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Full Length Research Paper

Fourier transform infrared (FTIR) spectroscopy for identification of *Chlorella vulgaris* Beijerinck 1890 and *Scenedesmus obliquus* (Turpin) Kützing 1833

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Fourier transform infrared (FTIR) spectroscopy was used in this study to identify and determine spectral features of *Chlorella vulgaris* Beijerinck 1890 and *Scenedesmus obliquus* (Turpin) Kützing 1833. Two cultures were grown in a chemically-defined media under photoautotrophic culture conditions isolated from eutrophic freshwater lake in Ankara. For FTIR analyses, a view from the transmission region between 4000 and 500 cm^{-1} on the microscope was chosen. All FTIR spectra showed a closely similar sequence of 11 distinct bands and were assigned a range of vibrationally active chemical groups, including residual water (–OH), lipid ($–CH_2$), cellulose (–C=O), protein (amide), nucleic acid (>P=O) and starch (–C–O). The nonparametric (Spearman) correlations revealed a high level of correlations between certain bands. Comparison of band intensities (normalized to amide I) using Mann-Whitney test demonstrated major differences in relative band intensities, with bands 1, 3 and 5 to 11 showing significant differences between the two algal species at the 99% significant level. While bands 8, 9 and 10 showed the highest variations (CV>30%) in *C. vulgaris*, bands 1, 2, 3 and 7 with the highest variations (CV>30%) in *S. obliquus*. The results show that FTIR technique has the potential to become applicable for the determination of single cell biomass composition from phytoplankton communities.

Key words: Microalgae, *Chlorella*, *Scenedesmus*, Fourier transform infrared (FTIR) analysis, infrared spectroscopy.

INTRODUCTION

Algae play key roles in nutrient cycling and energy-flow through aquatic ecosystems and are pivotal in the sequestration of inorganic nutrients (for example, carbon, nitrogen, and phosphorus) and transformation into organic forms (Murdock and Wetzel, 2009), accounting for approximately 50% of the total planetary primary productivity (Shelly et al., 2002). They are essential for maintaining aquatic food chains and are important determinants in models describing global climate change (Heraud et al., 2005). Traditional identification of micro-algae is achieved by microscopic studies. Identification results are questionable to a certain degree. Most methods used in measuring algal nutritional and physiological changes are limited to detecting whole community responses because of the relatively large quantity of material needed for analysis (Murdock and Wetzel, 2009). In the past few years, Fourier transform infrared (FTIR) spectroscopy has developed to become a very powerful and flexible technique for the differentiation and identification of microorganisms. The spatial resolution achievable with infrared microspectroscopy allows for the analysis of macromolecular pools in

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Table 1. Cell count, biovolume and chlorophyll *a* determined[1].

Culture	Cell count (c/ml)	Biovolume (µm^3)	Chlorophyll a (µg L^{-1})
C. vulgaris	2.7 x 10^7	47.71-65.4	4.53
S. obliquus	7.5 x 10^6	67.02-196.35	6.42

[1]Species counts are the mean value from four samples.

individual cells that allow species-specific measurements within heterogenous microscopic communities (Murdock and Wetzel, 2009; Dean and Sigee, 2006). FTIR spectroscopy has been widely used to provide information on a range of vibrationally active functional groups (including O–H, N–H, C=O, =C–H, –CH_2, –CH3, C–O–C and >P=O) in biological specimens (Mantsch and Chapman, 1996; Stuart, 1997; Sigee et al., 2002; Dean and Sigee, 2006; Dean et al., 2008a). Although the technique has been largely used with isolated macromolecules and molecular complexes such as nucleic acids (Liquier and Taillandier, 1996), proteins (Stuart, 1997), lipids (Lewis and McElhaney, 1996), polysaccharides (Brandenburg and Seydel, 1996), and tissue culture cells (Holman et al., 2000; Wetzel and Levine, 1999; LeVine and Wetzel, 1993), studies have also been carried out on whole organisms, including bacteria (Naumann et al., 1994, 1996; Sigee et al., 2002), marine algae (Giordano et al., 2001), higher plants (Stuart, 1997; Wetzel et al., 1998; Wetzel and Fulcher 1990; Koc and Wetzel 2007), fungi (Fischer et al., 2006; Kos et al., 2002) and yeast (Galichet et al., 2001; Wennign et al., 2002).

FTIR spectroscopy has successfully been established as a tool for reliably, quickly and easily identifying microalgae under routine laboratory conditions (Bastert et al., 1999). The technique has recently been used to study physiological changes in microalgae (Heraud et al., 1999; Giordano et al., 2001), discrimination of cyanobacterial strains (Kansiz et al., 1999), and a novel method for monitoring carbon allocation in phytoplankton (Dean et al., 2008b). Studies on algae sampled directly from the environment have used FTIR spectroscopy to demonstrate molecular diversity within species (Sigee et al., 2002), to discriminate between different algal species (Dean et al., 2007; Dean and Sigee, 2006).

The purpose of the present investigation was to use FTIR analysis to examine molecular diversity in freshwater phytoplankton. *Chlorella vulgaris* Beijerinck 1890 and *Scenedesmus obliquus* (Turpin) Kützing 1833 were selected as the organisms to be analyzed since they have FTIR absorption spectrum with well-defined bands and are important constituents of phytoplankton in eutrophic lakes in Turkey. In our analytical system, pure culture of *C. vulgaris* and *S. obliquus* were optically too dense to give clear FTIR spectra. The aim of this work was also to develop a standardized procedure for cultivation and sample preparation for the identification of microalgae and to compare this approach using FTIR.

MATERIALS AND METHODS

Strains and growth conditions

All experiments were carried out on batch cultures of *C. vulgaris* and *S. obliquus* which were isolated from a eutrophic freshwater lake in Ankara. Micromanipulation technique was used in isolating from microalgae (CSIRO, 2010). Strains were inoculated into Allen medium as indicated on the UTEX (2010) and incubated at 25±1°C. The cultures were grown in Allen Medium under photoautotrophic culture conditions. All axenic cultures were maintained in 50 ml flask medium containing 45 ml and were illuminated for 18 h photoperiod (6 h dark period) a day with white fluorescent tubes providing a photon flux density of 50 µmol photons m^{-2} s^{-1}, grown in semi-continuous mode and seven days' growth rate determined. The pH of the medium was adjusted to 6.8 with 1 N NaOH (Czerpak et al., 2003). The mean biovolume for individual algal cells was calculated from the geometrical formulas in (Wetzel and Likens, 2000; Willen, 1976). The number of cells fitting 16 squares on the Thoma slide was counted and the cell counts determined as described by Schoen (1988) and Guillard (1978). For the determination of chlorophyll *a*, samples were filtered through Whatman GF/F glass fiber filters, and the pigments extracted in 96% ethanol. Chlorophyll *a* was measured using a Shimadzu UV-1700 spectrophotometer and calculated for concentration according to (Arvola, 1981). Cell numbers, chlorophyll *a* and biovolume of these cultures are given in Table 1.

Processing for spectroscopy (using FTIR spectrometer with microscope)

Within 1 h of culture, aliquots of the axenic microalgae were centrifuged and re-suspended in distilled water. Droplets of the concentrated suspension were placed on 'Lowe-e' infrared reflectance slides (Kevley Technologies) and the sample dried in a stream of air (sterile laminar flow) at room temperature. The slides were subsequently stored in desiccators at room temperature prior to FTIR analysis. Infrared analysis was carried out at Bilkent University Institute of Materials Science and Nanotechnology, Ankara, Turkey, using a Vertex 70 with Hyperion Microscope fitted with a Bruker Tensor 37 FTIR spectrometer. A view from the microscope was chosen from the transmission region between 4000 and 5000 cm^{-1} wave number range, 4 cm^{-1} resolution and aperture of 20x20 µm square aperture, placed over a clear field (background) and 128 scans were taken as spectra. The specimens were examined and analysed in the dry state, with no mounting medium or coverslip. Statistical calculations were done using EXCEL and SPSS software (Sigee et al., 2002).

Spectral absorption bands were identified in relation to published information. Supporting information on band assignments was also obtained by analysing a range of pure biochemical standards (protein, nucleic acid, fatty acid and soluble carbohydrate) as detailed in Sigee et al. (2002). These bands were published FTIR spectra in relation to specific molecular groups (Table 2). Band contributions were postulated from residual water (band 1), proteins (bands 1, 4, 5, 6 and 7), lipids (bands 2, 3, 6 and 7), cellulose (band

Table 2. Tentative assignment of bands found in FTIR spectra of *C. vulgaris* and *S. obliquus*[1].

Band	Main peak (cm^{-1}) (*S. obliquus* in brackets)	Typical band assignment from the literature	Wavenumber range (cm^{-1})
1	3327 (3330)	Water *v*(O-H) stretching Protein *v*(N-H) stretching (amide A)	3029-3639
2	2928 (2926)	Lipid – carbohydrate Mainly $v_{as}(CH_2)$ and $v_s(CH_2)$ stretching	2809-3012
3	1737 (1737)	Cellulose–Fatty Acids *v*(C=O) stretching of esters	1763-1712
4	1649 (1637)	Protein amide I band Mainly *v*(C=O) stretching	1583-1709
5	1543 (1532)	Protein amide II band mainly δ(N-H) bending and *v*(C-N) stretching	1481-1585
6	1452 (1450)	Protein $\delta_{as}(CH_2)$ and $\delta_{as}(CH_3)$ bending of methyl, Lipid $\delta_{as}(CH_2)$ bending of methyl	1425-1477
7	1389 (1383)	Protein $\delta_s(CH_2)$ and $\delta_s(CH_3)$ bending of methyl Carboxylic Acid v_s(C-O) of COO^- groups of carboxylates Lipid $\delta_s(N(CH_3)_3)$ bending of methyl	1357-1423
8	1248 (1243)	Nucleic Acid (other phosphate-containing compounds) v_{as}(>P=O) stretching of phosphodiesters	1191-1356
9	1149 (1146)	Carbohydrate *v*(C-O-C) of Polysaccharides	1134-1174
10	1082 (1077)	Carbohydrate *v*(C-O-C) of polysaccharides Nucleic Acid (and other phosphate-containing compounds) v_s(>P=O) stretching of phosphodiesters	1072-1099
11	1036 (1024)	Carbohydrate *v*(C-O-C) of polysaccharides	980-1072

[1]Band assignment based on Benning et al. (2004), Giordano et al. (2001), Keller (1986), Naumann et al. (1996), Stuart (1997), Wong et al. (1991), Dean et al. (2007), Sigee et al. (2002), Maquelin and Kirchner (2002). FTIR, Fourier transform infrared.

3), nucleic acids (bands 8, 10) and carbohydrate (bands 2, 9, 10, 11). The manipulation of FTIR spectra was carried out using Bruker OPUS software and baseline-corrected, using the automatic baseline correct algorithm and normalized to amide I.

RESULTS

The initial cell count showed a rapid increase up to stationary phase on day 7. Cell biovolume and chlorophyll *a* concentration were observed during exponential phase (Table 1). The typical appearances of absorption spectra from air-dried cultures shown in Figure 1 had 11 clear bands over the wave number range 4000 to 5000 cm^{-1}. These bands were tentatively identified on the basis of reference standards (Sigee et al., 2002) and published FTIR spectra in relation to specific molecular groups (Table 2). Infrared absorption spectra that were closely similar to *C. vulgaris* and *S. obliquus* each containing 11 clear bands over the wave number range 4000 to 5000 cm^{-1} were generated.

Each peak was assigned a functional group. Protein spectra were characterized by two strong features at 1649 cm^{-1} (*C. vulgaris*) to 1637 cm^{-1} (*S. obliquus*) (amide I) and 1543 cm^{-1} (*C. vulgaris*) to 1532 cm^{-1} (*S. obliquus*) (amide II). These bands were due primarily, to C=O stretching vibration and a combination of N-H bending and C-N stretching vibrations in amide complexes, respectively. Lipid spectra were characterized by two sets

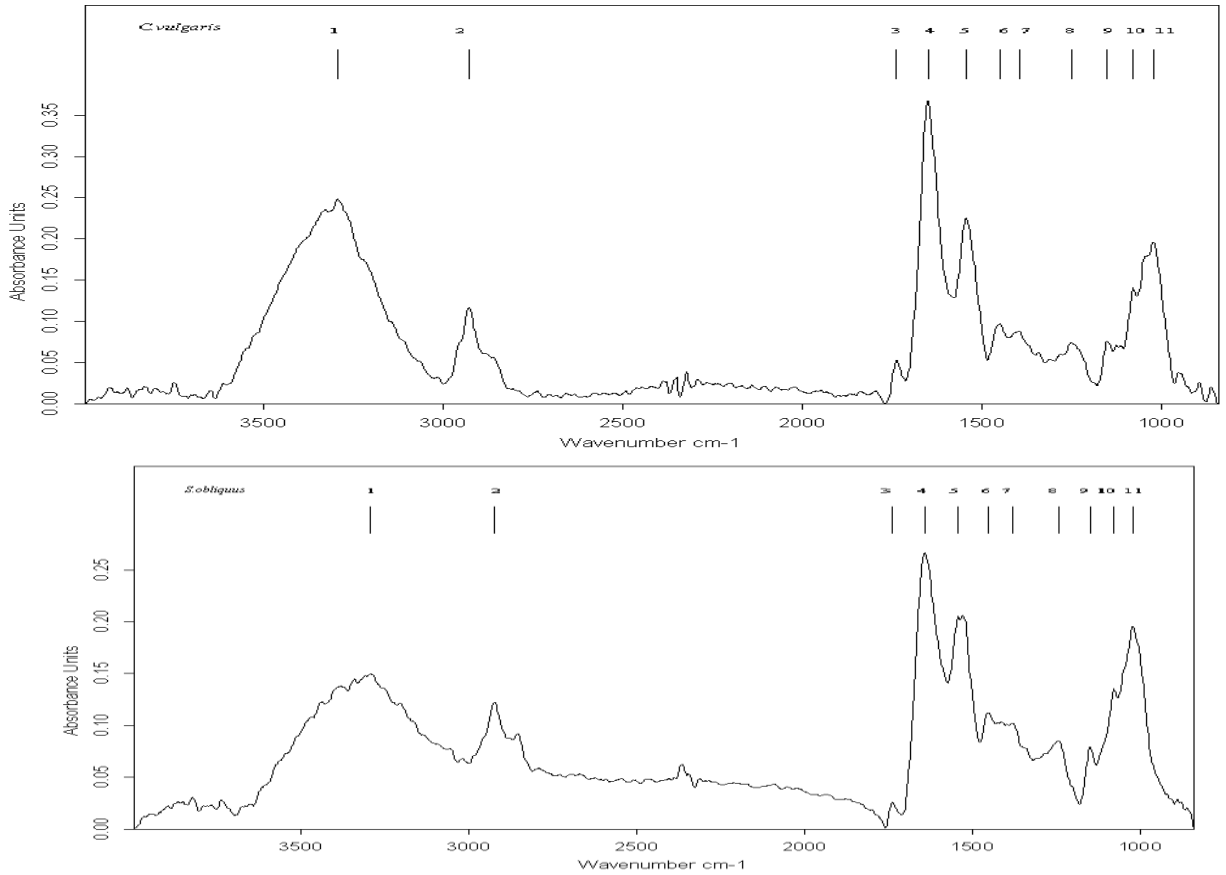

Figure 1. Infrared absorption spectra from *C. vulgaris* and *S. obliquus*. Band assignments are given in Table 2.

of strong vibrations, the C-H at 2928 cm^{-1} (*C. vulgaris*) to 2926 cm^{-1} (*S. obliquus*), and the C=O mode of the side chain from ester carbonyl group at 1737 cm^{-1}, carbohydrate absorption bands due to C-O-C of polysaccharides at 1149 cm^{-1} (*C. vulgaris*) to 1146 cm^{-1} (*S. obliquus*), 1082 cm^{-1} (*C. vulgaris*) to 1077 cm^{-1} (*S. obliquus*), 1036 cm^{-1} (*C. vulgaris*) to 1024 cm^{-1} (*S. obliquus*), respectively.

Table 3 shows band intensities for each species, together with the coefficient of variation (CV). In *C. vulgaris,* while band 5 (amide II) showed the smallest coefficient of variation (7%), probably as a result of being normalized to band four (amide I), bands 8, 9 and 10 showed the highest variations (CV > 30%). Other bands in these algae showed lesser variation than in *S. obliquus*, with CVs of between 12 and 28%. In *S. obliquus*, band 5 again, showed the smallest coefficient of variation. Bands 1, 2, 3 and 7 showed the highest variations (CV > 30%). Other bands had CVs of between 14 and 27%.

The comparison of band intensities (normalized to amide I) using Mann-Whitney test (Table 3) demonstrated major differences in relative band intensities between the two algal species, with bands 1, 3 and 5 to 11 showing significant differences between the algae at the 99% significance

Table 3. Comparison of band intensities, Mann-Whitney Test.

Band	*C. vulgaris*		*S. obliquus*		Significance
	Mean	CV	Mean	CV	
1	0.651	15	0.477	52	0.000*
2	0.313	26	0.417	48	0.029**
3	0.144	12	0.104	44	0.000*
5	0.620	7	0.806	14	0.000*
6	0.270	18	0.430	25	0.000*
7	0.230	26	0.392	32	0.000*
8	0.198	39	0.331	27	0.000*
9	0.202	40	0.303	25	0.000*
10	0.359	33	0.530	24	0.000*
11	0.507	28	0.721	18	0.000*

For each algal species, mean band intensities are shown as normalized data referenced to amide I band (n=33).

Table 4. Nonparametric correlations between band intensities in *C.vulgaris* and *S.obliquus*[1].

Band	1	2	3	4	5	6	7	8	9	10	11
1	__	**0.749**									
2	0.833	__				**0.568**	**0.621**				
3			__								
4				__							
5					__	**0.573**					
6					0.524	__	**0.789**	**0.866**			
7						0.873	__				
8						0.682	0.788	__	**0.763**	**0.547**	
9		0.384							__	**0.501**	**0.543**
10								0.614	0.636	__	**0.655**
11											__

[1]Correlation coefficients (significant at 99% probability level) are shown for Spearman (nonparametric) analysis (n=44). *C. vulgaris*, bold type on the upper half of table; *S. obliquus*, light type on the lower half.

level . Band 2 only showed a significant difference at the 95% level of probability.

The nonparametric (Spearman) correlations shown in Table 4 (99% probability level) reveal high levels of correlation between certain bands. In *C. vulgaris*, bands 1 and 2 shows a correlation. Bands 2, 6 and 7 show positive correlations with each other as do bands 6, 7 and 8, and also bands 8, 9 and 10. In *S. obliquus*, bands one and two are strongly correlated. Bands 8, 6 and 7 and 10, 8 and 9 are correlated.

Table 5 shows that though the two algal species had a similar band pattern with each band position having interspecific differences, the average position of bands 4, 5, 7, 8, 10 and 11 in *C. vulgaris* and *S. obliquus* differed by more than the 4 cm^{-1} resolution of the instrument. The other bands within the resolution were assumed to be insignificantly different.

DISCUSSION

Band assignments and relationships

Individuals of *Chlorella* and *Scenedesmus* generated FTIR spectra with clear bands (Figure 1). The molecular assignments of bands given in Table 2 are based on published data on phytoplankton, bacteria and the other biological materials. In this study, for both algae, the average positions belong to protein (amides I and II), lipid and carbohydrate absorption bands confirmed by the literature. In some spectra of both algae, bands were also seen at ≈1050 and ≈1012 cm^{-1}. These band positions match those attributed to the *v*(C-O-C) stretching of polysaccharides (Brandenburg and Seydel, 1996). In the research, this band position is attributed to band 11. According to a recent paper by Diem et al. (2000), these bands were observed at slightly different frequencies in

Table 5. Comparison of band positions.

Band	*C. vulgaris*			*S. obliquus*			Significance
	Mean (cm^{-1})	SD	n	Mean (cm^{-1})	SD	n	
1	3,326.16	10.73	45	3,266.28	441.81	46	0.366
2	2,927.62	2.25	45	2,925.76	8.01	46	0.135
3	1,736.09	1.50	45	1,737.54	7.16	46	0.184
4	1,649.16	2.34	45	1,634.72	11.23	46	0.000*
5	1,542.00	3.59	45	1,536.74	9.76	46	0.001*
6	1,444.73	45.35	45	1,449.78	8.36	46	0.460
7	1,388.33	16.24	45	1,379.13	7.90	46	0.001*
8	1,247.98	5.93	45	1,244.41	8.12	46	0.019*
9	1,149.02	4.08	45	1,147.20	4.73	46	0.052
10	1,081.70	12.72	44	1,074.74	8.54	46	0.003*
11	1,036.09	11.14	45	1,024.63	8.90	46	0.000*

*Band position significantly different at the 99% probability level.

different materials (that is, 1147, 1086 and 1025 cm^{-1}). While carbohydrates are the strongest absorbers between 1200 and 1000 cm^{-1}, several other classes of compounds, such as nucleic acids, have functional groups with absorption bands in the same region of the spectrum. The nutritional status of the cells is determined by comparing the strength of the protein absorption bands to that of the carbohydrate absorption bands for spectra within the cells (Hirschmugl et al., 2006).

As individual bands may have contributions from a number of molecular groups representing different macromolecular components, the high level of correlation between certain bands has been used in aiding band assignment. Thus, information on band assignments can be obtained from correlation. For both algae, the strong correlation was between bands 1 and 2. The close correlation between bands 1 and 2, which shows prominent peaks at 2900 and 3300 cm^{-1}, suggested that bands 1 and 2 were both due to the presence of carbohydrate. Correlation was seen between band 2 and the other carbohydrate peaks (9, 10 and 11) on both algae. In this case, band 2 was mainly to suggest that carbohydrates may not be very accurate for *C. vulgaris*. In *C. vulgaris*, the correlation between bands 2, 6 and 7 suggests that the major contribution to band 2 might not be from the strong C-H vibrational modes of carbohydrate but from lipids. In *C. vulgaris*, bands 9, 10 and 11 showed positive correlations with each other, while in *S. obliquus* the only correlation shown, was between bands 9 and 10. The major contribution to these bands was derived from carbohydrate.

On both algae, the strong correlation of bands 6 and 7 suggested that they were dominated by the $\delta_{as}(CH_3)$ and $\delta_s(CH_3)$ bending modes of methyl groups of protein (Dean et al., 2007). Other researchers have stated that bands 6 and 7 may have contributions from lipids (Benning et al., 2004) and carboxylic acid (Naumann et al., 1996; Giordano et al., 2001), respectively. In this study, for *C. vulgaris*, the close correlation between the peaks and the existence of correlation with band 2 (lipid-derived band) suggests that the contributions of lipids to these bands were high but for *S. obliquus*, might be low, due to lack of correlation with the peaks and band 2. Also, here in the research, if band 10 were due to the presence of nucleic acid, then it would have been expected to correlate with band 8 (≈1250 cm^{-1}), which was largely due to ν_{as}(>P=O) stretching, and correlation was observed in both algae. This suggests that nucleic acid was indeed, the significant contributor to this band. Also, for both *C. vulgaris and S. obliquus*, the correlation between bands 8 and 11 suggests that these bands were derived from different sources, such as carbohydrates and nucleic acids.

Population heterogeneity

Intraspecific variation in band intensity gives a measure of population heterogeneity, indicating how cellular concentrations of macromolecular components varied between algae taken from the same population (Dean et al., 2007).

In *C. vulgaris* and *S. obliguus,* band 5 (amide II) showed the smallest coefficient of variation (7 and 14% respectively). As the spectra were normalized to amide I, variations in band intensity of amide II were reduced accordingly. As bands 6 and 7 were derived largely from protein, both bands and other functional groups such as lipid (band 6) and carboxylic acid (band 7) might have contributed to variation in these bands, and should be affected by normalization to amide I. Bands 6 and 7 had coefficients of 18 and 26%, respectively in *C. vulgaris* and 25 and 32% in *S. obliguus* (Table 3). As stated, except 32%, these coefficients were lower than the other

coefficients of variation (especially, in bands 9, 10 and 11 in *C. vulgaris*, 1, 2 and 3 in *S. obliquus*). This might be due to the variations in the amount of protein, lipid, fatty acid and carboxylic acid and carbohydrate, protein, and partially lipid storage products stored by cells of *S. obliquus* and *C. vulgaris*. Band 8 (≈1248 cm^{-1} in *C. vulgaris* and ≈1243 cm^{-1} in *S. obliquus*), which is commonly attributed to the ν_{as}(>P=O) stretching of phosphodiester backbone of nucleic acid, had a coefficient variations of 39 and 27% suggesting that both cells showed wide variations in intracellular concentrations of nucleic acid.

Species comparisons

The highest intraspecific variations in band positions were observed, as exemplified by the high standard deviation in bands 1 (in *Scenedesmus*) and 6 (in *Chlorella*). However, in *C. vulgaris* and *S. obliquus*, only the position of bands 4, 5 (protein), 7, 8 (protein and nucleic acid), 10 and 11 (carbohydrate and nucleic acid), were significantly different at the 99% probability level. These differences may reflect the differing intermolecular interactions or molecular structure between the two algal species (Dean et al., 2007).

Band intensities also showed differences between the species with all except band 2. Particularly, prominent differences were observed in the intensity of band 11, with a mean of 0.721±235 in *S. obliquus* and 0.507±216 in *C. vulgaris*. The wide variation in *C. vulgaris* suggests that the heterogeneity in the relative concentration was wider than in *S. obliquus*.

As the spectra were normalized to amide I, the higher carbohydrate intensity in *S. obliquus* indicated a higher carbohydrate/protein ratio than in *C. vulgaris*. An FTIR study on *Scenedesmus* has also shown an increase in the carbohydrate/protein ratio response to increase on re-supply of phosphorus (Beardall et al., 2001). From the study, it could be said that, under equally- optimum growth conditions, the differences in the carbohydrate/ protein ratio may reflect the C allocation patterns difference between the two algae.

Conclusion

As a conclusion, macromolecular compositions of two algal species were determined with FTIR spectroscopy method. FTIR spectra can provide data on the cellular content of macromolecular pools, using small amounts of cell material; but the qualification or quantification of macromolecules in a complex biological sample may not always have the same results. Environmental factors and cell diversity are important variables. The changes in secondary metabolites or accumulation of polyphosphates in algal cells vary according to the quantity and quality of nutrients uptake and cell structure. Therefore, the results of both cell and/or molecular diversity studies under equal conditions, for example, cultivation or sample preparation, will be more decisive for discrimination.

It is pertinent to mention that further experiments need be carried out in order to authenticate the possibility of extending FTIR chemometrics technology to other phytoplankton classes of single cells taken from natural phytoplankton samples.

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