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Nitrogen recycling from fuel-extracted algal biomass: Residuals as the sole nitrogen source for culturing *Scenedesmus acutus*

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HIGHLIGHTS

- Algal fermentation residues (SABCr) were enriched in proteins and amino acids.
- SABCr could replace nitrate as the sole nitrogen source to grow *Scenedesmus acutus*.
- Substitution of SABCr for nitrate enhanced *Scenedesmus* growth and lipid production.
- Selected strains showed different abilities to assimilate organic nitrogen sources.

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ABSTRACT

In this study, the reuse of nitrogen from fuel-extracted algal residues was investigated. The alga *Scenedesmus acutus* was found to be able to assimilate nitrogen contained in amino acids, yeast extracts, and proteinaceous alga residuals. Moreover, these alternative nitrogen resources could replace nitrate in culturing media. The ability of *S. acutus* to utilize the nitrogen remaining in processed algal biomass was unique among the promising biofuel strains tested. This alga was leveraged in a recycling approach where nitrogen is recovered from algal biomass residuals that remain after lipids are extracted and carbohydrates are fermented to ethanol. The protein-rich residuals not only provided an effective nitrogen resource, but also contributed to a carbon "heterotrophic boost" in subsequent culturing, improving overall biomass and lipid yields relative to the control medium with only nitrate. Prior treatment of the algal residues with Diaion HP20 resin was required to remove compounds inhibitory to algal growth.
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1. Introduction

The use of algae in next generation biofuel approaches has received substantial attention in recent years; however, significant advances in culturing inputs and product yields are still required to improve the overall economics and sustainability of these feedstocks (Chisti, 2007; Hu et al., 2008; Work et al., 2013). A key parameter in enabling economic viability is inorganic nutrient recycling (Clarens et al., 2010; Pienkos and Darzins, 2009). In particular, nitrogen is not only undesirable in the final fuel product, it also represents a major cost and energy input for algal culturing (Lardon et al., 2009). Therefore, research into nitrogen recycling is necessary to improve the overall economics of algal biofuel platforms. Previous nitrogen recycling research efforts have included coupling algal growth with wastewater treatment (Pittman et al., 2011; Sacristan de Alva et al., 2013), and using the aqueous phase from hydrothermal liquefaction (HTL) as a biomass fertilizer (Biller et al., 2012; Garcia Alba et al., 2013). Recycling nutrients from methane digesters has also been investigated (Golueke and Oswald, 1959; Habig et al., 1984). In this study, it was assessed whether the nitrogen remaining in algal fermentation residues, generated after lipid extraction and then carbohydrate fermentation (Gao et al., 2012; Harun et al., 2010), could be effectively recycled and used to supplement, or even replace, nitrate addition to culture media.

A promising recent approach to algal biomass processing involves using dilute acid pretreatment to lyse algal cells and hydrolyze biomass for subsequent conversion to biofuels and/or high value products (Laurens et al., 2014; Martin et al., 2007). In this approach, algal biomass is treated with dilute sulfuric acid at moderately elevated temperatures (ca. 150 °C) to lyse cells and initiate biomass deconstruction; lipids are then separated from the water-soluble constituents, and the carbohydrates remaining in the aqueous phase are fermented to alcohols (or other potential

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carbohydrate-conversion products) to maximize utilization of algal biofuel feedstocks. After alcohol recovery, the remaining biomass is enriched in amino acids and peptides that could putatively be recycled back to algal growth media and used as a nitrogen source, provided the nitrogen sources in these processed residuals are bioavailable. Potential obstacles to this approach include: (i) the emergence of toxic byproducts generated during acid pretreatment at elevated temperature and/or fermentation processes that inhibit algal growth (Martin et al., 2007), (ii) transformations of amino acids to relatively non-metabolizable products, and (iii) the inability of many algae to effectively assimilate amino acids, peptides, and other organic nitrogen sources (Flynn, 1990; Rivkin and Putt, 1987).

This research was specifically focused on (i) identifying promising algal biofuel strains that could mobilize and utilize the nitrogen remaining in processed algal biomass, (ii) developing processes that would effectively allow the reutilization of nitrogen after the conversion of carbohydrates and lipids to commodity products, and (iii) determining whether the addition of these nitrogen sources, which are more reduced than nitrate, and residual carbon products would relieve the photosynthetic burden required for *de novo* amino acid synthesis and nitrate reduction, allowing more biomass to be generated and/or enabling increased metabolic partitioning to lipid and/or carbohydrate biosynthetic pathways.

2. Methods

2.1. Microalgal strains and culturing

Scenedesmus acutus (SCE 0401) and *Chlorella vulgaris* (LRB 1201) were provided by Arizona State University. These two strains were collected and are curated by the Arizona Center for Algae Technology and Innovation (AzCATI) at Arizona State University. *Nannochloropsis salina* (CCMP1776), *Nannochloropsis gaditana* (CCMP526) and *Nannochloropsis granulata* (CCMP535) were obtained from the National Center for Marine Algae and Microbiota.

S. acutus (SCE 0401) and *C. vulgaris* (LRB 1201) were grown in BG11 medium, whereas the *Nannochloropsis* strains were cultivated in f/2 medium (50% Boothbay seawater) supplemented with additional nitrogen (to 750 mg/L $NaNO_3$) and phosphate (to 50 mg/L $NaH_2PO_4 \cdot H_2O$) resulting in a modified f/2 medium. All precultures were maintained autotrophically in indicated growth media until densities reached ~4 g/L biomass (~1–2 weeks). Cultures were grown under continuous illumination of 110 μmol/m^2 s photosynthetically active radiation (PAR) using fluorescent light in a growth chamber (Multitron, AJ125BC) containing a 1% CO_2/air atmosphere and maintained at 26 °C. Culture flasks were agitated on a shaking platform (120 rpm). Cells were harvested by centrifugation (3716g), washed with nitrogen depleted media once and resuspended in indicated media at a biomass density of ~2 g/L. Biomass was assessed by filtering 3–5 mL of culture through pre-weighed 0.4 μm glass fiber filters (Pall Corporation, 28150-190), followed by washing 3 times with deionized water to remove media constituents. Filters were dried overnight at 80 °C, cooled to room temperature and then reweighed. Cell numbers were measured using a Z2 Coulter Counter (Beckman-Coulter).

2.2. Media nitrogen sources

Yeast extracts and amino acid mixtures were used as nitrogen sources and as proxies to algal residues in preliminary strain selection. 17 mM sodium nitrate is typically present in BG11 (8.82 mM nitrate in modified f/2) medium and was used as a positive control. Media without the addition of sodium nitrate (BG11−N or modified f/2−N) were used as negative controls. For media supplemented with yeast extracts, 1.4 g/L yeast extracts (BD Difco™ 2× YT broth, BD 244020) was added to both BG11 and BG11−N (or modified f/2±N) as an augmentation to, or replacement for, sodium nitrate. For BG11 plus amino acids (AA) or BG11−N+AA, Minimum Essential Medium (MEM) amino acids (Invitrogen, 11130-051), MEM non-essential amino acids (Invitrogen 11140-050) and L-glutamine solution (Invitrogen, 25030-081) were mixed at ratios of 1:2:0.1, and added to BG11 and BG11-N medium to make a final working concentration with the majority of the amino acids at ~0.5 mM.

Algal biomass fermentation residues are referenced here as Sustainable Algal Biofuels Consortium residuals (SABCr). It was produced from carbohydrate rich *S. acutus* (SCE 0401) biomass that was pretreated with dilute sulfuric acid (2% w/w) in a Jaygo reactor [130 L (Union, NJ)] at 150 °C for 0.5 h to lyse cells and hydrolyze portions of the biomass. Lipids were separated from the resulting sample slurry by centrifugation, and the saccharified liquid supernatant was fermented using *Saccharomyces cerevisiae* (D5A) to produce ethanol after supplementation with 5 g/L yeast extract and 10 g/L peptone to the fermenter (Laurens et al., 2014). The resulting fermentation broth was assessed for nitrogen content (see below), and added to BG11 or BG11−N as a potential nitrogen source for algal growth, resulting in the media BG11+SABCr or BG11−N+SABCr, respectively. These untreated fermentation residues were inhibitory to algal growth under the experimental conditions used. Therefore, fermentation residues were incubated at 65 °C for ~2 h with the Diaion™ HP20 (Supelco, 13607) polymer (styrene–divinylbenzene) resin at 6% (w/w) to remove large non-polar organics with putative antimicrobial activities, as described previously for other systems (In et al., 2005; Nogawa et al., 2001).

Fatty acid, protein and reducing carbohydrate levels remaining in the Diaion™ HP20-treated algal fermentation residuals were characterized, and its total nitrogen (TN) and organic carbon (TOC) contents were determined, as described below. The SABCr were diluted 1:15 into growth media (BG11+N or −N) to effectively replace the total nitrogen content (0.25 g/L) typically supplied as nitrate in BG11 medium, and to determine whether SABCr is able to serve as an effective nitrogen replacement for some algal strains in this medium. Diluted SABCr was also added to BG11 containing normal levels of nitrate as a control to determine whether SABCr contained toxic products, or whether this nitrogen augmentation could boost biomass productivities when nitrate and SABCr were provided.

2.3. Nitrogen and biochemical analysis

The TN and TOC in the culturing media were determined using fresh media at the indicated times of culturing, using a total organic carbon analyzer (TOC-L, Shimadzu). Briefly, cultures were centrifuged at the indicated times and the supernatant diluted (1:10 v:v) with deionized water prior to TN/TOC analysis. TN calibration was performed in the range of 0–50 ppm using potassium nitrate as a standard, and TOC was calibrated in the range of 0–100 ppm using potassium hydrogen phthalate (KHP) as a standard. TOC values were measured in the non-purgeable organic carbon (NPOC) mode.

Reducing carbohydrate levels were determined using the anthrone/sulfuric acid method, as described previously (Meuser et al., 2012; Morris, 1948). All samples were diluted 10-fold to reduce the culture biomass density to less than 1 g/L. 0.1 ml diluted samples and glucose standards (0, 100, 150, 200, 300, 400 μg/ml) were independently added to 0.9 ml of freshly prepared anthrone reagent containing 2 g/L anthrone and 71% sulfuric acid, in microcentrifuge tubes. All tubes with anthrone reagent were then immersed immediately in ice to quench reactivity

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