Algal Research 13 (2016) 311–317

Contents lists available at ScienceDirect

Algal Research

journal homepage: www.elsevier.com/locate/algal

Characterization of *Picochlorum* sp. use of wastewater generated from hydrothermal liquefaction as a nitrogen source

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ARTICLE INFO

Article history:
Received 1 July 2015
Received in revised form 6 October 2015
Accepted 20 November 2015
Available online xxxx

Keywords:
Algal biofuels
Hydrothermal liquefaction
Nutrient recycling
Proteomics

ABSTRACT

Picochlorum sp. strain *SENEW3* is a halotolerant green alga with high growth rates and the ability to utilize organic nitrogen sources including wastewater generated from hydrothermal liquefaction (HTL-WW). *Picochlorum* acclimates to the presence of HTL-WW. In a photosynthesis/respiration rate test, *Picochlorum SENEW3* showed a stress response to HTL-WW additions in a dose-dependent manner while cells pre-grown with HTL-WW had a greatly reduced response to additions. A quantitative proteomics tool, iTRAQ, was applied to assess *Picochlorum* global proteome changes in response to HTL-WW as a nitrogen source. From this approach, a total of 350 different proteins were identified across 2 biological replicates which were significantly up-regulated or down-regulated (average ratio of more than 1.2 or less than 0.8, at least one p-value of <0.05). Protease and oxidative stress enzymes were notably up-regulated. An aminopeptidase enzyme assay showed that, compared to controls, cells grown with 0.1% (vol) HTL-WW had 2.1-fold higher protease activity. An ascorbate peroxidase assay showed an 8.6-fold increase in exponential cells grown with 0.1% HTL-WW compared to controls. This study provides insights into the development of microalgae for algal biofuel production using HTL wastewater recycling [1].

1. Introduction

One of the biggest challenges facing the large-scale utilization of algae-based biofuels is to identify sources for the essential nutrients (mainly nitrogen and phosphorus) required for growth. The projected nutrient demands scale in proportion to the algal biomass production volume, leading to increasingly severe nutrient demand challenges at the higher target oil production levels [1]. Nutrients also have been reported as one of the major operational costs for algal biomass production. Based on a reconciled model from life cycle analysis (LCA) and techno-economic analysis (TEA), nutrients account for 10% of gross operation costs [2,3]. Beyond the operational cost, the LCA study shows that nutrients from fertilizer have an environmental cost, which is as high as 3.2 kg carbon dioxide per kilogram ammonia nitrogen [2]. Therefore, it would be valuable to pursue options for algal production that enable the effective capture and reuse of nutrients from the production cycle to improve overall life cycle performance [4–6].

Hydrothermal liquefaction (HTL) is a process that directly liquefies wet biomass to crude oil in the presence of water. It creates short-chain petroleum hydrocarbons as well as an aqueous phase and gaseous products. The petroleum hydrocarbons extractable by a non-polar solvent are referred to as crude oil. The aqueous phase, referred as HTL wastewater (HTL-WW) in this study, is rich in organic nitrogen and other nutrients. This would ideally be recycled to grow more algal biomass instead of being discharged, which might cause adverse impacts to the environment.

However, the potential of HTL-WW for nutrient recycling has not been well studied. Jena et al. showed the feasibility of recycling HTL-WW by growing *Chlorella minutissima* with diluted HTL-WW from *Spriulina* in deionized water [7]. Biller et al. demonstrated a closed loop system using recovered HTL-WW from the HTL process for algal growth of eukaryotic microalgae (*Chlorella vulgaris*, *Scenedesmus dimorphus*) and two cyanobacteria (*Spirulina platensis* and *Chlorogloeopsis fritschii*) [8]. It was also found that *C. vulgaris* grown in HTL-WW as the only nutrient source had higher biomass and fatty acid methyl ester yields compared to regular medium [9]. In a recent study, growth reduction was observed when *Desmodesmus* sp. was grown with HTL-WW, which was due to insufficient macro- or micro-nutrients other than N and P [10].

Picochlorum SENEW3 (SE3) was isolated from a small permanent pond in San Elijo Lagoon system in San Diego, California. The pond is subject to large seasonal fluctuations in salinity (1.7–108.3‰) via evaporation, precipitation, and tidal influx of seawater. *Picochlorum* is a slightly oval, nonflagellated green alga with an approximate mean size of 2 μm. The 18S rRNA sequence of this isolate showed very high percentage (99%) identity with a published 18S rDNA sequence of *Picochlorum* sp., which is classified as belonging to the Domain Eukarya, Kingdom Protista, Division Chlorophyta and Class Trebouxiophyceae

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http://dx.doi.org/10.1016/j.algal.2015.11.015
2211-9264/

[11,12]. As one of the smallest and most gene dense eukaryotic algae, *Picochlorum* SE3 encodes 7367 genes with 13.3 Mbp genome [13]. It has high growth rates (>0.5 day^{-1}) over broad temperature and salinity ranges as well as high oil contents [14]. It also shows ability to utilized organic nitrogen sources (manuscript in preparation). For all these reasons, *Picochlorum* SE3 may be a promising candidate for commercial algal biomass applications and an ideal model strain for genome and proteome studies.

Quantitative proteomic analyses provide an opportunity to enable large scale understanding of how *Picochlorum* SE3 adjusts its proteome and overall metabolism to HTL-WW as a nitrogen source. In order to confirm proteomic data and better understand the responses of *Picochlorum* SE3, enzyme assays were also performed. These data provide insights into finding, acclimating, or engineering algae to be part of an algal biomass recycling system.

2. Methods and material

2.1. Microalgal cultivation

One ml previously grown *Picochlorum* SE3 was inoculated in 1 l sterile f/2 growth medium or nitrate deficient f/2 medium containing 0.1% (vol) HTL-WW (Table 1). When cells under both treatments reached stationary phase, they were transferred to "bag" photobioreactors inside of a greenhouse with natural light. These photobioreactors were made using 16″ or 10″ 10 mil low density polyethylene (LDPE) tubing (Landsberg, Pasadena, CA) hung from pallet racks and filled with sterile-filtered 50 l of f/2 media or nitrate deficient f/2 medium with 0.1% (vol) HTL-WW. 3/8″ HDPE tubing with 4 drilled holes at the bend were inserted into each bag and used to bubble air plumbed in from a blower fan for aeration and mixing. Bubbling rate was adjusted for each bag using a ball valve. Environmental controls in the greenhouse attempted to maintain a temperature of 22 ± 2 °C. Both treatment conditions were duplicates.

Pond cultures were grown in 320 gal (1200 l) round stock tanks (Den Hartog Industries, Inc., Hospers, IA) with a final volume of 800 l media at a depth of approximately 40 cm. Ponds were inoculated with greenhouse bag cultures. Ponds were mixed using an air stone and airlifters constructed with PVC pipe connected to a blower fan. Nutrients were added when the nitrate and phosphate concentration were below 5 ppm. Pond cultures were harvested periodically using a continuous flow centrifuge at 3000 rpm. Chlorophyll was recorded daily using an Aquafluor portable fluorometer (Turner Designs, Sunnyvale, CA). Optical density (OD) readings were taken on a Thermo Spectronic 20 at 750 nm (Thermo Fischer Scientific, Waltham, MA). Biomass was measured before and after harvests based on the protocol recommended by the Algal Biomass Organization [15].

Table 1
Composition of HTL-WW sample used in this study.

Components	Concentration (ppm)
Nitrogen	
Total nitrogen	11,936
Nitrite	193 (4 mM)
Nitrate	75 (1 mM)
Ammonium	8966 (498 mM)
Total Kjeldahl nitrogen	4887
Carbon	
Total carbon	39,220
Total organic carbon	34,920
Inorganic carbon	4292
Phosphate and other nutrients	
Phosphate	3072 (32 mM)
Fluoride	1994 (105 mM)
Chloride	1109 (31 mM)
Sulfate	545 (6 mM)
Bromide	43 (1 mM)
Sodium	3507 (152 mM)
Potassium	3204 (82 mM)

2.2. Photosynthetic and respiration measurements

Photosynthetic and respiration responses to various HTL-WW concentrations were determined using a Clark oxygen electrode (Hansatech instruments LTD, UK) as described in Garbayo et al. [16]. Briefly, 2 ml of exponential phase *Picochlorum* SE3 culture were placed in the electrode chamber and treated with several light/dark cycles. At the beginning of each cycle, HTL-WW was spiked into the chamber to the final volume concentrations of 0.1%, 0.2%, 0.5%, 1%, 2% and 5%. The reaction chamber was illuminated with 100 μE/m^2 of LED light during the light cycles and maintained at 22 °C. Respiration was determined for the same sample by darkening the chamber containing the cells. Cell number was determined in a hemacytometer (Hausser Scientific Co., Horsham, PA). The photosynthetic and respiration measurements were obtained by normalizing the oxygen production or consumption rates in light or dark cycles to cell concentrations.

2.3. iTRAQ protein preparation and analysis

Cells for iTRAQ analysis were sampled from photobioreactors. 1 l exponential phase cells were collected when chlorophyll levels reached approximately 30 RFU (Stationary phase was 120 RFU). Cells were washed and re-suspended in approximately 4 ml of 100 mM MET. The concentrated cells were broken in a French Pressure Cell Press (SLM Instrument, Inc.). Total proteins of extract sample slurry were measured using Pierce BCA Protein Assay Kit (Thermo scientific, Rockford, IL) and stored at −80 °C for future analysis.

Protein samples were sent to the Department of Chemistry and Biochemistry, UCSD for further analysis. Briefly, protein samples diluted in TNE buffer (50 mM Tris pH 8.0, 100 mM NaCl, 1 mM EDTA) were boiled with RapiGest SF reagent (final concentration 0.1%, Waters Corp.) for 5 min. TCEP (Tris (2-carboxyethyl) phosphine) was added to 1 mM (final concentration) and incubated at 37 °C for 30 min. Subsequently, the samples were carboxymethylated with 0.5 mg/ml of iodoacetamide for 30 min at 37 °C followed by neutralization with 2 mM TCEP (final concentration). Proteins samples prepared as above were digested with trypsin (trypsin:protein ratio — 1:50) overnight at 37 °C. RapiGest was degraded and removed by treating the samples with 250 mM HCl at 37 °C for 1 h followed by centrifugation at 14,000 rpm for 30 min at 4 °C. The soluble fraction was then added to a new tube and the peptides were extracted and desalted using Aspire RP30 desalting columns (Thermo Scientific).

The four trypsinized samples were then pooled and fractionated using high pH reverse phase chromatography (HPRP-Xterra C18 reverse phase, 4.6 mm × 10 mm 5 μm particle (Waters)). The chromatography conditions were as follows: the column was heated to 37 °C and a linear gradient from 5 to 35% B (Buffer A—20 mM ammonium formate pH 10 aqueous, Buffer B—20 mM ammonium formate pH 10 in 80% Acetonitrile water) was applied for 80 min at 0.5 ml/min flow rate. A total of 48 fractions of 0.5 ml volume where collected for LC-MSMS analysis. Each of these fractions were analyzed by high pressure liquid chromatography (HPLC) coupled with tandem mass spectroscopy (LC-MS/MS) using nano-spray ionization. The nanospray ionization experiments were performed using a QSTAR-Elite hybrid mass spectrometer (ABSCIEX) interfaced with nano-scale reversed-phase HPLC (Tempo) using a 10 cm–180 μm ID glass capillary packed with 5-μm C18 ZorbaxTM beads (Agilent Technologies, Santa Clara, CA). Peptides were eluted from the C18 column into the mass spectrometer using a linear gradient (5–30%) of Acetonitrile at a flowrate of 550 μl/min for 100 min. The buffers used to create the ACN gradient were as follows: Buffer A (98% H_2O, 2% ACN, 0.2% formic acid, and 0.005% TFA) and Buffer B (100% ACN, 0.2% formic acid, and 0.005% TFA). MS/MS data were acquired in a data-dependent manner in which the MS1 data was