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Hydrogen production by *Clostridium acetobutylicum* ATCC 824 and megaplasmid-deficient mutant M5 evaluated using a large headspace volume technique

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ABSTRACT

Biohydrogen production is measured using a variety of techniques, ranging from low cost intermittent gas release methods where yields are usually reduced due to high partial pressures of hydrogen, to expensive respirometers that can eliminate pressure buildup. A new large headspace volume technique was developed that reduces the potential for hydrogen gas inhibition without the need for a respirometer. We tested this method with two strains of clostridia, *Clostridium acetobutylicum* ATCC 824 and its mutant M5 that lacks a megaplasmid responsible for butanol and acetone production, and a mixed culture (heat-treated sludge). The hydrogen yield using M5 (2.64 mol-H_2/mol-glucose) was 47% higher than that of the parent strain (1.79 mol-H_2/mol-glucose), and 118% larger than that obtained in tests with the sludge inoculum (1.21 mol-H_2/mol-glucose). The increased yield for M5 was primarily due to a decrease in biomass synthesis (38%) compared to the parent strain. Hydrogen yields measured using this new method were on average 14% higher than those obtained using a conventional respirometric method. These findings indicate enhanced biohydrogen production from the megaplasmid-deficient mutant of *C. acetobutylicum* ATCC 824, and that an intermittent gas-sampling technique can effectively measure high hydrogen gas by using a large headspace volume.

1. Introduction

Microbial biohydrogen production, through fermentative and photosynthetic pathways, offers a renewable strategy for hydrogen production. However, hydrogen gas yields are affected by the hydrogen partial pressure, which can be dependent on the technique used to measure gas production. Hydrogen gas production in batch tests using small laboratory bottle-type reactors is often measured through intermittent gas release [1] by a technique originally developed for methane gas production called the Owen method [2]. When using the Owen method for measuring methane gas production, build of methane gas does not affect gas production and thus the volume of the headspace relative to that of the liquid is not important. However, this method (40% headspace) has been shown to result in reduced hydrogen gas production

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compared to other methods that continuously release gas pressure [3], or that reduce hydrogen gas partial pressure through inert gas sparging [4] or application of a vacuum [5]. Hydrogen and carbon dioxide accumulates to high pressures in the headspace and inhibits net hydrogen production. For example, Van Andel et al. [6] found that decreasing the partial pressure of hydrogen increased the acetate/butyrate ratio and the production of hydrogen. Park et al. [7] demonstrated that stripping CO_2 from the headspace increased the net hydrogen production of a heat-treated inoculum by eliminating homoacetogenic hydrogen consumption. We hypothesized that modifying the Owen Method by using a large headspace volume, relative to that of the liquid culture, would remove the potential for gas product inhibition by reducing the partial pressure of these gases, thereby eliminating the need for relatively expensive gas respirometers.

Hydrogen can be produced from carbohydrates by various strains of bacteria [8], including members of the genera *Enterobacter* [9,10], *Bacillus* [11], *Citrobacter* [12], and *Clostridium* [12]. Mixed cultures that produce large yields of hydrogen that are prepared from soil and wastewater inocula usually contain mostly clostridia [13,14]. We therefore selected *Clostridium acetobutylicum* ATCC 824 as one of the inocula for our tests. This strain has been used in the commercial production of acetone and butanol [15], has a fully sequenced genome [16] and genetic system that makes it amenable to genetic manipulation, and it was also previously examined for biohydrogen production using other techniques [17]. However, strains selected on the basis of high solvent production may not provide the optimal genetic platform for hydrogen generation due to diversion of the electrons for solvent production compared to hydrogen gas production. The production of the reduced products butanol and ethanol are used to regenerate NAD^+ within the cells [18], but production of these compounds diverts electron flow from hydrogen generation. Solvent production in strain ATCC 824 is enhanced by the presence of a 192-kb megaplasmid that contains the genes of the solvent formation locus, which are responsible for butanol and acetone production [16,19]. *C. acetobutylicum* mutant strain M5 lacks this megaplasmid, but it has not been previously characterized for hydrogen production. We hypothesized that the lack of this megaplasmid could improve hydrogen yields through enhanced electron flow through acetate and hydrogen production.

We tested our hypotheses of increased hydrogen production using this large volume headspace technique using *C. acetobutylicum* ATCC 824 and its megaplasmid-deficient mutant M5 in order to examine the effect of removing the megaplasmid on hydrogen production. We also compared hydrogen yields by M5 in batch tests with the large headspace volume method to those obtained in continuous gas release tests using a respirometer. Continuous liquid flow reactor tests can produce 2 to 2.5 times more hydrogen than batch tests with mixed cultures [20]. We therefore also examined hydrogen production by M5 in continuous flow reactors. Hydrogen production by these two pure cultures was compared to that obtained using a heat-treated wastewater inoculum in order to compare our results to those previously obtained in batch-fed and continuous flow reactors with different hydrogen measurement methods [3].

2. Materials and methods

2.1. Cultures

C. acetobutylicum ATCC 824 was obtained from the American Type Culture Collection (ATCC). Strain M5 was kindly provided by E. Terry Papoutsakis, Northwestern University. An uncharacterized mixed culture capable of biohydrogen production was heat-treated to remove non-spore-forming bacteria (such as methanogens) as previously described [21] using anaerobic sludge obtained from the Penn State Wastewater Treatment Plant. Heat-treated sludge was stored in a sealed container at ambient temperature [3]. Glucose was added to a nutrient salt (NS) medium containing (per liter of distilled water): 1.0 g of NH_4Cl, 0.2 g of KH_2PO_4, 0.2 g of K_2HPO_4, 30 mg $MgCl_2 \cdot 6H_2O$, 2.5 mg of $FeCl_3$, 1.6 mg of $NiSO_4$, 2.5 mg of $CaCl_2$, 1.15 mg of $ZnCl_2$, 1.0 mg of $CoCl \cdot 6H_2O$, 0.5 mg of $CuCl_2 \cdot 2H_2O$, and 1.5 mg of $MnCl_2 \cdot 4H_2O$. The pH was buffered using 0.07 M 2-(N-morpholino)ethanesulfonic acid monohydrate (MES; J.T. Baker) adjusted to pH 6.2 using 1 M NaOH. Vials of each pure culture frozen stock suspension (ATCC 824 and M5) were thawed, immersed in a boiling water bath for 1 min, and transferred to tubes containing 20 ml of NS medium (7 g/liter glucose). For the mixed culture, 0.5 g of heated sludge was used in the same manner. These three master culture tubes were incubated at 30 °C for 3 days, and gas was released using autoclaved syringes. When gas production had ceased, the cultures were used as inocula for the batch tests.

2.2. Intermittent gas release batch tests

Hydrogen production with intermittent gas release was examined using 26.5-ml gas-tight test tubes (BellCo Glass Inc., Vineland, NJ). NS medium with different final glucose concentrations of 1, 3, 7, or 10 g/liter (each 250 ml) were sparged with nitrogen gas for 15 minutes and were put in an anaerobic chamber. The test tubes were filled with only 5 ml of the four different media. All the test tubes were capped with a rubber stopper and crimp-sealed. Concentrated L-cysteine solution (10 g/liter) was then added to each tube to produce a final concentration of approximately 84 mg/liter, and the test tubes were autoclaved for 15 min.

Batch tests were started by inoculating with 10 drops of the resuscitated cultures using 1-ml autoclaved syringes (21 G needle). The remaining headspace volume of the test tubes was 21.5 ml (81% headspace). These tests were conducted in constant temperature rooms (30 °C, except as indicated) in triplicate. During the incubation, the total gas volume was measured by releasing the pressure in the tubes using glass syringes that had been purged with nitrogen (1, 2, 5, 10, or 25 ml capacity; Perfektum Syringes; Popper & Sons, Inc.). After measuring the gas production volume, 0.1 ml of the headspace sample was taken using a gas-tight syringe for hydrogen measurement. Gas volume measurements were repeated over time until gas was no longer produced.

Separate batch tests with intermittent gas release were used to monitor biomass yield for ATCC 824 and M5 pure cultures. These tests were conducted in 26.5-ml gas-tight test

tubes (BellCo Glass Inc., Vineland, NJ) containing 15 ml NS medium buffered with 0.07 M MES adjusted to pH 6.2, with L-cysteine and 7 g/liter glucose. Tubes were inoculated with 0.375 ml of the resuscitated stock cultures (42% headspace), and samples were collected for initial dry weight biomass determination. Cultures were incubated at 30 °C, with periodic monitoring of absorbance at 600 nm. When growth ceased at approximately 60 h, samples were collected for a final biomass determination.

2.3. Continuous gas release batch tests

The effect of continuous gas release on hydrogen production was conducted as previously described [3]. Tests were run in duplicate and results averaged. Inocula (1%, v/v) from the master culture tubes were added to 159 ml serum bottles (Wheaton™ Scientific), which were then mixed using a stir bar on a respirometer stir plate (dial setting 2–3; Challenge Environmental Systems AER-200 Respirometer, Fayetteville, Arkansas) in a constant temperature room (30 °C). Gas production in the respirometer system was measured with a bubble meter calibrated according to the manufacturer's instructions.

2.4. Continuous liquid flow tests

Hydrogen production by strain M5 in continuous culture was investigated using a 2-liter fermentor (New Brunswick BioFlo 110). The reactor containing NS medium was autoclaved for 1 hr, cooled, and then sparged with filter-sterilized N_2 gas for 1 hr before being inoculated with 300 ml of strain M5 obtained from a batch culture test. L-cysteine (0.5 g/liter) was added to consume oxygen in the medium and lower the redox potential, and glucose (10 g COD/liter) was added as the electron donor. The solution pH was adjusted to 5.5 using 1 M KOH.

The reactor was initially operated in batch mode until the redox potential was reduced below −200 mV (~2 days). Then the reactor was switched to continuous mode at a hydraulic retention time (HRT) of 10 h and operated at 30 °C and a pH of 5.5 with glucose (10 g COD/liter). The feed bottle was continuously sparged with N_2 gas. Biogas production was measured using a soapfilm flowmeter, constructed with a graduated serological pipet, five times per sampling event for a total of fifteen measurements per HRT. Steady state conditions were assumed when hydrogen gas production was constant after at least 3 HRT. Headspace hydrogen and suspended biomass concentrations were measured in triplicate for each HRT, while volatile acid, alcohol, and glucose concentrations were measured once.

2.5. Analytical methods

Hydrogen in the headspace of test tubes and the fermentor was periodically measured using a gas-tight syringe (0.1 ml injection volume) and a gas chromatograph as previously described [21]. Hydrogen gas production in the intermittent gas release batch tests and the continuous culture test was calculated from headspace measurements of gas composition and the total volume of biogas produced at each time interval [3]. The concentrations of solvents (acetone, ethanol, propanol, and butanol) and organic acids (acetate, propionate, and butyrate) were analyzed by gas chromatography [22]. Glucose was analyzed using the phenol-sulfuric acid method for reducing sugars [23] following sample filtration (0.2 μm pore-diameter, Pall Corp., Ann Arbor, MI). Solution pH was measured using a pH probe and meter (Fisher Scientific accumet® model 10 and VWR SympHony). Conductivity was measured using a conductivity cell (Thermo Orion 011010) and meter (Thermo Orion, Model 115). Biomass dry weight was determined using the Total Suspended Solids technique [24].

3. Results

3.1. Intermittent gas release batch tests

Hydrogen yields in batch tests with intermittent gas release and a large headspace were 2.64 ± 0.02 mol-H_2/mol-glucose for the mutant M5, which was 47% larger than that obtained with the parent strain *C. acetobutylicum* ATCC 824 (1.79 ± 0.05 mol/mol; 7 g/liter of glucose, 30 °C) (Fig. 1). These values are both substantially larger than the yield of 1.21 ± 0.03 mol/mol obtained here using a heat-treated mixed culture, a yield that is consistent with previous results [21]. The large gas headspace method produced precise results, with standard deviations less than 2.7% based on triplicate samples. Decreasing the temperature from 30 °C to 20 °C did not affect the hydrogen yields for any of the three inocula (Fig. 1), but it significantly lowered the rate of hydrogen production by each culture and influenced the fermentation end products of the mixed culture.

Hydrogen production by each culture was complete within 45 h at 30 °C (Fig. 2), while it required 170 h at 20 °C (data not shown). For M5 and ATCC 824, the hydrogen concentration remained stable once the maximum concentration was reached. For the mixed culture, however, hydrogen was reabsorbed into solution and decreased to almost zero over the next 300 h. Maximum hydrogen concentrations in the headspace were 32.6%, 25.4%, and 17.6% for M5, ATCC 824, and the mixed culture, respectively (Fig. 2). These percent

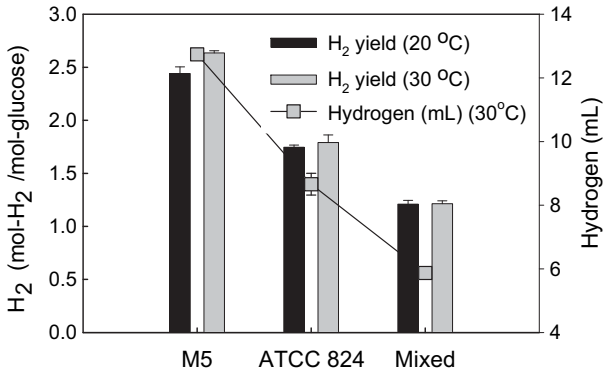

Fig. 1 – Hydrogen yield and production volume and effect of temperature (20 °C and 30 °C) on hydrogen yields with M5, ATCC 824, and mixed culture using the large volume headspace test. (Initial glucose = 7 g/liter; Error bars ± SD for experiments conducted in triplicate).

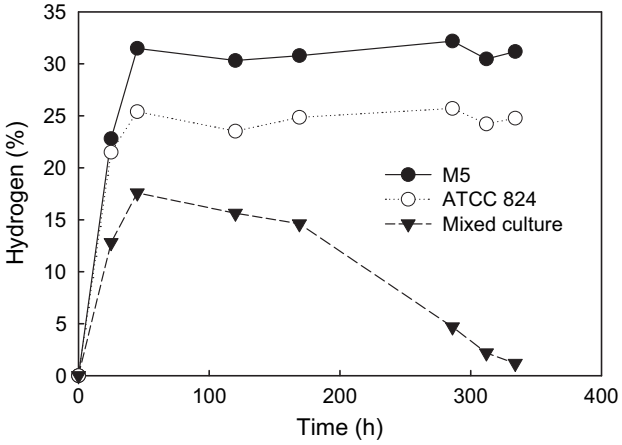

Fig. 2 – Hydrogen gas concentrations (%) in the headspace over time. (Initial glucose = 7 g/liter; 30 °C).

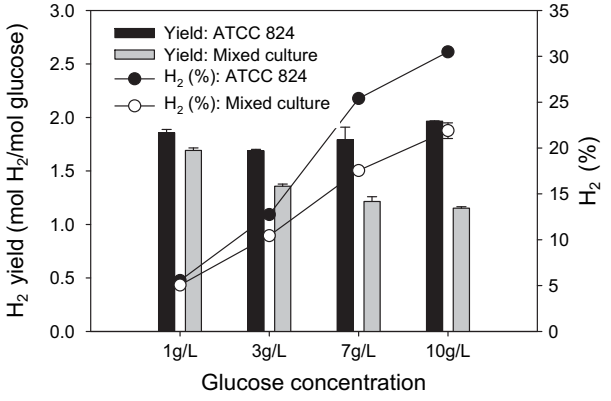

Fig. 3 – Hydrogen yield and hydrogen (% and ml) as a function of initial glucose concentration. (30 °C; Error bars ± SD for experiments conducted in triplicate).

concentrations are lower than the maximum concentrations typically achieved in batch tests (up to 72%; [21]) due to dilution in the large headspace. Hydrogen partial pressures were less than 56.5 kPa in all cases, equivalent to 12 ml of hydrogen in a headspace volume of 21.5 ml. Hydrogen in the headspace in abiotic controls (no inoculum; 60% hydrogen and 7 g/liter glucose) was constant over the same period of time, and in biotic controls (inoculum and nitrogen, no glucose) there was no measurable hydrogen production (data not shown).

The effect of glucose concentration on hydrogen yield and fermentation end product composition was tested using ATCC 824 and the mixed culture at glucose concentrations of 1, 3, 7, and 10 g/liter. For ATCC 824, hydrogen yields were independent of the initial glucose concentration over this range, averaging 1.83 ± 0.12 mol-H_2/mol-glucose (Fig. 3). For the mixed culture, however, hydrogen yield decreased from 1.69 to 1.15 mol-H_2/mol-glucose as the initial glucose concentration increased from 1 to 10 g/liter. Final hydrogen concentrations in the headspace of the mixed-culture tubes increased from 5.1% to 21.9% due to the higher glucose loading. These hydrogen concentrations in the headspace were lower than those observed using ATCC 824 at each respective initial glucose concentration.

After hydrogen production ceased, volatile fatty acids and solvents were measured in the test tubes inoculated with ATCC 824 (Fig. 4a) and the mixed culture (Fig. 4b). For ATCC 824, the predominant products were acetate and butyrate, produced in an average molar ratio of 0.68 ± 0.07. The ethanol concentration was nearly constant at 160 mg/liter, accounting for an appreciable fraction of the end products at 1 g/liter glucose but only a minor relative amount at 10 g/liter. The butanol concentration was consistently below 50 mg/liter, and propionate was not detected. For the mixed culture, the pattern of fermentation products as a function of glucose concentration was more varied (Fig. 4b). Ethanol and acetate were the most significant products at each glucose concentration. The ethanol concentration increased steadily with glucose concentration. The concentration of acetate, however, increased with glucose concentration up to 7 g/liter, but then decreased at 10 g/liter glucose. Butyrate concentrations increased with glucose, beginning as only a minor component at 1 g/liter glucose and increasing to just above 1000 mg/liter at 10 g/liter glucose. Propionate showed the same pattern as acetate, but it was present at considerably lower concentrations. The concentration of butanol was consistently low. Although M5 was not tested at various glucose concentrations, the fermentation products at 7 g/liter glucose were similar to those obtained with the parent strain (ATCC 824), with a comparable level of ethanol and a predominance of acetate and butyrate produced at an average molar ratio of 0.64 ± 0.02 (Fig. 5).

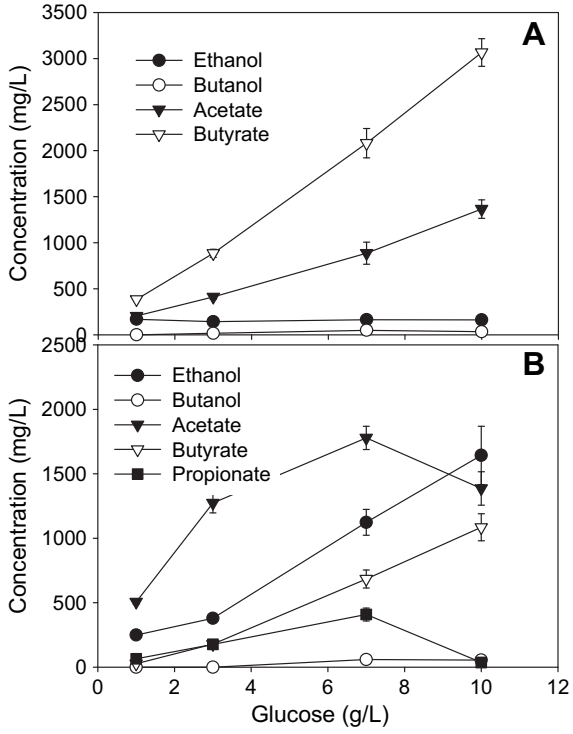

Fig. 4 – Alcohols and volatile fatty acids (VFAs) concentration as a function of initial glucose concentration using ATCC 824 (A) and mixed culture (B). (30 °C; Error bars ± SD based on triplicate measurements of the medium).

ATCC 824 and M5 showed similar fermentation products that did not vary with temperature. Expressing average fermentation products on the basis of chemical oxygen demand (COD), glucose was converted by ATCC 824 into 5% ethanol, 2% butanol, 12% acetate, 54% butyrate, and 15% hydrogen. For M5, the product distribution was 5% ethanol, <1% butanol, 14% acetate, 56% butyrate, and 22% hydrogen (Fig. 5). The trace detection of butanol in the M5 tests is likely just analytical noise, since strain M5 lacks the genes required for butanol formation [19] and the measured concentration was close to the quantitation limit. The temperature change did not affect the relative composition of alcohols, VFAs, and hydrogen for the pure cultures. For the mixed culture, the product composition changed depending on temperature. Most notably, ethanol increased between 20 and 30 °C from 19 to 30% of the COD, and acetate increased from 4 to 24%, with a commensurate decrease in butyrate from 50 to 16%.

Biomass production for ATCC 824 and M5 was measured in separate intermittent gas release culture tubes, due to the limited sample volume that could be spared from the large headspace volume test tube cultures. Absorbance monitoring of these two cultures revealed that ATCC 824 had a higher growth yield than M5 (Fig. 6). The biomass yields were 0.081 and 0.051 g dry weight/g COD for ATCC 824 and M5, respectively, based on the change in suspended dry weight over the course of the experiment. The addition of biomass to the COD balance, which respectively accounted for 12% and 7% of the added electrons for ATCC 824 and M5 using these measured yield values and a biomass dry weight molecular formula of $C_5H_7O_2N$ (i.e., 1.42 g COD/g dry weight), results in average COD recoveries of 100% and 104% for ATCC 824 and M5, respectively.

3.2. *Continuous gas release batch tests*

The same cultures used in the large headspace volume, intermittent gas release method were evaluated in a respirometric test. The maximum biogas production was obtained with M5 (102 ml) (Fig. 7). Biogas production was somewhat lower with ATCC 824 (72 ml) and was much lower with the mixed culture (46.7 ml). Following a lag phase of approximately 7 h, there was gas production for the next 6 h in all culture bottles. The maximum biogas production rates were 20.8 ml/h (M5), 15.1 ml/h (ATCC 824), and 13.7 ml/h (mixed culture), and glucose was almost completely consumed. Hydrogen yields were approximately 2.5 (M5), 1.6 (ATCC 824), and 1.1 (mixed culture) mol-H_2/mol-glucose, which were calculated assuming a constant hydrogen gas production of 62% based on a previous study [3]. The hydrogen yields measured by the large headspace volume, intermittent gas release method were on average 14% higher than these respirometrically determined yields.

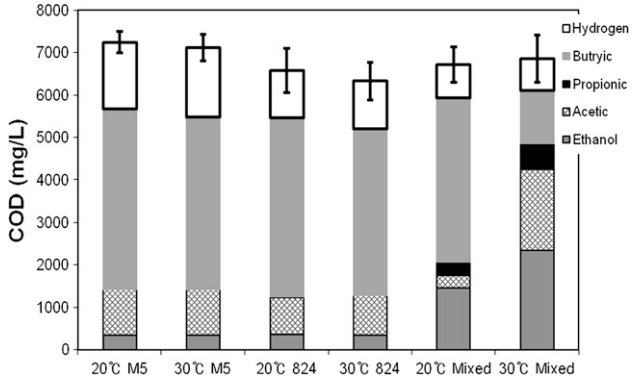

Fig. 5 – Chemical oxygen demands calculated from the product concentrations with M5, ATCC 824, and mixed culture at different temperature (20 °C and 30 °C). Initial glucose COD was 7466 mg/liter. All data presented are from averages of triplicate measurements of the medium, and the error bar represents the sum of the measurement standard deviations.

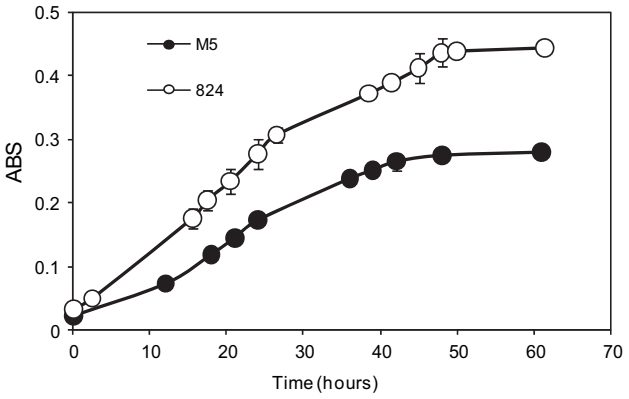

Fig. 6 – Growth of ATCC 824 and M5 in batch tests with intermittent gas release. (Initial glucose = 7 g COD/liter; 30 °C).

3.3. *Continuous liquid flow tests*

After a three-day acclimation period, continuous and steady hydrogen production was sustained by M5. Hydrogen yields, calculated from data on hydrogen production and total glucose consumption over 1 week of continuous operation,

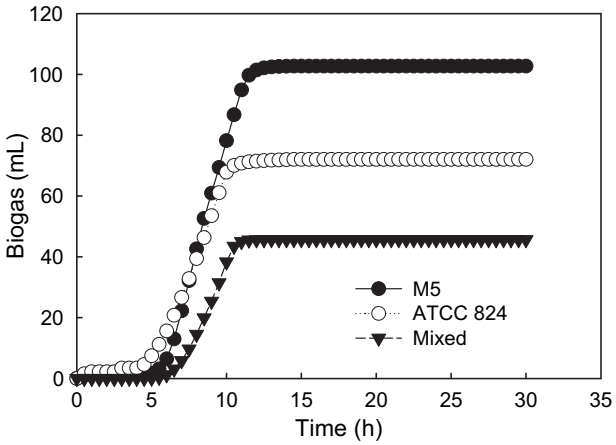

Fig. 7 – Biogas production from glucose (7 g/liter) by three different cultures over time in a respirometric test at 30 °C.

averaged 2.44 ± 0.16 mol H_2/mol glucose. An average hydrogen production rate of 9.39 ± 0.63 ml/min was obtained from a total gas production of 14.7 ± 0.9 ml/min, with a hydrogen gas phase concentration of $63.8 \pm 0.6\%$. The biomass concentration was 2.4 ± 0.3 g/liter at a glucose removal efficiency of $89.5 \pm 1.0\%$. The main fermentation byproducts were butyrate (2870 ± 60 mg/liter) and acetate (1320 ± 130 mg/liter). Ethanol and butanol concentrations were 71 ± 2 and 18 ± 11 mg/liter, respectively. This detection of butanol, which would account for only 0.47% of the electron equivalents in the added glucose, is likely just analytical noise close to the quantitation limit.

4. Discussion

The hydrogen yields obtained using the large headspace, intermittent gas release approach were higher for all three cultures than the continuous gas release respirometric results. The intermittent release method produced yields that were 9% and 10% higher for M5 and ATCC 824, respectively, and 17% higher yield for the mixed culture, than those obtained using a respirometer. The use of a large headspace in the intermittent gas release method reduces both the potential for adverse effects on gas production by high hydrogen and carbon dioxide partial pressures, as well as gas leakages from system connections and tubing, both of which may have contributed to the lower measured yields with the respirometric approach. Tests can also be conducted at a significantly lower cost and effort than with respirometric approaches. Screening a large number of cultures using a respirometer would be difficult since most respirometer systems manage only a few bottles at a time. Respirometric applications are more suitable when data is needed for kinetic parameters since gas production data are recorded automatically at preset time intervals.

In batch tests using M5 and the parent strain, neither of these cultures reconsumed hydrogen in the headspace. However, the mixed culture consumed all the hydrogen in the headspace within 300 h. Many hydrogenases catalyze the reversible oxidation of molecular hydrogen, and hydrogen uptake (hydrogen oxidation) is coupled to the reduction of electron acceptors. Potential electron acceptors in our tests were sulfate and carbon dioxide. ATCC 824 is not a homoacetogen, and thus we did not expect hydrogen reconsumption in pure culture tests. In mixed culture tests, some spore-forming homoacetogens and sulfate reducers likely survived heat treatment and consumed hydrogen [21].

Mutant M5 had a 47% higher hydrogen yield from glucose than its parent ATCC 824. However, based on a comparison of the electron balances for each culture, most of the additional electrons directed toward hydrogen production were not due to differences in soluble fermentation product distribution. ATCC 824 produced no acetone and only a very small amount of butanol under the experimental conditions used in this study. With an initial pH of 6.2 and 0.07 M MES buffer, the final pH of the ATCC 824 batch tests at 30 °C were 6.08, 5.78, 5.23, and 4.85 at glucose concentrations of 1, 3, 7, and 10 g/liter, respectively. The literature reports the lack of solvent production in cultures maintained at a constant circumneutral pH [25], a shift from acid production to alcohol and solvent production at or below pH 5.2 with batch cultures of *C. acetobutyricum* [26,27] and pH 4.6 with *Clostridium beijerinckii* [28], and butanol production with ATCC 824 at a high concentration of glucose (50 g/liter) and low buffer capacity (0.75 g/liter of KH_2PO_4 and 0.75 g/liter of K_2HPO_4) [29]. It appears that under conditions generated here, the duration of low pH and other incubation conditions were not sufficient to stimulate solvent production in these bacteria.

The COD balance indicates that 67% of the increased hydrogen yield with M5 can be attributed to a lower biomass yield with this strain (i.e., M5 produced 328 mg COD/L less biomass and 486 mg COD/L more hydrogen). An explanation for the 38% lower cell yield for M5 relative to ATCC 824 is not clear from the monitoring of fermentation products, as these two cultures produced very similar soluble products. The megaplasmid contributes to ATCC 824 second copies of genes related to PTS-type sugar transport, aldolase that cleaves fructose-1,6-bisphosphate in glycolysis, and thiolase that catalyzes the conversion of two acetyl-CoA molecules into acetoacetyl-CoA toward the production of butyrate or butanol [16]. Given the nearly identical catabolic end product yields, apart from hydrogen, the energetics of catabolism might be similar between these strains. The reorientation of electron fluxes through alternate pathways can be difficult to predict with single gene alterations, let alone with the presence or absence of a genetic element that encodes an estimated 178 polypeptides [16].

The conversion efficiency of glucose to hydrogen by M5 using the large headspace method was 66% (assuming a maximum 4 mol H_2/mol-glucose), compared to a 60% conversion efficiency obtained in a continuous mode at a 10-h HRT. With mixed-culture inocula, continuous flow reactors have been shown to have higher hydrogen conversion efficiencies (40–45%) [30] than batch cultures (25%) [3], which could be explained by community shifts in the continuous flow systems that include washing out some hydrogen-consuming bacteria at a short HRT (5–50 h HRT). However, this would not be relevant for the pure-culture M5 reactor, and hydrogen consumption was not observed with this strain. As in the comparison between intermittent and continuous gas release measurements, the slight difference in hydrogen yields between batch and continuous cultures could be explained by better hydrogen containment with the test tube cultures compared to the 2-liter fermentor.

5. Conclusions

The use of a high hydrogen headspace volume (81%) and intermittent gas release increased hydrogen yields on average between 9 and 17% compared to respirometric tests using pure and mixed cultures. Measuring hydrogen gas production with a large headspace has the advantage of minimal opportunities for gas leakage, as well as a reduction in the partial pressure of hydrogen and carbon dioxide compared to intermittent release tests using tubes with a much lower headspace volume (e.g., 40% with the Owen method). Our results also show that a megaplasmid-deficient mutant strain M5 of *C. acetobutylicum* ATCC 824 produced more hydrogen than the parent strain. While the reason for this higher hydrogen yield is not

completely clear, it was related to the significantly reduced biomass production by M5 compared to the parent strain. Our results demonstrate that the high headspace testing procedure is a useful and economical method for screening different types of cultures for hydrogen production.

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