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Short Communication

Optimization of renewable pinene production from the conversion of macroalgae *Saccharina latissima*

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HIGHLIGHTS

- *S. latissima* was used as a sugar source for fermentation of pinene.
- A cellulase and laminarinase mixture releases more glucose than laminarinase.
- Hydrolysates were used to produce pinene with a novel *Escherichia coli* system.

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ABSTRACT

Enzymatic hydrolysis of *Saccharina latissima* with laminarinase was compared to hydrolysis with different combinations of cellulase and hemicellulase enzyme mixtures. The hemicellulase mixture resulted in similar release of glucose, while the cellulase mixture released 40% more glucose than laminarinase alone. The combination of a laminarinase augmented with a cellulase mixture resulted in a 53% increase of glucose release from *S. latissima* than laminarinase. Increasing biomass loading above 4% (w/v) reduced the sugar yield. Resulting macroalgae hydrolysates were used as a carbon source for the production of pinene, making use of a novel two plasmid *Escherichia coli* system. The macroalgal hydrolysates were suitable for the novel microbial production of pinene with no further treatment and/or purification.

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1. Introduction

Both micro- and macro-algae are thought to minimally compete with established and projected food supplies and estimated to require 4–40 times less area than that projected for terrestrial biomass sources to generate significant amounts of renewable chemicals (Adams et al., 2009). Macroalgae has been commercially collected for many years in weights of kilotons in China, Philippines, Indonesia, Chile and numerous countries in Europe with over 16 megatons harvested world wide in 2006, and there are large areas of unused shorelines that have potential for algae cultivation (Roesijad et al., 2010; Vea and Ask, 2011). It has been estimated that using 0.09% of the potential United States offshore 'Exclusive Economic Zone' to grow macroalgae could replace 1% of the current United States fuel consumption (Roesijad et al., 2010). Further, countries with current macroalgae farms are developing low impact renewable cultivation methods providing guidance for sustainable farming (Ugarte and Sharp, 2012; Vea and Ask, 2011). World-wide commercial production of alginate was valued at 213 million in 2003, with potential for co-production of an easily fermentable sugar source of both mannitol and laminarin (Roesijad et al., 2010). The typical polysaccharide profile of Phaeophyceae (brown algae) includes alginate, fucoidan, mannitol and laminarin. The Phaeophyceae *Saccharina latissima* has been show to have some of the highest sugar content in the form of laminarin and mannitol (Adams et al., 2009).

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Mannitol is synthesized in algae as one of the major photosynthetic products, (Iwamoto and Shiraiwa, 2005) is found in quantities as high as 20–30% in brown algae. Laminarin is polysaccharide of (1,3)-β-D-glucan with β-(1,6) branching ending with either a mannitol or a glucose residue approximately 5000 kDa in size (Rioux et al., 2007). It is the primary storage of glucose in the algae, and the focus of fermentation attempts previously with use of a laminarinase (Ross et al., 2011, 2008).

Higher harvesting yields do not necessarily directly equate to higher fermentation yields; rather macroalgal composition plays an important role (Broch and Slagstad, 2011). The recoverable concentration of fermentable sugars (laminarin, mannitol, and alginate) has been shown to vary with harvest time; a 6-fold reduction in laminarin in *S. latissima* has been reported to monotonically decrease in harvests from August to December (Broch and Slagstad, 2011; Holdt and Kraan, 2011; Peteiro and Freire, 2009). The regional and seasonal growth differences, even within the same strain, can vastly change the methodology to optimize sugar production for use in biosynthesis. These parameters can impact potential yield and therefor extremely important for estimations of overall feasibility.

Phaeophyceae have been shown to have promise for biofuel production (ethanol and methanol (Ross et al., 2011, 2008)). Advances in understanding microorganism metabolism have allowed molecular engineering to produce novel and complex biofuels and chemicals, including butanol, fatty acid esters, methyl ethyl ketones and terpenes (Peralta-Yahya et al., 2012). Pinene is a terpene that is a high value chemical precursor for multiple commercial components, such as aromatic chemicals in fragrances, and is currently extracted as a byproduct from the wood pulp industry (Sarria et al., 2014). Components found in hydrolysates of biomass such as salts, phenols, or other materials can inhibit microbial growth and can have an impact on the efficiency of conversion during fermentation. Testing and use of developed microorganisms with biomass-generated hydrolysates is necessary to understand the biomass specific process hurdles that prevent commercialization. In the present work, *S. latissima* harvested off the Norwegian Coast was characterized and sugar production was evaluated. The hydrolysates were then used to produce pinene with an *E. coli*-based advanced biosynthesis pathway (Bokinsky et al., 2011; Peralta-Yahya et al., 2012) to demonstrate suitability of hydrolysate resulting from macroalgae.

2. Methods

2.1. Materials and preparation

Samples of *S. latissima* were collected off the coast of Norway in December 2010, July 2011 and August 2011. The samples were freeze-dried for 1–2 days. These samples were stored in 4 °C before and after shipping. Shipping of the freeze-dried algae to Joint BioEnergy Institute was done at room temperature. Moisture content was continually checked.

2.2. Total sugar analysis

General composition and structural carbohydrates of algae containing mannitol, glucose, xylose, fucose were determined according to the two-step acid hydrolysis procedure of the National Renewable Energy Laboratory (NREL, Sluiter, 2008). Carbohydrates were analyzed by high pressure anion exchange chromatography (HPAEC) on an ICS-3000 system (Dionex, Sunnyvale, CA) equipped with an electrochemical detector and a 4 × 250 mm CarboPac SA10 analytical column. 1 or 10 μL (depending on the concentration) of the sample was injected into the column and was eluted with 1 mM KOH for 14 min. The flow rate of the eluent was maintained at 1.2 mL per min. Standards were made for mannitol, glucose, xylose, fucose of 6, 10, 25, 50, and 100 μM.

2.3. Enzymatic saccharification

Enzymatic saccharification of algae samples was carried out at 50 °C and 150 rpm in a reciprocating shaker (Enviro-Genie, Scientific Industries, Inc.) in 50 mM citrate buffer (pH of 4.8). The glucan content in the solution was maintained at 5 g glucan per liter, unless otherwise noted. 20 mg protein per g glucan of Cellic® CTec2 (Novozymes) and 2 mg protein per g glucan of Cellic® HTec2 (Novozymes) and/or 0.1 U per 20 g glucan laminarinase (1 U per 31 mg Sigma, L5272), (Adams et al., 2009), were used for hydrolysis reactions unless otherwise noted. A 60 μL of the supernatant was taken at specific time intervals (0, 0.5, 1, 2, 5, 24, 48, 72 h) to monitor the hydrolysis reaction. The reducing sugars in the supernatant were measured using the 3,5-dinitrosalicylic acid assay, (DNS) or HPAEC.

Solutions of D-glucose were used as standards in the DNS and Amplex assays. All assays were performed with three replicates. Error bars show the standard error of three replicate measurements.

Each saccharification comparison was run concurrently with all samples in the same comparison to eliminate potential differences in temperature history or other parameters. The rate of hydrolysis was calculated based on the sugar released in the first 30 min of hydrolysis. The supernatant collected after 72 h of hydrolysis was analyzed with HPAEC for the monosaccharide composition. All assays were performed with three replicates, unless noted. It should be noted that the DNS assay does not account for the hydrolysis reaction stoichiometry of cellulose and hemicellulose upon complete hydrolysis.

2.4. Composition

Ash content was measured using the procedure of NREL (Sluiter et al., 2008). Protein was extracted from algae using glass bead homogenization followed by quantitation with Coomassie Blue (BioRad) with BSA standards. Alginate composition was measured using an adaptation of a multi-step process to get a relative quantifiable amount and was burned to adjust for the ash content of the material (Peña et al., 1997; Rioux et al., 2007). Briefly, alginate was extracted using the multistep process described in (Peña et al., 1997) with Na_2CO_3 and $CaCl_2$. The recovered alginate was then ashed at 575 °C for 3 h to account for the mineral content, the difference between the 105 °C dried weight and the ashed values were used to calculated the alginate concentration. Conductivity was measured using a pocket conductivity meter (B-173, Horiba, Edison, NJ), samples were diluted 10- and 100-fold and confirmed for linearity of measurement.

2.5. Confocal fluorescence imaging

S. latissima samples were cut with a razor blade from freeze-dried samples from the December 2010 harvest. These sections were stored at 4 °C until imaged. Slices were placed between a coverslip and slide with 150 μL buffered enzyme to wet each sample. Autofluorescence images during heating were collected with a Zeiss LSM 710 confocal system mounted on a Zeiss inverted microscope (Carl Zeiss Microscopy, LLC, Thornwood, NY). A 405 nm diode laser and a 488 nm argon laser were used for sequential excitation over a 410–759 nm range with 5×, 10× or 40× objectives. The resulting images were analyzed using the Zen software and reproduced in pseudo color (Zeiss).

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