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Using FTIR spectroscopy for rapid determination of lipid accumulation in response to nitrogen limitation in freshwater microalgae

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ABSTRACT

In this study Fourier transform infrared micro-spectroscopy (FTIR) was used to determine lipid and carbohydrate content over time in the freshwater microalgae *Chlamydomonas reinhardtii* and *Scenedesmus subspicatus* grown in batch culture in limiting concentrations of nitrogen (N). Both algae exhibited restricted cell division and increased cell size following N-limitation. FTIR spectra of cells in N-limited media showed increasing lipid:amide I and carbohydrate:amide I ratios over time. The use of lipid- and starch-staining dyes confirmed that the observed ratio changes were due to increased lipid and carbohydrate synthesis. These results demonstrate rapid metabolic responses of *C. reinhardtii* and *S. subspicatus* to changing nutrient availability, and indicate the efficiency of FTIR as a reliable method for high-throughput determination of lipid induction.

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1. Introduction

Biofuel production from renewable sources is widely considered to be one of the most sustainable alternatives to fossil fuels and a viable means to combat the environmental impact of fossil fuels on global warming (Hill et al., 2006). Microalgae have long been proposed as a potential renewable fuel source (Benemann et al., 1977; Oswald and Golueke, 1960) and extensive research funded by the US Department of Energy between 1978 and 1996 clearly validated this potential of microalgae (Sheehan et al., 1998). Microalgae are of particular interest as a sustainable source of biodiesel due to their ability to synthesise and accumulate significant quantities of lipids – up to approximately 80% of cell dry weight in some species (Chisti, 2007; Griffiths and Harrison, 2009; Sheehan et al., 1998). Under ideal growth conditions, many algal species produce saturated and unsaturated fatty acids which have potential nutritional value but are less ideal for biofuels, however, the synthesis of neutral lipids in the form of triacylglycerol can be induced in many species, for example under stress conditions, and these lipids are suitable precursors for biodiesel production (Hu et al., 2008; Miao and Wu, 2006).

Previous studies have demonstrated that nutrient stress conditions, such as nitrogen (N) starvation, phosphorus (P) starvation, urea limitation, and iron supplementation, can induce significant increases in lipid content in many microalgae species (Converti et al., 2009; Hsieh and Wu, 2009; Illman et al., 2000; Li et al., 2008; Liu et al., 2008; Pruvost et al., 2009; Rodolfi et al., 2009; Takagi et al., 2000; Tornabene et al., 1983). Commonly a 2- to 4-fold increase in lipid content has been observed in N-starved freshwater or marine microalgae such as in *Chlorella* and *Nannochloropsis* species (Illman et al., 2000; Rodolfi et al., 2009). Such studies therefore provide insights into the means by which mass culture conditions may be manipulated to increase oil production for biofuel applications. Despite significant lipid accumulation, microalgal biomass during nutrient stress is often reduced compared to cells grown under non-stressed conditions. A key aim of the algal biofuel research field is therefore to identify strains and mass culture conditions that provide high lipid yield coupled with high biomass productivity, and therefore increase overall lipid productivity.

Increasingly efficient and rapid methods of lipid detection are desirable. Methods of lipid detection include chromatographic-based separation and quantification of lipid classes (Krank et al., 2007) that require solvent extraction and fractionation but which are time-consuming. Another frequently used method makes use of the neutral lipid-staining fluorescent dye Nile Red. Nile Red can be used to image neutral lipid accumulation within cells or to quantify lipid biosynthesis by fluorescence spectroscopy (Cooksey et al., 1987; Elsey et al., 2007). However, Nile Red-based methods can also be time-consuming, are not as quantitative as chromatographic methods, and there may be variation in the efficiency of Nile Red accumulation into some algal species. An alternative approach is the use of Fourier transform infrared (FTIR) micro-spectroscopy. This is a method for whole organism analysis using intact cells which involves the measurement of infrared absorption in relation to a range of molecular vibrational modes

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(Murdock and Wetzel, 2009). Specific molecular groups can be identified by their absorption bands, allowing macromolecules (including proteins, lipids, carbohydrates, and nucleic acids) to be quantified.

A few studies have begun to demonstrate the potential of FTIR as a tool to identify changes in cellular components, including lipids, in response to a nutrient stress, such as low-N (Giordano et al., 2001; Heraud et al., 2005; Stehfest et al., 2005) and low-P (Dean et al., 2008; Sigee et al., 2007). In this study, we have used FTIR to investigate the effects of N-limitation on *Chlamydomonas reinhardtii* and *Scenedesmus subspicatus* grown in batch culture. We have examined how these microalgae species increase lipid and carbohydrate content following two different N-limitation stress treatments, and have validated the effectiveness of FTIR as an efficient method for determining lipid induction by comparison with the Nile Red method. Furthermore, this FTIR study extends on earlier observations of N-stressed algae (Giordano et al., 2001; Stehfest et al., 2005) by analysing the molecular response to N-availability with changes in growth phase during the batch culture cycle.

2. Methods

2.1. Cell cultivation

C. reinhardtii (CCAP 11/32A) and *S. subspicatus* (CCAP 276/20) were obtained from the Culture Collection of Algae and Protozoa, UK. Prior to the main experiment, algae were cultured in Jaworski's Medium (JM) to log phase, then filtered through a 1 μm filter membrane, washed in deionised water (to remove excess culture medium) and re-suspended in deionised water. An algal inoculum of 2 ml was then added to the experimental culture vessels (250 ml conical flasks) containing 200 ml of culture medium. The starting cell density was the same in all three treatments; 0.024×10^6 cells ml^{-1} for *C. reinhardtii*, and 0.18×10^6 cells ml^{-1} for *S. subspicatus*. The culture media consisted of unmodified JM with N (as NO_3) at concentrations of 19.6 mg l^{-1} (high-N culture) and modified JM, with N at concentrations of 3.0 mg l^{-1} (intermediate-N) and 0.8 mg l^{-1} (low-N). Each treatment consisted of triplicate flasks. Cultures were grown under a 16 h:8 h light dark cycle, at 25 °C, at a photon flux of approximately 150 μmol m^{-2} s^{-1}, on a orbital shaker at 120 rpm.

Cells were also grown in P limited media as a positive control, in order to validate the Nile Red staining (see below) of lipids. The culture media consisted of unmodified JM with P (as PO_4) at concentrations of 5.9 mg l^{-1} (high-P) and modified JM with P at concentrations of 0.05 mg l^{-1} (low-P). All other conditions were as described above.

2.2. Cell growth, nutrient, chlorophyll content, and protein analysis

Samples of cells and culture media were taken from the replicate flasks at regular intervals throughout the experiment. For cell counts, 1 ml of each algal culture was removed and preserved with 100 μl of Lugols' iodine prior to counting using a Sedgwick rafter slide and light microscope. For chlorophyll-a and nutrient analysis 5 ml of sample was filtered through a Whatman 0.45 μm cellulose acetate filter, with the deposit analysed for chlorophyll-a and the filtrate for NO_3–N and PO_4–P quantification. Chlorophyll-a was determined as described by Jespersen and Christoffersen (1987) following ethanol extraction. NO_3–N and PO_4–P in the culture media were analysed on a Skalar Sans Plus autoanalyser using standard methodology (Skalar Analytical). Cell volume was calculated by approximating the cells to a prolate spheroid for *S. subspicatus* and to a sphere for *C. reinhardtii* using the formulae given by Hillebrand et al. (1999). Total protein content was determined by Bradford assay following protein extraction by resuspending a frozen cell pellet in lysis buffer (50 mM Tris–HCl pH 8.0, 2% SDS, 10 mM EDTA, Sigma protease inhibitor mix) and performing three freeze–thaw cycles then incubating the cells for a further 1 h at room temperature before centrifugation at 13,000*g* for 20 min at 4 °C to remove cell debris.

2.3. FTIR spectroscopy

For FTIR spectroscopy a 0.5 ml sample was taken from each replicate flask for each treatment, mixed, centrifuged, the supernatant removed and the cells re-suspended in approximately 100 μm of distilled water 30 μl of which was then deposited on an 96 well silicon microplate, and oven-dried at 40 °C for 30 min. The plate was placed in a HTS-XT high-throughput microplate extension and FTIR spectra collected using a Bruker equinox 55 FTIR spectrometer, equipped with a mercury–cadmium–telluride detector cooled with liquid N_2. Spectra were collected over the wavenumber range 4000–600 cm^{-1}. Each sample was analysed in triplicate. Spectra were baseline corrected using the automatic baseline correction algorithm and were scaled to amide I. Principal component analysis (PCA) was performed using MATLAB.

2.4. Fluorescence microscopy and quantification

Neutral lipid-staining using Nile Red was performed essentially as described previously (Cooksey et al., 1987; Elsey et al., 2007). Cells were stained with 0.25 μg ml^{-1} Nile Red (9-diethylamino-5H-benzo[α]phenoxazine-5-one) (Invitrogen) solution in acetone and imaged by epi-fluorescence microscopy with a Leica DMR microscope using a 490 nm excitation/530 nm emission wavelength filter cube (Leica Microsystems). To obtain images of Nile Red fluorescence in conjunction with chlorophyll autofluorescence, Nile Red-stained cells were imaged using a broad-range UV excitation filter cube (340–380 nm excitation/>425 nm emission wavelength; Leica Microsystems). For starch-staining, cells were stained with Safranin O (Klut et al., 1989). Cells were stained with 0.02% Safranin O (Sigma) and were imaged by epi-fluorescence microscopy using a 436 nm excitation/480 nm emission wavelength filter cube (Chroma Technology). Relative fluorescence intensity of Nile Red and Safranin O staining was quantified on a fluorescence spectrometer (Jasco FP750) using 530 nm excitation and 575 nm emission (Nile Red), and 435 nm excitation and 480 nm emission wavelengths (Safranin O).

3. Results and discussion

3.1. Impact of N-limitation on algae biomass

The impact of N-limitation was examined on two biomass indicators, cell density and cell size. *C. reinhardtii* and *S. subspicatus* cells were grown in batch culture under N-replete and two N limiting conditions – an intermediate-N (3 mg l^{-1}) and low-N (0.8 mg l^{-1}) treatment. The high-N treatment (19.6 mg l^{-1}) was typical of a high-nutrient growth medium, in this case JM, and served as a reference for comparison with the N limiting experiments.

3.1.1. Cell density analysis

In all N treatments *C. reinhardtii* and *S. subspicatus* cell growth occurred with no distinctive lag phase but growth increased rapidly for the high-N treatments (Fig. 1a and b). All *C. reinhardtii* treatments entered stationary phase at the same time (day 21) although with significantly different cell counts, with the high-N

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