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Biomolecular transitions and lipid accumulation in green microalgae monitored by FTIR and Raman analysis

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ABSTRACT

Fourier transform infrared (FTIR) and Raman spectroscopic techniques were employed to analyze the biomolecular transitions and lipid accumulation in three freshwater green microalgal species, *Monoraphidium contortum (M. contortum)*, *Pseudomuriella* sp. and *Chlamydomonas* sp. during various phases of their growth. Biomolecular transitions and lipid [hydrocarbons, triacylglycerides (TAGs)] accumulation within the microalgal cells were identified using second derivatives of the FTIR absorption spectroscopy. Second derivative analysis normalized and resolved the original spectra and led to the identification of smaller, overlapping bands. Both relative and absolute content of lipids were determined using the integrated band area. *M. contortum* exhibited higher accumulation of lipids than the other two species. The integrated band area of the vibrations from saturated (SFA) and unsaturated lipids (UFA) enabled quantification of fatty acids. The percentage of SFA and UFA was determined using GC, FTIR and Raman spectroscopy. From the spectral data, the order of increasing concentration of SFA among the three microalgal species was *M. contortum* > *Chlamydomonas* sp. > *Pseudomuriella* sp. The spectral results on fatty acids were consistent with the separation of lipids by gas chromatography. The results emphasized the significance of FTIR and Raman spectroscopic methods in monitoring the biomolecular transitions and rapid quantification of lipids, without the need for extraction of lipids.

Keywords:

Vibrational spectroscopy; infrared; hydrocarbons; lipid; biodiesel; biofuel; bioenergy

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1. Introduction

In the last two decades, several researchers have explored the potential of microalgae as a source for biodiesel [1–4]. Microalgae are unicellular organisms, also known as phytoplankton that can normally grow in fresh water and in marine systems. Among their several taxonomic groups, green algae (Chlorophyceae), cyanobacteria (Cyanophyceae), golden algae (Chrysophyceae) and diatoms (Bacillariophyceae) have been reported as potential species for biofuel production [5,6]. Microalgae have gained a considerable interest across the world due to its ability to synthesize and accumulate more lipids and have become a significant source of third generation biofuels.

Lipids from microalgal species comprises of saturated and unsaturated fatty acids (mono and polyunsaturated fatty acids) that have long hydrocarbon chains with carboxylic acids. Under nutrient stress, algal cells alter their lipid biosynthetic pathways towards formation of neutral lipids called Triacylglycerides (TAGs) [7–9], which are made up of three fatty acids and a glycerol. The majority of fatty acids in TAGs are saturated (SFA) and monounsaturated (MUFA). Polyunsaturated fatty acids are enriched in polar lipids [10,11]. SFA and MUFA in microalgae can be an excellent source of biodiesel due to their high oxidative stability and lesser NO_X emissions in comparison to polyunsaturated fatty acids (PUFA), except that the high melting point of SFA can cause gelling of fuel in cold weather. [12,13]. The primary focus in algal biofuel research was to identify microalgal species that can produce significant amount of lipids in the form of TAGs.

Biomolecular composition of microalgal cells with particular interest on lipids have been quantified by numerous investigative techniques. Among them traditional analytical methods of lipid detection include chromatographic based separation and quantification of lipids such as gas chromatography (GC), high performance liquid chromatography (HPLC) and thin layer chromatography (TLC). These are powerful techniques for lipid analysis. However, the methods require extraction of lipids from algal biomass prior to analysis and are destructive, time consuming, also involves large amount of biomass, costly chemical reagents for analysis and therefore cannot be effectively used for screening large number of microalgal samples [14–16]. Another method for lipid detection using fluorescent dye called Nile Red staining has been used to visualize lipid accumulation within cells by fluorescence microscopy. However, it is affected by uneven dye uptake due to differences in cell wall composition among species. Nile red staining is not quantitative as chromatographic methods and cannot distinguish between neutral and polar lipids [5,8].

Rapid, efficient methods are desirable for lipid quantification in algal biomass for screening of species and growth conditions. Spectroscopic techniques such as Fourier transformed infrared spectroscopy (FTIR) and Raman spectroscopy have been reported to be non-destructive, fast and versatile tools to analyze algal biomass using minimal volume with much ease [17–19]. The analysis involves the measurement of absorption (FTIR)/scattering (Raman) intensity in relation to a range of molecular vibrational modes due to the presence of biomolecules, enabling quantification. [18,20,21].

Several researchers have demonstrated the use of either of these spectral techniques to analyze the microalgal cells. FTIR spectroscopy has been used to identify the biomolecules present in microalgae [22–24] and to determine lipid accumulation in response to nutrient stress like nitrogen and phosphorus or other environmental modifications [7,8,25]. Studies have reported the use of FTIR in evaluating and monitoring lipid accumulation and its extraction efficiency in other microorganisms such as oleaginous yeasts and fungi [26,27]. Second order derivatives of the absorption spectra have also been determined to enhance separation of the overlapping bands of the raw spectra and to do quantitative analysis [26,28]. FTIR spectroscopy has been shown to enhance the biological and ecological analysis of algae and to assess quantitative changes in the intracellular macromolecular pools of microalgae [17,18,23,29–32].

Raman spectroscopy and its imaging techniques have also been used to identify biochemical composition and to predict the nutrient status of microalgal cells [29,33]. In 2007, the Raman Research Group at Ghent, Belgium published a reference database of Raman spectra of biological molecules [34]. Apart from the qualitative analysis of the biochemical composition, Raman spectroscopy has also been used to do quantitative analysis of the degree of unsaturation of lipids in microalgae [35–37], and to evaluate the accumulation of lipids, especially triacylglycerides (TAGs) in microalgae [19,38–41]. Raman spectroscopy with chemometrics was applied for assessing the physiological responses and growth phases of different microalgae under environmental changes [42]. Statistical tools and multivariate analysis combined with FTIR and Raman spectral studies simplify the complexity of the spectral data [6,8,23,24,43,44].

Fewer studies have been reported on using both FTIR and Raman spectral analyses to study microalgal species [45]. Microalgal species like *Chlorella, Haematococcus, Dunaliella , Spirulina , Nannochloropsis, Scenedesmus, Chlamydomonas* and *Monoraphidium* have been reported as promising species for biodiesel applications through traditional lipid quantification methods [10,46,47]. Among them green algal species, *Chlorella* sp. has

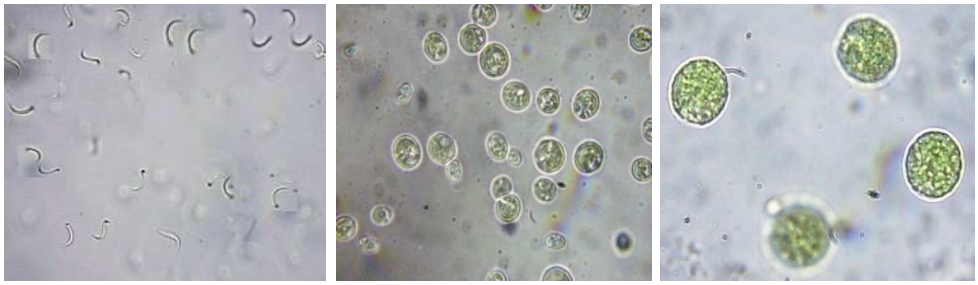
exhibited 2 to 4 fold increase in lipid content, under nitrogen starved condition [48]. *Chlamydomonas reinhardtii* and *Scenedesmus subspicatus* have exhibited 5 to 6 fold increase in lipid accumulation [8].

The present study aims to monitor the biomolecular transitions and lipid accumulation especially TAGs in three fresh water green microalgal species *Monoraphidium contortum (M. contortum)*, *Pseudomuriella* sp., and *Chlamydomonas* sp. during their various growth phases using FTIR and Raman spectral techniques for biodiesel application. The study aims to validate the spectral results with lipid quantification by GC and intends to emphasize the significance of these two spectroscopic methods in the rapid quantification of lipids, without the need for extraction of lipids.

2. Materials and Methods

2.1. Microalgal strains and culture conditions

Three freshwater green microalgal isolates, *Monoraphidium contortum (M. contortum)*, *Pseudomuriella* sp., and *Chlamydomonas* sp., (Fig. 1) were obtained from the Research centre in Botany and Microbiology, Lady Doak College, Madurai, Tamil Nadu, India. The cells were cultured in modified BG11 medium by adding inoculum with 0.15 optical density (OD) to the culture vessels containing 300ml of culture medium in triplicates. Batch cultures (static with intermittent shaking) were illuminated with a light intensity of 27 photons (µmol m^{-2} s^{-1}) at 25±2°C and a photoperiod of 12:12h light dark cycle was maintained throughout the study period. During incubation, the monospecificity of the cultures were verified periodically by microscopic observation and no evidence of contamination was found.



M. contortum ***Pseudomuriella* sp.** ***Chlamydomonas* sp.**

Fig.1. Optical microscopic images of the three freshwater green microalgal species

2.2. Biomass harvest for spectral analysis

The cells of *M. contortum*, *Pseudomuriella* sp., and *Chlamydomonas* sp., were harvested on their 10^{th}, 20^{th}, 30^{th} and 40^{th} day of growth. 30 ml of the culture from each triplicate flask was taken and centrifuged at 5000 rpm for 5 min. The supernatant was

discarded and the cells were washed with distilled water and were freeze dried at -55^{o}C to -85^{o}C using a lyophilizer Alpha 1-2 LD plus, CHRIST GmbH, Germany.

2.3. FTIR spectroscopy and measurements

Freeze dried biomass from each triplicate was equally weighed and mixed with KBr in the 1:100 ratio and were pelletized using a hydraulic pellet press. Infrared absorption spectra were recorded using a Thermo Scientific Nicolet 380 FTIR spectrometer, Thermo Electron Corporation, USA, in the Transmission E.S.P mode with 32 scans for each spectrum at 4 cm^{-1} resolution in the wave number range of 4000 - 500 cm^{-1}. Deuterated triglycine sulphate (DTGS) detector was used for detection. Spectra were recorded for three replicates per sample, resulting in 9 spectra at every phase of growth (10^{th} to 40^{th} day) for all three species yielding a total of 108 spectra. All spectra were normalized using the automatic baseline correction algorithm of Omnic 7.

2.4. Raman spectroscopy and measurements

Raman spectral measurements were carried out on the freeze dried biomass from each triplicate. They were equally weighed and placed in an eppendorf tube in front of the peak finder fibre optic video probe of a Modular 1064 Raman system, BaySpec, California, USA. The distance between the probe and the sample was maintained the same for all analysis. A total of 36 spectra were recorded for the three species in triplicate at the four intervals of their growth phases. Near IR laser of 1064nm with a laser power of 149mW was focused on the sample. Spectra were recorded in the spectral range of 200 – 3200 cm^{-1} at 10-15 cm^{-1} resolution with an exposure time of 5s for 10 scans. The exposure time was optimally chosen by ensuring that there was no laser induced damage for the algal biomass. Indium gallium arsenide (InGaAs) detector was used for detection. Each spectral acquisition was made with dark background subtraction and all spectra were normalized by baseline correction using Spec2020 software.

2.5. Post processing of spectral data

The acquired FTIR and Raman spectral data were processed using Origin Pro 8 software. All the spectra were smoothed using Savitzky – Golay method with a 10 point window. Second order derivatives of the FTIR and Raman spectra were obtained by using the differentiate method with derivative order 2. Integrated peak area was obtained from the second derivative spectra to determine the relative and absolute content of lipids.

2.6. Fatty acid quantification by Gas chromatography (GC)

Algal cells were harvested by centrifugation at 5000 rpm for 10 minutes and lipid extraction was carried out from the pellets based on modified Bligh and Dyer (1959) method. 5ml of MeOH/$CHCl_3$ (2:1) was added to 1ml of algal culture and vortexed. Centrifugation was done at 5000 rpm for 5minutes. The upper layer was discarded and the lower chloroform phase containing the extracted lipids was transferred into a new tube. Fatty acid methyl esters (FAME) were prepared from the extracted lipids by transmethylation with 2% sulphuric acid in methanol [7]. Anhydrous sodium sulphate was added to the layer to eliminate the water content. The obtained FAME was stored at -20^{0}C for GC analysis. Fatty acid analysis was performed using Gas Chromatograph 2010 Plus (Shimadzu, Japan) using Flame Ionization Detector (FID). Injector and Detector temperature was set at 225^{0}C and 250^{0}C respectively. One microlitre of the sample was injected in a split mode (35:1) at a flow rate of 184.9 ml/min with Nitrogen as the carrier gas onto a FAMEs-RTX-2330 column (length 105.0m, Film thickness 0.20 μm, total run time 40min). Peak areas were integrated using the GC solution software and the FAME were identified using fatty acid standards (Sigma, Supleco, 37 FAMEs).

3. Results and Discussion

3.1. FTIR analyses on the biomolecular transitions and lipid accumulation

FTIR absorption spectra of the three microalgal isolate *M. contortum*, *Pseudomuriella* sp., and *Chlamydomonas* sp., were analyzed for their biomolecular transitions and lipid accumulation at their various growth phases using second order derivative method. Second derivatives of the absorption spectra were determined as mentioned in Section 2.5. These spectra were plotted for the 10th to the 40th day of growth, in order to analyze the significant spectral changes due to the change in physiology in the algal species at every phase of their growth. The second derivative spectra of the microalgal species *M.cortortum (*Fig 2), *Pseudomuriella* sp. (Fig.3a) and *Chlamydomonas* sp.(Fig 3b) were reported along with a typical raw spectra of their stationary phase (30th day) of growth, to show the regions where spectral variations occur. The important biomolecular absorptions were indicated. Second derivatives resolved the overlapping bands in the raw spectra, removed the baseline errors and lead to the identification of small and adjacent absorption bands and enabled quantitative analysis [26,49].

The raw absorption spectra showed distinct absorption bands corresponding to different biomolecules such as carbohydrates, proteins and lipids over the wave number range

4000 – 500 cm^{-1}. The bands observed in the region 3050 to 2800 cm^{-1}corresponds to $\nu(CH_3)$ and $\nu(CH_2)$ asymmetric and symmetric stretches of hydrocarbons from lipids and the band at ~1745 cm^{-1} was due to carbonyl stretches from esters of fatty acids, mainly called as triacylglycerides (TAGs). These two regions are significant to monitor the lipid accumulation in microalgal species. Two characteristic bands of the amide groups of protein chains, amide I and amide II were observed at ~1653cm^{-1} and ~1550 cm^{-1} respectively. The region between 1250cm^{-1} and 1000 cm^{-1} was due to ν(C-O) stretches of polysaccharides from carbohydrate and ν(P=O) stretches of phospholipids and nucleic acids (Table 1) [8,20–22,30]. The intensity variations in the absorption spectra and in its second derivative spectra in the above regions enabled the rapid analyses of biomolecular transitions and lipid accumulation.

To better evaluate the spectral changes in the three species during their various phases of growth, Second derivatives of the FTIR spectra were analyzed in two ways. (i) Analysis on each species during their 10th to 40th day of growth, to identify the growth phase at which maximum lipid accumulation occurred. (Fig.2 and Fig.3) (ii) Analysis at every growth phases for the three species, to identify the maximum lipid accumulating species. (Fig.4 and Fig.5).

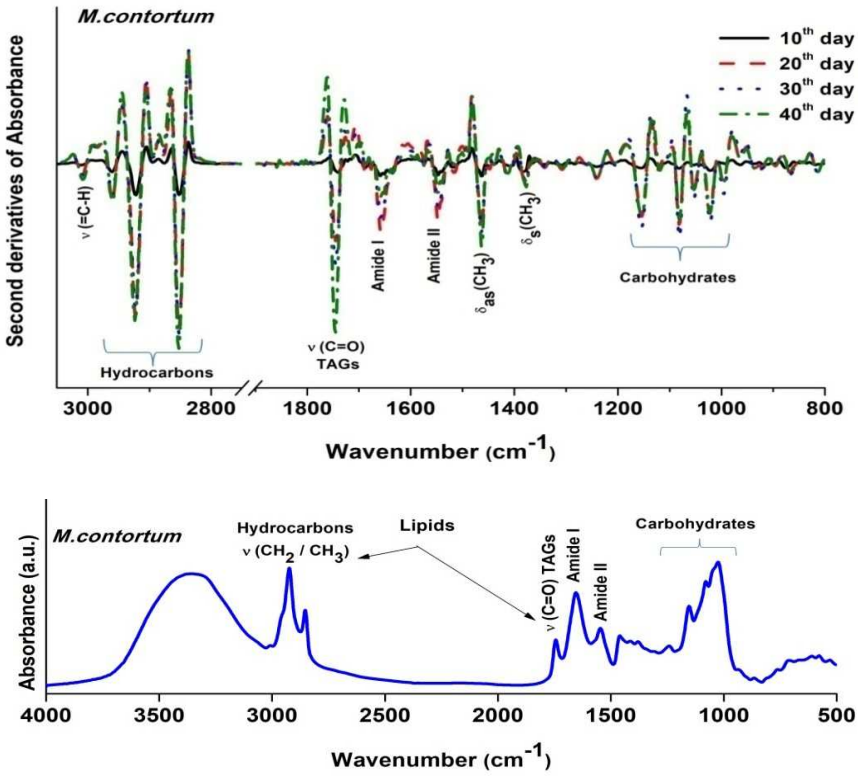


Fig.2. FTIR analyses of *M.contortum* at their various growth phases.
Second derivative spectra on the 10th to 40th day of growth along with the absorption spectra of the stationary phase of growth (30th day) are reported to show the regions of spectral variations. Each spectrum is an average of 9 replicates.

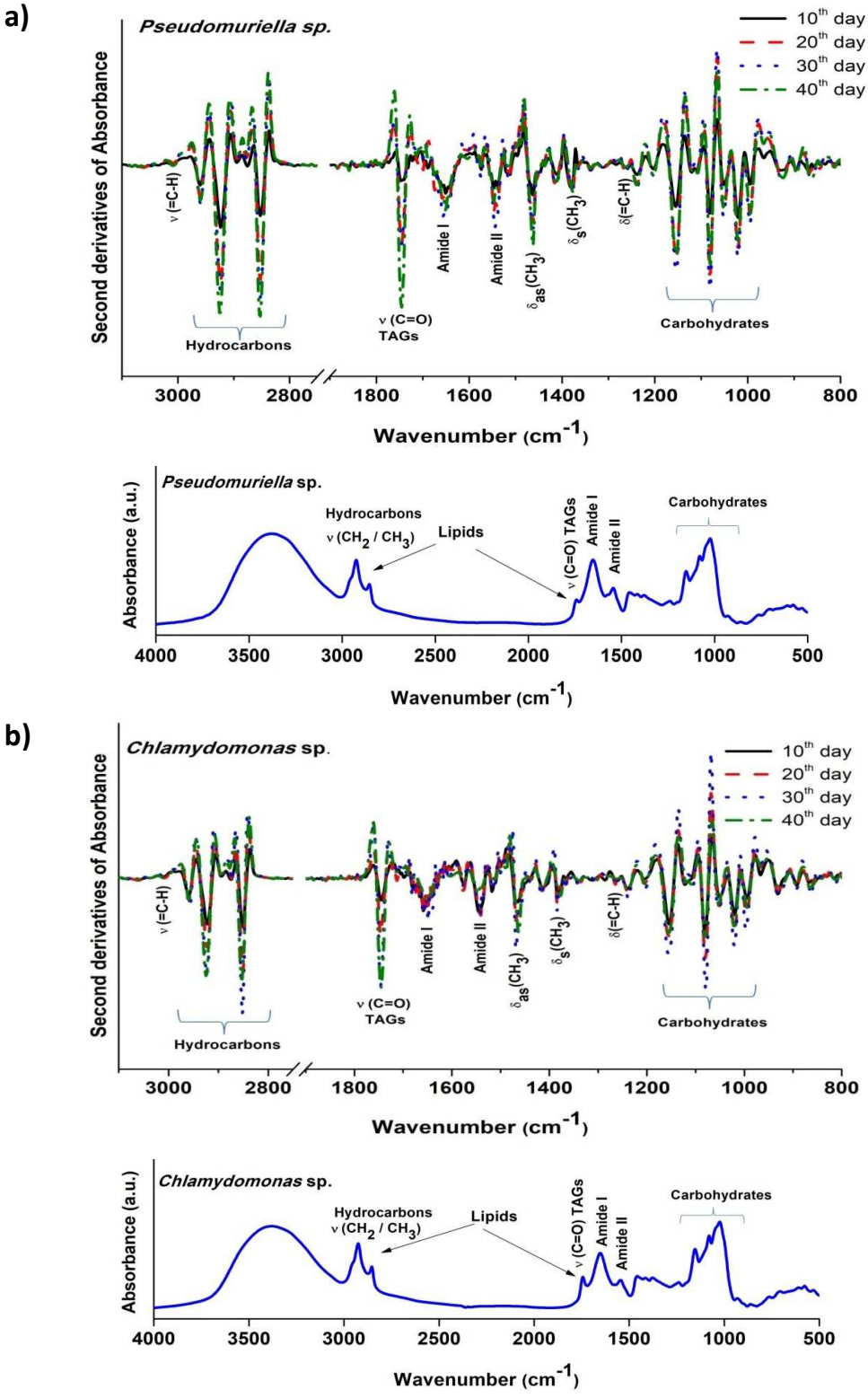


Fig.3. FTIR analyses of a) Pseudomuriella sp. and b) Chlamydomonas sp. at their various growth phases.

Second derivative spectra on the 10th to 40th day of growth along with the absorption spectra of the stationary phase of growth (30th day are reported to show the regions of spectral variations. Each spectrum is an average of 9 replicates.

3.1.1. Second derivative analysis on each species at their various growth phases:

On the examination of the second derivative spectra of all the three species shown in Fig.2 and Fig.3, the position of the bands corresponding to the biomolecules was observed to be almost the same (i.e. in the expected wavenumber range) at every phase of growth, but their intensity varied remarkably. In particular, the lipid bands from the hydrocarbons in the region 3050 to 2800cm^{-1} and the ν(C=O) esters of TAGs (neutral lipids) at ~1745cm^{-1} increased steadily from the 10th day to the 40th day (exponential phase to the declining phase of growth). While the intensity of the amide I (~ 1653 cm^{-1}) and amide II (~ 1550 cm^{-1}) bands of proteins and the carbohydrate bands increased till the stationary phase and decreased in the declining phase. The intensity variation in the spectral data represents the change in absorbance which is proportional to the concentration / cell population of the microalgae, according to the Beer Lambert's law. Thus the intensity variation observed in the spectra due to biomolecular transitions can be attributed to the metabolism of cells during their various phases of growth [26,28]. During the early exponential phase, when there is sufficient availability of nutrients, the microalgal cells divide, but as the cells enter the stationary phase, the nutrient availability becomes limited, the cell division stops, the algal cells start to accumulate and stores more energy in the form of lipids and carbohydrates. During the declining phase, lipid accumulation exceeds carbohydrates [9,32].

In addition to the identification of the growth phase with maximum lipid accumulation, several small overlapping bands significant to the saturated and unsaturated fatty acids of lipids were resolved in the second derivatives of the absorption spectra. The smaller bands at ~3008 cm^{-1} ν (= C-H) and ~ 1270 cm^{-1} δ(= C-H) were due to the stretching and bending vibrations of unsaturated fatty acids in the algal lipids. [21,26,50].

3.1.2. Second derivative analysis at each phase of growth for the three species:

Second order derivative of the FTIR spectra were analyzed in the two spectral regions, 3050 to 2800cm^{-1} and 1800 to 1000 cm^{-1} (Fig.4 and Fig.5) in the high lipid accumulating species during various phases of growth,

The region 3050 to 2800cm^{-1}:

The characteristic bands of hydrocarbons are the four bands at ~ 2960cm^{-1}($\nu_{as}CH_3$), 2920cm^{-1}($\nu_{as}CH_2$), 2870 cm^{-1}(ν_sCH_3) and 2850 cm^{-1}(ν_sCH_2) [22,26]. Second derivative analysis of the FTIR absorption spectra revealed these four bands. Fig.4a to Fig. 4d indicates the variations in the spectral intensity of these bands during the growth phase of the microalgal cells. The spectra of the 10th day of growth (Fig.4a) exhibited no significant

changes among the three species in the hydrocarbon region. However, from the 20th day to the 40th day of growth, drastic changes in the spectral intensity of the three species were observed (Fig.4b). Accumulation of lipids in the form of hydrocarbons was higher for *M. contortum* than for the other two species. The band at ~ 3008 cm^{-1} corresponded to the stretching vibrations ν (= C-H) from the unsaturated fatty acids was clearly visible in *M.contortum.* (Fig. 4b to Fig. 4d) [21,26]. However, this band was observed as a very weak band in *Pseudomuriella* sp., and *Chlamydomonas* sp. (Fig.3). This indicates the presence of unsaturated fatty acids (UFA) in the three algal species.

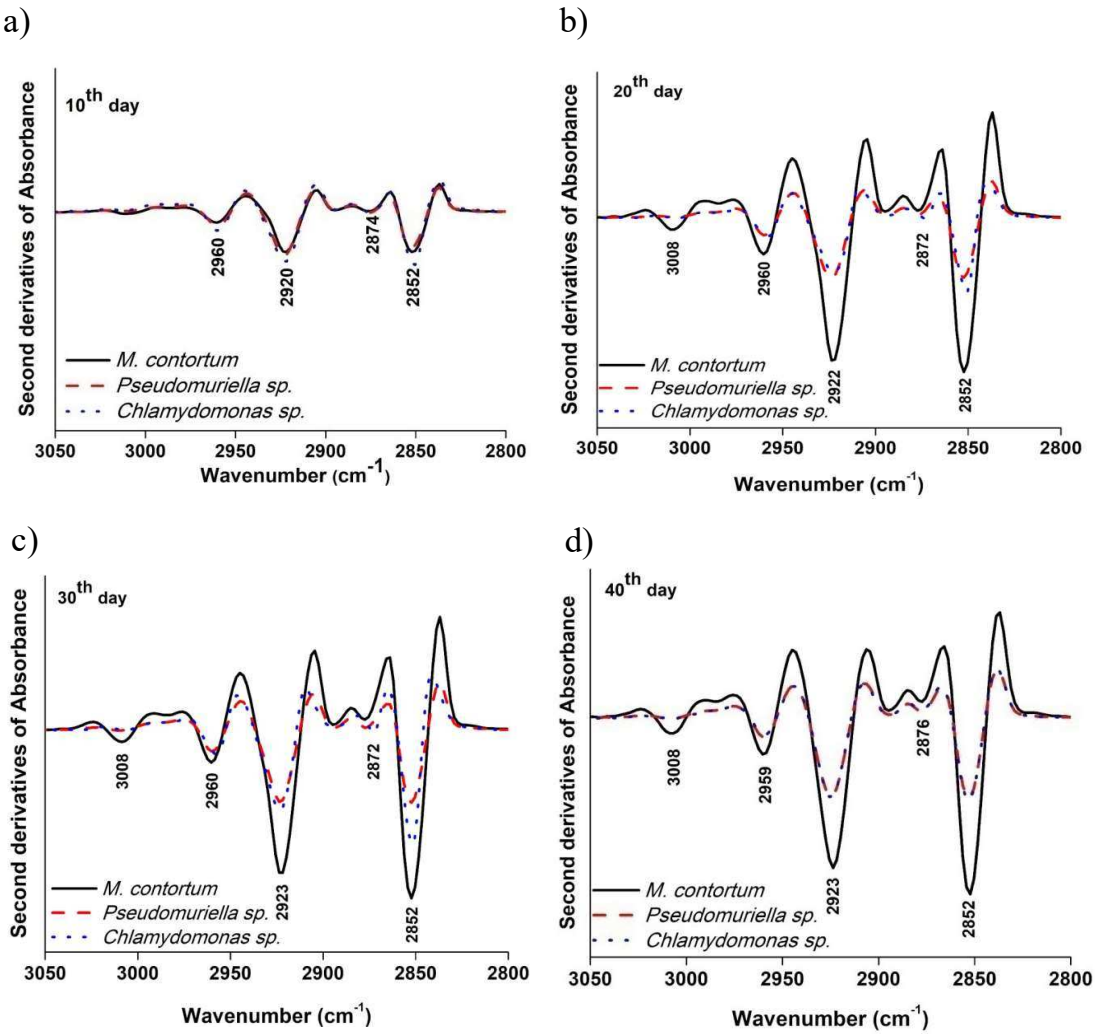


Fig.4. Second derivative spectra between 3050 and 2800 cm^{-1}of the three microalgal species on a)10th day b) 20th day c) 30th day and d) 40th day of growth

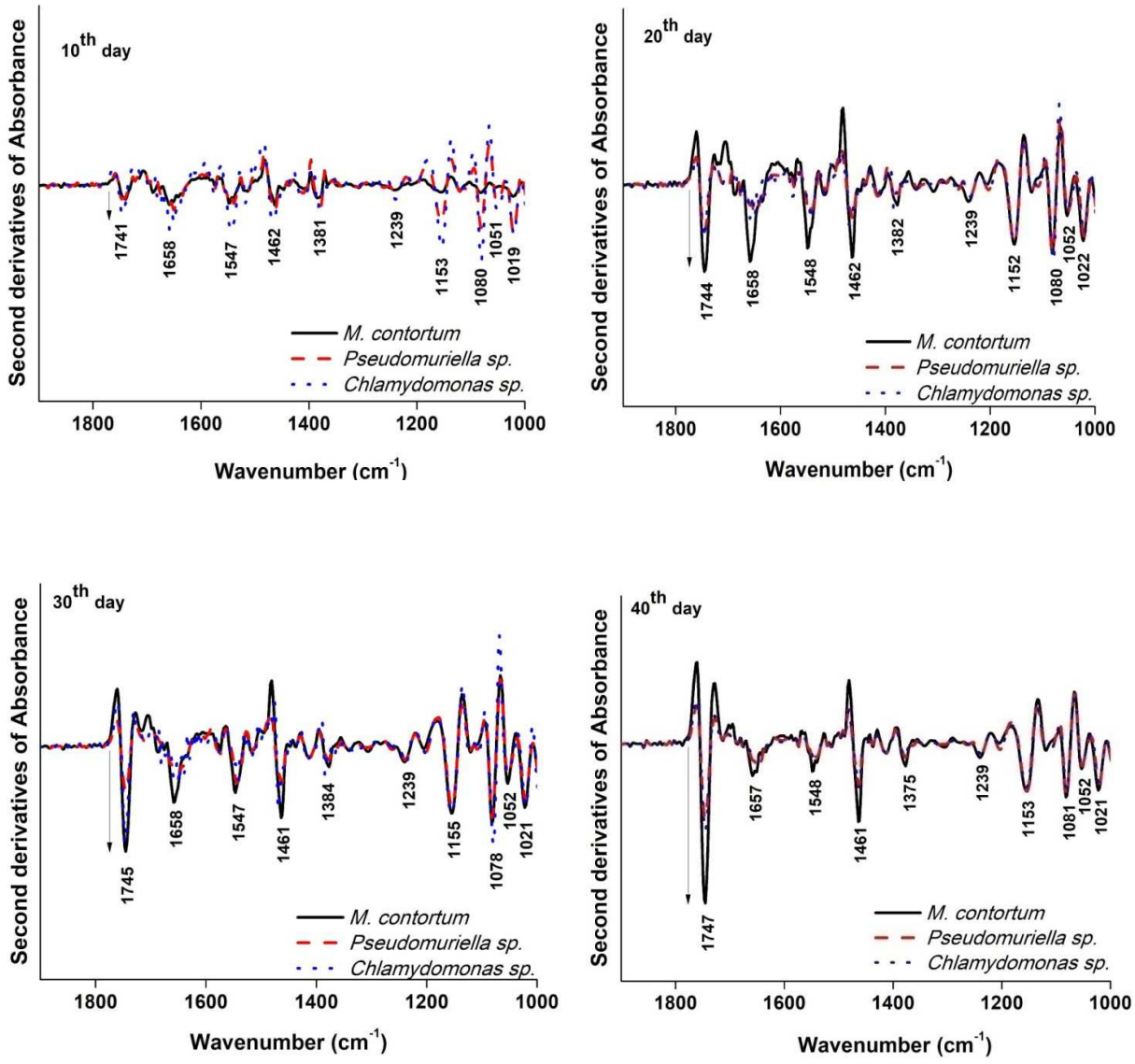


Fig. 5. Second derivative spectra between 1800 and 1000 cm^{-1} of the three microalgal species on a) 10th day b) 20th day c) 30th day and d) 40th day of growth. The arrow indicates the increasing TAGs.

The region 1800 to 1000cm^{-1}:

The region 1800 to 1000cm^{-1}, indicates 10 distinct bands, characteristic of various biomolecules. Bands due to lipids were ~1745cm^{-1} (νC=O), ~1462cm^{-1} ($\delta_{as}CH_3$), ~1380cm^{-1}(δ_sCH_3) and of proteins were ~1658cm^{-1}(amide I mainly νC=O), ~ 1547 cm^{-1} (amide II δN-H, νC-N). The small shoulder bands in the region 1200 - 1000cm^{-1} that correspond to the ν (C-O-C), ν (P=O) stretches of carbohydrates were resolved and observed as four bands ~1154cm^{-1}, ~1080cm^{-1}, ~1052cm^{-1}, ~1020cm^{-1} (Table 1). From the 10th day to

the 40th day of growth, the band at ~1745cm^{-1} from the neutral lipids (TAGs) exhibited remarkable changes in its intensity (Fig.5a to Fig 5d). Such predominant changes were not observed in the other lipid bands at ~ 1462cm^{-1} and ~ 1380cm^{-1}. This confirms that, these microalgal species are accumulating more triacylglycerides (TAGs) than the other forms of lipids, which is a good indicator for biodiesel production. Among the three species, increased accumulation of TAGs (Fig.5) was observed for *M.contortum* which indicate that the species is rich with saturated and monounsaturated fatty acids [10,11,47]. In addition to the changes in lipid bands, significant changes were observed for the protein bands at ~1658 and ~ 1547cm^{-1}. Fig 5c and Fig.5d clearly indicate the decrease in the intensity of two amide bands, which reflects the metabolism of the microalgal cells (Section 3.1.1). Thus, second derivative analysis vividly explains the biomolecular transitions and the lipid accumulation during the growth phases of microalgal cells.

3.1.3. Determination of lipid content from FTIR spectra

To evaluate the accumulation of lipids in the three microalgal species, relative and absolute content of lipids [hydrocarbons and triacylglycerides (TAGs)] were determined from their second derivative spectral data.

Relative content of lipids:

Relative content was obtained by normalizing the lipid bands with the protein bands [14,15,17,19]. The integrated band area of the region 3050 – 2800 cm^{-1} that corresponds to hydrocarbons (CH stretch) and the band area of TAGs (~1745 cm^{-1}) were normalized by the amide I band area (~1655 cm^{-1}) and their corresponding peak ratios were plotted during the 10th to 40th day of growth of the microalgal species as shown in Fig 6. An increase in the CH: Amide I ratio (Fig.6a) with an increase in the days of growth occurred for all the three species. During the declining phase of growth (40th day) all the three species exhibited a maximum of two to threefold increase in the relative hydrocarbon content. Similarly, the TAGs: Amide I ratio with the days of growth (Fig.6b) showed an increased accumulation of triacylglycerides for the microalgal species *M. contortum* and *Chlamydomonas* sp., from the initial value of around 0.5 to 4.3 and 3.8 respectively, resulting in a seven to eight fold increase during the declining phase of growth in comparison to *Pseudomuriella* sp. Previous studies on green algal species like *Chlamydomonas reinhardtii* and *Scenedesmus subspicatus* have exhibited 5 to 6 fold increase in lipid accumulation [8]

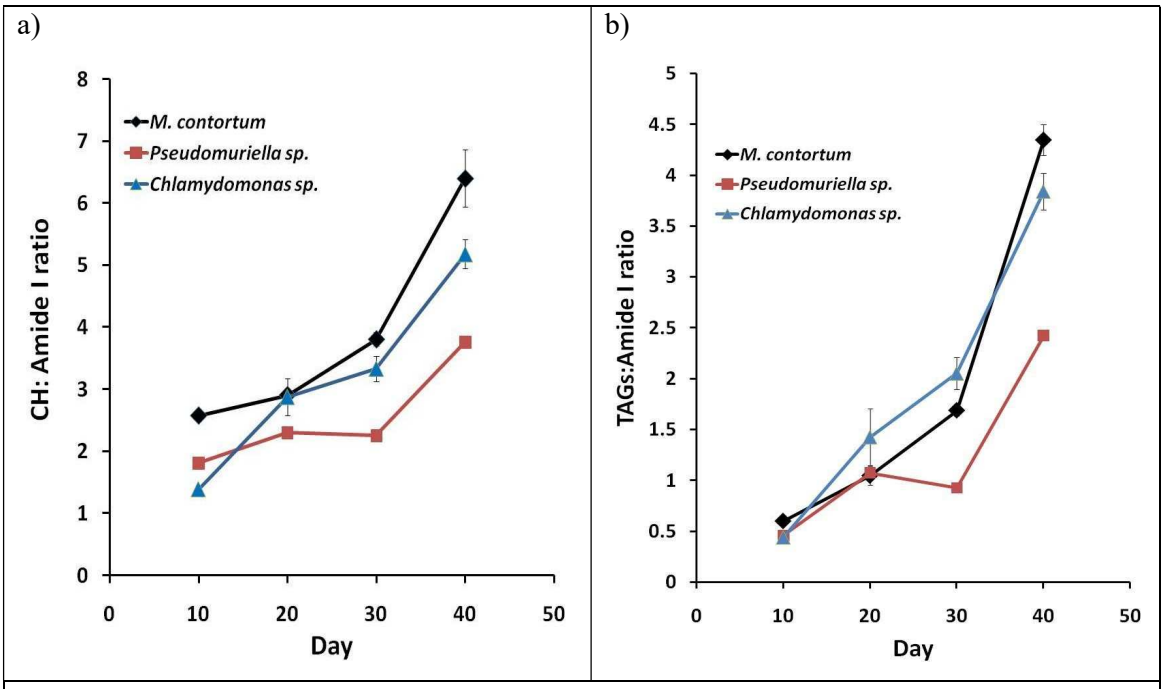


Fig.6. Relative content of a) hydrocarbons and b) triacylglycerides (TAGs) during the various phases of growth of the microalgal species. Each point is the mean (±SE) of 9 replicates.

Absolute content of lipids:

The integrated band area of amide I was used for the normalization of lipid bands to determine the relative lipid content. However, the intensity of the amide I band was also changing with the phases of growth of the microalgal species (Fig.2, Fig.3 and Fig 4). Therefore to exclude the impact of changing protein content, the integrated band area of the hydrocarbons (3050-2800 cm^{-1}) and TAGs (~1745 cm^{-1}) were normalized by a region with almost zero intensity (10^{-6}) in the second derivative spectra, which does not change with the phases of growth as well as with the species and have no characteristic bands . This results in the determination of the absolute content of hydrocarbons and TAGs. As the second derivative spectra itself are normalized, the integrated peak area represents the absolute content. Fig.7 shows the absolute content of lipids during the various growth phases of the microalgal species. The enhancement of lipid accumulation was observed for all the species with an increase in the days of growth. The absolute lipid content of *M.contortum* was distinctly higher than the *Pseudomuriella* sp. and *Chlamydomonas* sp. *M.contortum* exhibited nearly 6 fold increase in the hydrocarbons (0.7 to 3.9) and a thirteen fold increase in TAGs (0.2 to 2.6). The absolute content of lipid accumulation exactly matches with the second derivatives of the absorption spectra shown in Fig 4 and Fig.5. The increased amount of

TAGs signifies the predominance of saturated and monounsaturated fatty acids in the algal lipids of these species.

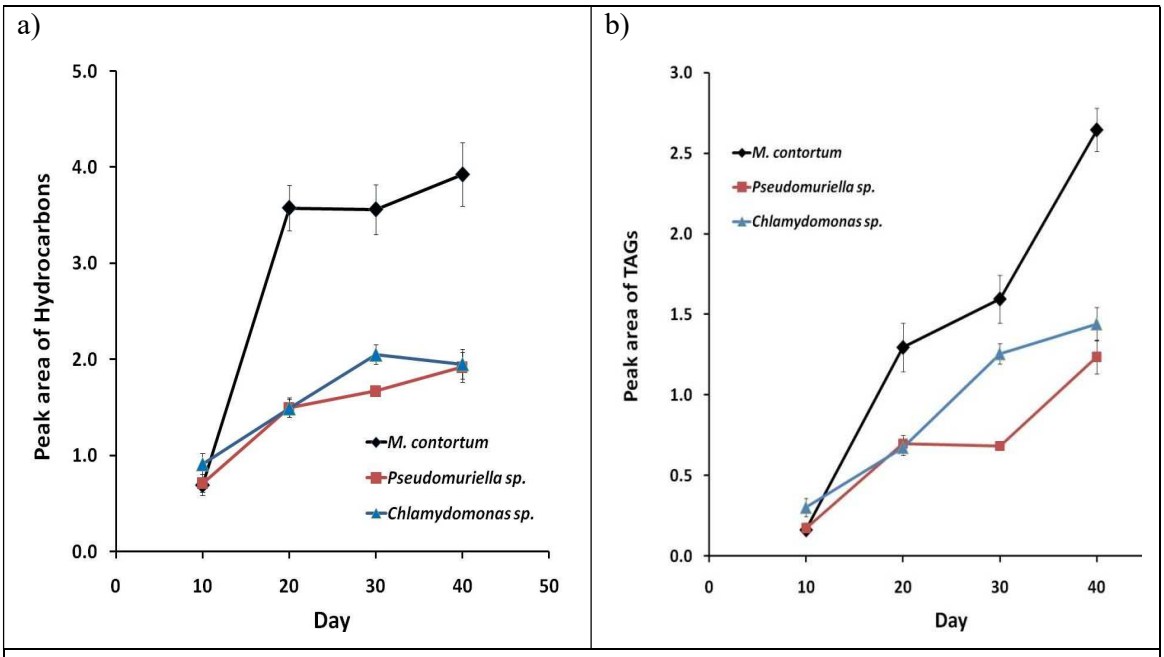


Fig.7. Absolute content of a) hydrocarbons and b) triacylglycerides (TAGs) during the various phases of growth of the microalgal species. Each point is the mean (±SE) of 9 replicates.

3.2. Raman spectral analysis on the lipid accumulation in green microalgal species

Raman spectra of the three green microalgal species *M. contortum*, *Pseudomuriella* sp., and *Chlamydomonas* sp., were analyzed during their stationary phase and reported with their second derivatives in Fig. 8. Raman spectral analysis provided additional information to the FTIR analysis on lipid accumulation. In FTIR analysis, lipid accumulation was monitored in the characteristic regions of hydrocarbons (3050 – 2800 cm^{-1}) and TAGs (~1745 cm^{-1}). TAGs are neutral lipids primarily made up of saturated (SFA) and monounsaturated fatty acids (MUFA) [10,11] and are the preferred compounds for biodiesel (Section 1). Raman spectra revealed significant bands that correspond to these saturated and unsaturated fatty acids in the algal lipids.

Strong bands were observed at ~ 2948cm^{-1} and ~ 2863cm^{-1} that corresponded to the asymmetric and symmetric stretches of hydrocarbons. A highly intense band was observed at ~1440 cm^{-1}, due to $\delta(CH_2)$ scissoring vibrations from the saturated fatty acids present in the algal lipids (Fig. 8a). In addition, the spectra showed bands corresponding to the other biomolecules such as carbohydrates and pigments like carotenoids and chlorophyll a,

represented in Table 1. The characteristic bands of proteins observed in the FTIR spectra at ~1658 cm^{-1} and ~1547 cm^{-1} were not prominent in the Raman spectra, because carbonyl stretches ν(C=O) are weak [39].

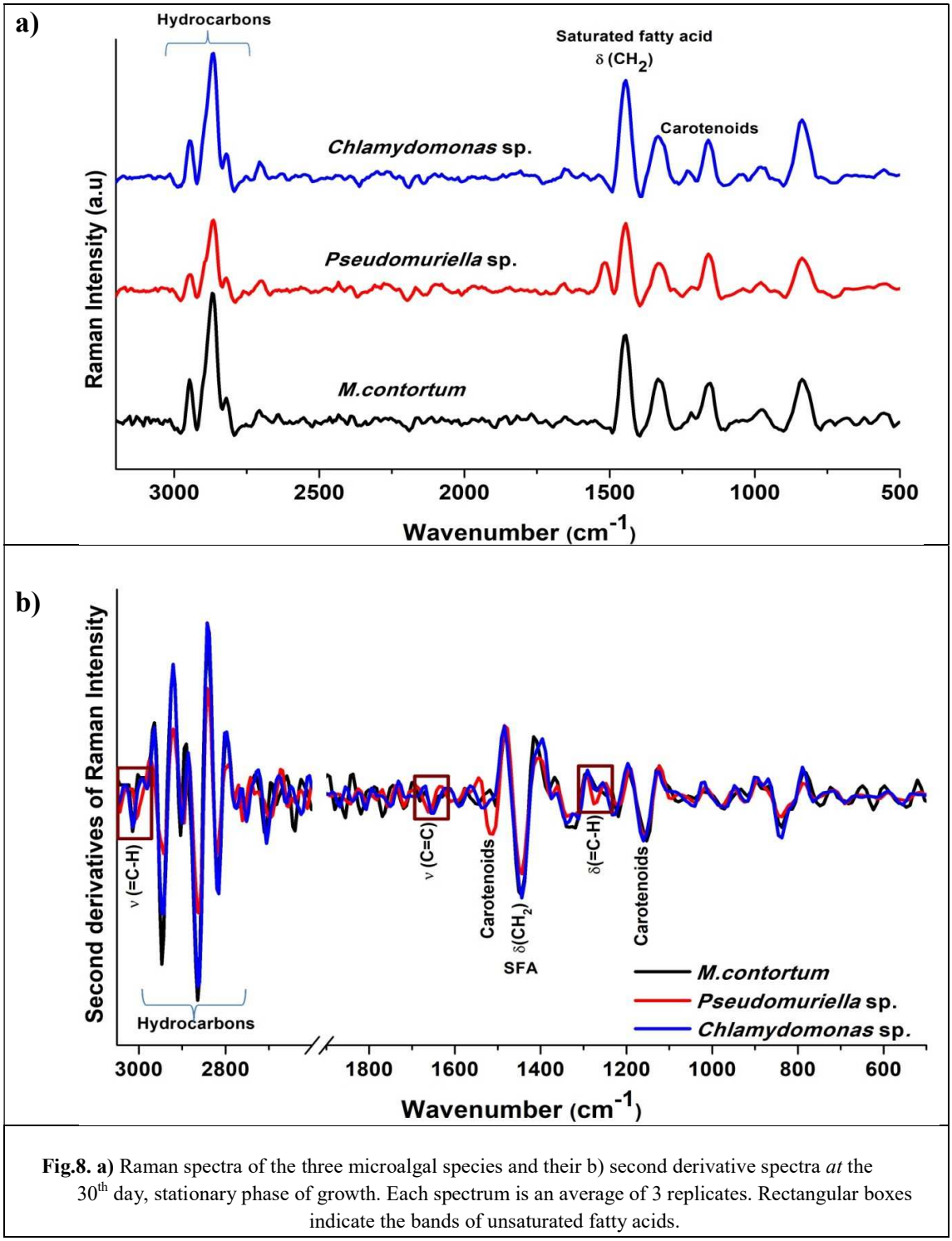


Fig.8. a) Raman spectra of the three microalgal species and their b) second derivative spectra *at* the 30th day, stationary phase of growth. Each spectrum is an average of 3 replicates. Rectangular boxes indicate the bands of unsaturated fatty acids.

The second derivative analysis of Raman spectra revealed the bands that correspond to the unsaturated fatty acids. The characteristic vibrations of lipid unsaturation are ν(=C-H) at ~3008 cm^{-1}, ν(C=C) at ~1655 cm^{-1} and δ(=C-H) at ~1270 cm^{-1} [21,34,50]. Second derivatives of the Raman spectra clearly showed the variation in the intensity of these vibrations for the three species (Fig. 8b). The stretching vibration of =C-H at ~3008 cm^{-1} was observed for all the three species, with maximum intensity for *M.contortum*, which was also confirmed in the FTIR spectra (Fig.2 and Fig.4). The C=C stretching vibration at ~1655 cm^{-1} was observed as small bands with similar intensities in all the three species. The band due to the =C-H deformation at ~1270 cm^{-1} was predominant in *Pseudomuriella* sp., than in the other two species. All these bands indicate the presence of unsaturated fatty acids (MUFA and PUFA) in the microalgal lipids. However, the smaller intensity of these bands in comparison to the saturated fatty acids at ~1440 cm^{-1} indicate that these three microalgae are rich with saturated fatty acids, and confirms the potential of these microalgae to be a good source for biodiesel. [34,36,38].

3.3. Fatty acid analysis by GC, FTIR and Raman spectroscopy

FTIR and Raman spectral analysis of the three microalgal species revealed the accumulation of lipids in the form of hydrocarbons, TAGs and fatty acids. To analyze the potential of these species to be a source for biodiesel, quantification of the fatty acids [saturated (SFA) and unsaturated (UFA)] is necessary. Gas chromatography (GC) is one of the conventional methods, widely used as a reliable technique for fatty acid analysis. However, the method requires extraction of lipids from algal cells. The alternative, effective methods to quantify lipids without the extraction are the FTIR and Raman spectroscopy.

Fig. 9a and Fig. 9b shows the second derivatives of the FTIR and Raman spectra of the three species during their stationary phase of growth. The rectangular boxes in the spectra indicate the regions of saturated and unsaturated fatty acids present in the algal lipids. The integrated band area for the characteristic vibrations of unsaturated lipids = C-H stretch (~3008 cm^{-1}), C=C stretch (~1655 cm^{-1}) and = C-H deformation (~1270 cm^{-1}) were calculated. The band area for the vibration ν(C=C) were not calculated in the FTIR spectra, because, they are generally weak in IR and also the same region attributes to the carbonyl stretch (C=O) of amide I, which is a strong band in FTIR. The characteristic vibration of TAGs (~1745 cm^{-1}) comprise of SFA and MUFA. The band area of TAGs and the total band area of UFA were determined. This enabled to quantify the SFA and UFA (Fig9a and

Fig.10b) of the three species from the FTIR spectra. The percentage of SFA from the FTIR spectra was 93 for *M.contortum,* 83 for *Chlamydomonas* sp. 74 for *Pseudomuriella* sp. Similarly, from the Raman spectra, the total UFA was determined by summation of the three lipid unsaturation regions. SFA was calculated from the integrated band area at ~1440 cm^{-1} that corresponds to CH_2 scissoring vibrations (Fig. 9b and Fig.10c). The percentage of SFA from the Raman spectra was 80 for *M.contortum,* 75 for *Chlamydomonas* sp. 71 for *Pseudomuriella* sp. The enhancement in the percentage of SFA indicates the suitability of these species for biodiesel application.

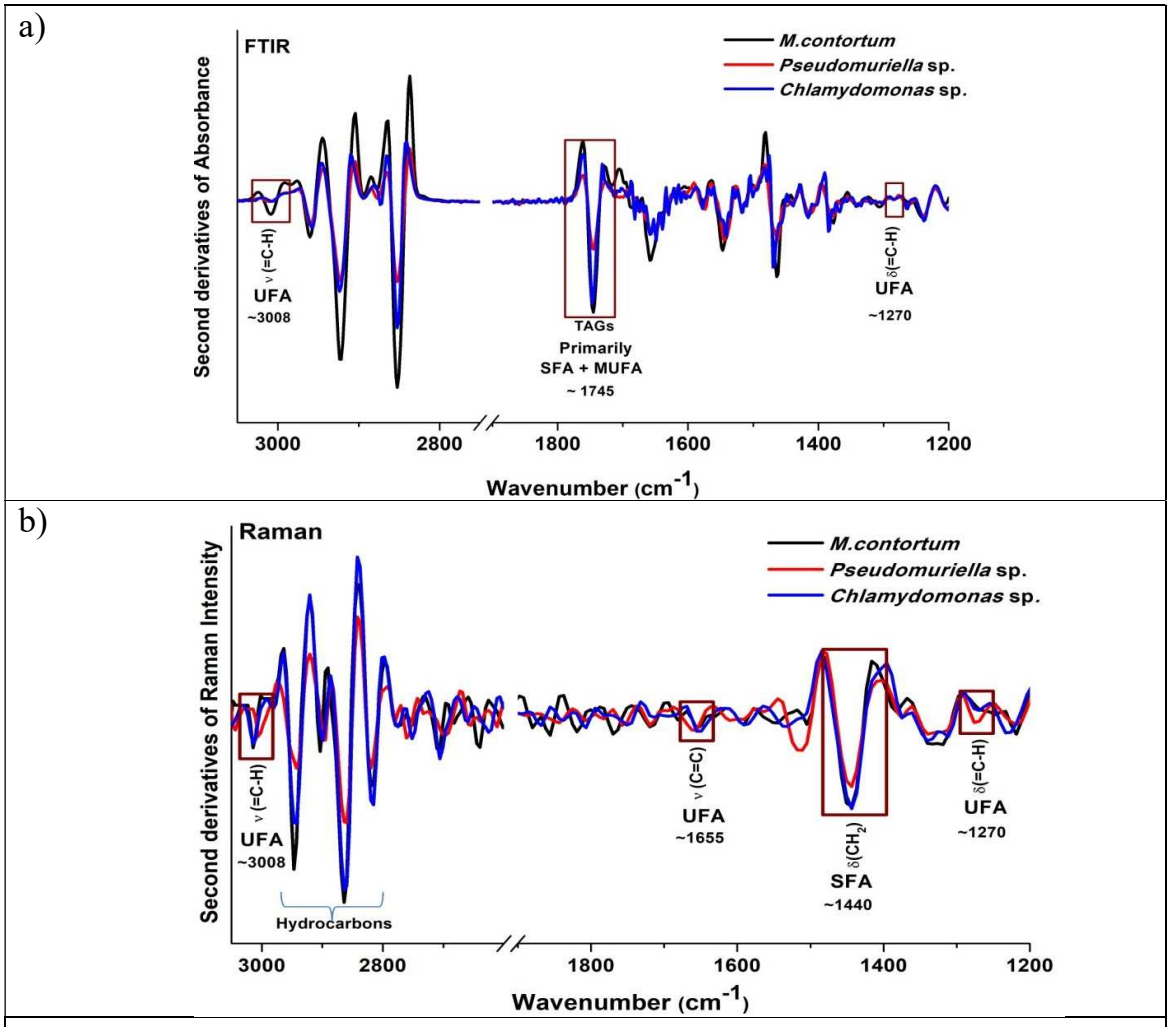


Fig.9 Saturated and unsaturated fatty acid regions in the a)FTIR and b)Raman spectra of the three microalgal species. The rectangular boxes indicate the regions.

Colour should be used for this figure in print

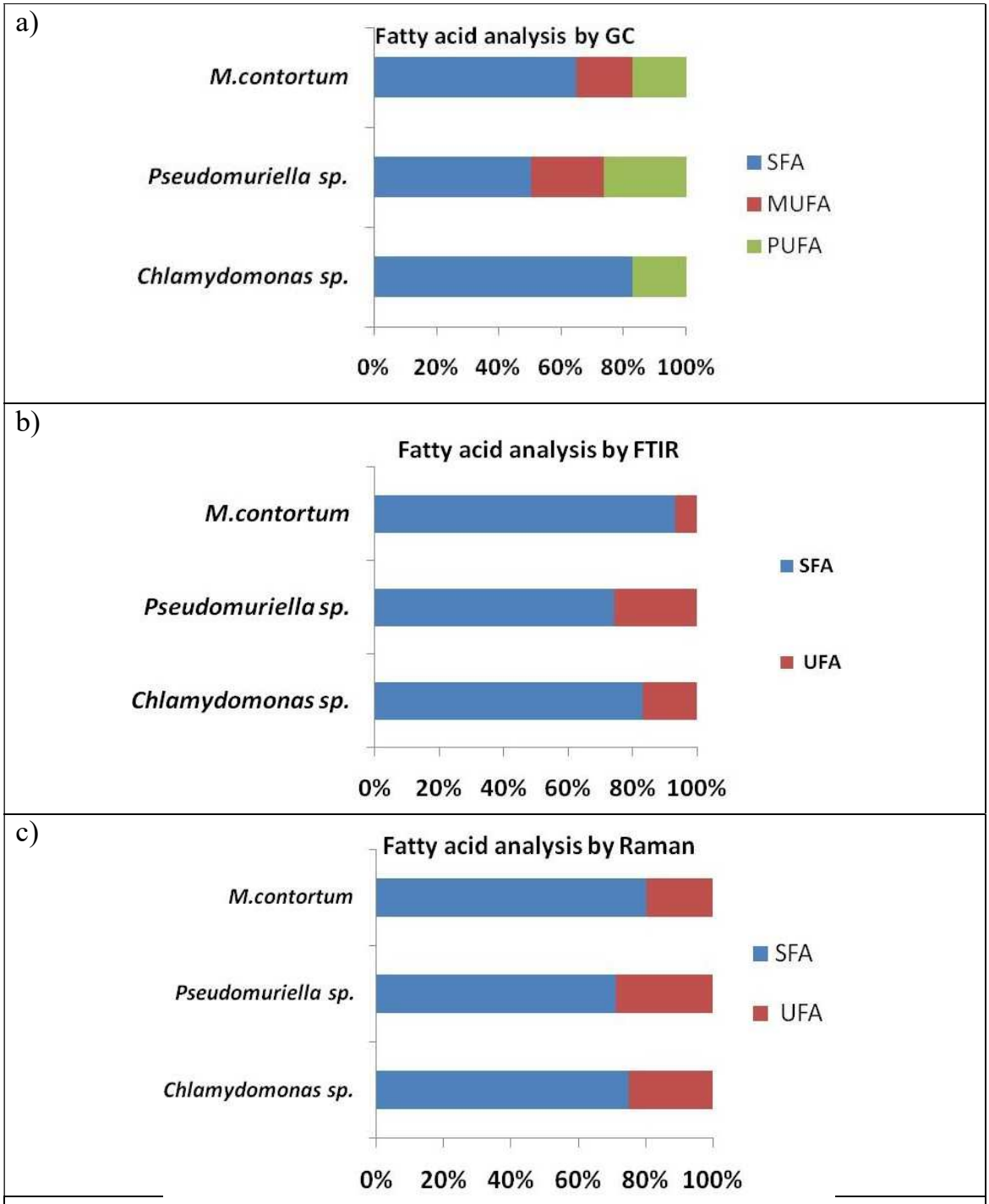


Fig.10 Fatty acid analysis by a) Gas chromatography b) FTIR and c) Raman Spectroscopy, recorded on the 30th day growth of the microalgal species.

The results of GC analysis for the three microalgal species on their 30th day of growth (Fig.10a) showed maximum SFA for *Chlamydomonas sp*. However, a total of SFA and MUFA (preferred components for biodiesel) in the microalgal species were high for *M.contortum.* The spectral results on the fatty acids showed an increasing concentration of SFA in the microalgal species in the order, *M. contortum* > *Chlamydomonas* sp. > *Pseudomuriella* sp. The results obtained from FTIR and Raman analysis were consistent with GC analysis. Thus quantification of fatty acids by the FTIR and Raman spectroscopic methods was fast, reliable and non- destructive.

4. Conclusion

Spectroscopic investigations were performed to analyze the biomolecular transitions and accumulation of lipids within the microalgal cells. Three green microalgal species, *M. contortum, Pseudomuriella* sp*.,* and *Chlamydomonas* sp. were examined during their various phases of growth. FTIR absorption spectra and its second derivatives reflected the biomolecular transitions that occurred within the cells due to physiology. Second derivative analyses of the FTIR and Raman spectra normalized and resolved the original spectra and lead to the identification of even smaller overlapping bands. Relative and absolute lipid contents of the species were calculated using the integrated peak area of the lipid regions. *M.contortum* exhibited higher accumulation of lipids in the form of hydrocarbons and TAGs than *Chlamydomonas* sp. and *Pseudomuriella* sp. Raman spectroscopy revealed significant bands that corresponds to the saturated (SFA) and unsaturated fatty acids (UFA) in the algal lipids. Fatty acid analysis was performed by GC, FTIR and Raman to identify the potential species with higher SFA and MUFA for biodiesel application. In the spectroscopic methods, the integrated band area for the characteristic vibrations of unsaturated lipids, = C-H stretch (~3008 cm^{-1}), C=C stretch (~1655 cm^{-1}) and = C-H deformation (~1270 cm^{-1}) and saturated lipids TAGs (1745 cm^{-1}), CH_2 deformation (1440 cm^{-1}) were determined from the second derivatives of the FTIR and Raman spectra. This enabled to quantify the percentage of SFA and UFA. The order of increasing concentration of SFA in the microalgal species was *M. contortum* > *Chlamydomonas* sp. > *Pseudomuriella* sp. The results obtained from FTIR and Raman spectral analysis were consistent with the separation of lipids by Gas chromatography. The enhancement in the percentage of SFA indicates the suitability of these indigenous species for biodiesel application. In conclusion, FTIR and Raman spectroscopy enabled better understanding of biomolecular transitions and a rapid and easy assessment of lipid accumulation, especially to quantify fatty acids without the need for the extraction of lipids.

Table 1: FTIR and Raman band assignments of *M. contortum*, *Pseudomuriella* sp. and *Chlamydomonas* sp.

M. contortum		***Pseudomuriella* sp.**		***Chlamydomonas* sp.**		**Band Assignments**
Infrared Wavenumber cm^{-1}	**Raman Wavenumber cm^{-1}**	**Infrared Wavenumber cm^{-1}**	**Raman Wavenumber cm^{-1}**	**Infrared Wavenumber cm^{-1}**	**Raman Wavenumber cm^{-1}**	
3364 (br, vs)		3379 (br, vs)		3376 (br, vs)		ν (OH) stretches from water molecules
3008(w)	3008(m)	3008(vw)	3008(m)	3008(vw)	3008(m)	ν(= CH) stretches from unsaturated lipids
	2948 (m)		2949 (m)		2945(m)	$\nu_{as}(CH_3)$ asymmetric stretches
2923 (vs)		2924 (vs)		2924 (vs)		$\nu_{as}(CH_2)$ asymmetric stretches — Hydrocarbons
	2866 (vs)		2863 (vs)		2863(vs)	$\nu_{s}(CH_3)$ symmetric stretches
2850 (sh, m)		2852 (sh, m)		2852 (sh, m)		$\nu_{s}(CH_2)$ symmetric stretches
1746 (sh, m)		1745 (sh, m)		1746 (sh, m)		ν(C=O) stretches of esters from lipids -Triacylglycerides (TAGs)
1657 (s)	1654 (w)	1650 (s)	1667 (w)	1652 (s)	1656 (w)	Amide I from proteins (IR), cis ν(C=C) from unsaturated lipids (Raman)
1546 (m)		1542 (m)		1543 (m)		Amide II from proteins
			1514 (m)			Carotene, ν(C=C)
1463 (sh, m)		1462 (sh, m)		1459(sh, m)		$\delta_{as}(CH_3)$ asymmetric bend from lipids
	1445 (vs)		1441 (vs)		1442(vs)	δ (CH_2) scissoring from saturated lipids
1374 (w)		1377 (w)		1374 (w)		$\delta_{s}(CH_3)$ symmetric bend from lipids and proteins
	1331 (s)		1331(s)		1331 (s)	δ (CH_2) deformations from carbohydrates and ν(C-N) stretches, δ (C-H)deformations from Chlorophyll a
1270(vw)	1270(w)	1270(w)	1270(m)	1270(w)	1270 (w)	δ(= CH) deformations from unsaturated lipids
1239 (w)		1242 (w)		1243 (w)		$\nu_{as}(P=O)$asymmetric stretches from nucleic acids
	1218 (w)		1218 (w)		1220 (w)	δ (C-H) ring bend from nucleic acids
1154 (sh, m)	1153(s)	1155(sh, m)	1159 (s)	1152(sh, m)	1158(s)	ν (C-O-C)stretches from carbohydrates (IR), Carotene (Raman)
1082 (sh, s)		1082 (sh, s)		1078 (sh,s)		δ (C-O-H) deformation, ν (C-O-C) stretches of polysaccharides from carbohydrates, $\nu_{s}(P=O)$ symmetric stretches from nucleic acids
1052 (sh, m)		1051 (sh, m)		1052 (sh, m)		
1022 (sh, m)		1020 (sh,,m)		1019 (sh, m)		

Abbreviations: vw, very weak, w, weak; m, medium; s, strong; vs, very strong; sh, shoulder; br, broad
References:[8,20–22,30]

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Competing Financial Interests

The authors declare no competing financial interest

References

[1] Y. Chisti, Biodiesel from microalgae, Biotechnol. Adv. (2007) 294–306. doi:10.1016/j.biotechadv.2007.02.001.

[2] L. Gouveia, A.C. Oliveira, R. Congestri, L. Bruno, A.T. Soares, R.S. Menezes, N.R.A. Filho, I. Tzovenis, Biodiesel from microalgae, Microalgae-Based Biofuels Bioprod. from Feed. Cultiv. to End-Products. 25 (2017) 235–258. doi:10.1016/B978-0-08-101023-5.00010-8.

[3] F. Alam, S. Mobin, H. Chowdhury, Third Generation Biofuel from Algae, Procedia Eng. 105 (2015) 763–768. doi:10.1016/j.proeng.2015.05.068.

[4] R.S. Gajraj, G.P. Singh, A. Kumar, Third-Generation Biofuel: Algal Biofuels as a Sustainable Energy Source, in: Y.Y. Kumar A., Ogita S. (Ed.), Biofuels Greenh. Gas Mitig. Glob. Warm., Springer India, New Delhi, 2018: pp. 307–325. doi:10.1007/978-81-322-3763-1_17.

[5] P.J.L.B. Williams, L.M.L. Laurens, Microalgae as biodiesel & biomass feedstocks: Review & analysis of the biochemistry, energetics & economics, Energy Environ. Sci. 3 (2010) 554–590. doi:10.1039/b924978h.

[6] L.M.L. Laurens, E.J. Wolfrum, Feasibility of Spectroscopic Characterization of Algal Lipids : Chemometric Correlation of NIR and FTIR Spectra with Exogenous Lipids in Algal Biomass, (2011) 22–35. doi:10.1007/s12155-010-9098-y.

[7] A.P. Dean, J.M. Nicholson, D.C. Sigee, Impact of phosphorus quota and growth phase on carbon allocation in Chlamydomonas reinhardtii: An FTIR microspectroscopy study, Eur. J. Phycol. 43 (2008) 345–354. doi:10.1080/09670260801979287.

[8] A.P. Dean, D.C. Sigee, B. Estrada, J.K. Pittman, Using FTIR spectroscopy for rapid determination of lipid accumulation in response to nitrogen limitation in freshwater microalgae, Bioresour. Technol. 101 (2010) 4499–4507. doi:10.1016/j.biortech.2010.01.065.

[9] K.K. Sharma, H. Schuhmann, P.M. Schenk, High lipid induction in microalgae for biodiesel production, Energies. 5 (2012) 1532–1553. doi:10.3390/en5051532.

[10] L. Rodolfi, G.C. Zittelli, N. Bassi, G. Padovani, N. Biondi, G. Bonini, M.R. Tredici, Microalgae for oil: Strain selection, induction of lipid synthesis and outdoor mass cultivation in a low-cost photobioreactor,

Biotechnol. Bioeng. 102 (2009) 100–112. doi:10.1002/bit.22033.

[11] P.-L. Shen, H.-T. Wang, Y.-F. Pan, Y.-Y. Meng, P.-C. Wu, S. Xue, Identification of Characteristic Fatty Acids to Quantify Triacylglycerols in Microalgae, Front. Plant Sci. 7 (2016). doi:10.3389/fpls.2016.00162.

[12] M.D. Redel-Macías, S. Pinzi, M.F. Ruz, A.J. Cubero-Atienza, M.P. Dorado, Biodiesel from saturated and monounsaturated fatty acid methyl esters and their influence over noise and air pollution, Fuel. 97 (2012) 751–756. doi:10.1016/j.fuel.2012.01.070.

[13] Y. Cao, W. Liu, X. Xu, H. Zhang, J. Wang, M. Xian, Production of free monounsaturated fatty acids by metabolically engineered *Escherichia coli,* Biotechnol. Biofuels. 7 (2014) 1–11. doi:10.1186/1754-6834-7-59.

[14] T. Lewis, P.D. Nichols, T.A. McMeekin, Evaluation of extraction methods for recovery of fatty acids from lipid-producing microheterotrophs, J. Microbiol. Methods. 43 (2000) 107–116. doi:10.1016/S0167-7012(00)00217-7.

[15] E.G. Bligh, W.J. Dyer, A rapid method of total lipid extraction and purification, Can. J. Biochem. Physiol. 37 (1959) 911–917. doi:10.1139/o59-099.

[16] M. Axelsson, F. Gentili, A single-step method for rapid extraction of total lipids from green microalgae, PLoS One. 9 (2014) 17–20. doi:10.1371/journal.pone.0089643.

[17] B. Wood, P. Heraud, M. Kansiz, J. Beardall, M. Giordano, D. McNaughton, Fourier transform infrared spectroscopy as a novel tool to investigate changes in intracellular macromolecular pools in the marine microalga *Chaetoceros muellerii* (Bacillariophyceae), J. Phycol. 37 (2001) 271–279. doi:10.1046/j.1529-8817.2001.037002271.x.

[18] H. Wagner, Z. Liu, U. Langner, K. Stehfest, C. Wilhelm, The use of FTIR spectroscopy to assess quantitative changes in the biochemical composition of microalgae, 566 (2010) 557–566. doi:10.1002/jbio.201000019.

[19] H. Wu, J. V. Volponi, A.N. Parikh, B.A. Simmons, A.E. Oliver, S. Singh, In vivo lipidomics using single-cell Raman spectroscopy, Proc. Natl. Acad. Sci. 108 (2011) 3809–3814. doi:10.1073/pnas.1009043108.

[20] J.N. Murdock, D.L. Wetzel, FT-IR Microspectroscopy Enhances Biological and Ecological Analysis of Algae, Appl. Spectrosc. Rev. 44 (2009) 335–361. doi:10.1080/05704920902907440.

[21] P. Larkin, Infrared and Raman spectroscopy: Principles and Spectral Interpretation, 1st Editio, Elsevier, 2011. http://www.chemistry.uoc.gr/lapkin/Infrared_and_Raman_Spectroscopy__Principles_and_Spectral_Interpretation.pdf.

[22] B.H. Stuart, Infrared Spectroscopy: Fundamentals and Applications, John Wiley & Sons, Ltd, Chichester, UK, 2004. doi:10.1002/0470011149.

[23] A.M.A. Pistorius, W.J. Degrip, T.A. Egorova-zachernyuk, Monitoring of Biomass Composition From Microbiological Sources by Means of FT-IR Spectroscopy, 103 (2009) 123–129. doi:10.1002/bit.22220.

[24] D.C. Sigee, A. Dean, E. Levado, M.J. Tobin, Fourier-transform infrared spectroscopy of Pediastrum duplex: Characterization of a micro-population isolated from a eutrophic lake, Eur. J. Phycol. 37 (2002) 19–26. doi:10.1017/S0967026201003444.

[25] R. Miglio, S. Palmery, M. Salvalaggio, L. Carnelli, F. Capuano, R. Borrelli, Microalgae triacylglycerols content by FT-IR spectroscopy, (2013) 1621–1631. doi:10.1007/s10811-013-0007-6.

[26] D. Ami, R. Posteri, P. Mereghetti, D. Porro, S.M. Doglia, P. Branduardi, Fourier transform infrared spectroscopy as a method to study lipid accumulation in oleaginous yeasts, Biotechnol. Biofuels. 7 (2014) 1–14. doi:10.1186/1754-6834-7-12.

[27] K. Forfang, B. Zimmermann, G. Kosa, A. Kohler, V. Shapaval, FTIR Spectroscopy for Evaluation and Monitoring of Lipid Extraction Efficiency for Oleaginous Fungi, (2017) 1–17. doi:10.1371/journal.pone.0170611.

[28] L. Rieppo, S. Saarakkala, T. Närhi, H.J. Helminen, J.S. Jurvelin, J. Rieppo, Application of second derivative spectroscopy for increasing molecular specificity of fourier transform infrared spectroscopic imaging of articular cartilage, Osteoarthr. Cartil. 20 (2012) 451–459. doi:10.1016/j.joca.2012.01.010.

[29] P. Heraud, J. Beardall, D. McNaughton, B.R. Wood, In vivo prediction of the nutrient status of individual microalgal cells using Raman microspectroscopy, FEMS Microbiol. Lett. 275 (2007) 24–30. doi:10.1111/j.1574-6968.2007.00861.x.

[30] K. Stehfest, J. Toepel, C. Wilhelm, The application of micro-FTIR spectroscopy to analyze nutrient stress-related changes in biomass composition of phytoplankton algae, 43 (2005) 717–726. doi:10.1016/j.plaphy.2005.07.001.

[31] Y. Meng, C. Yao, S. Xue, H. Yang, Bioresource Technology Application of Fourier transform infrared (FT-IR) spectroscopy in determination of microalgal compositions, Bioresour. Technol. 151 (2014) 347–354. doi:10.1016/j.biortech.2013.10.064.

[32] J. Mayers, K.J. Flynn, R.J. Shields, Rapid determination of bulk microalgal biochemical composition by Fourier-Transform Infrared spectroscopy, (2013). doi:10.1016/j.biortech.2013.08.133.

[33] Y.Y. Huang, C.M. Beal, W.W. Cai, R.S. Ruoff, E.M. Terentjev, Micro-Raman spectroscopy of algae: Composition analysis and fluorescence background behavior, Biotechnol. Bioeng. 105 (2010) 889–898. doi:10.1002/bit.22617.

[34] J. De Gelder, K. De Gussem, P. Vandenabeele, L. Moens, Reference database of Raman spectra of biological molecules, J. Raman Spectrosc. 38 (2007) 1133–1147. doi:10.1002/jrs.1734.

[35] O. Samek, A. Jonáš, Z. Pilát, P. Zemánek, L. Nedbal, J. Tríska, P. Kotas, M. Trtílek, Raman microspectroscopy of individual algal cells: Sensing unsaturation of storage lipids in vivo, Sensors. 10 (2010) 8635–8651. doi:10.3390/s100908635.

[36] O. Samek, P. Zemánek, A. Jonáš, H.H. Telle, Characterization of oil-producing microalgae using Raman spectroscopy, Laser Phys. Lett. 8 (2011) 701–709. doi:10.1002/lapl.201110060.

[37] D. Jaeger, C. Pilger, H. Hachmeister, E. Oberländer, R. Wördenweber, J. Wichmann, J.H. Mussgnug, T. Huser, O. Kruse, Label-free in vivo analysis of intracellular lipid droplets in the oleaginous microalga *Monoraphidium neglectum* by coherent Raman scattering microscopy, Sci. Rep. 6 (2016) 2–10. doi:10.1038/srep35340.

[38] S.K. Sharma, D.R. Nelson, R. Abdrabu, B. Khraiwesh, K. Jijakli, M. Arnoux, M.J. O'Connor, T. Bahmani, H. Cai, S. Khapli, R. Jagannathan, K. Salehi-Ashtiani, An integrative Raman microscopy-based workflow for rapid in situ analysis of microalgal lipid bodies, Biotechnol. Biofuels. 8 (2015) 1–14. doi:10.1186/s13068-015-0349-1.

[39] A. Holzenburg, S. Vitha, J. Laane, T.P. Devarenne, T.L. Weiss, H.J. Chun, S. Okada, Raman Spectroscopy Analysis of Botryococcene Hydrocarbons from the Green Microalga *Botryococcus braunii* , J. Biol. Chem. 285 (2010) 32458–32466. doi:10.1074/jbc.m110.157230.

[40] V. Baeten, Raman spectroscopy in lipid analysis, Lipid Technol. 22 (2010) 36–38. doi:10.1002/lite.200900082.

[41] T. Wang, Y. Ji, Y. Wang, J. Jia, J. Li, S. Huang, D. Han, Q. Hu, W.E. Huang, J. Xu, Quantitative dynamics of triacylglycerol accumulation in microalgae populations at single-cell resolution revealed by Raman microspectroscopy, Biotechnol. Biofuels. 7 (2014) 1–12. doi:10.1186/1754-6834-7-58.

[42] S. He, S. Fang, W. Xie, P. Zhang, Z. Li, D. Zhou, Z. Zhang, J. Guo, C. Du, J. Du, D. Wang, Assessment of physiological responses and growth phases of different microalgae under environmental changes by Raman spectroscopy with chemometrics, Spectrochim. Acta Part A Mol. Biomol. Spectrosc. 204 (2018) 287–294. doi:10.1016/j.saa.2018.06.060.

[43] T. Driver, A.K. Bajhaiya, J.W. Allwood, R. Goodacre, J.K. Pittman, A.P. Dean, Metabolic responses of eukaryotic microalgae to environmental stress limit the ability of FT-IR spectroscopy for species identi fi cation, 11 (2015) 148–155. doi:10.1016/j.algal.2015.06.009.

[44] F. Vogt, L. White, Spectroscopic analyses of chemical adaptation processes within microalgal biomass in response to changing environments, Anal. Chim. Acta. 867 (2015) 18–28. doi:10.1016/j.aca.2015.02.005.

[45] J. Rüger, N. Unger, I.W. Schie, E. Brunner, J. Popp, C. Krafft, Assessment of growth phases of the diatom *Ditylum brightwellii* by FT-IR and Raman spectroscopy, Algal Res. 19 (2016) 246–252. doi:10.1016/j.algal.2016.09.007.

[46] C. Bogen, V. Klassen, J. Wichmann, M. La Russa, A. Doebbe, M. Grundmann, P. Uronen, O. Kruse, J.H. Mussgnug, Identification of *Monoraphidium contortum* as a promising species for liquid biofuel production, Bioresour. Technol. 133 (2013) 622–626. doi:10.1016/j.biortech.2013.01.164.

[47] G.P. Holbrook, Z. Davidson, R.A. Tatara, N.L. Ziemer, K.A. Rosentrater, W. Scott Grayburn, Use of the microalga *Monoraphidium sp*. grown in wastewater as a feedstock for biodiesel: Cultivation and fuel characteristics, Appl. Energy. 131 (2014) 386–393. doi:10.1016/j.apenergy.2014.06.043.

[48] A.M. Illman, A.H. Scragg, ScienceDirect - Enzyme and Microbial Technology : Increase in Chlorella strains calorific values when grown in low nitrogen medium, Enzyme Microb. Technol. 27 (2000) 631–635.
http://www.sciencedirect.com/science/article/pii/S0141022900002660%0Apapers2://publication/uuid/B7537DAC-F40B-4395-9341-739FEEA48F97.

[49] Michael R. Whitbeck, Second Derivative Infrared Spectroscopy, Appl. Spectrosc. 35 (1981).

[50] K. Czamara, K. Majzner, M.Z. Pacia, K. Kochan, A. Kaczor, M. Baranska, Raman spectroscopy of lipids: A review, J. Raman Spectrosc. 46 (2015) 4–20. doi:10.1002/jrs.4607.