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Biological Hydrogen Production From Nitrogen-Deficient Substrates

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ABSTRACT: Dark fermentation of biomass using mixed bacterial cultures is one approach to producing renewable H_2. The objective of this work was to determine if this approach could be applied to N-deficient feedstocks using an N_2-fixing mixed culture. A mixed culture produced up to 240 mL H_2/g glucose (1.9 mol H_2/mol glucose) from a medium initially lacking combined N. Yields from sugarcane were also promising: 170 mL H_2/g volatile solids (7.5 mmol H_2/g volatile solids). This approach could reduce economic and environmental costs of fermentative H_2 production, provide combined N for subsequent bioconversion stages, and improve effluent suitability for subsequent uses.

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KEYWORDS: hydrogen; fermentation; nitrogen; dinitrogen fixation; anaerobic

Introduction

Hydrogen gas has the potential to be a useful energy carrier in a wide range of applications through the use of fuel cells, and is expected to become more important in the future (Dunn, 2002; Elam et al., 2003; Hoogers, 2003). However, to avoid consumption of fossil fuels or contribution to atmospheric CO_2, H_2 must be produced by renewable means, such as from biomass. One approach to renewable H_2 production is the use of mixed bacterial cultures to produce H_2 from biomass via dark fermentation (Hallenbeck, 2005). Most of the research done in this area has focused on pure sugars (Chen et al., 2001; Oh et al., 2004; Yu and Mu, 2006) and much less work has been done directly with plant materials (Fan et al., 2006; Roychowdhury et al., 1988).

Microbial conversion of biomass into H_2 requires a supply of inorganic nutrients, including N. For many potential feedstocks, endogenous N is limiting to microbial growth. Addition of N will generally incur costs and consume fossil fuel. This problem could potentially be solved through the use of N_2-fixing bacteria. Free-living N_2-fixing bacteria are present in genera of both facultative and obligate anaerobes that produce H_2 through fermentative pathways, for example, *Klebsiella* and *Clostridium* (Das and Veziroglu, 2001; Prescott et al., 2002). The objective of this work was to determine the technical feasibility of fermentative H_2 production from N-deficient substrates using a mixed N_2-fixing culture.

Materials and Methods

All experiments utilized 160 mL serum bottles as batch reactors. Reactors were incubated at 35°C, and accumulated biogas was removed and measured using syringes. Similar approaches have been used previously for anaerobic studies (Owen et al., 1979). Methane, CO_2, and H_2 concentrations in biogas samples were determined using two Gow-Mac Series 580 gas chromatographs with thermal conductivity detectors. For CH_4 and CO_2, helium was used as the carrier gas, with a 6 ft × ¼-inch Porapak Q column. For H_2, a 5 ft × 1/8-inch Carboxen 1,000 60/80 column was used with N_2 as the carrier gas. Cumulative hydrogen production was calculated as the sum of H_2 removed and H_2 remaining in the reactor headspace. All volumes were corrected to dry conditions (assuming biogas was saturated with water) and adjusted for temperature (to 0°C).

An anaerobic N_2-fixing H_2-producing mixed culture was developed through a simple enrichment process. Effluent from a laboratory scale anaerobic digester (fed dairy cattle manure) and primary sludge from a plant that processes recycled paper were used to inoculate a defined medium that lacked combined N. Nitrogen-deficient waste streams from

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pulp and paper plants can contain active N_2-fixing bacteria (Gapes et al., 1999; Gauthier et al., 2000). A 0.1% manure inoculum and a 0.6% sludge (wet mass basis) inoculum were used to limit the initial combined N concentration to <20 mg N/kg medium.

The defined medium followed Kim et al. (2004), Sparling et al. (1997), and Kraemer and Bagley (2005), with modifications and contained (per kg medium): glucose, 5.0 g; KH_2PO_4, 24 g; K_2HPO_4, 2.0 g; $MgCl_2 \cdot 6H_2O$, 0.20 g; $CaCl_2 \cdot 2H_2O$, 0.20 g; $Na_2SO_4 \cdot 10H_2O$, 0.40 g; $FeSO_4 \cdot 7H_2O$, 25 mg; $CoCl_2 \cdot 6H_2O$, 2.5 mg; H_3BO_4, 0.50 mg; $MnSO_4 \cdot 1H_2O$, 0.50 mg; $Na_2SeO_4 \cdot 10H_2O$, 0.50 mg; $NiCl_2 \cdot 6H_2O$, 0.30 mg; $ZnSO_4 \cdot 7H_2O$, 0.50 mg; $CuSO_4 \cdot 5H_2O$, 0.30 mg; $Na_2MoO_4 \cdot 2H_2O$, 0.50 mg; $NaWO_4 \cdot 2H_2O$, 0.50 mg; and $NaVO_3$, 0.050 mg. The relative concentrations of K_2HPO_4 and KH_2PO_4 were selected on the basis of chemical speciation modeling to provide a pH of 5.5, which was confirmed by measurement.

After inoculation and flushing with N_2 (>10 volume changes), reactors showed H_2 production within 1 day. After biogas production stopped, new reactors were started with a 10% inoculum supplied from the original reactors. This process was repeated more than five times before setting up the reactors used to generate the results shown here. Therefore, the concentration of combined N derived from the original inoculum was very small in the experiments described below (i.e., <0.001 mg N/kg medium).

Hydrogen production was quantified using reactors with 40 g of the defined medium, including a 10% inoculum. To confirm the presence of N_2 fixation, biogas production was compared among reactors with and without 0.9 g NH_4Cl/kg medium (236 mg N/kg medium), flushed with N_2 or He as described above. Additionally, quantification of N_2 fixation was done by measuring total N in the reactors originally containing no combined N following Standard Methods (Eaton et al., 1995). All conditions were studied in triplicate. Following successful results with glucose, one trial was run using sugarcane (*Saccharum* sp.) at a concentration of 1% (TS basis) in the defined medium. These reactors were also studied in triplicate and were flushed with N_2. Sugarcane was purchased from a local grocery store (distributor: World Variety Producer, Inc., Los Angeles, CA) and was peeled and ground in a food processor. Samples were analyzed for total solids (TS), volatile solids (VS), and total N following Standard Methods (Eaton et al., 1995). Statistical analyses were done using SAS v. 9.1 (SAS Institute, Cary, NC).

Results and Discussion

Reactors containing the glucose medium that lacked combined N showed H_2 production within 1 day after the initial inoculation. Results shown here are from reactors inoculated after more than five transfers following the original reactors. Total H_2 production from the N-free medium represents a yield of 220–240 mL H_2/g glucose (1.8–1.9 mol H_2/mol glucose), differing slightly between trials (Fig. 1A). Methane was not detected at any time. Maximum H_2 production rates were >1.5 L H_2/kg-d (per unit reacting mass). The normalized concentration of H_2 (i.e., excluding N_2 and H_2O) in the biogas from these reactors ranged from 50% to 56%, with a balance of CO_2.

Comparison of biogas production between reactors with N_2 and those with He confirmed that N_2 fixation was responsible for supplying combined N; with the N-free medium, reactors flushed with He showed very little biogas production (Fig. 1B). (H_2 could not be quantified in reactors with He, since He and H_2 co-elute from the column used in this work.) When N was added as NH_4Cl, total biogas production was similar between reactors with and without N_2 (Fig. 1B). Compared to reactors with NH_4Cl, those with

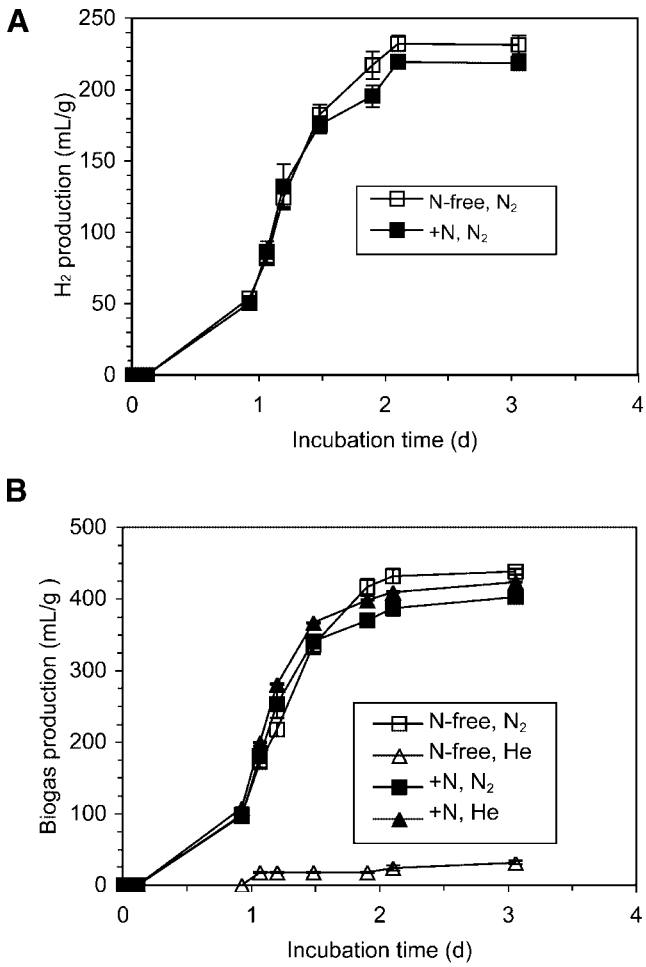

Figure 1. **A**: Cumulative H_2 production from glucose reactors flushed with N_2, per g glucose added. **B**: Cumulative total biogas production from glucose reactors, per g of glucose added. Reactor medium either contained no combined N (N-free) or NH_4Cl (+N) and reactors were flushed with N_2 or He as indicated. For parts A and B, bars represent standard error. All volumes are corrected to dry 0°C values.

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N_2 as the only source of N showed similar or greater biogas and H_2 production (Fig. 1). Reactors with NH_4Cl had slightly lower pH values at the end of the incubation than reactors with only N_2, with mean values of 4.31 (standard error, $SE = 0.019$, $n = 6$) and 4.46 ($SE = 0.013$, $n = 3$), respectively ($P < 0.0001$ by a linear contrast). This difference may have been due to assimilation of NH_3. Hydrogen yields from the N-free reactors are toward the higher end of the range for mixed mesophilic cultures from glucose, but greater yields have been reported (Chen et al., 2001; Morimoto et al., 2004; Oh et al., 2004; Park et al., 2005; Yu and Mu, 2006).

The final concentration of combined N in reactors that were started with N_2 as the only source of N was 22 mg N/kg medium ($SD = 2.1$ mg N/kg medium), which translates into an average rate of N_2 fixation over the 3.1 d incubation of 6.4 mg N/kg medium-d (after subtracting the contribution of the inoculum). Given an initial glucose concentration of 5.0 g/kg medium, the yield of combined N was 0.0044 g N/g glucose.

These results clearly show that H_2 production by dark fermentation via mixed cultures is possible from feedstocks lacking combined N. The anticipated application of this work is the production of H_2 from low-N substrates, such as energy crops. Results from sugarcane were encouraging. Sugarcane had a high VS content (98.0%, $SD = 0.24\%$, $n = 3$) and a very low N concentration (0.047%, $SD = 0.004\%$, $n = 3$). Production of H_2 was initiated within 1 day and rates of H_2 production exceeded 1.4 L H_2/kg-d (per unit reacting mass). The normalized concentration of H_2 in biogas ranged from 48% to 58% with a balance of CO_2. Methane was not detected at any time. A H_2 yield of 170 mL H_2/g VS (7.5 mmol/g VS) ($SD = 3$ mL H_2/g VS) was observed, which is relatively high compared to studies using other biomass materials and is similar to yields from pure sugars (see previous references).

The approach used here could eliminate the cost of supplementing N-deficient feedstocks with combined N, while also producing effluent enriched in N. Even with high yields, fermentative H_2 production from biomass yields much less energy than does biomethanation, due to both lower yields (L/g VS) and a lower energy content per mole (see previous references and Gunaseelan, 1997; Padro and Putsche, 1999). To improve the feasibility of fermentative H_2 production, it is necessary to utilize organic acids that are also produced, for example, to produce additional H_2 via photofermentation or for CH_4 production via biomethanation (Barbosa et al., 2001; Hallenbeck, 2005; Hallenbeck and Benemann, 2002; Kraemer and Bagley, 2005). Depending on its availability, combined N from N_2 fixation in a dark fermentation stage could serve as an N source for further microbial conversion. The final effluent from a two-stage process would have an improved fertilizer value compared to the original feedstock.

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