

## Rapid Determination of Lipid Accumulation Under Sulfur Starvation in *Chlamydomonas reinhardtii* Microalga Using Fourier Transform Infrared (FTIR) spectroscopy

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### ABSTRACT

*Chlamydomonas reinhardtii*, the most commonly studied model organisms in microalgal kingdom, is known to be a rich lipid producer. Here, a naturally isolated *Chlamydomonas reinhardtii* strain was cultivated phototrophically at Erlenmeyer scale in BG11 culture medium. After reaching to stationary growth phase, a sulfur limited regime was applied. The lipid content of *C. reinhardtii* microalga, was determined using Fourier transform infrared micro-spectroscopy (FTIR) method in Erlenmeyer flasks in normal and sulfur starved conditions. After sulfur limitation the microalgal cells were significantly decreased (1.582 g L<sup>-1</sup>) in comparison with grown cells in sulfur rich medium (4.484 g L<sup>-1</sup>). The results indicate an elevated lipid: amide I in sulfur rich medium (1.66-fold) compared with sulfur rich medium. Gravimetric analysis of the total lipid contents, as a conventional method, confirmed that the lipid accumulation (36.98%) was due to elevated lipid biosynthesis. The results open a new way to direct quantification of total lipids in more microalgal strains using FTIR as a reliable and robust method.

**Keywords:** Biofuel, *Chlamydomonas reinhardtii*, Fourier transform infrared spectroscopy, Lipid, Sulfur starvation.

### INTRODUCTION

Anthropogenic greenhouse gas (GHGs) emissions which has brought up the global warming and climate change, dwindling reserves of crude oil, concerns regarding to energy security and growing fossil fuel costs has mitigated worldwide attention to produce a secure, sustainable and environmentally friendly renewable energy source<sup>1-3</sup>. Photosynthetic microorganisms including microalgal strains has been regarded as promising candidates for bioenergy production, CO<sub>2</sub> fixation, waste water treatment, waste heat consumption and bulk chemicals production as well<sup>4-7</sup>. Microalgae could synthesize high values of lipids under different environmental stress condition including light, temperature, salinity, pH or nutrient limitation regimen<sup>8,9</sup>. Nitrogen, and sulfur starvation has proven<sup>10</sup> to induce a vast range of cellular and biochemical response mechanisms such as biomass and lipid enhancement in *Chlamydomonas reinhardtii*, a photosynthetic microalga with biodiesel production potential<sup>11</sup>. Compared with N-deprived *C. reinhardtii* cells, S-deprived cells accumulate more lipid and triacylglycerol (TAG), with elevated cell volumes and growth rates<sup>12</sup>. Hence, it could be suggested that S deprivation strategy could be a useful biochemical engineering method to enhance production of the potentially useful lipids for biodiesel production in *C. reinhardtii*. Fourier Transform Infra-Red (FTIR)

spectroscopy method has recognized as a robust method for rapid identification of lipid and fatty acids in different microalgal strains such as *Chlorella* during nitrogen starvation. It has been established as a new confirmation to more common methods such as fluorescent staining<sup>13,14</sup>, spectroscopic<sup>15</sup> or chromatographic methods<sup>16,17</sup> for lipid determination. In this study a novel analytical method was proposed which combines microalgal biotechnology and analytical chemistry for rapid detection of lipids in normal and S-starved *C. reinhardtii* culture. Mentioned method is based upon the detection of active carbon double bonds, to avoid time consumption. We also propose a new approach to biochemically distinguish both forms of starved and normal cells in *C. reinhardtii* culture using FTIR Spectroscopy. In addition, the microalgal growth rates in normal and starvation media, and the lipid components in the total obtained biomass in each cultivation mode were investigated. The results of this study might be useful in optimization studies scale-up or industrial process as a robust method for lipid identification during biodiesel production procedure.

### MATERIALS AND METHODS

*Microalgal strain, media and cultivation condition*

*C. reinhardtii* (Chlorophyta, Chlorophyceae) was obtained from the Microalgal Culture Collection of Shiraz University of Medical Sciences (MCCS), Shiraz, Iran. In

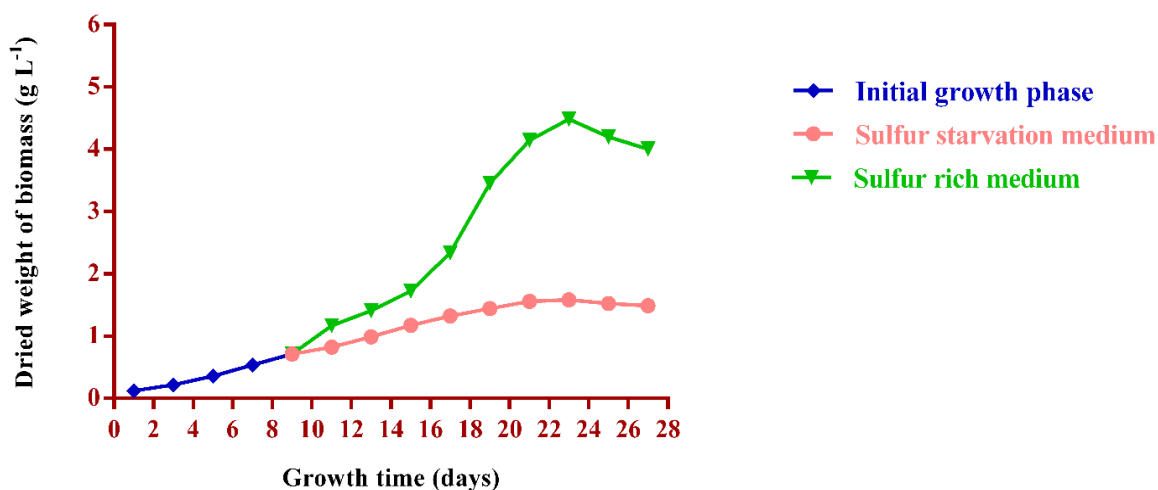


Figure 1: Growth rate of *C. reinhardtii* under sulfur rich and sulfur starvation media in shake flask scale during 28 days of study using dry cell weight method.

Table 1: The observed critical bands in FTIR spectroscopy, the attributed functional groups and the wavenumber ranges ( $\text{cm}^{-1}$ ) obtained from lipid analysis of *C. reinhardtii*.

Observed band in FTIR spectroscopy	Wavenumber range ( $\text{cm}^{-1}$ )
$\nu$ (C=O)	amide I, $1655 \text{ cm}^{-1}$
$\delta$ (N-H)	amide II, $1545 \text{ cm}^{-1}$
$\delta_{\text{as}}$ ( $\text{CH}_2$ )	$1455 \text{ cm}^{-1}$
$\delta_{\text{as}}$ ( $\text{CH}_3$ )	$1455 \text{ cm}^{-1}$
$\nu_s$ (C-O)	$1380 \text{ cm}^{-1}$
The lipid band	$1740 \text{ cm}^{-1}$

the first phase of study (10 days), a sulfur rich medium, BG11<sup>18</sup> was used for microalgal growth and cultivation. After entering the mid logarithmic phase, cell was filtered using a 1 mm filter membrane and then washed two times using an isotonic normal saline solution in aseptic conditions using laminar air flow. Fresh culture medium (100 mL) both from normal and sulfur starved culture medium was added to the obtained biomass. Both experiments were performed in three independent Erlenmeyer flasks. Culture study was performed for another 18 days. All experiments were performed in 500 mL Erlenmeyer flasks at  $25^\circ\text{C}$  in an orbital incubator shaker (PECO, Iran), with agitation rate at 130 rpm and at the light intensity of  $60 \text{ mol m}^{-2} \text{ s}^{-1}$ .

#### Cell growth measurement

The microalgal growth rates were monitored every two days during 28 days of study using dry cell weight method<sup>19</sup>. *C. reinhardtii* cells (5 mL) were isolated aseptically and then centrifuged for 5 min at  $6000 g$ . The obtained cell pellets were washed twice with an isotonic normal saline solution ( $0.09 \text{ g NaCl L}^{-1}$ ) and centrifuged again to remove the possible impurities. After it, the microalgal pellets were dried at  $88^\circ\text{C}$  overnight for cell dry weight determination.

#### Lipid determination

After 28 days of cultivation, the total lipids were extracted and quantified gravimetrically<sup>20</sup>. Briefly, 1 g of *C. reinhardtii* biomass was boiled for 2 min in isopropanol to

inhibit the unwanted lipase activity which reduces the yield of lipid extraction. It was dissolved in a mixture of solvents composed of chloroform-methanol (1:2); and butylated hydroxytoluene (BHT) (0.05% w/v). In next step, the potassium chloride aqueous solution (0.88 w/v), 5 mL of chloroform and 0.8 mL of double distilled water, were added to the previous mixture. After vigorous shaking, the obtained solvent phase was then collected and dried using nitrogen gas. Finally, the extracted lipids were determined and expressed as % w/w in total biomass.

#### FTIR spectroscopy

For FTIR spectroscopy a Bruker, Vertex 70, FTIR Spectrometer (Bruker company, Billerica, Massachusetts, USA) was used. 1.5 mg of dried biomass obtained from each cultivation mode and 150 mg of KBr were used for KBr pellet formation. It was then inserted in the mentioned FTIR spectrometer apparatus equipped with a data acquisition mode based upon two channel mode with 24-bit dynamic range running in parallel way which are integrated to the detector. FTIR spectra were monitored in  $4000\text{--}600 \text{ cm}^{-1}$  wavenumber range and scaled regarding the amide I band. The experiment was performed in triplicate.

#### Statistical analysis

To assess the significance of the obtained results, IBM SPSS software version 22.0 (Armonk, NY: IBM Corp.) and GraphPad prism version 6.00 (GraphPad Software, La Jolla California, USA) were employed. In ANOVA analysis, the statistical different levels at 5% were considered as significant.

## RESULTS AND DISCUSSION

### Impacts of S-deprivation on microalgal biomass and growth rate

To monitor the microalgal growth pattern, dry cell weight methods was exploited. The growth analysis was performed in both normal physiologic conditions and also in S starvation experiment. As depicted in Fig. 1, the initial three days were considered as the lag phase, and after it the logarithmic growth phase was observed in 3<sup>rd</sup> to 10<sup>th</sup> day of cultivation. Both the normal physiologic and starved microalgal cells entered the stationary phase at the same

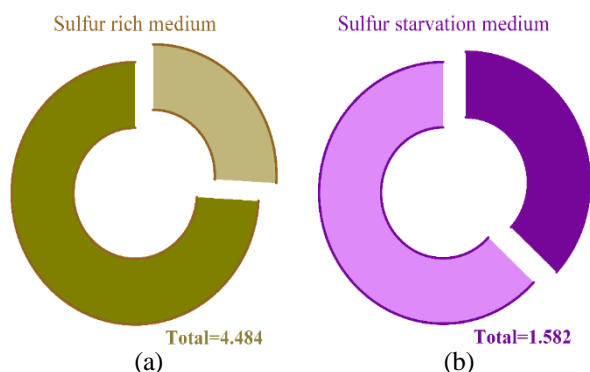


Figure 2: The total lipid content of each identified microalgal strain (% w/w) in the final obtained biomass in sulfur rich (a) and sulfur starved (b) media.

day (day 10). The initial dry cell weight of microalga was found to be  $0.121 \text{ g L}^{-1}$ . After 10 days of primarily culture normal physiologic conditions, the microalgal dry cell weights were reported as  $0.713 \text{ g L}^{-1}$ . After commencing the sulfur deprivation experiment, with adding the fresh BG11 medium, the microalgal growing trend was continued with a characteristic sigmoidal pattern in both cultivation modes. Although, the sulfur rich medium displayed a greater slope in comparison with sulfur starvation mode. During another 18 days of cultivation experiment, trends of cell growth in the nutrient rich culture ( $4.484 \text{ g L}^{-1}$ ) was 283.44% higher than sulfur deficient ( $1.582 \text{ g L}^{-1}$ ) conditions. Findings of this study were in agreement with some previous reports in *C. reinhardtii* about the effects of sulfur starvation on reducing the microalgal biomass production<sup>10</sup>.

#### Impacts of S-deprivation on lipid production

To examine the effects of sulfur limitation on a naturally isolated strain of *C. reinhardtii*, two different culture media with and without sulfur were employed in 28 days of study. As presented in Fig. 1, the total amounts of biomass in sulfur rich and sulfur starved culture media were reached to  $4.484 \text{ g L}^{-1}$  and  $1.582 \text{ g L}^{-1}$ , accordingly. In the same period, the total lipid content in *C. reinhardtii* cells were found to be  $1.163 \text{ g L}^{-1}$  (sulfur rich medium) (Fig. 2a) and  $0.585 \text{ g L}^{-1}$  (sulfur starved medium) (Fig. 2b). On the other word, after 28 days of cultivation, the obtained biomass was composed of 25.94% (in sulfur rich medium) and 36.98% (in sulfur starved medium) lipids. Based upon the results, higher amount of lipid production was observed in sulfur starved medium. Besides, the results indicated the importance of appropriate sulfur levels for biomass and lipid production. In suitable conditions for microalgal growth, various strains normally produce different classes of fatty acids including saturated and unsaturated ones with great industrial, nutritional and pharmaceutical potentials. Unfortunately, the saturated ones are not suitable for biofuel production, although, in stress conditions, the cell tend to increase neutral lipid biosynthesis, mainly as TAGs which are considered as practical candidates for green energy production. Furthermore, nutrient deprivation is assumed to initiate lipid accumulation in various strains microalgae<sup>8</sup>. Here, we also confirmed the increased lipid biosynthesis in sulfur

starved cells of *C. reinhardtii*. Microalgae exhibit a promising potential for biodiesel production<sup>1,21</sup>. Despite having numerous advantages over crop plants, microalgae are associated with a number of technical, biological and economical hindrances that prevent algal biodiesels from effectively replacing petroleum-derived fuels<sup>6</sup>. The predominant biological problem is the low lipid contents displayed by many microalgal species. Under stress conditions, most microalgae direct their metabolism to the production of non-polar lipids, and especially TAG, which are used as storage molecules and rapidly degraded for energy production when optimal conditions arise. The results of the current study also confirmed the possibility of using starvation strategy to achieve higher cell densities for biodiesel production.

#### Lipid detection using FTIR spectroscopy

Microalgae tend to accumulate lipids and especially neutral lipids like triacylglycerol (TAGs) in stress conditions. Chromatography and fluorescent staining using Nile Red are considered as standard methods to investigate the amounts of lipid accumulation in microalgal cell. Although, the mentioned methods have major limitations. They are faced with some technical difficulties, time consuming, and sometimes with unwelcomed sensitivity and efficiency. Based upon this fact, in the current study, we assessed the possibility of using FTIR spectroscopy method as a robust and cheap method with desirable sensitivity and accuracy to examine the occurred changes in carbon allocation patterns during different metabolic and physiologic changes in the *C. reinhardtii* culture medium in both studied situations. As presented in Table (1), FTIR spectroscopy analysis of the studied microalgal strain showed six noticeable changes in absorption bonds in  $2100\text{-}800 \text{ cm}^{-1}$  wavenumber range. In this context, two bands including  $1740 \text{ cm}^{-1}$  standing for  $\nu$  (C=O) or stretching of ester groups which mainly exist in fatty acids and lipids and although the  $1200\text{-}940 \text{ cm}^{-1}$  region accompanying with  $\nu$  (C-O-C) or stretching of carbon-oxygen-carbon groups occurring specilay in glycosylated lipids were of special interest. Moreover, based upon the present biochemical standards and previously performed studies, the observed bands were attributed to special functional groups. Bands were assumed to  $\nu$  (C=O) showing the stretching of amide groups (amide I, ca.  $1655 \text{ cm}^{-1}$ );  $\delta$  (N-H) bending of amide groups (amide II, ca.  $1545 \text{ cm}^{-1}$ );  $\delta_{\text{as}}$  ( $\text{CH}_2$ ) and  $\delta_{\text{as}}$  ( $\text{CH}_3$ ) bending of methyl groups (ca.  $1455 \text{ cm}^{-1}$ );  $\nu_s$  (C-O) for stretching of COO- moiety (ca.  $1380 \text{ cm}^{-1}$ ). The lipid band ( $1740 \text{ cm}^{-1}$ ) compared with amide I band was monitored to determine the relative content of total lipids in normal and S starved microalgal cells (Fig. 3). In both experiments, an elevated lipid: amide I ratio was observed. The mentioned ratio was about 0.3 for both normal and S starved cells respectively at the beginning of the cultivation. After 12 days of additional cultivation, the microalgal cells grown in sulfur rich medium showed no significant increase in lipid: amide I band ratio in the first six days (10<sup>th</sup>-16<sup>th</sup> day of cultivation) followed by a vast increase up to 0.85 in 23<sup>rd</sup> day of experiment (Fig. 3). The S starved cells displayed more increase in the mentioned ratio and reached

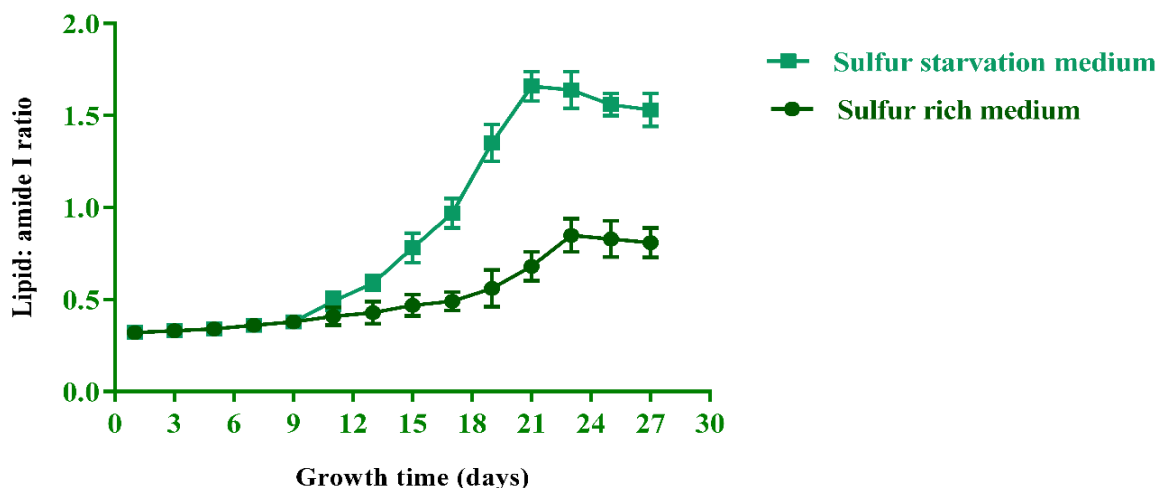


Figure 3: The observed changes in the lipid: amide I ratio in *C. reinhardtii* cells during 28 days of cultivation in sulfur rich and sulfur starved media.

up to 1.66 from just ten initial days of starvation study (10<sup>th</sup>-20<sup>th</sup> day of cultivation). After reaching to its maximum values, the lipid: amide I ratio seem showed a plateau in 22<sup>nd</sup>-28<sup>th</sup> day of starvation experiment (Fig. 3). The observed trend implied that there is a possible limitation in lipid accumulation amounts in *C. reinhardtii* cells due to S starvation. The results of FTIR spectroscopy analysis in normal and S starved culture media confirmed an elevated level for lipid: amide I ratio during the time. Exploiting the conventional gravimetric method for lipid analysis confirmed the described changes in ratio because of the amplified lipid biosynthetic activity. Hence, finding of the current study, proved the quick metabolic changes due to sulfur deprivation in *C. reinhardtii* cells. More importantly, it demonstrated the capability of using FTIR spectroscopy as a robust and valuable method for rapid and sensitive determination of lipids. Some researchers have evaluated the possibility of using FTIR spectroscopy as a versatile tool to determine the occurring changes in lipid contents in different microalgal cells during nutrient starvation including nitrogen or phosphorous limitation<sup>22-24</sup>. In the present study, we also demonstrated the possibility of using FTIR spectroscopy method to identify the influence of sulfur starvation *C. reinhardtii* cells in shake flask experiment. Moreover, we have validated the usefulness of this newly emerged method as a versatile and acceptable way for identification of lipids in comparison with the conventional gravimetric methods. Additionally, the FTIR method could determine the changes in lipid contents earlier than other mentioned methods for lipid determination. Because, FTIR monitors the molecular changes due to nutrient starvation during the cultivation experiment.

## CONCLUSION

Our study confirmed that FTIR spectroscopy could be employed as a versatile and reliable method for quantitative measurement of lipids and other related biological macromolecules in microalgal culture. The obtained results from FTIR analysis were confirmed using gravimetric method for lipid determination. Besides, we showed the usefulness of nutrient deprivation strategy to

increase the lipid production yields for biodiesel production and other applications.

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