

Use of FTIR Spectroscopy and PLS-Regression in Monitoring Biomolecules in *Spirulina platensis* during its Growth in an Internally-Illuminated Photobioreactor

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This study developed a rapid and non-destructive analytical method for biochemical analysis of the *Spirulina* biomass via FTIR spectroscopy and PLS regression. It was then used to monitor the biochemical changes in the biomass during an 8-day cultivation period in a 3-L internally-illuminated concentric-tube airlift photobioreactor at different light intensities (69, 110, and 166 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$). It was found that PLS-regression have better predictive power than multipoint regression. It also yielded statistically similar results with conventional biochemical methods. This new method reduced the time for sample preparation and eliminated the extraction of target biomolecule for analysis. It was also found to be valuable in monitoring the changes in the total protein, carbohydrate and lipid content (% w/w of total biomolecules) in *Spirulina* biomass during its growth. It was observed that the protein content of the biomass decreased initially, which was compensated by an increase in the carbohydrate content. This may imply that biomass production may be driven by production of more carbohydrates during the early period of growth. Protein content then started to increase until the third day of growth and remained constant thereafter. Carbohydrate and lipid content also remained constant from the third day of growth. The lowest light intensity was found to have the highest protein content (60.4 ± 0.87 %w/w) but it has the lowest overall biomass productivity (0.090 ± 0.017 g L⁻¹ d⁻¹). While at the highest light intensity, protein content was slightly lower (57.3 ± 0.777 %w/w) than that of the lowest light intensity. But it has the highest overall biomass productivity (0.141 ± 0.007 g L⁻¹ d⁻¹).

1. Introduction

Conventional biochemical methods in quantifying biomolecules such as proteins, carbohydrates, and lipids in algal biomass are usually time-consuming due to the series of steps that need to be undertaken during the analysis. These steps usually require sample preparation and extraction steps before measurement can be done. In extracting the target analyte, conventional biochemical methods often require high temperature that may be destructive to the sample. For example, the proteins are extracted from the biomass at high temperature using alkaline solution or a detergent-containing buffer. To prevent underestimation of protein content, several extraction steps are necessary to ensure maximum extraction of protein. In carbohydrate analysis via the phenol-sulfuric acid method, breaking down the polysaccharides and oligosaccharides to their constituent monosaccharides often uses strong acids and often takes four hours in boiling water baths. These methods also often require toxic and corrosive reagents that are harmful to human health and the environment.

These methods are also sensitive to other interfering substances. For example, Lowry assay for protein analysis is sensitive to the presence of carbohydrate, glycerol, and EDTA that are typically present in algal extracts or in extraction buffers (Quiang and Richmond 2013). Detergents like sodium dodecyl sulfate and Triton X-100 may also interfere with color development in Bradford protein assay. Overestimation of protein may also result due to high presence of arginine and/or phenylalanine that bind disproportionately with the Coomassie Brilliant Blue G250 dye (Quiang and Richmond 2013). Phenol-sulfuric acid method for

carbohydrate analysis is also a colorimetric method that presence of pigments in the sample may interfere with the analysis. Removal of these interfering substances may be required prior to analysis.

Aside from these limitations, conventional biochemical methods are limited to detecting whole communities of algae that a large quantity of materials is needed and analysis at a single-cell level may be difficult (Murdock and Wetzel 2009). Moreover, results from such methods are only accurate to a certain degree (Duygu et al. 2012). Characterization of algal composition and monitoring algal growth, therefore, need a fast and sensitive method, preferably at the scale of single cells (Rüger et al. 2016).

Fourier-Transform Infrared (FTIR) spectroscopy coupled with chemometric techniques can be used to develop a method alternative to conventional methods. FTIR has been used in characterization of products from biomass, not only microalgae. It has been used to identify functional groups in the cellulose isolated oil palm empty fruit branch (Shanmugarajah et al. 2015). It has also been used, together with Scanning Electron Microscope (SEM) and X-ray Diffraction (XRD), to study the structural changes in açai seeds untreated and treated with sulfuric acid and sodium hydroxide (Oliveira et al. 2016).

FTIR spectroscopy in algal characterization can overcome the limitations of conventional methods (Stehfest et al. 2004). It has the potential for determining single-cell biomass composition (Duygu et al. 2012). It is highly reliable, fast, and sensitive to species-specific changes in molecular composition (Bartošová, Blinová, and Gerulová 2015). In FTIR spectroscopy, the presence of proteins, carbohydrates, and lipids in algal biomass can be determined by identifying their characteristic bands such as the amide band in proteins, C-C and C-O bands in carbohydrates, and C=O ester band in lipids. Furthermore, quantification can be done by relating the absorbance and the spectral structure of these functional groups to known concentrations of standards.

The simplest model that linearly relates absorbance to concentration is the Beer-Lambert's law. Linear regression can be done on the spectral peak absorbance or peak area against concentration. However, linear regression on spectral data may not be highly reliable since such data often contain numerous, noisy, and strongly correlated predictor variables (Höskuldsson 1988). Partial least squares (PLS) regression is good in this situation especially where there are many variables but not necessarily many samples or observations. It also gives the maximal reduction in covariance of the data in order to give the minimum number of variables necessary to build a reliable prediction model (Höskuldsson 1988).

Bataller and Capareda (2018) developed a novel method in quantifying total proteins, carbohydrates and lipids in *Spirulina* using FTIR-ATR (attenuated total reflectance) spectroscopy and Partial Least Square (PLS) – regression. The authors found that results of the model are statistically similar to the results of conventional biochemical methods. They also found that PLS-regression produced better results than multipoint regression, which is a least square method. The authors then concluded that this novel method can be used as an alternative to conventional methods which are time consuming and may be destructive to the algal sample. Since this method is rapid, it can be used to monitor the daily changes in the biochemical composition of algal samples.

Most studies on algal cultivation determined the biochemical composition only after harvest. To the extent of our knowledge, there are only a few or no study on algal growth that monitored daily composition of the algae. Thus in this study, FTIR spectroscopy and PLS-regression method, developed by Bataller and Capareda (2018), was used to quantify and monitor the daily total protein, carbohydrate, and lipid content of *Spirulina platensis* as they grow in a 3-L internally-illuminated concentric-tube airlift photobioreactor.

2. Methodology

2.1 Cultivation of *Spirulina platensis*

Spirulina platensis was grown at different light intensities – 69, 110, and 166 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ in a 3-L internally-illuminated concentric tube airlift photobioreactor (PBR). The internal lighting of the photobioreactor was provided by cool white light-emitting diodes (LEDs) with 12V DC input (3 Watts per foot) and 164 lumens per foot output. It was placed inside a glass chamber at the center of PBR. The light intensity was measured one inch from the reactor's light chamber using a quantum PAR meter (Hydrofarm® LGBQM Quantum PAR Meter) and was controlled by an LED dimmer. Initial biomass concentration (0.05 g L^{-1}) and air flow rate (0.3 vvm) were kept constant. The filtered air is not supplement with CO_2 .

The stock culture of *Spirulina* was obtained from the BioSeparations Laboratory, Texas A&M University, USA. The growth medium used is the Zarrouk's medium adjusted to pH = 9.2 – 9.5. Prior to inoculation, the optical density of the stock culture was determined and the volume of Zarrouk's medium and stock culture were determined to make 3 L of 0.05 g L^{-1} *Spirulina* aliquot. These amounts were poured into three PBRs that served as the three replicates of each run. Air provided to the PBR was filtered using a $0.2 \mu\text{m}$ air venting filter and was maintained at 0.9 L min^{-1} (0.3 vvm). *Spirulina* was allowed to grow for eight days or until the stationary phase was reached and the temperature was maintained at $30 \text{ }^\circ\text{C}$.

2.2 Monitoring daily growth of *Spirulina platensis*

A 60-ml sample was taken from each PBR right after inoculation, after 5 h, and after 24 h, thereafter for 8 days. The sample's optical density was read at 680 nm using a UV-Vis spectrophotometer (VWR UV-Vis Scanning UV-3100). From the growth curve developed, the specific growth rate, overall and daily volumetric biomass productivities were determined. The remaining sample was then pipetted to three 15-ml glass centrifuge tubes for protein, carbohydrate and lipid content measurement using the FTIR and PLS regression method described in Section 2.3.

The whole *Spirulina* culture was then harvested after 8 days or until the stationary phase was reached. Its final volume was measured, centrifuged and washed twice with deionized water in Beckman-Coulter Allegra™ 25R Centrifuge at $4633 \times g$ and at 5°C . The pellets were then dried at 60°C in an oven until constant weight. And, the mass of the dried biomass was obtained.

2.3 FTIR spectroscopy and PLS-regression for biochemical analysis

After optical density measurement, *Spirulina* samples were transferred to 15-mL glass centrifuge tubes and were centrifuged at $1,717 \times g$ for 10 mins. The pellets were washed twice with deionized water to remove residual salts and precipitates. After washing, the pellets were resuspended by vortex in a volume of deionized water to concentrate or dilute the sample to 1.5 absorbance. Five microliters from each mixture was pipetted to the FTIR (Shimadzu IRAffinity-1) ATR (MIRacle™ Single Reflectance Attenuated Total Reflectance) sample cell. The biomass was dried by nitrogen purge at 2.0 L min^{-1} for 5 mins. The sample was then scanned in the FTIR using Happ-Genzel apodization at $4,000\text{-}600 \text{ cm}^{-1}$ wavenumber range and 4 cm^{-1} resolution for 45 scans. Three spectral measurements were obtained for each sample. The protein, carbohydrate, and lipid content of the sample were obtained using the PLS-regression model developed by Bataller and Capareda (2018).

3. Results and Discussion

Light intensity is one of the major factors that affect the growth of photosynthetic organisms such as microalgae. Low light intensity may suppress growth due to insufficient source of energy needed for photosynthesis (photolimitation). On the other hand, severe light intensity may cause photo-oxidation and pigment bleaching (photoinhibition). Both cases result to low biomass yield. Optimal light intensity must be utilized for every culture grown at specific conditions and reactor geometry to obtain maximal biomass yield. Thus, growth parameters and biochemical changes in *Spirulina* was monitored at different light intensities (69, 110, and $166 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$).

Table 1 presents a summary of the growth parameters for each light intensity. Results show that growth performance of *Spirulina* generally increases with light intensity for the light intensities tested in terms of the specific growth rate, final biomass concentration after 8 days cultivation, and overall and maximum daily biomass concentration. This indicates that there is no reduction in the overall photosynthetic capacity in *Spirulina* caused by excessive light intensity at $166 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$. *Spirulina* still has the potential to give better growth performance at higher light intensities. However, experiments at higher light intensities were not performed since $166 \mu\text{mol m}^{-2} \text{ s}^{-1}$ is the maximum light intensity that the light source can provide. Soletto *et. al* (2008) reported that photoinhibition threshold for *Spirulina* ranges from $150\text{-}230 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$.

The specific growth rates observed in this study were significantly higher than those observed by Converti *et al.* (2006) who utilized a 5.5-L 0.5-in. diameter combined airlift-tubular photobioreactor in growing *Spirulina*. Converti *et al.* (2006) obtained $\mu = 0.17 \text{ d}^{-1}$ for cultures grown at $120 \mu\text{mol m}^{-2} \text{ s}^{-1}$ compared to $\mu = 0.63 \text{ d}^{-1}$ obtained in this study for the $110 \mu\text{mol m}^{-2} \text{ s}^{-1}$ culture. The reason may be due to the higher initial biomass concentration in Converti's study (0.15 g L^{-1} compared to 0.05 g L^{-1} in this study), wherein there would be less photon dose per cell mass at higher biomass concentration. It could also be due to the effect of internal lighting in this study where cells can utilize light irradiance more efficiently than when light is provided externally. This is supported by the fact that photosynthetic efficiency observed in this study was also higher than those observed by Converti *et al.* (2006). The difference in the geometry of reactors used may also have contributed to the different specific growth rates observed. However, Converti *et al.* (2006) reported better biomass productivity, which may be due to the shorter light path of their reactor (about 0.5 in.); whereas, the light path from the light chamber to the inner diameter of the draft tube of the reactor in this study is 1.0 in. The shorter light path may have prevented shading effect enabling the reactor to support higher biomass concentrations. In this study, shading effect might have become significant just after a few days of growth since it was observed that growth slowed down just after 4-5 days.

Table 1. Summary of growth parameters of *Spirulina platensis* cultivated at various light intensities in a 3-L short tube internally-illuminated concentric-tube airlift photobioreactor. Air flow rate was maintained at 0.3 vvm and the initial biomass concentration was 0.05 g L⁻¹.

Growth Parameters	Light Intensity ($\mu\text{mol m}^{-2} \text{s}^{-1}$)		
	69	110	166
Specific growth rate, μ (d^{-1})	0.485 ± 0.021	0.630 ± 0.008	0.664 ± 0.035
Overall biomass productivity, Q_x ($\text{g L}^{-1} \text{d}^{-1}$)	0.090 ± 0.017	0.115 ± 0.003	0.141 ± 0.007
Max. daily biomass productivity, ($\text{g L}^{-1} \text{d}^{-1}$)	0.160 ± 0.048	0.185 ± 0.006	0.217 ± 0.013
Final biomass concentration, (g L^{-1})	0.777 ± 0.142	0.989 ± 0.024	1.172 ± 0.056
Final biomolecule content (% w/w)			
Protein	60.4 ± 0.87	56.2 ± 1.94	57.3 ± 0.777
Carbohydrate	36.1 ± 0.53	39.8 ± 2.20	38.8 ± 1.69
Lipid	3.4 ± 0.54	3.96 ± 0.550	3.91 ± 0.910

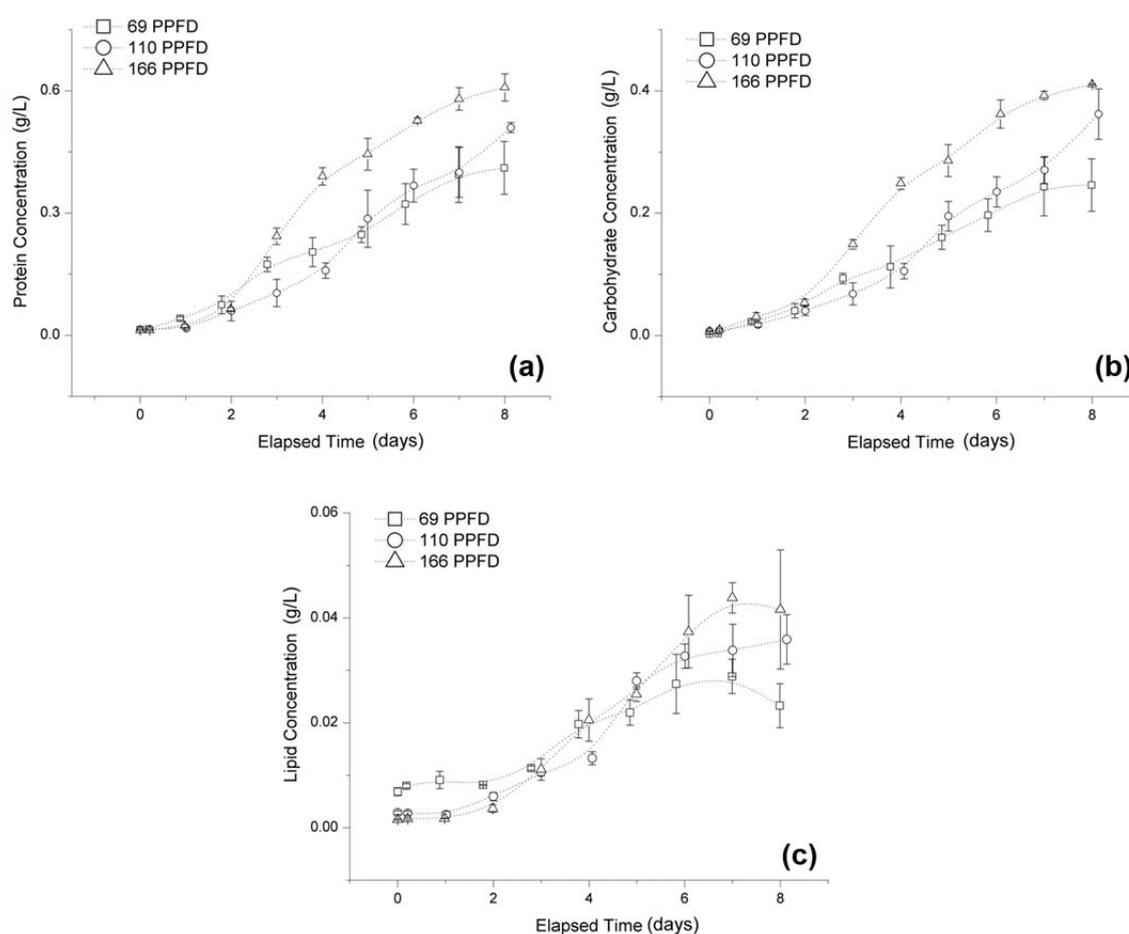


Figure 1. Time profile of (a) protein concentration, (b) carbohydrate concentration, and (c) lipid concentration of *Spirulina platensis* cultivated at various light intensities.

It was also observed that there is only a slight increase in the final biomass concentration, specific growth rate and biomass productivity from 110 to 166 $\mu\text{mol m}^{-2} \text{s}^{-1}$ compared to the increase in these parameters from 69 to 110 $\mu\text{mol m}^{-2} \text{s}^{-1}$. According to Soletto *et al.* (2008), biomass can grow rapidly at higher light intensity if supplied with sufficient amount of carbon. Since cultures were grown on same growth medium containing the same amount of carbon source, cultures grown at higher light intensity that have higher growth rate may have

less carbon available per cell mass. Also, since cell density tend to become higher at higher light intensity, mutual shading may have prevented the cells to obtain higher increase in growth rate.

The time profiles of protein, carbohydrate, and lipid production of *Spirulina* are shown in Figure 1. Daily measurements of the biochemical content of *Spirulina* was made possible by the FTIR and PLS-regression method, which gives fast results. Similar with biomass concentration, the biomolecule concentration of *Spirulina* generally increases with time. They increase only slightly during the first two days of growth; then increase rapidly, thereafter. The biomolecule production then slowed down during the last three days of cultivation. This trend is attributed to the daily biomass production since protein, carbohydrate and lipid comprise the microalgal biomass. Figure 1 also shows that biomolecule production increase with light intensity. *Spirulina* cultures grown at $166 \mu\text{mol m}^{-2} \text{s}^{-1}$ have the highest protein, carbohydrate and lipid productivity and final concentration among the light intensities tested; while, those grown at $69 \mu\text{mol m}^{-2} \text{s}^{-1}$ have the lowest. This result is also attributed to the effect of light intensity on biomass production wherein the highest biomass yield and productivity was observed at the highest light intensity tested.

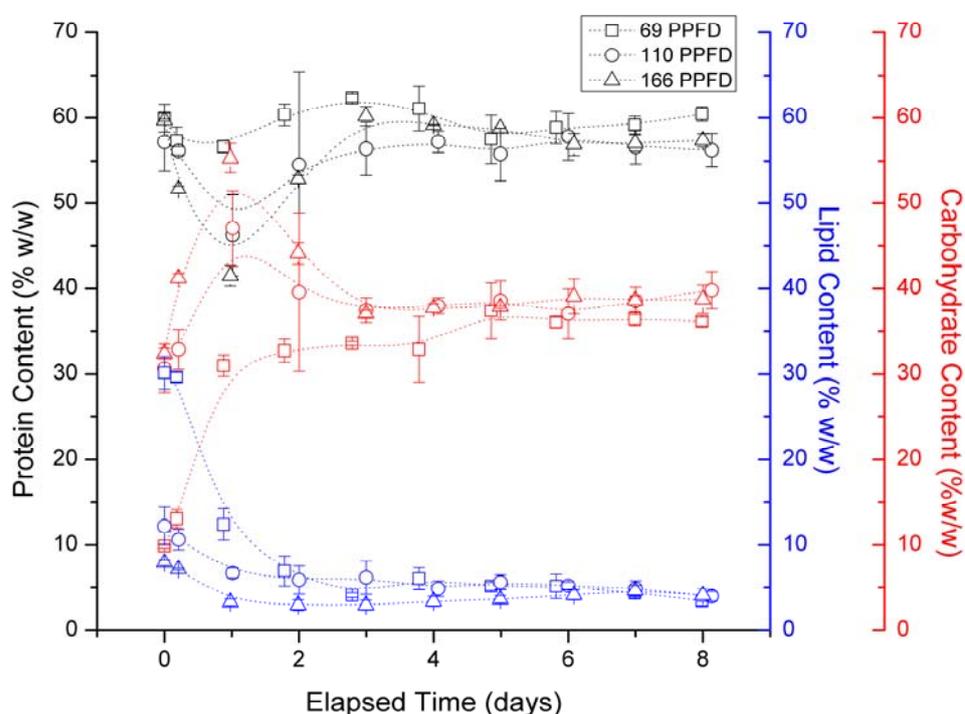


Figure 2. Time profile of biomolecule content (black – protein content, red – carbohydrate content, and blue – lipid content) of *Spirulina platensis* cultivated at various light intensities (open square - $69 \mu\text{mol m}^{-2} \text{s}^{-1}$, open circle - $110 \mu\text{mol m}^{-2} \text{s}^{-1}$, and open triangle - $166 \mu\text{mol m}^{-2} \text{s}^{-1}$). PPFD is photosynthetic photon flux density ($\mu\text{mol photons m}^{-2} \text{s}^{-1}$). The biomolecule contents are expressed in terms of %w/w of total organic material.

The daily changes in the biochemical content of *Spirulina*'s protein, carbohydrate and lipid content (as % by weight of total organic material) were also presented in Figure 2. The percent by weight is expressed in terms of the total organic material only so that its value is not influenced by the growth medium's inorganic components. It was observed that the protein content of the biomass decreased initially, which was compensated by an increase in the carbohydrate content. This shows biomass production was driven by production of carbohydrates possibly for the energy requirement of the cells to perform various cellular activities during the early stage of growth. Biomolecule content remained constant as the cells become more acclimatized to culture environment. There is no study available in literature, to the extent of the authors' knowledge, that have monitored the daily biochemical changes in microalgae may be due to the large amount of time and labor required by conventional biochemical methods. Most studies only measure the initial and final biochemical content.

4. Conclusion

FTIR spectroscopy and PLS-regression — a fast, non-destructive and species-specific analytical method — allowed for the daily monitoring of biochemical changes in *Spirulina platensis*. Monitoring the biomass composition has been difficult in the past since conventional biochemical methods require several steps in the analysis including sample preparation and extraction of target analyte. Together with UV-Vis spectrophotometry, one can comprehensively characterize the growth of microalgal cultures in terms of specific growth rate and biomass and product yield and productivity. This novel method was also successfully used in testing the effect of light intensity on the growth of *Spirulina* in a 3-L internally-illuminated concentric-tube airlift photobioreactor.

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