

# Investigation of Differences in the Cultivation of *Nannochloropsis* and *Chlorella* species by Fourier-transform Infrared Spectroscopy

Bernadett Kiss<sup>1</sup>, Szilveszter Gergely<sup>1</sup>, András Salgó<sup>1</sup>, Áron Németh<sup>1\*</sup>

<sup>1</sup> Department of Applied Biotechnology and Food Science, Faculty of Chemical Technology and Biotechnology, Budapest University of Technology and Economics, H-1111 Budapest, Műegyetem rkp. 3, Hungary

\* Corresponding author, e-mail: [naron@f-labor.mkt.bme.hu](mailto:naron@f-labor.mkt.bme.hu)

Received: 15 July 2018, Accepted: 08 October 2018, Published online: 20 November 2018

## Abstract

The increasing use of energy in the world is leading to the exhaustion of fossil fuels, so novel alternative solutions have to be found to meet our needs. One solution is renewable raw materials extracted from algae. The use of microalgae is widespread, in addition to energy formation, their biomass can also be utilized as food and other valuable components of them, e.g. amino acids, vitamins and minerals can be used in drugs and cosmetics. Due to their boundless diversity and components, they have become the focus of an ever-increasing number of research areas. Different processes can induce changes in their nutritional content, so optimizing the conditions used during their cultivation is important to produce the desired product. In our study different isolates of microalgae, namely *Nannochloropsis* sp. and *Chlorella vulgaris*, were studied using Fourier-transform infrared (FT-IR) spectroscopic analysis. Variations in the spectra of a given species were studied under different cultivation conditions.

## Keywords

Chlorella, Nannochloropsis, algae, FT-IR

## 1 Introduction

The applications of microalgae are spreading. Technological developments may result in the applications of algae becoming more economical in many fields, from bioenergetics to uses in the food industry. Their oil content, which could be used to produce biodiesel, has resulted in them becoming the focus of much research. Microalgae-based carbohydrates consist mainly of cellulose and lignin-free starch, thus providing carbon sources with fermentation for industry [1-7].

Besides the fuel industry, certain microalgae such as *Nannochloropsis*, *Tetraselmis*, *Isochrysis*, *Thalassiosira* and *Chaetoceros* are valuable nutritional supplements. Their long-chain fatty acid content, e.g. docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), makes them important in the diet of humans. Algae are sources of functional food in the healthy diets of humans because they contain sterols, *polyunsaturated fatty acids* (PUFAs) and other functional ingredients [1-12].

Biomass from algae is also used in the treatment of ulcers and in cosmetics [13-14]. Their composition of bioactive substances makes them suitable in the production of

medicines [15-19]. Preparations of microalgae are also used in agriculture for nutrition and leaf fertilization [1, 20-21]. Their carotenoid content, namely zeaxanthin, astaxanthin, canthaxanthin and lutein, has a beneficial effect of stimulating many repair and defense mechanisms in the human body to protect, for example, eyesight [16, 22-27]. Their composition also consists of many essential amino acids that the human body needs [28, 22].

The production of products intended for human consumption requires much more stringently controlled conditions, so they are produced in closed reactors. When producing biofuels, this is unnecessary, so open pond cultivation is also appropriate [1, 26, 29-30].

The monitoring of algae cultivation is has several aspects. The best methods are capable of obtaining information not only about cell concentration, but even about changes in the intracellular composition of cells. One of the most current methods is to use the non-destructive techniques of Raman and IR spectroscopy, because of their insensitivity to the disturbing water signal, generally present on other IR spectra [31]. However, little

information is available on Universal Attenuated Total Reflection (UATR)-based IR measurements for the cultivation of algae in the literature. Therefore we decided to examine the applicability of an FT-IR (UATR) equipment beside application of different strains, cultivation techniques, aeration and carbon dioxide supplementation. These results provide basic data for the future monitoring of biomass, namely its amount and composition, even online during cultivation.

## 2 Materials and Methods

### 2.1 Strains

*Nannochloropsis* and *Chlorella* green algae species, which can be classified as eukaryotic microalgae, were investigated. Two isolates of *Chlorella vulgaris*, one from Tihany (T) and the other from Hamburg (H), were examined.

### 2.2 Media

For spectroscopy investigations *Nannochloropsis* was cultured on the medium f/2 [26] and *Chlorella* was cultured on the medium BG-11 [32]. For comparative fermentation, inocula were grown in 200-200 ml of the corresponding media. The fermentation was conducted in a total volume of 700 mL. The f/2 medium contained 1-1 mL of 75 gL<sup>-1</sup> NaNO<sub>3</sub>, 5 gL<sup>-1</sup> NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O and 0.5ml of both trace and vitamin solutions. The trace solution contained 3.15 g of Fe<sub>2</sub>Cl<sub>3</sub>·6H<sub>2</sub>O, 4.36 g of Na<sub>2</sub>EDTA·H<sub>2</sub>O as well as 1-1 mL of 9.8 gL<sup>-1</sup> CuSO<sub>4</sub>·5H<sub>2</sub>O, 6.3 gL<sup>-1</sup> Na<sub>2</sub>MoO<sub>4</sub>·2H<sub>2</sub>O, 22.0 gL<sup>-1</sup> ZnSO<sub>4</sub>·7H<sub>2</sub>O, 10.0 gL<sup>-1</sup> CoCl<sub>2</sub>·6H<sub>2</sub>O and 180.0 gL<sup>-1</sup> MnCl<sub>2</sub>·H<sub>2</sub>O solutions per liter. 1 L of vitamin solution contained 200 mg of thiamine, 10 ml of 0.1 gL<sup>-1</sup> biotin and 1 mL of 1.0 gL<sup>-1</sup> cobalamin. The medium BG-11 contained 1.5 g NaNO<sub>3</sub>, 1 mg Na<sub>2</sub>MgEDTA, 66 mg Fe(NH<sub>4</sub>)<sub>2</sub>(SO<sub>4</sub>)<sub>2</sub>·6H<sub>2</sub>O,

6 mg citric acid, 36 mg CaCl<sub>2</sub>·2H<sub>2</sub>O, 75 mg MgSO<sub>4</sub>·7H<sub>2</sub>O, 40 mg K<sub>2</sub>HPO<sub>4</sub>·3H<sub>2</sub>O, 20 mg Na<sub>2</sub>CO<sub>3</sub> and 1 ml of trace element solution A5 per liter. Solution A5 contained 2.86 gL<sup>-1</sup> H<sub>3</sub>BO<sub>3</sub>, 1.81 gL<sup>-1</sup> MnCl<sub>2</sub>·4H<sub>2</sub>O, 0.222 gL<sup>-1</sup> ZnSO<sub>4</sub>, 0.079 gL<sup>-1</sup> CuSO<sub>4</sub>·5H<sub>2</sub>O, 0.05 gL<sup>-1</sup> CoCl<sub>2</sub>·6H<sub>2</sub>O and 0.391 gL<sup>-1</sup> NaMoO<sub>4</sub>·2H<sub>2</sub>O. Deionized distilled water (Milli-Q®, EMD Millipore, Darmstadt, Germany) was used for the preparation of media, which were heat sterilized in an autoclave at 121 °C and under 1 bar of overpressure for 20 minutes.

### 2.3 Cultivations

A comparative experiment of the three species was conducted in a 1 L bioreactor (Biostat Q®, B. Braun Biotech International, Melsungen, Germany) with a working volume of 700 mL, under alternating 16-hour illuminated and 8-hour dark cycles. The illuminance value was 900 lux, which was provided by a conventional 60 W bulb. Some fermentations were conducted in batch mode (see Table 1) and others in repeated-batch mode, which mean that after a cultivation time of 485 hours ca. 80 % of the broth was removed and the same volume of fresh BG-11 medium added.

A pH electrode and a thermometer were installed in the fermenters. No adjustment of the pH was required because the pH values varied within a suitable range for growth (pH 6.5-9). Homogeneous mixing was assured by a magnetic stirrer. Aeration was varied according to Table 1. Fermentors were sterilized at 121 °C for 20 minutes in an autoclave. The comparative experiments of three strains were run in parallel for 818 hours in three identical fermentors. Two samples were taken per day to monitor the fermentations.

Experiments were also conducted to study the effect of cultivation techniques on infrared spectra, besides in the shaking flasks of the aforementioned bioreactor

**Table 1** The properties of experiments

Strain	Notation	Conditions	Volume (mL)	Aeration flow rate (minL <sup>-1</sup> )	CO <sub>2</sub> (%)	Illumination type
<i>Chlorella vulgaris</i> (Hamburg)	H1	flask cultivation, batch	200	-	-	natural
	H2	bioreactor fermentations, repeated batch	700	0.2	-	bulb
	H3	bioreactor fermentations, repeated batch	700	0.2	-	bulb
<i>Chlorella vulgaris</i> (Tihany)	T1	non-stirred aerated column reactor, batch	150	0.2	-	bulb
	T2	bioreactor fermentations, repeated batch	700	0.2	-	bulb
	T3	bioreactor fermentations, repeated batch	700	0.2	-	bulb
	T4	bioreactor fermentations, batch	700	0.2	-	bulb
	T5	bioreactor fermentations, batch	700	0.2	1	bulb
	T6	bioreactor fermentations, batch	700	0.2	5	bulb
	T7	bioreactor fermentations, batch	700	0.6	5	bulb
<i>Nannochloropsis</i> sp.	N3	bioreactor fermentations, batch	700	0.2	-	bulb

experiments (labelled as H1 in Table 1) and non-stirred aerated bubble column (labelled as T1 in Table 1). A summary of our experiments and sample markings is given in Table 1.

After 818 h (on the 34<sup>th</sup> day), the culture of the isolate from Tihany was further tested with carbon-dioxide enrichment. Carbon dioxide was mixed into the airflow at a concentration of 1 and 5 % whilst the rate of the airflow varied between 0.2 and 0.6 minL<sup>-1</sup> (Table 1).

## 2.4 Sample preparations and measurements

To follow the fermentations in the shaking flasks and different bioreactors, the optical density was measured by a spectrophotometer. A centrifuged (almost cell-free) supernatant, cell suspension, filtered fermentation broth (completely cell-free) and dried biomass were used for IR spectroscopy measurements from the fermented broth.

### 2.4.1 Optical density

Measurements of optical density (OD) were performed to monitor the course of the experiments by taking 1.5 ml samples. The OD was measured by a spectrophotometer at 560 nm in a 3-fold dilution in triplicates. Because the media only contained very small amounts of inorganic salts, distilled water was used as a blind reference.

### 2.4.2 Fourier-transform infrared (FT-IR) spectroscopy

The cultured biomasses were tested by a FT-IR spectrometer with attenuated total reflection (ATR) technique. Different harvesting and cultivation types were compared based on IR spectra.

To elaborate on an appropriate method of measuring IR, 4 different sample preparation techniques were compared for all fermentations as follows: 1) the IR spectra of 100-100 ml of the complete final fermentation broths (media+cells) were recorded, 2) the remaining fermentation broths were concentrated twenty-fold by centrifugation (5000rpm, 4°C, 20min., Janetzki K23D). The concentrated cell suspension was membrane-filtered (0.2mm) and filter cakes were removed then vacuum-dried (130 mbar, 30°C, Memmert vacuum oven VO200, Germany). The IR spectra of three dried samples were recorded from all fermentations: 2a) vacuum-dried biomass, 2b) vacuum-dried biomass grinded in a mortar and 2c) the filter membrane that still contained the rest of the filter cake. For blank measurements, pure (empty) media were used. Three parallel measurements were made from each sample. According to previous experience, solid samples should be grinded because of the inhomogeneity and inertia of

the ATR with the diamond/ZnSe composite crystal having been subjected to a large degree of variance in the parallel measurements. The IR spectrum was recorded at a resolution of 4 cm<sup>-1</sup> in the 4000–650 cm<sup>-1</sup> wavenumber range, but in aqueous samples only between 1500 and 1000 cm<sup>-1</sup> and in solid ones between 1800 and 800 cm<sup>-1</sup> were evaluated for both aqueous and solid samples. The main purposes of these wavenumber ranges were to avoid the water absorption bands resulting from IR absorption and highlight the so-called fingerprint range [32]. IR spectra were collected by a PerkinElmer Spotlight 400 FT-IR/FT-NIR spectrophotometer and its associated Universal Attenuated Total Reflection (UATR) sample treatment unit (PerkinElmer, Inc., Waltham, MA, USA) using a compressive force of 100 N. For the evaluation of IR spectra, Statistica 12.5 software (StatSoft, Inc., Tulsa, OK, USA) was used. For the analysis of IR spectra, the principal component analysis (PCA) and cluster analysis (CA) were selected from multivariate data analysis techniques [33].

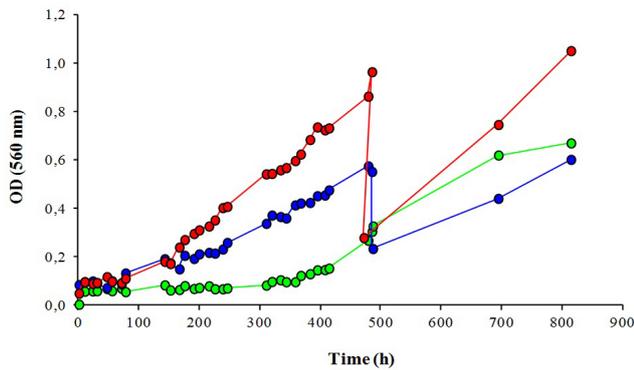
## 3 Results and Discussion

The goal of our work was to examine the applicability of FT-IR in terms of the detection of differences in intracellular compositions originating from different cultivation methods and isolates.

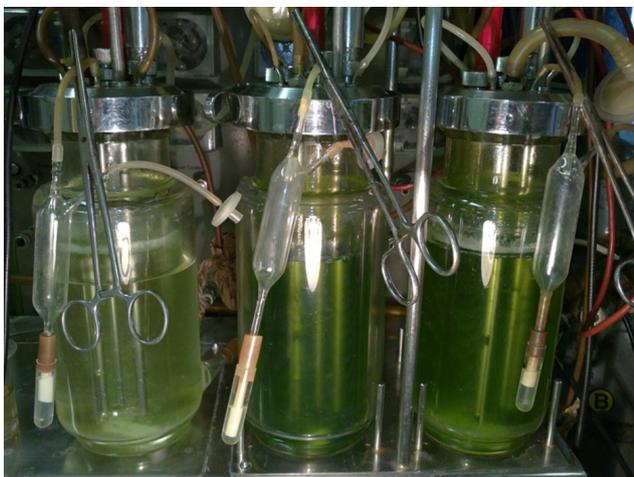
A comprehensive fermentation experiment was conducted with three different microalgae strains (isolates of *Chlorella vulgaris* from Tihany and Hamburg in addition to *Nannochloropsis sp.*) in 3 parallel batch fermentations. Optical density data measured off-line are presented in Fig. 1. These fermentations not only provided biomasses for FT-IR investigations, but were also used to select the highest biomass-producing strain for further cultivation tests that apply different aeration rates and CO<sub>2</sub> enrichment. According to Fig. 1 the fastest growth was exhibited by the isolate from Tihany which also produced the highest OD value that was also visible and exhibited the darkest green color (Fig. 2)

The final concentration of dry biomass achieved in the experiments correlates with the results of the OD measurements, i.e. *Nannochloropsis sp.*: 0.21 gL<sup>-1</sup>, *Chlorella vulgaris* (Hamburg): 0.23 gL<sup>-1</sup>, and *Chlorella vulgaris* (Tihany): 0.37 gL<sup>-1</sup> dry biomass yield.

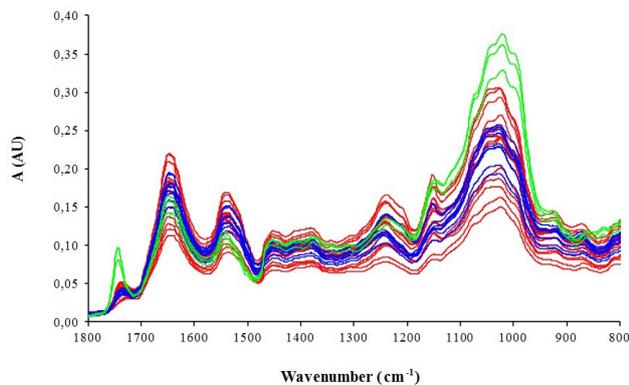
Since water exhibits a dominant signal on IR spectra, dissolved and/or suspended aqueous material cannot be evaluated (data not shown). Therefore, only information-rich spectral fragments (1800-800 cm<sup>-1</sup>) of dried solid samples are presented (Fig. 3).



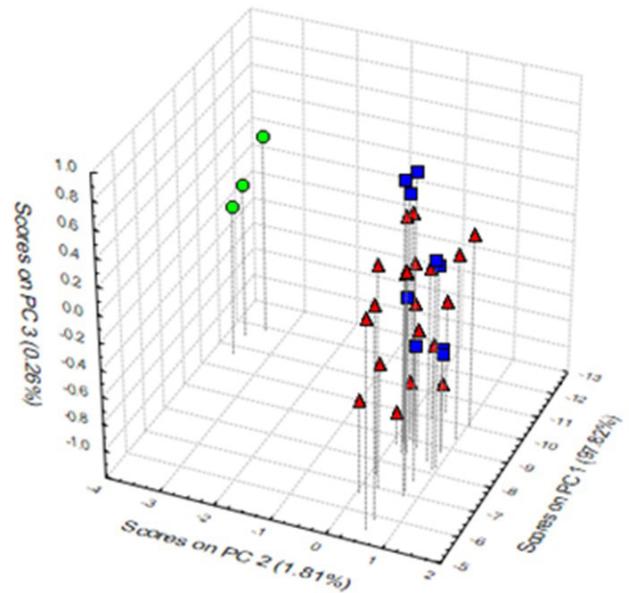
**Fig. 1** OD results of parallel fermentations: *Nannochloropsis* sp. (N3) ●, *Chlorella vulgaris* (Tihany) (T3) ●, *Chlorella vulgaris* (Hamburg) (H3) ●



**Fig. 2** Fermentations in bioreactors: *Nannochloropsis* sp. (N3, left), *Chlorella vulgaris* (Tihany) (T3, middle), *Chlorella vulgaris* (Hamburg) (H3, right)



**Fig. 3** FT-IR spectra in the range of 1800-800  $\text{cm}^{-1}$  wavenumbers *Nannochloropsis* sp. (N3) —, *Chlorella vulgaris* (Tihany, T1-T7) —, *Chlorella vulgaris* (Hamburg, H1-H3) —



**Fig. 4** The complete FT-IR spectra of the sample group. The first three score plots of principal components (PC1-3) of the PCA in the range of 1800-800  $\text{cm}^{-1}$  wavenumbers: *Nannochloropsis* sp. (N3) ●, *Chlorella vulgaris* (Tihany, T1-T7) ▲, *Chlorella vulgaris* (Hamburg, H1-H3) ■

In the examined region, the spectroscopic differences between the two genera can be seen even before multivariate data analysis: for example, at 1740  $\text{cm}^{-1}$ , the spectra of the *Nannochloropsis* species exhibit sharply separated peaks (black arrow on Fig. 3). Many articles reported that this absorption range is correlated with lipids. [7-8, 33-34]. The analysis of the IR spectra using PCA followed by the illustration of its output, so-called score values, shows clear differences between the species (Fig. 4). In the case of IR spectra, the first three principal components (PCs) usually describe almost 100 % of spectroscopic variability, subsequently the first three major components were examined. In terms of the second principal component (PC2), the sample group of *Nannochloropsis* separates sharply.

The weight function of PC2 (Fig. 5) shows characteristic peaks at four typical absorption bands which are denoted by black arrows in Fig. 5 (around 1740, 1645, 1530 and 980  $\text{cm}^{-1}$ ).

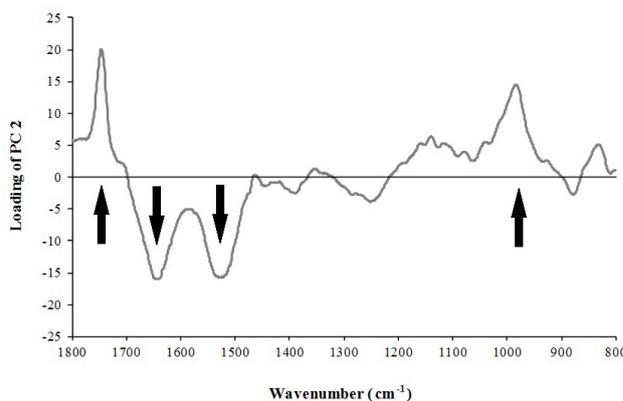
These peaks can be linked to specific vibrations of lipids, proteins and carbohydrates [7-8, 32-34] (Table 2).

The peak height of these absorption bands was determined as the relative amount of each major chemical component of green algae, i.e. lipids, proteins and carbohydrates, which are nutritional key components in each experiment (Fig. 6).

**Table 2** Summary of the vibration of specific groups according to their wavenumbers

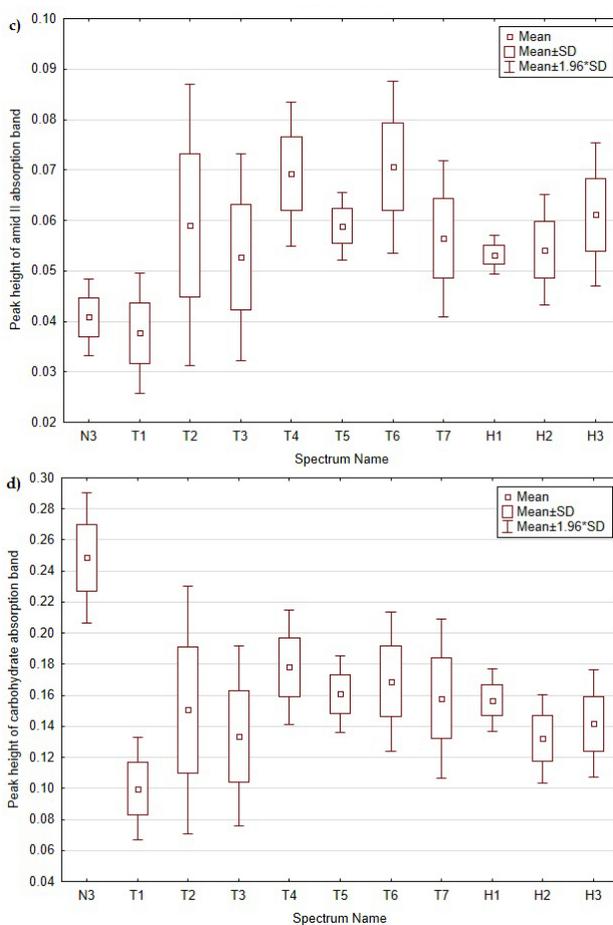
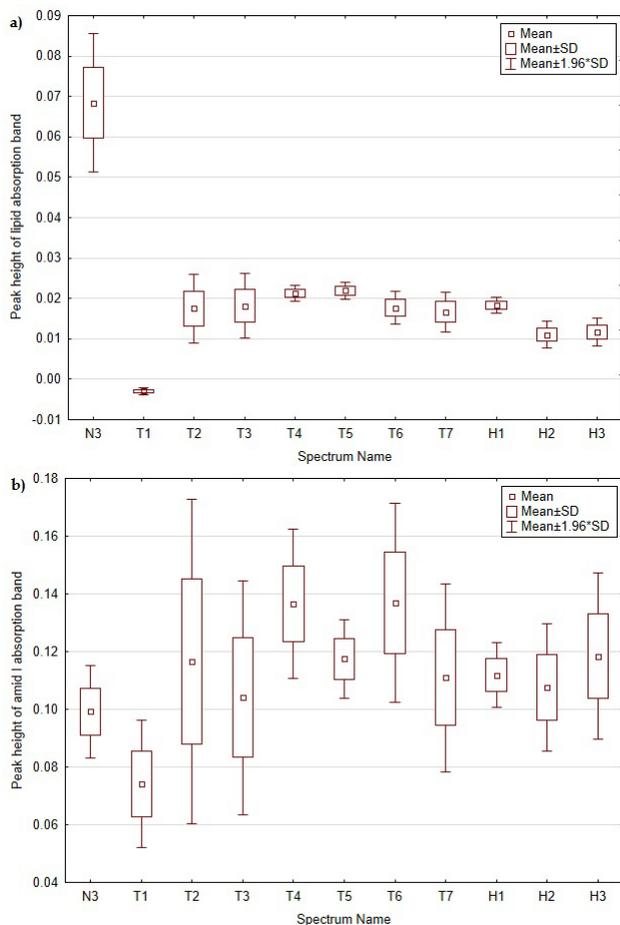
Wavenumber	Group vibration
~ 1740 cm <sup>-1</sup>	vC=O ester group vibration lipids and fatty acids
~ 1650 cm <sup>-1</sup>	vC=O amide group vibration proteins (amide I)
~ 1540 cm <sup>-1</sup>	δN–H amide group vibration proteins (amide II)
~ 1200-900 cm <sup>-1</sup>	vC–O–C vibration carbohydrates

The nutritional profiles reflect well both the isolates, i.e. N, H and T, and the different culture conditions. The latter is well illustrated by the lower lipid content of the experiment in the bubble column reactor (T1), or the higher lipid content of the shake flask experiments (H1) with *Chlorella vulgaris* green algae from Hamburg (H series) (Fig. 6(a)). It should be noted that while the difference in lipid content between the isolates, i.e. N, H and T, is significant at a confidence level of 95 % (Fig. 6(a)), the contents of



**Fig. 5** Weight function of PC2 generated based of all recorded spectra between 1800-800 cm<sup>-1</sup> wavenumbers.

protein and carbohydrate show only minor differences. However, different species and cultivations have resulted in diversity that can still be observed (Fig. 6(b)-(d)). The highest correlation coefficient (r), not surprisingly, is the protein content defined by the vibrations of amides I and II (0.97) during the complete cross-correlation of peak



**Fig. 6** The peak height of the lipid (a), protein (b-amide I, c-amide II) and carbohydrate (d) absorption bands of the FT-IR spectra of green algae cultivated in different experiments

heights that reflect nutritional properties. Smaller, but still significant,  $r$  values (0.87) are shown by the correlation values of lipid and carbohydrate content.

Since the isolate of *Chlorella vulgaris* from Tihany exhibited the highest final OD, it was selected for further studies. In the following, only the relative position of the isolate from Tihany was studied in the PCA. It can be stated that the different types of cultivation are well separated, forming distinctive groups (Fig. 7).

The T1 samples (●) were extracted from the bubble column reactor. The other samples (T2-T4 ▲, T5 ■, T6 ◆ and T7 \*) were cultured in the same Biostat bioreactor. The batches T2-T4, which were not subject to atmospheric change, are more distinct from samples that were exposed to different concentrations of CO<sub>2</sub> by changing the aeration rate (T5-T7). The non-stirred aerated experiment (T1) is likely to be significantly separated due to the differences in content (Fig. 6) discussed earlier. This is confirmed by the weight function of PC1 (Fig. 8(a)), where high lipid, protein and carbohydrate absorption sites are obtained at high local extremes. The weight function for PC2 (Fig. 8(b)) yields the maximum extremes within the range of 1250-1100 cm<sup>-1</sup> wavenumbers. Many reports have revealed that bicyclic monoterpenes (~1214 cm<sup>-1</sup>, C-O-C) and pectin (~1150 cm<sup>-1</sup>, C-O-C) exhibit absorption in this region based on vibrations of the C-O-C group, which can be associated with subunits of carbohydrate. Thus, the altered atmospheric composition affects the carbohydrate content of algae biomass [21, 26, 33-34].

#### 4 Conclusion

Conclusions from the evaluation revealed that both the type of fermentation and the set of conditions affect the intracellular content of microalgae. Based on the FT-IR spectra, the greatest difference was observed in the varieties of lipids and carbohydrates. The results also demonstrated that the sensitivity of FT-IR spectroscopy is excellent for monitoring the cultivation of algae and fermentation conditions.

It was concluded that FT-IR spectroscopy is a fast, reliable and appropriate technique to detect qualitative differences in the compositions of microalgae. For a quantitative application of FT-IR spectroscopy, reference laboratory testing is necessary.

Therefore, with the help of the available FT-IR spectroscopic technique, appropriate reference laboratory tests, e.g. Soxhlet extraction and Dumas total nitrogen determination, and a sufficient amount of biomass it will be possible to create and validate quantitative calibration between

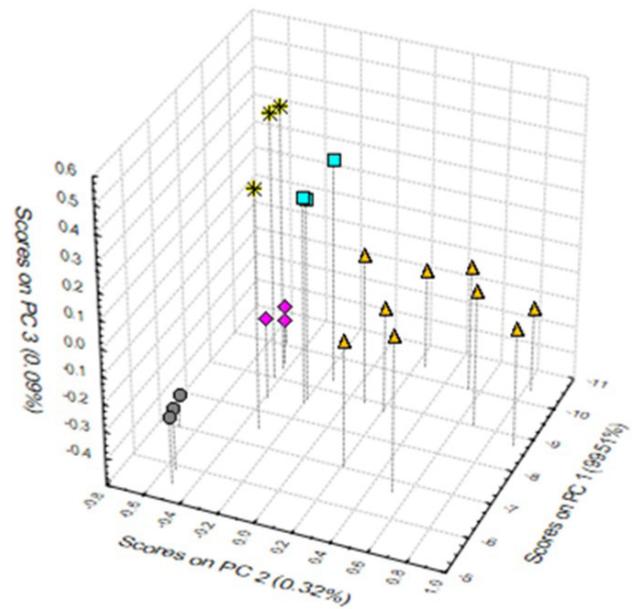


Fig. 7 Score plot of PCA from different spectra of Tihany isolate T1 ●, T2-T4 ▲, T5 ■, T6 ◆, T7 \*.

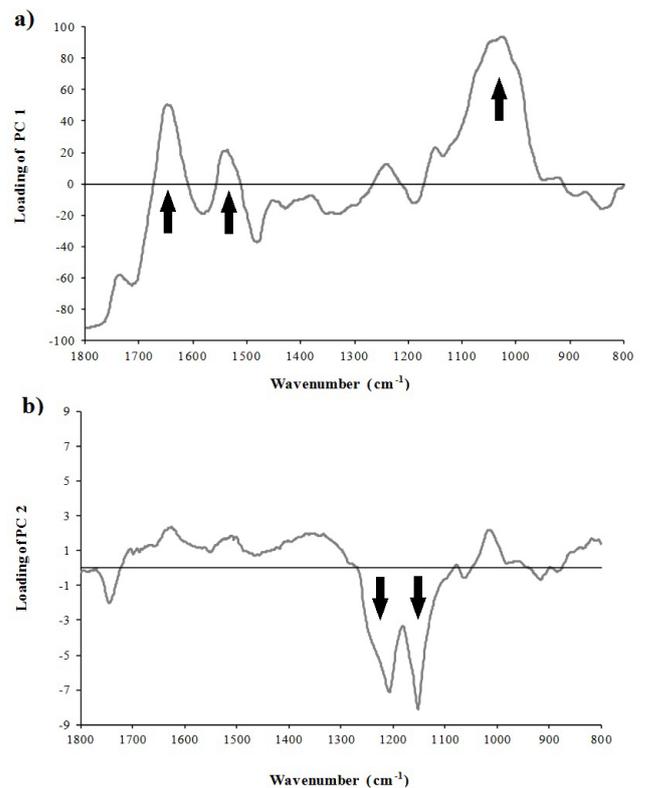


Fig. 8 Weight function of PCA : a) for PC1 and b) for PC2 in case of Tihanyi isolate within the range of 1800-800 cm<sup>-1</sup> wavenumbers

reference data determined both by spectroscopy and a reference method. This will both speed up quantitative determination of the content of algae-based products and make the monitoring of fermentations more effective.

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