015) [60] have developed the NMR technique for characterization and determination of lipid content and fatty acid profile of microalgal cultures. NMR method for neutral lipid estimation is a rapid and economical means for lipid estimation of algal cultures. It is a non-destructive method of lipid estimation. This method can be used for both qualitative and quantitative analysis of lipids. The basic principle of this technique is related to the magnetic properties of the atomic nuclei. The nucleus of every atom is electrically charged and each nucleus of the elemental isotopes has its characteristic spin. For a particular nucleus, two spin states exist when an external magnetic field is applied. The difference in the energy between these spin states differs with the magnetic field applied and the nucleus which is being studied. When a sample is subjected to irradiation with radiofrequency energy exactly to the spin state separation of the particular nuclei, it leads to the excitation of the set of nuclei from its lower energy state to its higher energy state (Xu *et al.,* 2015) [72].

Time Domain (TD)-NMR is the cheaper and the simplified version of the traditional NMR technique. The frequency range of 2 to 25 MHz is commonly used to operate it (Falch *et al.,* 2006) [20]. It mainly focuses on the relaxation times of the hydrogen nuclei in different phases of the samples. Gao *et al.* (2008) [23] applied the Spin-echo NMR pulse sequence for separating lipid hydrogen nuclei signals from others. Recently, benchtop NMR spectroscopy devices have emerged to improve accessibility and cost. Using this, lipid estimation can be done without disturbing the biological processes of the algal cells (Bouillauda *et al.,* 2019) [8]. Algal lipid estimation *via* NMR provides additional information with high accuracy and precision (Landkhorst and Chang, 2018) [38].

Gravimetric method

It is one of the oldest methods of lipid estimation. The procedure involves the extraction of lipids from the microalgal cells followed by quantification. The two-step extraction process involves methanol, chloroform, and water for phase separation and quantification. The health and safety issues, as well as environmental concerns due to the use of hazardous chemicals like methanol and chloroform, are considered as serious problem (Elsey *et al.,* 2007; Breil *et al.,* 2017) [18; 9]. This method stands as a reference for newly emerging methods (Chen and Wu, 2011) [14]. Many new techniques and instruments have emerged due to certain disadvantages of this method like time-consuming, labor-intensive, requires a large number of samples and procedure involves certain hazardous chemicals. So, it cannot be used for large-scale purposes (Gong and Jiang, 2011) [26].

Chromatographic techniques

Chromatography is a technique used in the separation of a mixture of chemical substances into its components. Among many types of chromatographic techniques High Performance Liquid Chromatography, Gas Chromatography Mass Spectrometry and Thin Layer Chromatography are widely used in lipid estimations. These techniques are usually coupled with other techniques for accuracy which include spectroscopy. The separation principle lies in the differing affinities of each component of the mixture loaded for the matrix.

TLC: The absorbent material used is silica which has high quality in bonding. Quantitative estimation can be done by direct recovery from the loaded plate or after recovering the individual components from the plate (Patel *et al.,* 2019) [52]. On the chromoplate, silica gel is most commonly used as the stationary phase (solid) and the loading component (sample) acts as the mobile phase (liquid). Several modifications like using Magnesium silica, Boric acid, Silver nitrate, etc. as stationary phases have been done to improve the resolution (Peterson and Cimmings, 2006) [53].

HPLC: It is the most widely used technique among other chromatographic methods. Advantages include high specificity, sensitivity, good resolution, and speed. This method decreases the sample exposure to atmospheric oxygen, which remains as an advantage over TLC (Peterson and Cimmings, 2006) [53]. The additional advantage is that it is a non-destructive method and samples can be collected later (Gomes *et al.,* 2018) [25].

GC: Lipid profiling using GC is usually coupled with Mass Spectroscopy (MS). The samples should be pre-treated to increase its polarity volatility before injection. To avoid contamination, splitless injections are used (Fusuhashi and Weckwerth, 2013) [22]. This method is used to differentiate the volatile compounds with semi-volatile compounds. GC coupled with MS (GC-MS) has a high spectral resolution (Gomes *et al.,* 2018) [25].

Conclusions

There are many methods for lipid estimation from algae and each method has its advantages and disadvantages as mentioned in Table 3. If rapid screening of algal lipid is required, Nile red spectrofluorometric method can be used. The quantification of algal lipid depends mainly on the method used *viz.,* conventional and non-conventional method. The non-conventional methods like NMR, spectroscopy, etc are more accurate and less time consuming compared to conventional methods.

Table 3: Advantages and Disadvantages of various methods of lipid estimation

S. No	Methods	Advantages	Disadvantages
1	Nile Red staining	• Fast screening of potential oleaginous microalgae • Stains intracellular neutral lipids (TAG)	• Limited photostability • Interference with chlorophyll • Difficulty of permeation for some species
2	BODIPY 505/515 Staining	Crosses cell membrane easily. Do not bind to cytoplasmic compartments other than lipid bodies (green fluorescence) and chloroplast (red fluorescence) Insensitive to pH and polarity of the environment	• Background fluorescence of the dye in the medium • Failure to quantify neutral lipids between rich and low oil strains
3	Sudan black B staining	• Stains neutral lipids and phospholipids	• The thickness of the section may affect the intensity of the staining
4	Sulpho-Phospho-Vanillin (SPV) method	• Requires only a small amount of target sample	• Time intensive and costly
5	MALDI TOF MS	• Simple, fast and sensitive technique. • Sample impurities, such as salts and detergents are tolerated to high extent.	• Individual Phospholipid classes (Phosphatidylcholine [PC], Sphingomyelin [SM], Lysophosphatidylcholine [LPC]) are detected with different sensitivities. • Signal suppression of lipids with lower ion yield
6	Raman Spectroscopy	• Non-destructive • No sample preparation needed • Raman spectra are acquired quickly within seconds • Not interfered by water	• Do not detect lipid composition in large algal cultures (only detects in single cells) • sample heating through the intense laser radiation can destroy the sample • Fatty acid profiling cannot be done
7	Near Infrared Reflectance Spectroscopy (NIRS)	• Non-destructive and Non invasive • Low-cost optical technique • No pretreatment of sample is needed • Method also works in presence of interfering substances such as glass and plastic containers • Neutral and polar lipids can be detected	• Fatty acid profiling cannot be done • Strict sample temperature control required • Lack in structural interpretative value (difficult to identify unknowns)
8	Nuclear Magnetic Resonance (NMR)	• Non-destructive • Direct,quantitative analysis of virtually all	• Expensive equipment. • Relatively low sensitivity.

	spectroscopy	Phospholipids • Continuous lipid analysis from live algal cells under different conditions • Neutral and polar lipids can be detected	• High concentrations of detergent needed • Crowded spectra in the case of lipid mixtures.
9	Thin Layer Chromatography (TLC)	• Simple, fast and inexpensive • Non volatile compounds can be seperated	• Low resolution • Very difficult to differentiate individual fatty acids
10	High Performance Liquid Chromatography (HPLC)	• Individual lipid classes can be differentiated • Fatty acid profiling can be done • Higher resolution and speed of analysis	• More expensive • Lack of universal detector • Less separation efficiency than GC
11	Gas Chromatography (GC)	• Higher resolution and short time analysis • Highly established for fatty acid compositional analysis of lipids • Fatty acid profiling can be done • Using Mclafferty rearrangement mass fragmentation can be detected	• Purification and processing of samples are required • Only volatile and thermally stable compounds can be separated.
12	Hyper spectral imaging technique	• Creates images using hundreds and thousands of narrow band • Higher level of spectral detail in hyperspectral images gives better capability to see the unseen	• Cost and complexity • Large data storage capacities are needed

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Compliance with ethical standards

Conflict of interest Author (s) declares that they do not have any conflict of interest.

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