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Characterization of plant carbon substrate utilization by *Auxenochlorella protothecoides*

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ABSTRACT

Algae hold great potential as a source of renewable fuel due to their ability to produce refinery-compatible diesel and jet fuel precursors. Significant effort has been made to maximize productivity under photoautotrophic growth conditions; however, little progress has been made to discover and understand reduced carbon assimilation pathways or enzymatic degradation of complex carbon substrates in algae. We purport that utilization of plant-based carbon substrates in addition to photosynthesis (mixotrophic growth) for biochemical assimilation into biomass, biofuels, and bioproducts, can increase cultivation productivity and improve the economic viability of algal-derived biofuels. Herein we report that a freshwater production strain of microalgae, *Auxenochlorella protothecoides* UTEX 25, is capable of directly degrading and utilizing non-food plant substrates, such as switchgrass, for cell growth. Glycome profiling of plant substrates before and after addition to *A. protothecoides* cultures demonstrates the utilization of xyloglucans. Genomic, proteomic and transcriptomic analyses revealed the identity of many enzymes that are hypothesized to be involved in complex carbohydrate degradation, including several family 5 and 9 glycosyl hydrolases. This work paves the way for future designer engineering of plant-carbon utilization to further improve productivity of algal production strains.

1. Introduction

Auxenochlorella protothecoides UTEX 25, a freshwater microalga, produces large quantities of lipids [1–3], that can be directly converted to fuel. *A. protothecoides* can grow mixotrophically on simple sugars to increase biomass productivity [3], but feeding sugar to algae is not economically viable. Furthermore, adding sugar monomers to open ponds will inevitably cause rampant contamination by heterotrophic organisms. A potential cost-effective and energy-efficient alternative is the utilization of raw or minimally-treated (e.g., acid-treated, heat-treated) lignocellulosic feedstocks: trees, grasses, and agricultural residues [4]. These feedstocks are expected to increase in abundance over time which would further lower cost [5]. Whereas current technology allows these plant substrates to be processed into alcohols with *low* energy density, using these substrates as feedstocks for algae converts them to *high* energy density algal lipids with highly reduced carbon chains compatible with existing transportation fuel infrastructure (e.g., jet fuel, diesel, and gasoline). A recent investigation into hydrothermal liquefaction of blended pine-microalgal feedstocks suggests that blends of lignocellulosic and algal feedstocks may improve overall yields [6]. It is proposed that the synergistic effect of amine chemistry on lignocellulosic biopolymers results in the observed increase in conversion efficiency. This would allow for direct processing of the mixotrophic cultures with downstream production enhancements.

The extent to which algae can degrade the three polymers present in lignocellulose (cellulose, hemicelluloses, and lignin) is not known. A previous study has shown that *Chlamydomonas reinhardtii* is capable of degrading cellulose [7]. However, the degradation of complex lignocellulose has not been explored. Potential glycosyl hydrolases, enzymes that hydrolyze the glycosidic bonds in glycans such as cellulose and hemicelluloses, and their associated domains can be found in the deposited genome and transcriptome sequences of algae; however, no algal glycosyl hydrolases involved in the deconstruction of plant substrates have been identified. In the current study, we present the first

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example of algae degradation and utilization of untreated plant substrate, the putative genetic and molecular mechanism(s) behind this degradation, and identify potential glycosyl hydrolases that may be involved in plant deconstruction.

2. Materials and methods

2.1. Cultivation

Auxenochlorella protothecoides UTEX 25 was obtained from the UTEX Culture Collection of Algae (https://utex.org/). Wild-type *Chlamydomonas reinhardtii* (CC2677) was obtained from *Chlamydomonas* Genetic Center. Cultures were maintained on Sueoka's high salt (HS) media [8] on agar plates under constant light at room temperature. Cultures were replated on HS as necessary to maintain culture viability. For carboxymethylcellulose (CMC) degradation studies, cells were plated on HS media agar plates with 0.1% w/v CMC (Sigma) and grown in constant light or constant dark for 1 week. Culture plates were flooded with 0.2% w/v Congo Red (Sigma) in water, incubated for 20 min, decanted, and then destained with repeated washings of 1 M NaCl to determine the degree of CMC degradation.

In growth with plant substrate experiments, plant matter (raw *Pinacum virgatum* (switchgrass), 25% H_2O_2 pretreated switchgrass, ionic liquid pretreated switchgrass, raw *Eucalyptus grandis* (eucalyptus), ionic liquid pretreated eucalyptus, untreated disintegrated *Betula pendula* (silver birch) wood chips, soda-pulped disintegrated silver birch wood chips, liquid hot water pretreated *Zea mays* (corn) stover, ammonia fiber expansion (AFEX) pretreated eucalyptus top portion, raw *Solidago canadensis* (goldenrod) biomass, and extractive ammonia fiber expansion (EAFEX) pretreated corn stover) (all biomass provided to S. Pattathil courtesy of Idaho National Laboratory and Bruce Dale, Great Lakes Bioenergy Research Center, Michigan State University) was added to HS media. For initial glycomic analysis, triplicate 50 mL cultures of HS media with and without (control) 0.2% w/v plant substrate and with and without (control) *A. protothecoides*, inoculated from a liquid culture at an algae cell OD_{750} of 0.02, were grown at room temperature on a shaker in a 12/12 h light/dark cycle at ~80 μmol/$m^{-2}s^{-1}$ light intensity. Triplicate liquid samples of 50 mL were shipped to the University of Georgia Complex Carbohydrate Research Center for glycome analysis.

Hemicellulose (xylan and xyloglucan) enriched extracts were extracted from plant substrates with 4 M KOH as previously described [9]. For hemicellulose analyses, triplicate 100 mL cultures of HS media with and without (control) 0.01 g xylan extracts of raw switchgrass, ionic liquid pretreated switchgrass, 25% H_2O_2 pretreated switchgrass, raw eucalyptus, and ionic liquid pretreated eucalyptus with and without (control) *A. protothecoides* at a starting OD_{750} of 0.037 were grown in constant dark or constant minimal light (~25 μmol·m^{-2}·s^{-1}) and constant shaking at 22 °C for two weeks. 50 mL of each of these cultures was sent for glycome analyses. The remaining culture volume was centrifuged, and algae pellets were stored at −80 °C for proteomic analyses.

To examine growth with raw switchgrass, triplicate 50 mL cultures of *A. protothecoides* inoculated at around 4×10^5 cells/mL were grown with and without (control) switchgrass (0.2% w/v) at room temperature on a shaker in a 12/12 h light/dark cycle at ~80 μmol·m^{-2}·s^{-1} light intensity. Cell density was determined daily for all samples with a hemocytometer. All experiments were performed in triplicate and the standard deviations and a significance value (*p*-value) of the three biological replicates were determined. After 2 weeks of growth, culture samples were filtered to remove plant debris for metabolite 1D ^{1}H NMR analysis and fatty acid methyl ester (FAME) analysis for determination of algal lipids. Specific growth rate of flask cultures was calculated with the following equation: $\mu = \ln(N_2/N_1)/(t_2 - t_1)$, where μ is the specific growth rate, and N_1 and N_2 are the biomass at time 1 (t_1) and time 2 (t_2), respectively during the exponential growth phase.

For transcriptomic analysis, 50 mL cultures of HS with *A. protothecoides* at OD_{750} of 0.03 were grown in triplicate with and without (control) 0.1% w/v CMC (Sigma) in flasks under constant light or constant dark. After one week of growth, cells were pelleted and stored at −80 °C prior to RNA extraction and sequencing.

2.2. Metabolite 1D ^{1}H NMR

Analysis of cellulose and CMC conversion was conducted on an Avance 400 MHz NMR spectrometer (Bruker). Triplicate samples of HS media with switchgrass or CMC alone (control), algae alone (control), and switchgrass or CMC with algae, were incubated in constant light and shaking for one week. Cells and insoluble biomass were pelleted by centrifugation at 14,000 × *g* for 3 min and the supernatants were diluted by addition of 20% v/v D_2O. Trimethylsilylpropionic acid (Sigma) was used as an internal standard at 1 g/L. Water suppression was conducted using the Watergate W5 pulse sequence with double gradient echo [10], with a d1 relaxation time of 10 s, 4 dummy scans and 8 sample scans.

2.3. FAME analysis

Lipids were determined as FAME by gas chromatography coupled with flame ionization detection (GC/FID) according to Van Wychen et al. [11]. Acid-catalyzed transesterification to quantify the total fatty acids in the biomass was performed by treating 5–10 mg of freeze-dried algae biomass (from triplicate cultures) containing 25 μL of 10 mg/mL methyl tridecanoate (C13:0ME) as the internal standard with 200 μL of chloroform:methanol (2:1, v/v) and 300 μL of 0.6 M HCl:methanol. The samples were heated at 85 °C for 1 h and FAMEs were back extracted with 1 mL of hexane. The hexane extracts were analyzed with an Agilent 7890A Series GC/FID. 2 μL injections at a 10:1 split ratio were loaded onto a DB-WAX column (30 m length × 0.25 mm inner diameter × 0.25 μm film thickness (Agilent Technologies, Santa Clara, CA)). Helium was used as the carrier gas at a flow rate of 1 mL/min. Initial column temperature was 100 °C. Then, the temperature was ramped to 200 °C at 25 °C/min and held for 1 min and again ramped to 242 °C at 1.5 °C/min and held for 1 min (35 min total). The inlet and the detector temperatures were 250 and 280 °C, respectively. Chromatographic signals were matched to a GLC 461C 30-component FAME standard mix (Nu-Chek Prep, Inc., Elysian, MN), and FAME quantification was performed by internal calibration using C13:0ME as the internal standard.

2.4. Glycome profiling

To determine the type of cell-wall glycans in plant substrates before and after *A. protothecoides* growth, glycome profiling was performed as previously described [9]. Briefly, sequential extracts of the Alcohol Insoluble Residues (AIR) from various plant biomass samples was completed using increasingly harsh reagents to isolate cell wall components on the bases of the relative tightness with which they are integrated onto the cell walls followed by screening of these extracts with a comprehensive suite of cell wall glycan directed monoclonal antibodies (mAbs) that can monitor glycan epitopes comprised in most major non-cellulosic matrix glycans. The comprehensive suite of mAbs employed in glycome profiling were procured from laboratory stocks (CCRC, JIM and MAC series) at the Complex Carbohydrate Research Center (available through CarboSource Services; http://www.carbosource.net) or from BioSupplies (Australia) (BG1, LAMP).

2.5. Proteomic analysis

Single pellets (one per treatment) were shipped to Kendrick Labs (Madison, WI) for analysis by 2D gel electrophoresis. Two-dimensional electrophoresis was performed according to the carrier ampholine