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Hydrogen Production by the Thermophilic Bacterium *Thermotoga neapolitana*

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Abstract

Virtually all members of the order *Thermotogales* have demonstrated the ability to produce hydrogen; however, some members of this order produce considerably greater quantities than others. With one representative of this order, *Thermotoga neapolitana*, we have consistently obtained accumulation of 25–30% hydrogen with 12–15% carbon dioxide as the only other prominent product in the batch reaction. In contradistinction to information widely disseminated in the literature, we have also found that most members of this order tolerate and appear to utilize the moderate amounts of oxygen present in the gaseous phase of batch reactors (6–12%), with no apparent decrease in hydrogen production. Hydrogen accumulation has been widely reported to inhibit growth of *Thermotogales*. While this may be true at very high hydrogen tensions, we have observed log phase bacterial morphology (rods) even in the presence of 25–35% hydrogen concentrations. To maximize hydrogen production and minimize production of hydrogen sulfide, inorganic sulfur donors are avoided and the cysteine concentration in the medium is increased. We and others have demonstrated that different members of the order *Thermotogales* utilize a wide variety of feedstocks, including complex carbohydrates and proteins. Thus, it appears that organisms within this order have the potential to utilize a variety of organic wastes and to cost-effectively generate hydrogen.

Index Entries: *Thermotogales; Thermotoga neapolitana;* hydrogen generation; microaerophiles.

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Introduction

The United States currently consumes approx 3.6 trillion cubic feet of hydrogen gas annually, while the worldwide consumption is about three times that amount *(1,2)*. The US demand for hydrogen gas is expected to grow by 40% within the next 5 yr. Most of this hydrogen gas is made from synthesized gas that comes either from the reformation of natural gas or from the gasification of coal *(3,4)*. These processes are costly and environmentally problematic. Therefore, society must develop economical, continuous, environmentally friendly methods of hydrogen production. Biological generation of hydrogen as an end product or byproduct of the metabolism of biologic organisms has been proposed as one means of producing needed hydrogen *(5)*.

Investigators have attempted to produce biohydrogen for many years, generally using one of three different types of metabolic processes: (1) photosynthetic unicellular organisms that utilize either nitrogenase or hydrogenase reactions to produce hydrogen, (2) fermentative bacteria that produce hydrogen anaerobically, and (3) various stepwise processes in which a combination of bacteria of one population predigests more complex organic molecules to make a less complex feedstock of simple organic molecules that can be subsequently used by hydrogen-producing organisms *(6–9)*. Only modest success has been achieved so far because organism growth is end product (or byproduct) inhibited by catabolites produced in the medium, hydrogen gas production is inhibited because growth slows with increasing hydrogen concentrations, the range of feedstocks the organisms can use is prohibitively narrow, the concomitant production of undesirable gases is high, or the rate of hydrogen gas production is endogenously low *(5)*.

Building on earlier information obtained by other investigators, we have taken a different approach to the biogeneration of hydrogen and have tested the hydrogen generation capability of 15 different strains of extremophilic bacteria of the order *Thermotogales*. *Thermotogales* is an order composed of rod-shaped, Gram-negative, nonsporulating bacteria that have a loose surrounding membrane or "toga." In 1986, Huber et al. *(10)* identified the first member of this order, *Thermotoga maritima* (DSM 3109). At about the same time, a second extremophilic bacterium, *Thermotoga neapolitana* (Deutshe Sammlung von Mikroorganismen [DSM] 4359, American Type Culture Condition [ATCC] 49049), was identified by Belkin, and coworkers *(11,12)*. More than 20 different members of the order *Thermotogales* in at least five different genera *(13)* are currently known. These organisms have been isolated from a variety of environments: freshwater and marine hot springs, hot sulfur springs, near the mouths of marine black smokers, and hot oil wells. All of these organisms have been isolated from environments in which the temperature is significantly elevated. Most of the *Thermotogales* can withstand elevated pressures as well; however, our laboratory studies confirm earlier reports *(10–14)* that

they grow quite readily at atmospheric pressure as long as the temperature is elevated. Most species, including *T. neapolitana*, are reported in the literature to be obligate anaerobes *(10–14)*; however, we present evidence to the contrary below. Many species of the *Thermotogales* appear to reduce S^0 or other sulfur compounds, although sulfur reduction does not appear to be required for the growth of *T. neapolitana*. In any event, the relationship between growth and sulfur utilization by the *Thermotogales* is currently unclear *(14)*.

We cultured and tested 15 different species in the order *Thermotogales* and confirmed results in the literature indicating that during bacterial metabolism these species characteristically produce hydrogen. Most reports in the literature do not give a clear indication of the amount of hydrogen produced, or the potential utility of these organisms for commercial production of hydrogen. We have found that the amount of hydrogen generated can be highly variable, depending on the *Thermotogales* species used and the degree of optimization of the organisms for production of hydrogen. However, we observed that under microaerophilic conditions, most species of the *Thermotogales* produce significant amounts of hydrogen during incubation.

Our studies concentrated on optimization of hydrogen production using *T. neapolitana* because this organism appears to be particularly robust and adaptable to varying conditions and to utilization of different primary carbon sources. Earlier researchers demonstrated that *T. neapolitana* is the only member of this order that can grow on a medium that does not contain proteins or other substances that are poorly defined *(15)*, and that it can utilize a wide variety of substrates.

Preserved cultures were purchased from either DSM or ATCC. Prior to inoculation of a new batch culture vessel, a sample of the stock culture was grown on a medium that did not contain a carbon source other than the yeast and trypticase that was part of the medium's formulation. In this way, we ensured that carbohydrate levels were not artificially elevated by inoculation of the new batch culture vessel with a carbon-rich medium. Serum bottles with rubber septa sealed with crimped lids were used as batch reactors.

Materials and Methods

The following medium (modified from ATCC 1977 medium recommended for *T. elfeii*) was used to prepare the batch reactors for culture of *T. neapolitana*: 1.0 g of NH_4Cl, 0.3 g of K_2HPO_4, 0.3 g of KH_2PO_4, 0.2 g of $MgCl_2 \cdot 6\,H_2O$, 1 g of $CaCl_2 \cdot 2\,H_2O$, 10.0 g of NaCl, 0.1 g of KCl, 1.0 g of Cysteine HCl, 2.0 g of yeast extract, 2.0 g of Trypticase, 10.0 mL of vitamin solution (DSM medium 141), 10.0 mL of trace element solution (DSM medium 141), and 1.0 L of H_2O. The initial pH of the medium was adjusted to 8.5 at 20°C with NaOH. Trizma base (1×10^{-4} M) was sometimes added to help maintain the pH in an optimal range for longer periods during

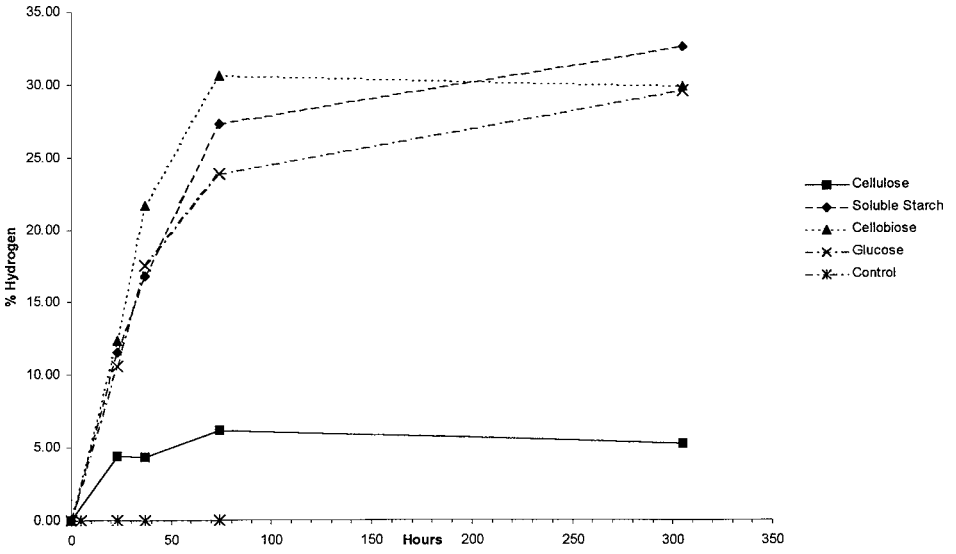


Fig. 1. Hydrogen production by *T. neapolitana* grown on different primary carbon sources.

incubation. A primary carbon source (such as 0.25 g/50 mL of cellulose, cellobiose, soluble starch, or glucose) was also added.

The medium was always prepared aerobically. Fifty milliliters of the forementioned medium was aliquoted into serum bottles, allowing 110 mL of headspace for gas accumulation. Excess oxygen was removed by heating the batch reactors containing medium and substrate to 100°C while sparging its contents (2–10 min) with nitrogen gas. Sparging time was varied to achieve predetermined concentrations of oxygen between 1 and 12% in the headspace. (The effects of different oxygen concentrations are discussed later). Serum bottles were then sealed, capped, and sterilized.

The headspace was sampled using gastight syringes and assayed to determine the amount of oxygen in the headspace using a Hewlett-Packard Gas Chromatograph. Thereafter, the batch reactor was inoculated with 1 mL of the bacterial stock solution using sterile tuberculin syringes. The batch reactor was then incubated at 70°C. All gas measurements were made at room temperature using the gas chromatograph . Glucose measurements were made with a handheld glucometer from Bayer and confirmed with a glucometer from Nova Biomedicals. Cells were counted using a counting chamber in association with SigmaScan image analysis software and confirmed later using a Beckmann Coulter Counter.

Results

As already noted, we have tested several different carbon sources for their ability to support production of hydrogen gas. Figure 1 shows an example of the variable hydrogen generation results obtained with differ-

ent primary carbon sources. For our more complete analysis (discussed later), only the results obtained when glucose was the primary carbon source are examined since growth on glucose is widely reported in the literature. This allows our results to be easily compared with those reported by other investigators.

Typical results are found in Figures 2A,B, which shows the relationship between initial oxygen concentration in the headspace and hydrogen generation with respect to time. In Figure 2A, the average (n = 6) initial oxygen level in the headspace was >11%, and the average concentration of hydrogen in the headspace after the experiment exceeded 28%. In a similar set of experiments (n = 3), in which the initial oxygen level in the headspace was quite low, hydrogen generation was also low (12%). In fact, oxygen concentrations in the headspace appeared to rise modestly over time when the initial oxygen level in the headspace was very low (Fig. 2B) The reason for and significance of this is currently under investigation.

Our experiments indicate that not all of the decrease in initial oxygen in the headspace of the batch reactor can be related directly to utilization by microorganisms, but that a certain amount of oxygen depletion might be expected, particularly since the incubation temperature is 70°C. Figure 3A–C shows the relationship of oxygen depletion to hydrogen and carbon dioxide generation. Figure 3aAshows the results of control experiments, indicating oxygen levels (dotted line), accumulation of hydrogen (open bar), and carbon dioxide (shaded bars) for uninoculated batch reactors containing a glucose substrate. In Fig. 3A no hydrogen and very little carbon dioxide were produced. Overall, a decrease in oxygen of about 3% (n = 5) in the batch reactor headspace was seen. A similar response (about 2% decrease in oxygen, n = 3) was observed when the inoculated batch reactor contained no glucose as a substrate but did contain trypticase and yeast extract as a carbon source (Figure 3B). Note the differences seen are within sampling error for the system. However, in Fig. 3C, inoculated batch reactors (n = 6) contained glucose (Fig. 3C) and oxygen (dotted line) levels of about 6% in the headspace at the start of the batch reaction, and oxygen depletion was nearly complete over the course of the experiment. In addition, after 62 h, the hydrogen concentration averaged >23% and the carbon dioxide level also increased. The ratio of hydrogen to carbon dioxide approached 2:1. It is unlikely that this observed oxygen depletion can be attributed to leakage through the septum because hydrogen, a much smaller molecule, remains contained within the mildly pressurized batch reactor headspace while the oxygen concentration in the batch reactor decreases and remains low.

In an attempt to determine whether these organisms might be capable of catabolic processes requiring (and utilizing) oxygen, we tested the ability of malonate, a competitive inhibitor of the trichloroacetic acid cycle enzyme succinic dehydrogenase, to block the catabolic processes associated with hydrogen production. Figure 4A,B presents the results of those tests. In the presence of 0.104 g/50 mL of malonate, hydrogen generation

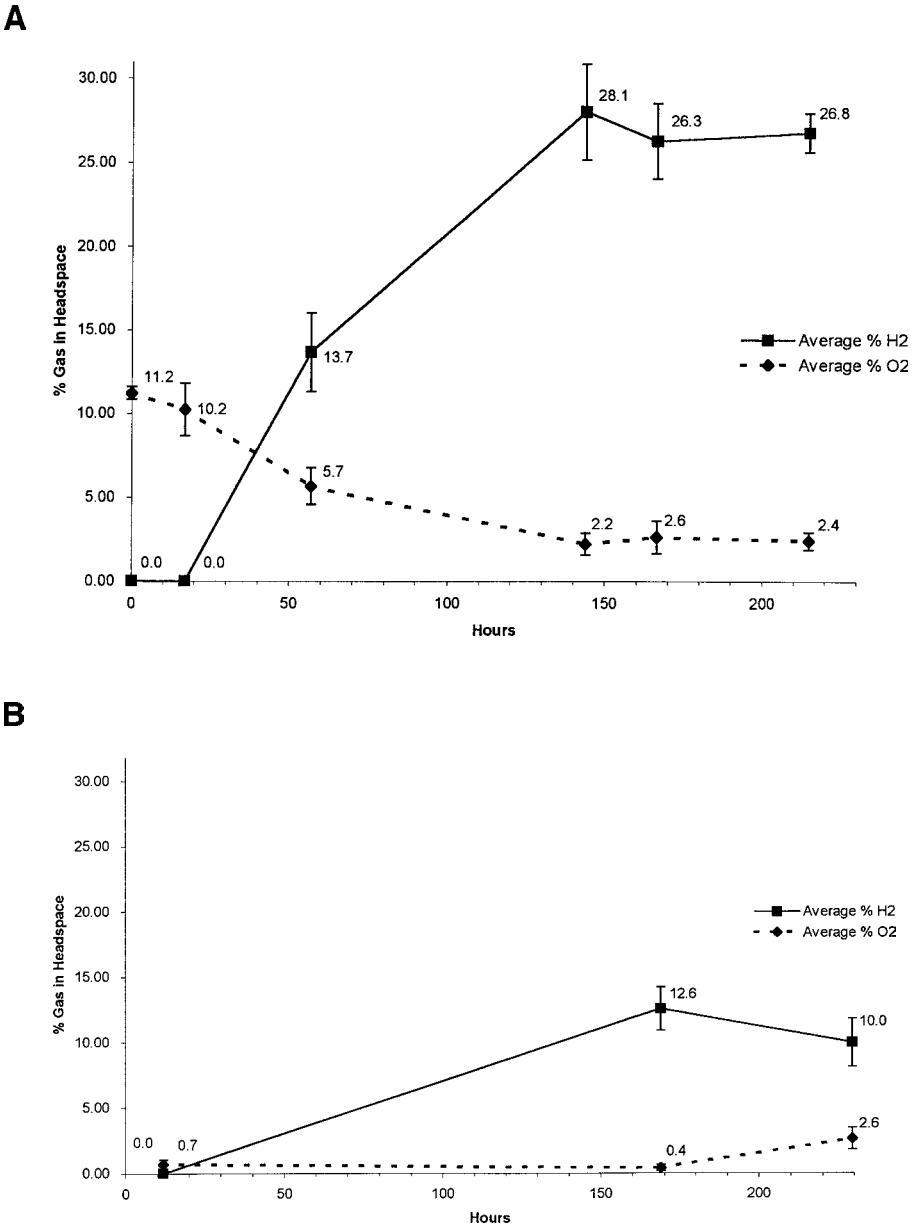


Fig. 2 (**A**) Relationship of headspace hydrogen and oxygen concentrations vs time when initial oxygen concentration was high. The percentage of hydrogen (■) increased to an average of 28.05% while the percentage of oxygen (◆) decreased from >11% to <3% over the same period of time (n = 6). (**B**) Percentage of hydrogen and oxygen concentrations vs time when initial headspace oxygen concentration was low. The maximum hydrogen (◆) increased to an average of less than half of that seen in (A), while the initial average percentage of oxygen (■) was <1 initially and actually increased slightly over extended periods of time (n = 3). The reason for the observed increase in oxygen after extended periods of low oxygen are uncertain, but commonly seen. The etiology of this is currently under investigation.

was completely inhibited for >40 hours. After that time, it is postulated that free malonate is no longer available to block catabolism, and thus rapid hydrogen generation ensues. Ion chromatographic studies are currently under way to verify that free malonate is not found in the medium once hydrogen production is no longer blocked. Note also that oxygen depletion does not begin in malonate-treated samples until hydrogen generation begins, and that oxygen concentration continues to rapidly decrease when hydrogen generation is increasing.

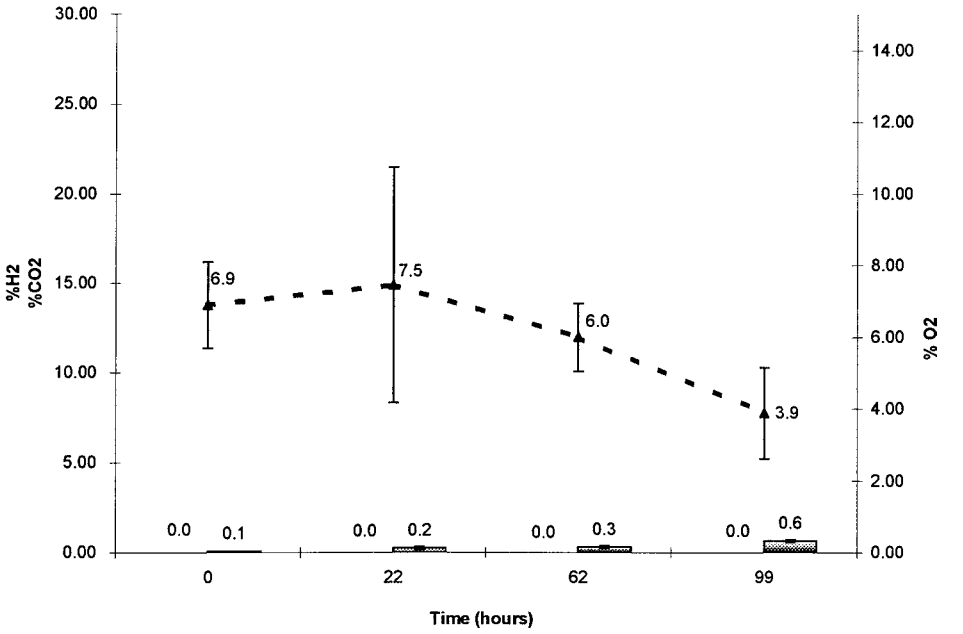
The rates and maximal concentrations of hydrogen generated can vary greatly depending on the primary carbon source and the initial oxygen concentration in the headspace. The maximum hydrogen generation rate we have recorded for any one specific batch experiment to date is given in Table 1. In another single experiment using *T. neapolitana* grown on soluble starch (Table 2), 28.47 mL of hydrogen gas was produced in 39 h. Using the ideal gas laws to calculate the production rate yields 6.63 mL/L headspace/h, 2.98×10^{-5} mol/h or 5.97×10^{-4} mol/L fluid/h. Because this was a batch experiment of short duration, the bacteria were still in the log phase of growth; this tells us little about what to expect from these bacteria in the stationary growth phase.

In a test to estimate yields (Table 3), we obtained hydrogen gas accumulations in the headspace of up to 28.51% in 50-mL batch experiments incubated for periods of 6 d (144 h). The calculated hydrogen production in the 110-mL headspace for this particular sample was 1.28×10^{-3} mol with a calculated rate of production of 1.98 mL/L headspace/h, or 8.9×10^{-6} mol/h. Based on the amount of glucose present before and after the experiment, and the amount of hydrogen produced, the calculated yield of this process was estimated to be 71 ± 24%, thus exceeding the Thauer limit anticipated for fermentations *(16)*. Because *T. neapolitana* has the ability to grow using only proteins as a carbon source, the end-product hydrogen gas produced in the absence of glucose as a substrate was subtracted from the total hydrogen output prior to calculating yield. We also counted the number of cells at the start and end of the experiment and found the increase in the cell number to be greater than 35.4 ± 3.1 times. This increase was correlated with absorbance changes and measured glucose concentrations before and after the batch reactor experiment. Thus, calculated yield values do not include increases in bacterial cell number.

Discussion

Most investigators have grown members of the order *Thermotogales* as strict anaerobes and have developed elaborate methods to remove all oxygen from the environment, forcing only fermentative catabolism. However, based on our results, *T. neapolitana*, appears to be a microaerophile, capable of utilizing reduced levels of oxygen and generating hydrogen as an end product. A finding of oxygen tolerance/utilization is not unprecedented since the only other thermophilic bacterial order that has

A


%H2
%CO2
% O2
Time (hours)
0
22
62
99
6.9
7.5
6.0
3.9
0.0
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0.0
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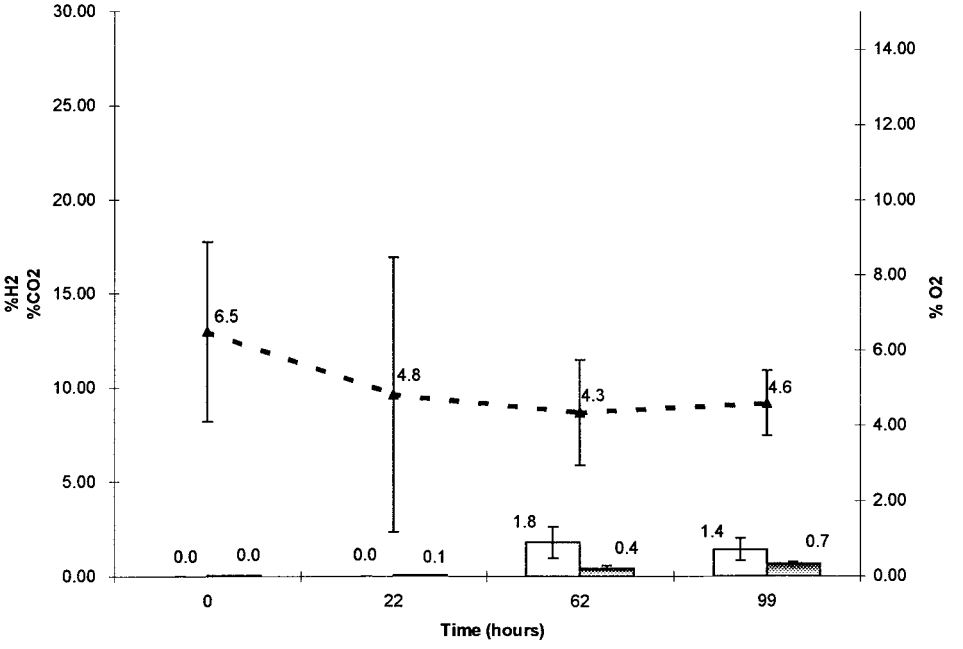
B


%H2
%CO2
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Time (hours)
0
22
62
99
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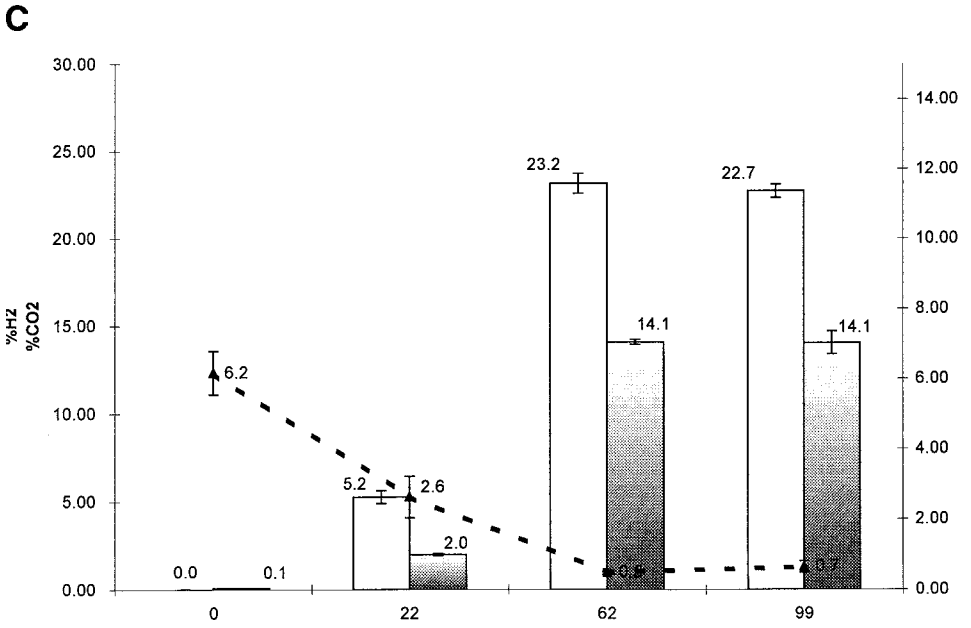

Fig. 3 (**A**) Relationship of average percentage of hydrogen (open bar: not seen when no hydrogen produced), carbon dioxide (shaded bars), and oxygen (solid bars) with respect to time when no inoculum was added to batch reactor (n = 5). Some oxygen depletion occurred in the absence of metabolizing cells; however, no hydrogen and very little carbon dioxide was produced without inoculation and incubation of the medium. (**B**) Relationship of average percentage of hydrogen (open bars), carbon dioxide (shaded bars), and oxygen (dotted line) with respect to time when no glucose was added to the batch reactor (n = 3). Some of the yeast and trypticase added to the batch reactor were capable of generating modest amounts of hydrogen and carbon dioxide. However, no hydrogen and very little carbon dioxide were produced without inoculation and incubation of the medium. (**C**) Relationship of average percentage of hydrogen (open bars), carbon dioxide (shaded bars), and oxygen (dotted line) when the batch reactor both contained glucose and was inoculated with bacteria (n = 6).

been identified as phylogenetically closely related to *Thermotogales*, the *Aquifex-Hydrogenobacter* group, includes more than one microaerophile *(17,18)*. Beh et al. *(19)* have shown that *Aquifex pyrophilus* (a microaerophile) is capable of shifting its metabolic behavior depending on oxygen availability. With oxygen available, its utilization by the metabolic behavior of *T. neapolitana* might also be predicted to shift metabolic behavior to a more energy-efficient catabolic pattern resulting in elevated hydrogen production and greater yields of metabolic hydrogen. Thus, oxygen removal may be responsible for a failure of earlier investigators to obtain high rates of hydrogen production. One avenue of future work will be to determine to what extent other facets of the metabolic behavior of *T.neapolitana* also vary with increased oxygen availability.

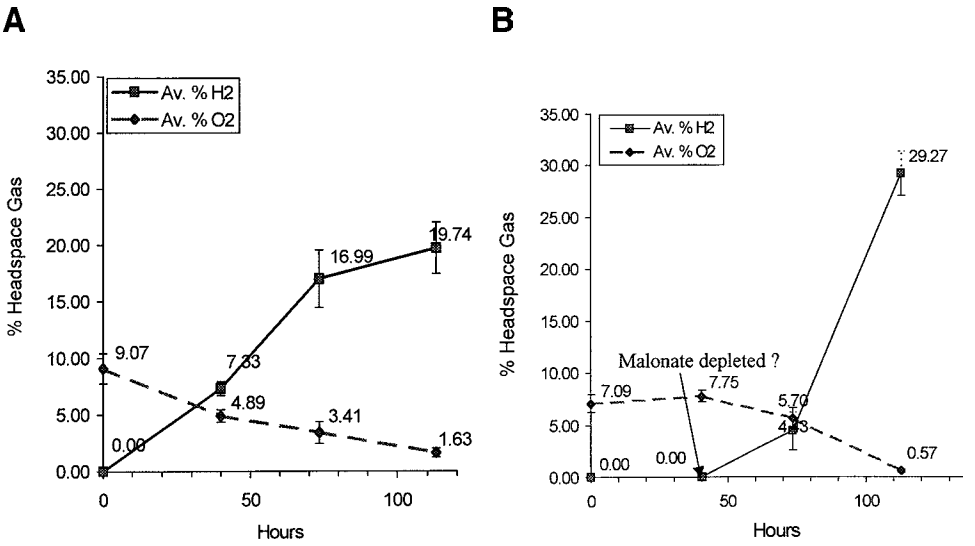

Fig. 4. Effect of malonate on generation of hydrogen gas and oxygen depletion: (**A**) Hydrogen generation in the absence of malonate (n = 7). (**B**) Hydrogen generation in the presence of 0.104 g/50 mL of malonate (n = 7). The oxygen depletion pattern mirrored hydrogen generation, and both were affected by malonate.

Rates of hydrogen generation can vary greatly for a variety of reasons other than varying oxygen levels in the headspace. Factors such as sampling time, pH, buffering capability of the medium, components of the medium, and genetic heterogeneity also contribute to variable rates of hydrogen generation. These confounding factors have been thoroughly examined and will be considered in a separate publication (in preparation). Even with other factors present that are known to affect results, the effects of different oxygen concentrations are readily apparent. Future work will attempt to decipher the roles of the various factors and optimize both rates and yields.

Meeting the future worldwide demand for hydrogen will require the development of continuous processes for hydrogen generation and efficient use of inexpensive waste materials as primary carbon sources. We are currently developing such a process. Preliminary experiments using a continuous stirred-tank reactor in batch mode indicate that after 6 wk of growth at constant temperature and pH, the organisms are still capable of producing significant amounts of hydrogen.

Acknowledgments

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Table 1
Greatest Rate of H_2 Output Produced to Date During a Batch Experiment [a]

Final H_2 %	Elapsed time (h)	Headspace volume (mL)	Fluid volume (mL)	H_2 partial pressure (atm)	Final volume of H_2 (mL)	H_2 product (mol)	H_2 production rate (mL/L headspace/h)	H_2 production rate (mol/h)	H_2 production rate (mL/L fluid/h)
25.86	39	110	50	.259	28.47	1.16×10^{-3}	6.63	2.98×10^{-5}	5.97×10^{-4}

[a]Elapsed time reflects the time from the start of inoculation and incubation at 70°C. These calculations utilize the ideal gas laws and assume that the initial pressure in the batch reactor was 1 atm and that the temperature of measurement was 25°C. The initial H_2 concentration was zero. Soluble starch was the primary carbon source.

Table 2
H_2 production by *T. neapolitana* Sample Used to Determine Cell Counts, Absorbance Measurements, and Yields Shown in Table 3[a]

Final H_2 %	Elapsed time (h)	Headspace volume (mL)	Fluid volume (mL)	H_2 partial pressure (atm)	Final volume of H_2 (mL)	H_2 product (mol)	H_2 production rate (mL/L headspace/h)	H_2 production rate (mol/h)	H_2 production rate (mL/L fluid/h)
31.05	144	110	50	.311	34.16	1.40×10^{-3}	2.16	9.71×10^{-6}	1.94×10^{-4}
28.51	144	110	50	.285	31.36	1.28×10^{-3}	1.98	8.91×10^{-6}	1.78×10^{-4}

[a]The first row shows the total hydrogen gas generated, while the second shows the values obtained when the percentage of hydrogen generated by utilization of the trypticase and yeast added to the media have been subtracted. Rates were calculated using the ideal gas laws and assuming that the initial pressure is 1 atm and the temperature at which gases were assayed is 25°C. Bacteria were grown with glucose as the primary carbon source.

Table 3
Estimated *T. neapolitana* H_2 Gas Generation and Biomass Increase in 144 h

Glucose used (mg/50 mL)	Initial count[a] (cells/mL)	Initial A_{420}	Final count[a] (cells/mL)	Final A_{420}	Cell count (final/initial)	Calculated % H_2 product yield[b,c]
27 ± 9	$7.293 \times 10^8 \pm 3.8$ %	0.013 ± 0.002	$2.58 \times 10^{10} \pm 8.2$%	0.320 ± 0.002	35.4 ± 3.1	71 ± 24

[a] Direct cell counts were compared using a newly developed computerized direct counting technique, and measurements of absorbance for comparison. The standard deviations obtained using the direct counting technique are higher than desirable; however, these numbers are considered to be conservative since it is likely that some of the bacteria were distributed out of the plane of focus and not counted.

$$C_6H_{12}O_6 + 6\ H_2O = 6\ CO_2 + 12\ H_2$$

[b] Based on the above equation, moles of glucose = 2.7×10^{-2} g of glucose/1.802×10^2 g of glucose/mol; yield = moles of H_2/(12 × moles of glucose) = 1.498×10^{-4} mol of glucose = 71%. Similarly, the calculated hydrogen production yield represents a very conservative estimate. Absorbance was measured using a Perkin-Elmer Lambda 3b spectrophotometer.

[c] The large error indicated here reflects uncertainty of glucose measurements.

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