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 and some members of this order produce considerably greater quantities than the rest. With one representative of this order, *Thermotoga neapolitana,* we have consistently obtained accumulations of 25-30% hydrogen with carbon dioxide as the only other prominent byproduct of the batch reaction (12-15%). In contradistinction to information widely disseminated in the literature, we have also found that most members of this order tolerate and appear to utilize moderate amounts of oxygen in the gaseous phase of the batch reactor (6-12%), with no apparent decrease in hydrogen production. Hydrogen accumulation has been widely reported to inhibit growth of members of the *Thermotogales*. While this may be true at very high hydrogen tensions, we have noted hydrogen generation during both the log and stationary phase. To maximize hydrogen production and minimize production of hydrogen sulfide, inorganic sulfur donors are avoided and the cysteine concentration in the medium is increased. Different members of the order *Thermotogales* have been demonstrated by the authors and by others to utilize a wide variety of feedstocks, including complex carbohydrates and proteins. Our results suggest that organisms within this order have the potential to utilize a variety of organic wastes and to cost-effectively generate significant quantities of hydrogen gas.

Introduction

The U.S. currently consumes approximately 3.6 trillion cubic feet of hydrogen annually, while the worldwide consumption is about three times that amount (Heydorn *et al* 1998, Padro *et al* 1999). The U.S. demand for hydrogen is expected to grow by 40% within the next five years. According to recent DOE hydrogen program overviews, most of this hydrogen gas is currently made from synthesis gas that comes either from the reformation of natural gas or from the gasification of coal. These processes are costly and environmentally problematic. Therefore, society must develop economical, continuous, environmentally friendly methods of production. Biological production of hydrogen as an endproduct or byproduct of the metabolism of biological organisms has been proposed as one means of producing needed hydrogen (Zaborsky 1997).

Investigators have attempted to produce bio-hydrogen 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 (Kaplan *et al*. 1998, Vatsala 1990, Weaver 1990, Melis *et al.* 2000). Only modest success has been achieved so far because: (1) organism growth is end-product (or byproduct) inhibited by catabolites produced in the medium, (2) hydrogen gas production is inhibited because growth slows with increasing hydrogen concentrations, (3) the range of feedstocks the organisms can use is prohibitively narrow, (4) the concomitant production of undesirable gases is high, or (5) the rate of hydrogen gas production is endogenously low (Kaplan *et al*. 1998).

Building on information obtained by earlier investigators, we have taken a different approach to the biogeneration of hydrogen, and have tested the hydrogen generation capability of fifteen different strains of extremophilic bacteria of the order *Thermotogales*. *Thermotogales* is an order composed of rod-shaped, gram-negative, non-sporulating bacteria that have a loose surrounding membrane or "toga." Huber *et al*. (1986) identified the first member of this order, *Thermotoga maritima* (DSM 3109), in 1986. At about the same time, a second extremophilic bacterium, *Thermotoga neapolitana* (DSM 4359, ATCC 49049), was identified by Belkin *et al.* (1986), and later expanded upon and classified as a member of the *Thermotogales* by Jannasch *et al.* (1988.) More than twenty different members in more than five different genera (Huber and Stetter 1992) are currently known. These organisms have been isolated from a variety of environments; freshwater and marine hot springs, hot sulfur springs, near the mouth of marine black smokers, and hot oil wells. All of these organisms have been isolated from environments where 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 *Thermotoga neapolitana*, are reported in the literature to be obligate anaerobes *(10-14)*; however, we present evidence to indicate that they can also grow in the presence of low concentrations of headspace oxygen. 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 (Adams 1994).

We cultured and tested fifteen different species in the order *Thermotogales* and confirmed literature results 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 hydrogen production. We have found that the amount of hydrogen generated can be highly variable, depending upon the *Thermotogales* species used and the degree of optimization of the organisms for hydrogen production. 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 *Thermotoga neapolitana* because this organism appears to be particularly robust and adaptable to varying conditions and to utilization of different primary carbon sources. Earlier workers showed that *T. neapolitana* is the only member of this order that can grow on a medium that does not contain proteins or other substances which are poorly defined (Childers *et al.* 1992), and that it can utilize a wide variety of substrates.

Preserved cultures were purchased from either DSMZ 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 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 for 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*: NH_4 Cl, 1.0 gm: K_2 HPO_4, 0.3 gm: KH_2PO_4 , 0.3 gm: $MgCl_2$ x 6 H_2O, 0.2 gm: $CaCl_2$ x 2 H_2O, 0.1 gm: NaCl, 10.0 gm: KCl, 0.1 gm: Cysteine HCl, 1.0 gm: Yeast Extract, 2.0 gm: Trypticase, 2.0 gm: Vitamin Solution (DSMZ medium 141), 10.0 ml: Trace Element Solution (DSMZ medium 141), 10.0 ml: H_2O, 1.0 L. The initial pH of the medium was adjusted to 8.5 @ 20°C with NaOH. Trizma base (1.0E-04M) can be added to help maintain pH in an optimal range for longer periods of time during incubation. A primary carbon source (0.25 g/50 ml cellulose, cellobiose, starches, or glucose) was also added.

The medium was always prepared aerobically. Fifty ml. of the above medium was aliquoted into serum bottles, allowing 110 ml. 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 of between 1 and 12% in the headspace and then the actual oxygen concentration in the bottle was tested using a gas chromatograph. (The effects of different oxygen concentrations are shown below). Serum bottles were then sealed, capped and sterilized.

The headspace was routinely sampled using gas-tight syringes during the course of the experiment using a Hewlett-Packard Gas Chromatograph (GC). One ml of a stock bacterial inoculum solution

was transferred to the fresh medium using tuberculin syringes. The serum bottle reactors were then incubated at 70°C. All gas measurements were made at room temperature using the GC. Glucose measurements were made with a hand-held glucometer produced by Bayer and confirmed with a glucometer produced by Nova Biomedicals. Cell counts were done using a counting chamber in association with Sigma counting software, and confirmed using a Beckmann-Coulter counter. Optical density was also measured on the same samples for which actual cell counts were obtained.

Results

As noted above, 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 different primary carbon sources. For our more complete analysis (below), only the results obtained when glucose was the primary carbon source will be examined since growth on glucose is widely reported in the literature. This allows our results to be readily compared with those reported by other investigators.

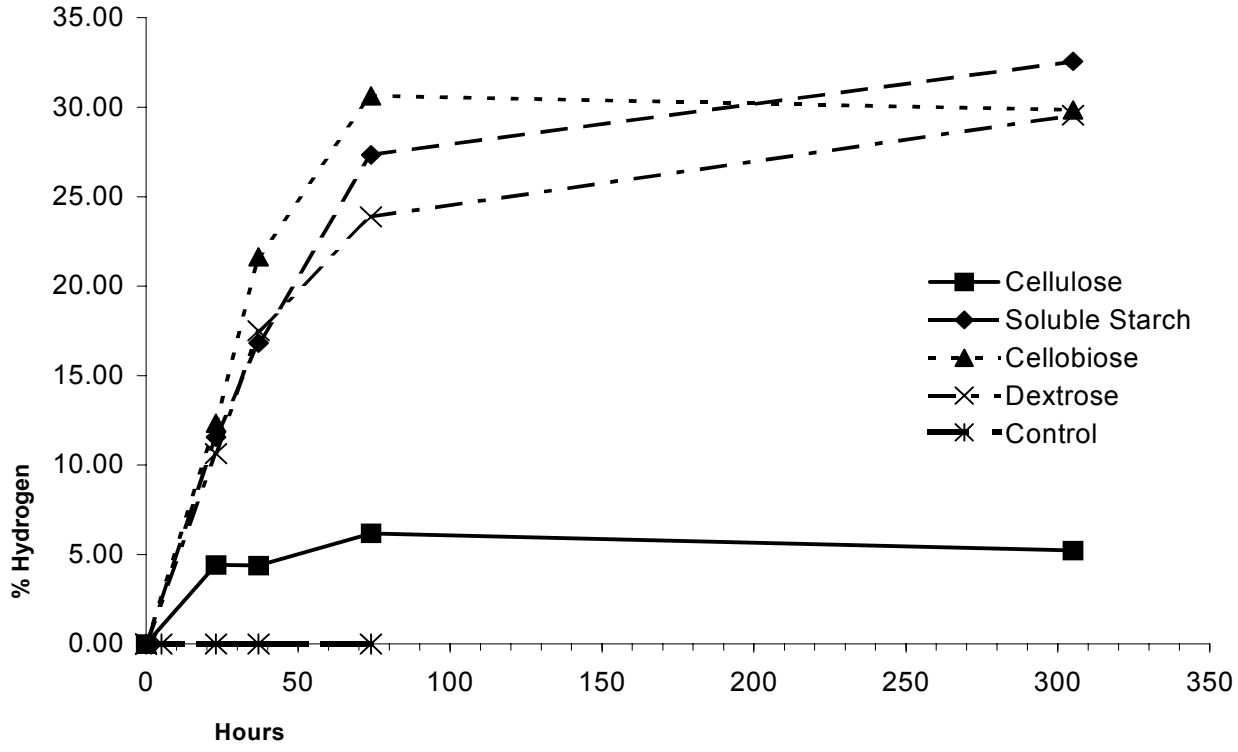
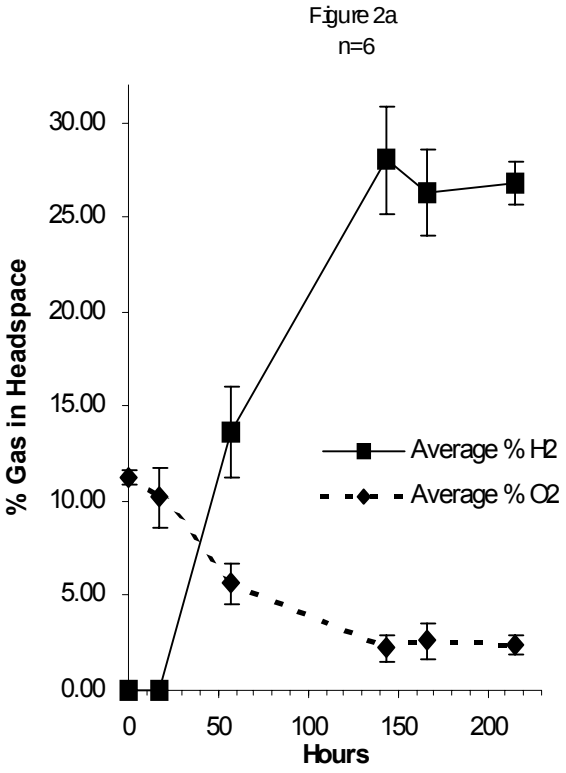


Figure 1: Hydrogen generation from various carbon sources

A comparison between the hydrogen generation seen when the oxygen concentration is slightly elevated and when the oxygen is very low can be found in Fig. 2 a & b. In Fig. 2a, the average (n=6) initial oxygen level in the headspace was greater than 11%, and the average concentration of hydrogen in the headspace after the experiment exceeded 28%. In a similar set of experiments (n=3), where the initial oxygen levels 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).



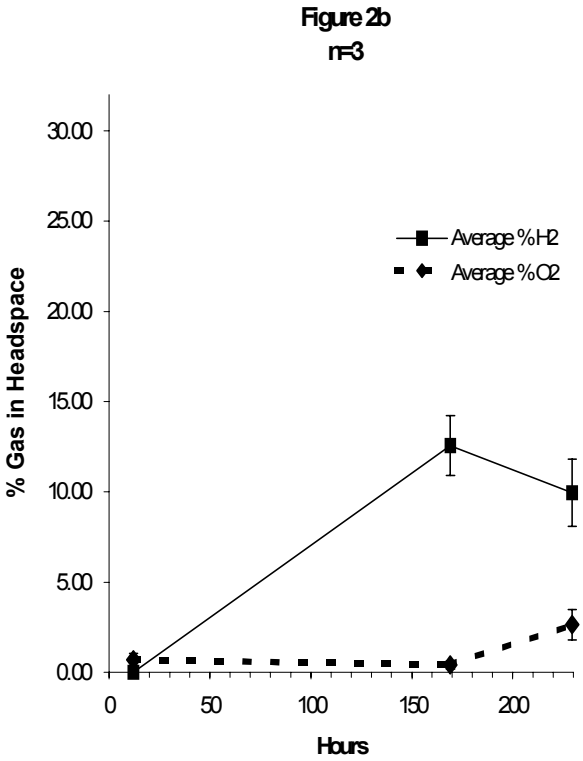


Figure 2a: Relationship of headspace hydrogen and oxygen concentrations vs time when the initial oxygen concentration was high. The percent hydrogen (✸) increased to an average of 28.05% while the percent oxygen (▼) decreased from greater than11% to less than 3% over the same period of time(n=6).

Figure 2b: Percent hydrogen and oxygen concentrations vs time when the initial headspace oxygen concentration was low. The maximum hydrogen (✸) increased to an average of less than half of that seen in Fig. 2a, while the initial average percent oxygen (▼) was less than 1 initially and actually increased slightly over time.

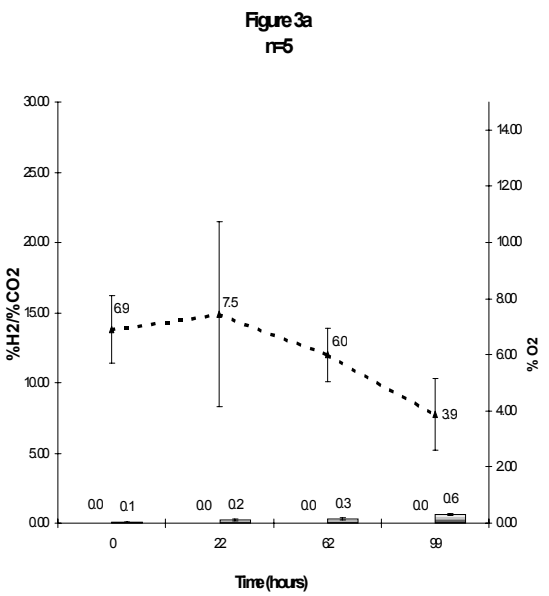
Our experiments indicate that not all of the initial oxygen decrease in the headspace of the batch reactor can be related directly to utilization by microorganisms. Figure 3 a-c shows the relationship of oxygen depletion to hydrogen and carbon dioxide generation. Figure 3a shows the results of control experiments, indicating oxygen levels (□), accumulation of hydrogen (clear column), and carbon dioxide (shaded column) for uninoculated batch reactors containing a glucose substrate. No hydrogen and very little carbon dioxide were produced. Overall, an oxygen decrease of about 3% (n=5) in the batch reactor headspace was seen. A similar response (about 2% oxygen (□) decrease, 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 *(Fig. 3b)*. Note that under these circumstances, a small amount of hydrogen and carbon dioxide were produced. However, in inoculated batch reactors (n=6) containing glucose *(Fig. 3c)* and oxygen (□) levels of about 6% in the head space at the start of the batch reactor, oxygen depletion was nearly complete over the course of the experiment. In addition, after 62 hours, the hydrogen concentration averaged over 23% and the carbon dioxide level also increased. The ratio of hydrogen to carbon dioxide approached a ratio of 2:1. It is unlikely that this oxygen depletion results from leakage of the septum because hydrogen is a much smaller molecule and it remained contained within the headspace while the oxygen concentration decreased.

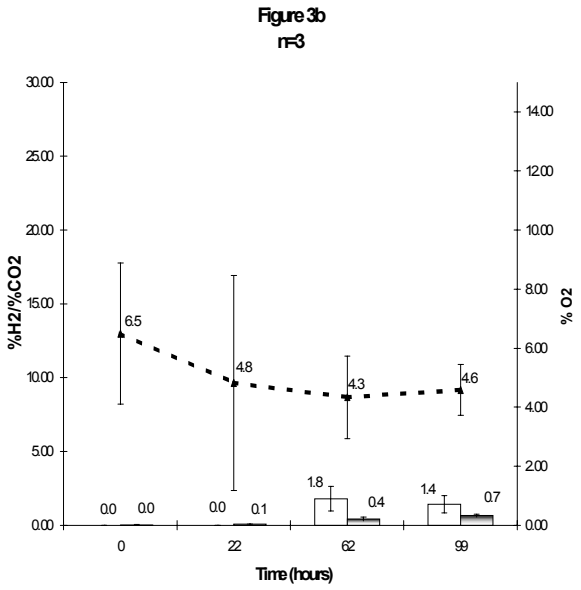
In order to determine if these organisms might be capable of catabolic processes requiring (and possibly utilizing) oxygen, we tested the ability of malonate, a competitive inhibitor of the TCA enzyme succinic dehydrogenase, to block the catabolic processes that are associated with hydrogen production.

The results of those tests are shown above *(Fig. 4a-b)*. In the presence of 0.104 g/50 ml malonate hydrogen generation was completely inhibited for over 40 hours. After that time, it is postulated that free malonate is no longer available to block catabolism, and thus rapid hydrogen generation ensues. It should also be noted that oxygen depletion does not begin in malonate treated samples until hydrogen generation begins, and that oxygen concentration continues to decrease when hydrogen generation is ongoing.

Discussion

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 shown in Table 1 below. In a single experiment using *T. neapolitana* grown on soluble starch, 28.47 ml of hydrogen gas was produced in 39 hours. Using the ideal gas laws to calculate the production rate yields 6.63E+00 ml/l headspace/hr, 2.98E-05 mol/hr or 5.97E-04 mol/l fluid/hr. Because this was a batch experiment of short duration, the bacteria were still in the log phase of growth, so this tells us little about what to expect from these bacteria in the stationary growth phase.





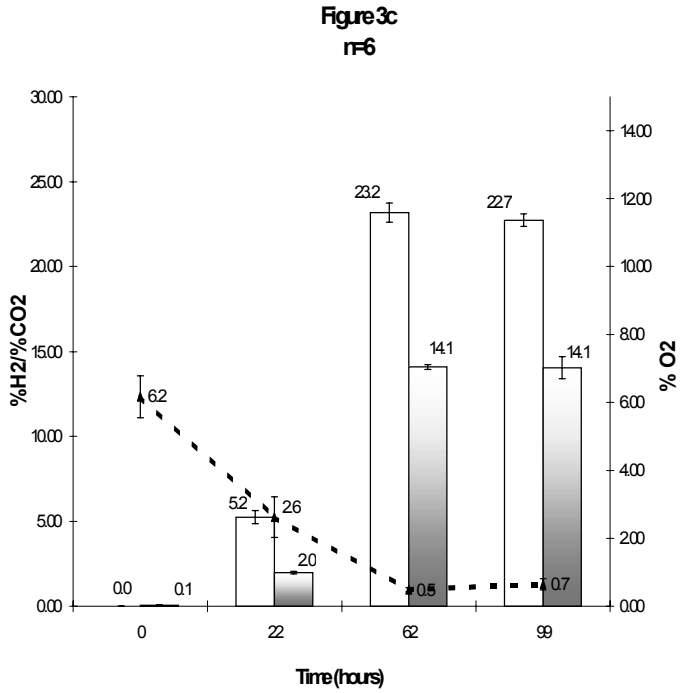


Figure 3a: Relationship of average percent hydrogen (clear bar), carbon dioxide (shaded bar), and oxygen (□) with respect to time when no inoculum was added to the batch reactor. Some oxygen depletion occurred in the absence of metabolizing cells; however, no hydrogen and very little carbon dioxide was produced without inoculation and incubation of the medium.

Figure 3b: Relationship of average percent hydrogen (clear bar), carbon dioxide (shaded bar), and oxygen (□) with respect to time when no glucose was added to the batch reactor. 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 was produced without inoculation and incubation of the medium.

Figure 3c: Relationship of average percent hydrogen (clear bar), carbon dioxide (shaded bar), and oxygen (□) when the batch reactor both contained glucose and was inoculated with bacteria.

Fig 4a: No Malonate
(n=7)

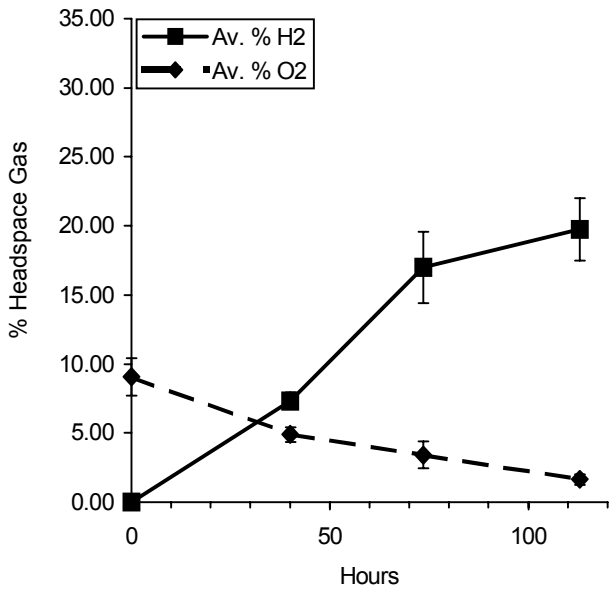


Fig 4b: 0.104 g/l Malonate
(n=7)

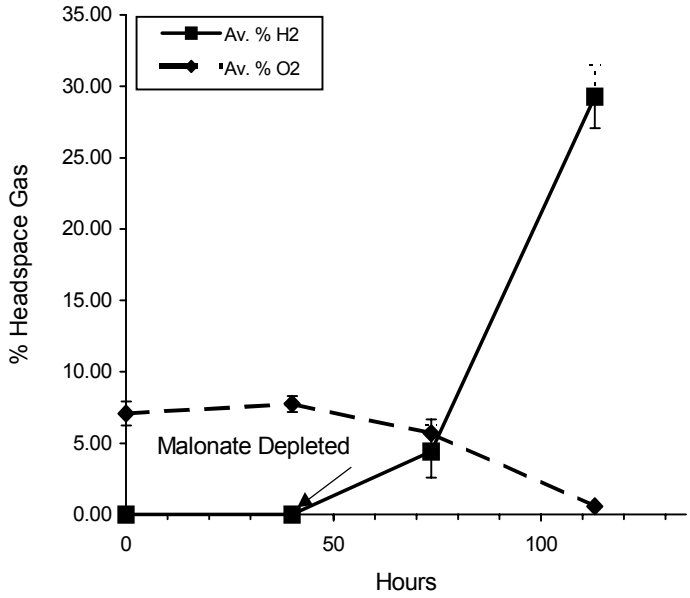


Figure 4. Effect of malonate on hydrogen gas generation and oxygen depletion.
Figure 4a shows hydrogen generation in the absence of malonate.
Figure 4b shows hydrogen generation in the presence of 0.104g/50 ml malonate. Oxygen depletion pattern is also affected by malonate block of hydrogen generation

The greatest rate of hydrogen output produced to date during a batch experiment is shown below in Table 1. 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 experiment was one atmosphere and that the temperature of measurement was 25°C. The initial H_2 concentration was measured to be zero. Soluble starch was the primary carbon source.

Table 1: Rates of Hydrogen Output

Final % H2	Elapsed Time (hrs)	Head-space Volume (ml)	Fluid Volume (ml)	H2 Partial Pressure (atm)	Final Vol H2 (ml)	Moles H2 Product	H2 Production Rate (ml/l head-space/hr)	H2 Production Rate (mol/hr)	H2 Production Rate (mol/l fluid/hr)
25.86	39	110	50	.259	28.47	1.16E-03	6.63E+00	2.98E-05	5.97E-04

In Table 2 we have shown a test of potential efficiency. We obtained hydrogen gas accumulations in the headspace of up to 28.51% in 50 ml batch experiments incubated for periods of six days (144 hours). The calculated hydrogen production in the 110 ml headspace was 1.28E-03 moles with a calculated rate of production of 1.98E+00 ml/l headspace/hour, or 8.9E-06 moll/hour. Based on the amount of glucose present before and after the experiment, and the amount of hydrogen produced, the calculated efficiency of this process was estimated to be 71 $\pm$ 24% or greater, thus exceeding the Thauer limit anticipated for fermentations (Thauer *et al.* 1977). Because *T. neapolitana* has the ability to grow using only proteins as a carbon source, the endproduct hydrogen gas produced in the absence of glucose as a substrate was subtracted from the total hydrogen output prior to calculation of efficiency. We also counted the number of cells at the start and end of the experiment and found the increase in cell number to be greater than 35.4 $\pm$ 3.1 times. This increase was correlated with absorbance changes and measured glucose concentrations before and after the batch reactor experiment (Table 3). Thus, calculated efficiency values do not include increases in bacterial cell number.

Hydrogen production by *T. neapolitana* sample used to determine cell counts, absorbance measurements and efficiency shown in Table 2b. The first line of this table shows the total hydrogen gas generated while the second row shows the values obtained when the % 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 initial pressure is one atmosphere and the temperature at which gases were assayed was 25°C. Bacteria were grown with glucose as the primary carbon source.

Table 2: Potential Efficiency

Final % H_2	Elapsed Time (hrs)	Head-space Volume (ml)	Fluid Volume (ml)	H_2 Partial Pressure (atm)	Final Vol H_2 (ml)	Moles H_2 Product	H_2 Production Rate (ml/l head-space/hr)	H_2 Production Rate (mol/hr)	H_2 Production Rate (mol/l fluid/hr)
31.05	144	110	50	.311	34.16	1.40E-03	2.16E+00	9.71E-06	1.94E-04
28.51	144	110	50	.285	31.36	1.28E-03	1.98E+00	8.91E-06	1.78E-04

Estimated *T. neapolitana* hydrogen gas generation and biomass increase in 144 hours, shown in Table 3. Comparison of direct cell counts 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. Similarly, the calculated hydrogen production efficiency represents a very conservative estimate. Absorbance was measured using a Perkin/Elmer Lambda 3b spectrophotometer.

Table 3: Calculated Production Efficiency

Glucose Used (mg/50 ml)	Initial Count (cells/ml)	Initial Abs_{420}	Final Count (cells/ml)	Final Abs_{420}	Cell Count (Final/Initial)	Calc.* % H2 Prod.Eff.
27 ± 9	7.293E+08± 3.8 %	.013±.002	2.58E+10± 8.2%	.320±.002	35.4± 3.1	71± 24

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

- Moles glucose = 2.7E-02g glucose/1.802E+02 g glucose/mole;
- Efficiency = Moles H_2 / (12 x Moles glucose) = 1.498E-04 moles glucose = 71%;
- ** Error reflects uncertainty of glucose measurements.

Most investigators have studied these organisms as strict anaerobes, and have developed elaborate methods to remove all oxygen from the environment, forcing only fermentative catabolism. While *T. neapolitana* does grow anaerobically, it also appears to be able to grow and produce significant amounts of hydrogen when provided low levels of oxygen. Based on our results, *T. neapolitana*, appears to be a microaerophile, capable of utilizing reduced levels of oxygen and generating hydrogen as an endproduct. A finding of oxygen tolerance/utilization is not unprecedented since the only other thermophilic bacterial order that has been identified as phylogenetically closely related to *Thermotogales*, the *Aquifex-Hydrogenobacter* group, includes more than one microaerophile (Kelly and Adams 1994, Shiba 1985). Beh *et al.* (1993) has shown that *Aquifex pyrophilus* (a microaerophile) is capable of shifting its metabolic behavior depending on oxygen availability. With oxygen availability and utilization, *T. neapolitana* metabolic behavior might also be predicted to shift to a more energy efficient catabolic pattern resulting in elevated hydrogen production and greater metabolic efficiency. 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.

Given the findings presented above, a remaining factor that needs to be accounted for is the fate of the excess oxygen generated by this process. The original media contains a considerable amount of ammonium chloride, and when the media is used by the bacteria the solution turns a bright yellow color. The possibility exists that nitrate and/or nitrite ions that were not present in the initial media may be produced, and that nitrogen may accept the oxygen generated. This possibility is one major focus for our further research and clarification.

Hydrogen generation rates 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, and genetic heterogeneity also contribute to variable hydrogen generation rates. These confounding factors (pH, buffers, media components, etc.) have been thoroughly examined and will be considered in a separate publication (in preparation). Even with confounding factors present, the effects of differing oxygen concentrations clearly affect hydrogen generation most dramatically. Future work will continue to decipher the roles of the various factors and optimize both rates and efficiency.

Meeting the future worldwide demand for hydrogen will require 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 (CSTR) in batch mode, indicate that after six weeks of growth at constant temperature and pH, the organisms are still capable of producing significant amounts of hydrogen.

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