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Fourier Transform Infrared Spectroscopy (FT-IR) Analysis and Morphological Studies on *Nannochloropsis Oculata* and *Tetraselmis Chuui*

K. VIJENDREN, Y. UEMURA, X. B. TAN, N. T. THANH, M. NURLIDIA*

Centre for Biofuel and Biochemical Research, Universiti Teknologi PETRONAS, 32610 Bandar Seri Iskandar, Perak, Malaysia.

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Abstract: Lipid extraction was conducted on two marine microalgae namely, *Nannochloropsis oculata* and *Tetraselmis chuui*. The Fourier Transform Infrared spectroscopy (FT-IR) was utilized to identify the cell composition of both marine microalgae before and after the lipid extraction. The microalgae were also examined morphologically by utilizing optical microscope and Scanning Electron Microscope (SEM). Microscopic studies show that *N. oculata* as a spherical and non-motile cell, whereby, *T. chuui* is an oval shaped and motile cell. Also, *T. chuui* appears three times larger than *N. oculata* cells. The FT-IR analyses on cell composition dynamics demonstrate the presence of carbohydrate, lipid and protein. Significant reduction in the peak area at wavenumber 1740 cm^{-1} was observed after lipid extraction on microalgae which strongly suggests that lipid has been completely extracted from the microalgae. FT-IR serves as an easy, fast and reliable technique to monitor changes in biochemical composition of marine microalgae.

Keywords: *Nannochloropsis oculata, Tetraselmis chuui*, marine microalgae, FTIR, infrared spectroscopy, SEM, lipid extraction.

1. INTRODUCTION

Biodiesel is produced worldwide from a wide variety of raw materials which include soybean, rapeseed, sunflower, palm, corn, and microalgae (Ramos. *et. al.,* 2009). It has been widely accepted that microalgae are among the highest lipid producing organism. The high photosynthetic ability of microalgae enables them to capture carbon dioxide and produces large amount of lipid in the cells. However, the lipid content varies among microalgae species (Singh. *et.al.,* 2014). In addition, the fatty acid composition of different microalgae species also varies significantly.

Generally, microalgae are composed of carbohydrate, protein and lipid in their cells (Meng. *et. al.,* 2014). Various methods are available to identify and quantify the substances which results in different extraction efficiency (Teo. *et. al.,* 2014). In the case of lipid extraction, gravimetric quantification, nile red, Folch and Bligh and Dyer methods are commonly used (Feng. *et. al.,* 2013), (Iverson. *et. al.,* 2001).These methods rely mainly on large amount of biomass sample extraction in order to achieve accurate quantification. However, it is unsure if the lipid has been completely extracted from the cell. In addition these conventional methods are relatively time consuming and tedious besides involving hazardous organic solvents which are harmful to the environment.

On the other hand, Fourier Transform Infrared Spectroscopy (FT-IR) has been utilized for determination of lipid, protein and carbohydrates as these substances have their own absorbance at specific frequency in the mid infrared regions between 4000 and 400 cm^{-1} (Feng. *et.al.,* 2013), This is a method using intact cells which involves the measurement of infrared absorption in relation to a range of molecular vibrational modes (Dean. *et. al.,* 2010). In addition, FT-IR can also be utilized to determine the relative concentration of macromolecules such as carbohydrates, lipids, proteins and nucleic acids in the biological cells (Giordano. *et. al.,* 2001). FT-IR spectroscopy is suitable for rapid, high throughput screening of microalgae composition.

In the present study, FT-IR was used to analyze the variation in composition of lipid before and after the lipid extraction from *N. oculata* and *T. chuui* on various harvesting days to identify the efficiency of lipid extraction. In addition, the morphological study of both marine microalgae was also conducted by using conventional optical microscope and scanning electron microscope (SEM). Biodiesel is produced worldwide from a wide variety of raw materials which include soybean, rapeseed, sunflower, palm, corn, and microalgae. It has been widely accepted that microalgae are among the highest lipid producing organism.

*Department of Chemical Engineering, University Teknologi PETRONAS, 32610 Bandar Seri Iskandar, Perak, Malaysia

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2. MATERIALS AND METHODS

2.1 Microalgae Cultivation and Extraction

Nannochloropsis oculata and *T. chuui* were purchased from Algaetech International Sdn. Bhd. Small scale culture was conducted in 500 ml culture flask containing F2 medium in artificial seawater (Guillard. *et. al.,* 1962). (Kester. *et. al.,* 1967). for both the microalgae. During the exponential phase, the microalgae were then transferred into 5.0 L glass bottle containing F2 medium at 23 ± 2 ^{0}C. All the cultures were continuously illuminated with fluorescent lamps approximately at 5000 lux and aerated with air at 3.0 L/min. At day 9, 12 and 14, 300 ml of culture were dispensed for biomass harvesting. Day 9, 12 and 14 are log, stationary and decline phase respectively for *N. oculata* and *T. chuui* as described in (Vijendren. *et. al.,* 2015). The biomass were washed by centrifugation at 3600 rpm for 20 min and dried in oven at 105 ^{0}C for 24 hours before the dry mass is measured.

2.2 FT-IR Spectroscopy

Approximately 1.0 mg of dried microalgal biomass was placed on FT-IR attenuated total reflection (ATR) ZnSe crystal. Spectra were collected over the wavenumber from 4000 to 600 cm^{-1} on Nicolet iS10 FT-IR spectrometer. The absorbance spectra were collected at a spectral resolution of 4 cm^{-1} with 20 scans for each sample. Background correction scans of ambient air were made prior to each sample scan. The peaks were identified by using the software Omnic (version 9, Thermo Fisher Scientific).

2.3 Light Microscopy and Scanning Electron Microscopy

The microalgae cells were placed on a clean glass slide and covered with a cover slip. Then, the specimens were observed under optical microscope (Carl Zeiss Axio Scope A1). The cells were further studied under scanning electron microscope.

Fresh microalgal biomass was fixed for 24 hours in glutaraldehyde prepared in artificial sea water (ASW) and then washed with ASW. The sample was then dehydrated by using increasing concentration of ethanol. The dehydrated tissues were immersed in hexa methy disilazane (HMDS) for 20 minutes. The HMDS were decant and the specimen were left to dry in the desiccator for 24 hours. The specimens were then mounted on SEM specimen stub coated with gold particles, there after observed under Scanning electron microscope (Hitachi TM3030).

Table 1: Main absorption bands of *Nannochloropsis oculata* and *Tetraselmis chuui* from the FT-IR

Band	Peak Wave number (cm^{-1})	Band assignment	Functional group
1	3285	$_{v}$O-H, $_{v}$N-H	Water, protein
2	2920	$_{vas}$CH$_2$, $_{vs}$CH$_2$	Methylene group
3	2850	$_{v}$CH$_2$, $_{v}$CH$_3$,	Methyl and methylene group of lipid and carbohydrate
4	1740	$_{v}$C=O	Ester of lipid and fatty acids
5	1630	$_{v}$C=O	Amide I carbonyl of protein
6	1535	$_{\delta}$N–H	Amide II of protein
7	1445	$_{\delta as}$CH$_2$,$_{\delta}$CH$_3$	Methyl and methylene group of protein
8	1375*	$_{\delta s}$CH$_2$,$_{\delta s}$CH$_3$, $_{vs}$C=O	Methyl and methylene group of protein and carboxylic group
9	1240	$_{vas}$P=O	Phosphodiester bond of nucleic acid and phospholipid
10	1150	$_{v}$C-O-C	Polysaccharides of carbohydrates
11	1070	$_{v}$C-O-C	Carbonyl bond of polysaccharide and nucleic acid
12	1025	$_{v}$C-O-C	Polysaccharides of carbohydrates

V = symmetric stretching, Vas = asymmetrical stretching, d = symmetric deformation (bend), das = asymmetric deformation (bend). Band assignments are based on references; (Mayers. *et.al.,* 2013). (Ponnuswamy. *et.al.,* 2013). * This band does not appear for Tetraselmis chuui.

3. RESULTS AND DISCUSSION

3.1 Microalgae FT-IR spectra

Fourier transform infrared spectroscopy has been used to simultaneously estimate protein, lipid and carbohydrate content in biomass originating from various microbiological sources (Pistorius. *et.al.,* 2009). (Duygu. *et.al.,* 2012). However, many factors such as the complexity of the spectra, overlapping of the absorption peaks as well as the sample preparation process must be taken into consideration. FT-IR has provided an easy method for determination of lipid content.

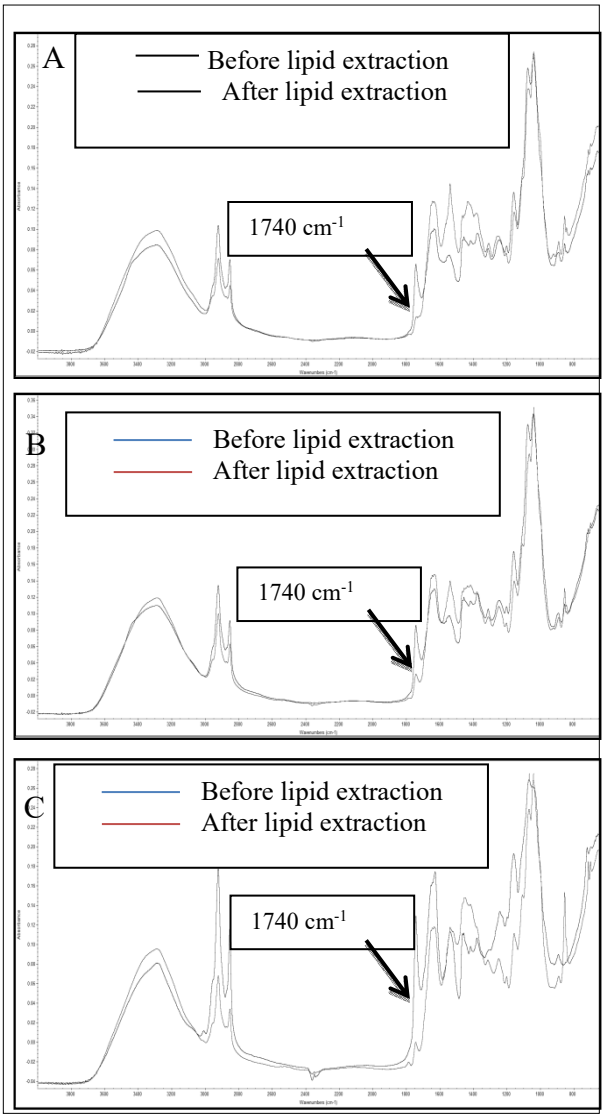


Fig. 1: FTIR spectra of *Nannochloropsis oculata* measured on (A) Day 9, (B) Day 12 and (C) Day 14

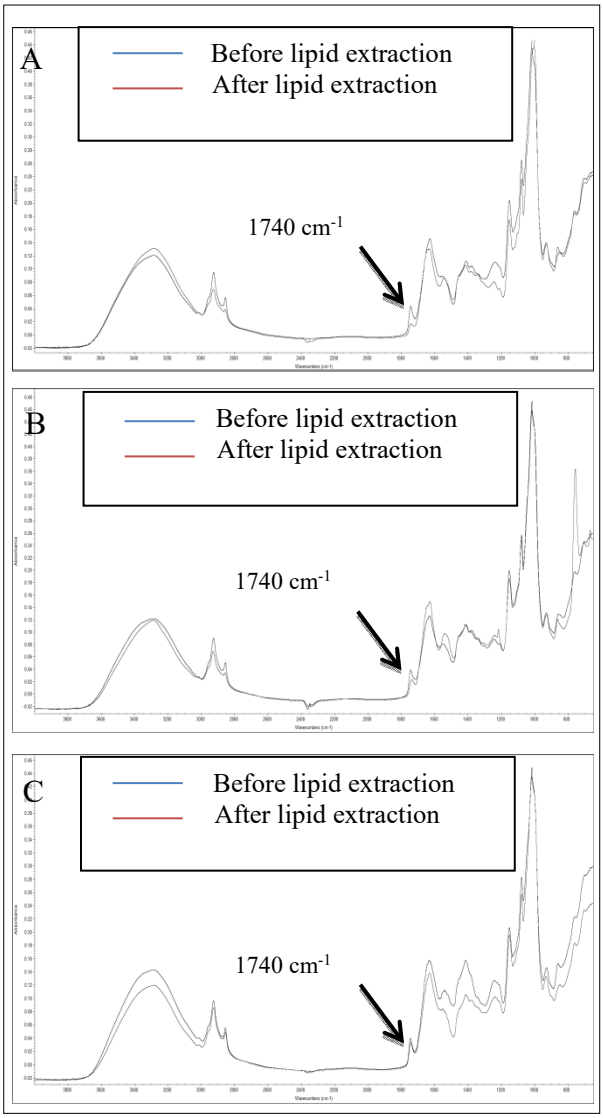


Fig. 2: FTIR spectra of *Tetraselmis chuui* measured on (A) Day 9, (B) Day 12 and (C) Day 14

Due to that reason, FT-IR is very useful to determine microalgae even with low lipid content. Microalgae with high lipid content are the main criteria to obtain the biofuel candidates. This study has also investigated the ability of the lipid extraction method on *N. oculata* and *T. chuui*.

In addition,. (Mayers. *et. al.,* 2013). have shown that *Nannochloropsis* sp. is composed of lipid, carbohydrate and protein at different fraction during the cultivation. FT-IR spectra of *N. oculata* and *T chuui* exhibited 12 major distinctive absorption bands (Table 1) over the wavenumbers 4000 – 600 cm^{-1}. These bands were assigned to specific molecular groups on the basis of biochemical standards. Spectra band at ~1630 cm^{-1} attributes to amide I carbonyl (vC=O) stretching of amides from protein, while ~1535 cm^{-1} attributes to amide II (δN–H) bending of amides from proteins. δas(CH_2) and δas(CH_3) bending of methyl from proteins are shown by the peaks at ~1445 cm-1. δs(CH_2) and δs(CH_3) bending of methyl and vs(C-O) stretching from COO- carbonyl groups were assigned to ~1375 cm^{-1} peak. These peaks in the FT-IR spectra show the presence of protein molecules in microalgae cells. On the other hand, bands at ~1150, ~1070 and ~1025 cm^{-1} were shown to characterize the carbonyl (C-O) stretching from carbohydrates. Carbohydrates represent a complex group in algal biomass consisting of a long, branched polysaccharides and large reserves of storage carbohydrates such as starch and glycogen . This study have observed similar band peak to that observed by which used bovine serum

albumin, starch and palmitic acid as a standard representative for protein, carbohydrate and lipid respectively.

Three bands are of particular interest for lipid determination which include 2920, 2850 and 1740 cm^{-1}. Among these band 1740 cm^{-1} is the most important as it is associated with (vC=O) of ester groups primarily from lipids and fatty acids. While the band 2920 and 2850 cm^{-1} are methyl and methylene in the lipid. Same observation was also reported by Feng *et al* 2013 However, the neutral amino acid of microalgae protein molecules also had methyl group with stretching vibration at 2800 – 3000 cm^{-1}. Giordano et al. has reported that band 1740 cm^{-1} strongly correlates to the concentration of lipid in the sample. On figure 1, FT-IR spectra obtained for *N. oculata* shows gradual increase in the band 1740 cm^{-1} from day 9 to day 14 of cultivation. This corresponds to increasing lipid content in the *N. oculata* cells. Interestingly, the spectrum after the lipid extraction shows negligible peak intensity at the same peak. This indicates that majority of lipid present in the microalgal cells have been extracted.

On the other hand, FT-IR spectra obtained for *T. chuui* **(Fig. 2)** shows low peak intensity at wavenumber 1740 cm^{-1}. This data is coherent with determination of lipid content done by conventional lipid extraction (Bligh. *et. al.,* 1959). (Data not shown), whereby *T. chuui* contains lower lipid content than *N. oculata*. In short, the overall spectral shape of FT-IR collected for *N. oculata* and *T. chuui* were similar, however the peak intensities are different at particular wavenumbers indicating different concentration of macromolecules in both microalgae. In addition, it was also shown that approximately the entire lipid present in the cells was extracted.

3.2 Optical Microscopy and Scanning Electron Microscopy

Photographs in Figure 3 shows that microalgae *N. oculata* does not live in the form of colonies. Each individual cells size is in the range from 3.1 µm to 3.9 µm. The *N. oculata* cells are green in colour, unicellular and spherical in shape. The green colour of the cells is due to the presence of chlorophyll in the cells (Boussiba. *et.al.,* 1987). Similarly, *T. chuui* in **(Fig. 4)** does not live in the form of colonies. However the size of each individual cell is much bigger than *N. oculata* which ranges from 11.8 µm to 12.5 µm. On the other hand, *T. chuui* cells contain semi-transparent cytoplasm with round shaped and green colour organelle in the centre. It is also unicellular and oval in shape. In addition, oral groove and anal groove were also observed on *T. chuui* cells. Interestingly, *T. chuui* is a motile cell which progress forward by swirling.

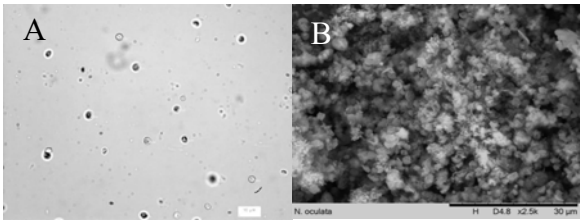


Fig.3: Microscopic image of *Nannochloropsis oculata* observed with (A) Optical microscope and (B) Scanning electron microscope

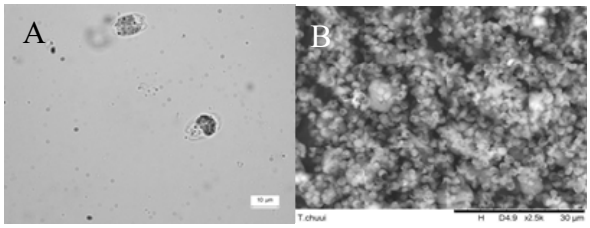
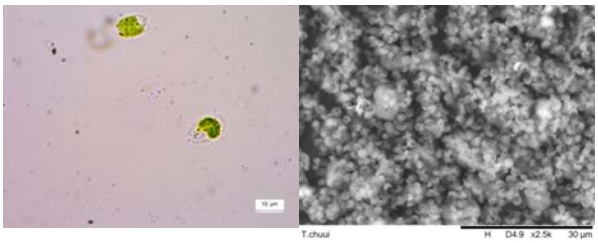


Fig.4: Microscopic image of *Tetraselmis chuui* observed with (A) Optical microscope and (B) Scanning electron microscope.



4. CONCLUSION

The FT-IR spectroscopy has shown that lipid extraction on both *Nannochloropsis oculata* and *Tetraselmis chuui* was successful. Increasing FT-IR band at 1740 cm^{-1} confirms that the lipid content in the *N. oculata* increases from day 9 to day 14. The results obtained from FT-IR are also comparable to conventional method for lipid determination. Therefore FT-IR serves as an easy, fast and reliable technique to monitor biochemical changes of marine microalgae.

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