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Mass spectrometry-based metabolomics of value-added biochemicals from *Ettlia oleoabundans*

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ABSTRACT

Microalgae are promising feedstocks for biofuels and other value-added biochemicals. *Ettlia oleoabundans*, a non-model green microalga, produces increased levels of key precursors for lipid-based biofuels under nutrient stress conditions. Here, we introduce a method for analysis of hydrophobic metabolites in *E. oleoabundans* and apply this method to microalgae grown under different nutrient conditions. We combined targeted analysis using gas chromatography/tandem mass spectrometry (GC–MS/MS) with targeted and untargeted metabolomics using liquid chromatography-quadrupole-time-of-flight mass spectrometry (LC-Q-ToF MS). Our method allows simultaneous detection of compounds from major lipid families, and other value-added biochemicals using less biomass, hence allowing analysis of larger number of samples to be processed simultaneously. In this study, we showed that 202 metabolites were enhanced or depleted under nutrient stress and annotated 163 of these species. A complex regulation of different classes of metabolites under nutrient stress was observed. Overall, triacylglycerols and sulfoquinovosyldiacylglycerols were accumulated, chlorophylls were depleted, and diacylglycerols were differentially regulated at the molecular level when grown under limited nutrient conditions. The method that we developed will serve as a template for targeted and untargeted metabolomics in *E. oleoabundans* and other non-model microalgae to understand biochemical regulations under different experimental conditions.
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1. Introduction

The Department of Energy (DoE) Algal Biofuels Technology roadmap fully engages algal research and development, and addresses challenges associated with producing algal biofuels at commercial scale [1]. On this track, it is critical to adjoin all environmental sustainability components algae offers with prospects of economical feasibility to compete with petroleum based fuels [2]. To achieve commercially applicable rates of return on algal biofuels, economic feasibility models suggest that there is a need to produce value-added chemicals within an integrated biorefinery scheme [3–5]. This requires targeting not only lipids as biodiesel feedstock, but also other biomolecules having higher value per dry biomass weight with potential applications such as food additives, health supplements, and pharmaceuticals [6]. Key examples of value added algal products include phytosterols [7], carotenoids [8], and vitamins [9]. Phytosterols are steroid alcohols that function in cell proliferation and signal transduction in microalgae [10]. The commercial interests in algal phytosterols are due to their potential for lowering total and low-density lipoprotein cholesterol [7]. Carotenoids, such as β-carotene, lycopene, and

Abbreviations: C–MS/MS, gas chromatography- tandem mass spectrometry; LC-Q-ToF MS, liquid chromatography- quadrupole-time-of-flight mass spectrometry; DoE, U.S., Department of Energy; ASP, Aquatic Species Program; N−, nitrogen deplete; N+, nitrogen replete; FAs, fatty acids; PGs, phosphatidylglycerols; PEs, phosphatidylethanolamines; PIs, phosphatidylinositols; MGMGs, monogalactosylmonoacylglycerols; DGDGs, digalactosyldiacylglycerols; SQDGs, sulfoquinovosyldiacylglycerols; DAGs, diacylglycerols; TAGs, triacylglycerols; MeOH, methanol; $CHCl_3$, chloroform; H_2O, water; IPA, isopropanol; HPLC, high performance liquid chromatography; ESI, electrospray ionization; +ESI, positive mode; −ESI, negative mode; N_2, nitrogen gas; MPP, Mass Profiler Professional; FDR, false discovery rate; MS/MS, tandem mass spectrometry; 13C12-PCB-52, ^{13}C-polycholinated biphenyl; SRM, selected reaction monitoring; SIM, selected ion monitoring; PBRs, photobioreactors; PAs, phosphatidic acids; PSs, phosphatidylserines; PCs, phosphatidylcholines; MGDGs, monogalactosyldiacylglycerols; LC-Q-ToF MS/MS, liquid chromatography- quadrupole-time-of-flight tandem mass spectrometry; MAGs, monoacylglycerols; PSI, photosystem I; PSII, photosystem II; NMR, nuclear magnetic resonance.

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zeaxanthin, have wider consumer applications and are utilized in food coloring/nutrition and cosmetics [6]. Vitamins C and E are also among the high-value products pursued from algae [11]. Vitamin E, synthesized only by photosynthetic organisms, is a lipid-soluble metabolite with antioxidant and radical scavenging activities [11].

Expanding on value-added biochemical portfolios often requires exploration and utilization of novel algae species, including unique non-model species. Among >3000 species of algae isolated by the DoE Aquatic Species Program (ASP), fifty-one varieties were characterized as potential high-value strains [12]. However, cellular and molecular characterization of these non-model species still lags behind established model organisms such as *Chlamydomonas reinhardtii*, *Chlorella variabilis*, *Micromonas*, *Nannochloropsis gaditana*, *Nannochloropsis oceanica*, *Ostreococcus tauri*, *Phaeodactylum tricornutum*, *Thalassiosira pseudonana*, and *Volvox carteri* [13]. In this work, we provide the first comprehensive metabolomics analysis of the non-model green microalgae species *Ettlia oleoabundans* (formerly known as *Neochloris oleoabundans*). *E. oleoabundans* is a Chlorophyceae class green microalga with unique characteristics. Originally isolated from a sand dune ecosystem, it can be grown both in freshwater [14] and seawater [15,16] suggesting a strong adaptability to different growth conditions and is a high lipid producer [17–20]. Generating and analyzing high throughput metabolomics data will assist in improving our knowledgebase in the novel non-model microalgae domain and gradually enable *E. oleoabundans* to be considered as a candidate model species.

Here, we provide a step-by-step methodology, from sample preparation to data analysis, for untargeted and targeted metabolomics [21–23] of *E. oleoabundans* utilizing liquid chromatography quadrupole-time-of-flight mass spectrometry (LC-Q-ToF MS) and gas chromatography tandem mass spectrometry (GC–MS/MS). Nutrient variations, especially nitrogen, are known to affect primary and secondary metabolisms in algae and were used in this study as a strategy to optimize yields of lipids and other value-added biochemicals [24–27]. For this reason, we focused on comparative analysis of nitrogen deplete (N−) and nitrogen replete (N+) growth conditions to test the metabolomic coverage we can achieve with the method we developed. Our protocols allowed simultaneous detection and comparative analysis of major lipid families, including fatty acids (FAs), phosphatidylglycerols (PGs), phosphatidylethanolamines (PEs), phosphatidylinositols (PIs), monogalactosylmonoacylglycerols (MGMGs), digalactosyldiacylglycerols (DGDGs), sulfoquinovosyldiacylglycerols (SQDGs), diacylglycerols (DAGs) and triacylglycerols (TAGs) in addition to key biofuel feedstock lipids and other hydrophobic metabolites such as chlorophylls, terpenes, phytosterols, and vitamins. Following this methodology, we analyzed the changes in metabolite composition in *E. oleoabundans* under N− conditions and found that TAGs and plant specific lipids such as SQDGs strongly accumulated, whereas chlorophylls and DGDGs were depleted in N−. The broad coverage of information obtained at the molecular level paves the way towards experimental designs where molecule-specific interpretation of the changes in metabolite composition will lead to detailed understanding of the metabolic networks.

2. Materials and methods

2.1. Materials

LC-MS grade methanol (MeOH) and HPLC grade isopropanol (IPA) were purchased from EMD Millipore (Billerica, MA). Formic acid, ammonium formate and ammonium hydroxide were purchased from Sigma Aldrich (St. Louis, MO). HPLC grade chloroform ($CHCl_3$) was purchased from Honeywell Burdick & Jackson (Muskegon, MI). Distilled water was provided by a Milli-Q purification system. A detailed list of all standards purchased for LC-Q-ToF MS and GC–MS/MS are presented in supporting information. Glass beads (0.5 mm) were purchased from Bertin Technologies (Montigny, France). Stock solutions of the standards were prepared in $CHCl_3$ and stored at −80 °C.

2.2. Microalgae seeding, growth, and harvesting

E. oleoabundans (UTEX 1185) was obtained from Culture Collection of Algae at The University of Texas at Austin. Algal cultures were maintained and grown axenically with Modified Bold 3N growth medium [28]. For each experimental condition, five biological replicates were used. Cultures were grown in 500 mL glass photobioreactors (PBRs) operated at room temperature (25 °C ± 2 °C), and with a 14:10 h light:dark cycle of exposure to fluorescent light (32 W Ecolux, General Electric, Fairfield, CT) at a photosynthetic photon flux density of 110 μmol-photon m^{-2} s^{-1}. PBRs were continuously aerated with sterile, activated carbon filtered air at a flow rate of 200 mL/min using a mass flow controller (Cole-Parmer Instrument Company, IL). No additional CO_2 was provided to air supply.

For experimental conditions, *E. oleoabundans* was grown under nitrogen replete (N+) (7.1 mM, 603.46 μg/mL), and nitrogen deplete (N−) (0.71 mM, 60.35 μg/mL) conditions as the total [NO_3^-] was adjusted in the growth media. Five biological replicates, harvested from different PBRs, were used for each condition. The nitrate concentration of culture media was determined daily by NECi Microplate Nitrate Kit (Cat # M-NTK-301, Lake Linden, MI). Algal growth was monitored by the optical density measured daily at 730 nm using a microwell-plate reader (SpectraMax i3, Molecular Devices, CA). The microalgae were harvested, centrifuged, decanted, and frozen at −80 °C until further analyses. Different aliquots, from each biological replicate, were prepared for biomass calculations, determination of total lipid composition and metabolite extraction. The biomass corresponding to each sample is reported in Table S1.

2.3. Metabolite extraction

Approximately 30–60 mg of microalgae biomass was spiked with 700 μL of MeOH containing squalene-*d*6, estrone-*d*4 and oleic acid-*d*9. The final concentrations of standards were 300–660 ng/mL for squalene-*d*6 and estrone-*d*4 and 8–14 μg/mL for oleic acid-*d*9 depending on the biomass of each sample. These isotope-labeled standards were used to calculate extraction efficiencies. The percent recoveries are reported in Table S2. Glass beads (250 mg) were added to this solution and vortexed five times (1 min each, with 1 min incubation on ice between the cycles). This suspension was transferred into a glass dounce homogenizer and the beads were rinsed with additional 400 μL of methanol by vortexing for an additional cycle; all the extracts were combined. This solution was then mixed with $CHCl_3$ and H_2O (MeOH/$CHCl_3$/H_2O, 1:2:1), and homogenized on ice (×40 strokes). After homogenization, the solution was centrifuged at 4 °C, 3000 ×*g* for 40 min, and the organic layer was separated from the aqueous layer and the protein disk. To improve extraction efficiency, the remaining protein disk and the aqueous layer was extracted two more times using additional $CHCl_3$. All organic layers were combined and evaporated to complete dryness. The remaining metabolite residues were normalized based on biomass and resuspended in $CHCl_3$ accordingly. The normalized concentration was 9.20 mg of biomass/mL. The hydrophobic metabolite extracts were stored in −80 °C until analysis.

2.4. Sample preparation and analysis

For untargeted profiling and targeted analysis of plant lipids, FAs and DAGs, original solutions were used. For targeted analysis of TAGs, a 20-fold dilution was carried out for each sample. The injection volume was 30 μL corresponding to metabolites extracted from 0.28 mg or 0.014 mg (for TAG analysis) biomass before extraction.

2.5. LC-Q-ToF MS analysis of extracts

Analyses were carried out using an HPLC-Q-ToF MS system equipped with an Infinity 1260 HPLC (Agilent Technologies) consisting

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