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Hydrogen and volatile fatty acid production during fermentation of cellulosic substrates by a thermophilic consortium at 50 and 60 °C

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ABSTRACT

The purpose of this study was to characterize the effect of temperature and cellulosic substrates on fermentative metabolites, H_2 production, and community successions in an anaerobic, cellulolytic consortium, TC60. Pyrosequencing analysis indicated that the consortium was predominated by *Thermoanaerobacter* and *Clostridium* spp. Metabolite production was analyzed with four cellulosic substrates at 4 kg/m^3. Triplicate cultures of each substrate were incubated at 50 or 60 °C. The main fermentation products (H_2, CO_2, ethanol, and acetate) were monitored over time. The ANOVA model for production rates showed a significant temperature effect ($P < 0.05$) on all products. Increased temperature promoted higher H_2, CO_2, and ethanol yields while acetate yields were only affected prior to 24 h of incubation. In addition to individual effects discerned in the model, ANOVA indicated significant interactions between the substrate and temperature. These interactions have not been previously recognized in the literature for cellulolytic and hydrogen-producing microorganisms.

1. Introduction

Hydrogen is a potential form of alternative energy that has the advantage of being carbon neutral and having a high heat index. There are multiple biological processes to generate hydrogen including dark fermentation by anaerobes (Hallenbeck, 2009; Hallenbeck and Ghosh, 2009; Lee et al., 2010). Dark fermentation entails the formation of short chain fatty acids, especially acetate, following glycolytic pathways and the coupled recycling of reducing equivalents with the generation of H_2 by hydrogenases. The formation of acetate not only regenerates reducing equivalents but also produces ATP via substrate level phosphorylation. Many fermentative pathways are sensitive to undissociated acids, pH, H_2 partial pressure, and metal ions (Chong et al., 2009). Dark fermentation provides a feasible route for maximizing H_2 production by genetic engineering and bioprocess optimization. Cellulose, an abundant renewable resource in the form of plant biomass, is composed of glucose subunits which readily enter fermentative pathways. Tying H_2 production with fermentation of cellulosic feedstocks could provide efficient production from renewable biomass resources.

H_2 production has been investigated using pure cultures as well as with enriched consortia. The order *Clostridiales*, which includes *Clostridium thermocellum* being arguably the most studied member, are well known cellulolytic H_2 producers (Levin et al., 2006). Many studies have focused on pure cultures or isolated enzymes, but microbial consortia provide advantages compared to their pure culture counterparts: a wider array of hydrolytic and catabolic enzymes, including hydrogenases (Cui et al., 2009; Lo et al., 2009; Kongjan et al., 2010). To select for the appropriate consortium, a selective pressure must be applied and maintained to enrich for the most efficient cellulolytic organisms capable of H_2 production.

Environmental sources for cellulolytic, H_2 producing consortia often contain organisms that consume or prevent maximum H_2 yields such as methanogens, sulfate reducers, and lactic acid bacteria. These organisms are all present in diverse, synergistic anaerobic communities. In vitro, H_2 consuming anaerobes must be inhibited or eliminated in order to maximize H_2 yields (Nath and Das, 2004). The first step is to choose the appropriate medium that eliminates electron acceptors that are associated with the use of H_2 as an electron donor. However, one such electron acceptor, CO_2, cannot be eliminated from the headspace; homoacetogens and hydrogenotrophic methanogens utilize CO_2 with H_2 as the reductant. In addition to media design, other selective measures can be used such as heat treatment. This is a common technique that eliminates all non-sporeformers, including methanogens and many homoacetogens, by pretreating the inoculum at 100 °C for 10–60 min. Many known cellulolytic, H_2 producers, such as

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Clostridium and *Bacillus* spp., are spore-formers and can survive heat treatment. Another way to eliminate unwanted organisms is to inhibit their growth with low pH. For example, many methanogens grow only between pH 6.0 and 8.0, whereas H_2 producing clostridia can grow well outside this range (Whitman et al., 2006). Moreover, maximum H_2 yields may be obtained by minimizing sources of inhibition such as butyric and acetic acids (Van Ginkel and Logan, 2005; Zheng and Yu, 2005) and high partial pressure of H_2 (Claassen et al., 1999; Logan et al., 2002). In the thermophilic environment, factors affecting cellulose metabolism in microbial consortia rather than pure cultures are poorly characterized. Among them, temperature effects in the thermophilic range and different types of cellulosic substrates have not been systematically examined in the previous literature.

In this study, the effects of temperature and different cellulosic substrates on metabolite production were examined using a cellulolytic, H_2 producing microbial consortium which was previously enriched on cellulose at 60 °C, TC60. Four different cellulosic substrates were used to culture TC60 at 50 and 60 °C. Metabolite production was monitored by measuring changes in the yields and production rates of H_2, CO_2, ethanol, and acetate over time along with reducing sugar analysis. The diversity of the consortium was also characterized by pyrosequencing analysis of 16S rRNA gene sequences. The data were analyzed with ANOVA to define interactions of the cellulosic substrate metabolism with temperature.

2. Methods

2.1. Culture and experimental set-up

The microbial consortium (TC60) originated from the interior of an active compost pile. The original culture was maintained at 55 °C for several years on microcrystalline cellulose and milled corn stover. Approximately 9 months prior to this experiment, the substrate was switched to 4 kg/m^3 microcrystalline cellulose (Sigmacell, Type 20, Sigma–Aldrich, St. Louis, MO) and the consortium, designated as TC52, was incubated at 52 °C. The optimal incubation temperature of TC52 was screened over a temperature range of 35–75 °C using a thermal gradient incubator (Terratec Australia, Margate, TAS, Australia) with temperature increments of 1.5–2 °C. In light of the results from the temperature gradient, TC52 was subsequently enriched and maintained at 60 °C in medium that contained (per m^3): 2 kg trypticase, 1 kg yeast extract, 4 kg Na_2CO_3, 0.23 kg K_2HPO_4, 0.18 kg KH_2PO_4, 0.36 kg NH_4Cl, 0.04 kg NaCl, 0.09 kg $MgSO_4{\cdot}7H_2O$, 0.06 kg $CaCl_2{\cdot}2H_2O$, 2 g $CoCl_2{\cdot}6H_2O$, 0.09 g $NiCl_2{\cdot}6H_2O$, 0.16 g $Na_2SeO_3{\cdot}5H_2O$, 0.25 kg cysteine–HCl, and 0.25 kg $Na_2S{\cdot}9H_2O$. Microcrystalline cellulose (4 kg/m^3 Sigmacell, Type 20) was the substrate during maintenance of the consortium. This new enrichment culture at 60 °C was designated TC60. Serum bottles (120 cm^3) were filled with 60 cm^3 medium including cellulose, degassed with N_2, sealed with butyl rubber stoppers, and inoculated with TC60 (10% v/v). Subcultures were maintained anaerobically under N_2 headspace on a shaker at 180 rpm (Carver et al., 2010, 2011).

Four substrates were tested with TC60 at 50 and 60 °C (±0.5 °C): microcrystalline cellulose Sigmacell Type 20 and 50 (Sigma–Aldrich), fibrous cellulose (Sigma–Aldrich), and 5 × 5 mm pieces of filter paper (cellulosic filters, Grade 1, Whatman, Piscataway, NJ) at a concentration of 4 kg cellulose/m^3 unless otherwise noted. Each substrate was run in separate triplicate sets at either 50 °C or 60 °C. Triplicate sets of controls containing no cellulose were included for each temperature. Undefined components in the medium, including yeast extract and trypticase, provided an additional 1.12 g total-C/l and 0.26 g total-N/l, which contributed to background metabolite production.

2.2. Analysis of chemical oxygen demand and soluble sugars

Chemical oxygen demand (COD) was monitored using a standard kit for measurements at 150–1000 mg COD/l (LCK114, Hach Lange, Loveland, CO). Digestion at 148 °C and measurement of absorbance were completed according to the manufacturer's instructions (Hach Co.). Soluble sugars were determined using a phenol–sulfuric acid method designed for samples containing suspended solids (Finger and Strutynski, 1975). Filtered (0.2 μm) samples were diluted to a final volume of 2 cm^3, combined with 1 cm^3 of 5% phenol and 5 cm^3 concentrated H_2SO_4, mixed well, cooled to ambient temperature, and absorbance was measured at 490 nm. It should be noted that residual cellulose could not be accurately measured because of the rheology of the system; i.e., problems with sampling of non-homogeneous suspensions.

2.3. Metabolite analyses

In order to ensure accurate measurements of gaseous metabolites at the first sampling point, excess gas was expelled from serum bottles upon temperature normalization. At each time point, gas overpressures in serum bottle cultures were measured immediately upon removal from the incubator and then wasted. To minimize gas composition changes or drastic changes in the temperature, samples were tested within 2 min of overpressure measurements. Samples of headspace gas (0.2 cm^3) were manually injected into a gas chromatograph (GC) with a sterile syringe. Gaseous metabolites were measured as described previously with a Shimadzu GC-2014 equipped with a Porapak N column (2 m length × 2 mm ID, Sigma–Aldrich) and a thermoconductivity detector (Carver et al., 2010, 2011). Chromatographs were analyzed with GC Solution Analysis software (Shimadzu Corp., Columbia, MD).

Following GC measurements, liquid samples were collected anaerobically, filtered if necessary, and stored at −20 °C. Metabolites in the liquid phase were monitored via a Thermo Scientific Focus GC equipped with an AS 3000 Series II autosampler, flame ionization detector, and Trace TR-FFAP capillary column (30 m × 0.32 mm × 0.25 μm, Thermo Scientific, Rockford, IL). Filtered supernatants were diluted 50% with deionized water and acidified to pH < 2 with concentrated formic acid. Crotonate (1 kg/m^3) and *n*-propanol (0.6 mm^3/cm^3) were used as internal standards. Samples were stored at −20 °C until analyzed. Prior to injection, samples were equilibrated to 22 ± 2 °C. The GC was run in a split mode (1:40, flow rate 100 cm^3/min) with He as a carrier gas (2.5 cm^3/min). Injector and detector temperatures were set at 200 and 230 °C, respectively. The oven program was 90 °C for 1.5 min, 30 °C/min ramp to 180 °C, and held at 180 °C for 2 min. Ethanol and butanol along with acetic, propionic, isobutyric, butyric, isovaleric, and valeric acid standards were prepared with GC quality (⩾98%) reagents (Sigma–Aldrich) and analyzed in all samples.

2.4. Statistical models and analyses

Production rates for metabolites were determined using linear regression in JMP8 software (SAS Institute Inc., Cary, NC). All data points were used unless they had a negative value due to a standard curve limitation. H_2 production over time did not follow a linear trend but after a square transformation of individual H_2 measurements, linear regression could be completed.

Production rates and apparent yields (mmol/g substrate added), henceforth called yields, were analyzed via separate analysis of variance (ANOVA) models and multiple comparative pairwise Tukey tests (confidence of 95%) in JMP8. The effect of temperature and substrate on product yields for H_2, CO_2, ethanol, and acetate