# H₂ production and carbon utilization by *Thermotoga neapolitana* under anaerobic and microaerobic growth conditions

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## Abstract

H₂ production by *Petrotoga miotherma*, *Thermosipho africanus*, *Thermotoga elfii*, *Fervidobacterium pennavorans*, and *Thermotoga neapolitana* was compared under microaerobic conditions. Contrary to these previously reported strains being strict anaerobes, all tested strains grew and produced H₂ in the presence of micromolar levels of O₂. *T. neapolitana* showed the highest H₂ production under these conditions. Microscopic counting techniques were used to determine growth curves and doubling times, which were subsequently correlated with optical density measurements. The Biolog anaerobic microtiter plate system was used to analyze the carbon source utilization spectrum of *T. neapolitana* and to select non-metabolized or poorly metabolized carbohydrates as physiological buffers. Itaconic acid was successfully used as a buffer to overcome pH-induced limitations of cell growth and to facilitate enhanced production of CO-free H₂.

## Introduction

When grown under strict anaerobic conditions, bacteria belonging to different genera including *Clostridium*, *Caldicellulosiruptor*, *Ruminococcus*, *Dictyoglomus*, *Anaerocellum*, and *Spirocheta* are capable of producing H₂ (Adams 1990, Balch & Wolfe 1976), the most sought-after and ultimately the optimal non-fossil, clean fuel. One of the most recently identified orders of H₂-producing bacteria is Thermotogales. The first isolated member of this order, *Thermotoga maritima*, was not identified until 1986 (Huber *et al.* 1986). Thirty two additional members of this order have since then been isolated and described in the National Center for Biotechnology Information (NCBI) taxonomy database. All members of the order Thermotogales are non-sporulating, Gram-negative,

rod-shaped, anaerobic thermophiles or hyperthermophiles (Schroder *et al.* 1994). Like *Tga. maritima*, which was isolated from hot water, all other members of this order were isolated from hot liquids.

All members of the order Thermotogales characterized to date produce H₂ as a product of their catabolism (Belkin *et al.* 1986, Childers *et al.* 1992, Van Niel *et al.* 2002, Van Ooteghem *et al.* 2002). However, contrary to what has been described for microorganisms belonging to other orders, we observed that one particular member of this order, *Thermotoga neapolitana*, efficiently produces H₂ gas under microaerobic conditions (Van Ooteghem *et al.* 2002). This occurs despite *Tga. neapolitana* being described as a heterotrophic, extremely thermophilic, obligate anaerobe (Belkin *et al.* 1986). The primary goal of

this work was to independently confirm earlier controversial reports of high $H_2$ production by members of the order Thermotogales under microaerobic conditions, and to further determine if other members of this order were capable of producing $H_2$ under similar conditions. We defined optimal growth and $H_2$ production conditions for *Tga. neapolitana* and systematically identified non-catabolized substrates by using the Biolog AN Microplate system, both as an identification tool and to screen for carbon source utilization. The substrate utilization data were used to select non-metabolized or poorly metabolized carbohydrates, such as itaconic acid, as physiological buffers to overcome pH-induced limitations of cell growth and to enhance $H_2$ production.

## Materials and methods

### Thermotogales strains and growth conditions

The $H_2$ and $CO_2$ production of *Petrotoga miotherma* (DSM 10691), *Thermosipho africanus* (DSM 5309), *Thermotoga elfii* (DSM 9442), *Fervidobacterium pennavorans* (DSM 9078), and *Tga. neapolitana* ATCC 49049) was compared under microaerobic conditions (see Table 1 for critical growth parameters). Cultures were grown in serum bottles containing 50 ml growth medium and a headspace of approx. 110 ml. *P. miotherma* and *F. pennavorans* were grown in DSM medium 718 and 740, respectively, while all other strains were grown in a modified version of ATCC 1977 medium which contained per liter: 1 g $NH_4Cl$, 0.3 g $K_2HPO_4$, 0.3 g $KH_2PO4$, 0.2 g $MgCl_2 \cdot 6H_2O$, 0.1 g $CaCl_2 \cdot 2H_2O$, 10 g NaCl, 0.1 g KCl, 1 g cysteine hydrochloride, 2 g yeast extract, 2 g trypticase, 5 g glucose, pH 8.5. The medium was supplemented with 10 ml of filter sterilized trace element and trace vitamin solutions (see DSMZ Medium 141). Aliquots of modified ATCC 1977 medium (50 ml) were pipetted into serum bottles. To create a reduced $O_2$ atmosphere (6 to 8% $O_2$ instead of 20%), the serum bottles were placed in a hot oil bath (98 °C), heated for 10 min, and then sparged for 5 min with $N_2$. Sparged bottles were immediately capped with a butyl rubber stopper, sealed and sterilized by autoclaving. To provide a 2% (v/v) inoculum, 1 ml culture was withdrawn from a freshly grown culture or a glycerol stock and injected directly into the recipient bottle through the septum. Cultures were grown at a preset incubation temperature without agitation (Van Ooteghem *et al.* 2002) unless indicated.

### Gas analysis

Gas concentrations were measured as volume % (v/v) of total gas in the serum bottle headspace. The cultures, incubated at elevated temperatures were allowed to cool to room temperature prior to gas sampling and analysis. The headspace was sampled using gastight syringes and assayed by gas chromatography using, where appropriate, thermal conductivity detectors. The gas mass balance total was within ±2% in each experiment.

### Analysis of microbial growth data

Growth rates and population densities were determined by direct counting using a Leitz phase-contrast microscope (40× magnification). When cell density was very high, sterile culture medium was used to dilute the sample. Direct counts were correlated with turbidity measured at 600 nm. Sampling was performed every 2 h on 3 replicates.

### Preparation of AN microplates

Biolog AN (anaerobic) microplates were purchased from Biolog Inc. (Hayward, CA). Cultures of *Tga. neapolitana* were grown in modified ATCC 1977 medium for 48 h to late growth phase. Fifty ml aliquots of the initial culture were centrifuged at 800 *g* for 15 min to remove the remaining glucose. The supernatant was removed with a sterile Pasteur pipette and the pellet was resuspended in sterile pre-warmed (65 °C for 15 min) modified ANIF solution (3 ml per pellet) which contained per liter: 4 g NaCl, 0.3 Pluronic F-68, 0.2 g Phytagel, 0.84 g $NaHCO_3$, pH 7.

The cells were harvested by centrifugation (12 min at 800 g), the supernatant was removed and the pellets were resuspended in 2 ml warm ANIF solution. This carbon source-free bacterial suspension was subsequently used to inoculate the Biolog AN microplate according to the manufacturer's instructions.

### AN Microplate Incubation Procedures

Microplates were incubated in either (1) Bio-Bag Type A environmental chambers (Becton Dickinson, Cockeysville, MD), which contain a catalyst that reduces the $O_2$ content while producing $H_2$ gas; or (2) two layers of zip-lock plastic bags (one inside the other) where both bags were flushed and filled with 99.99% $N_2$. For both incubations, a damp paper towel was used to maintain humidity and reduce fluid loss by

*Table 1.* Medium and cultivation conditions for different members of the order Thermotogales.

| Species | NaCl content (g l$^{-1}$) | NH$_4$Cl content (g l$^{-1}$) | Incubation temperature (°C) | Basic medium[a] |
|---|---|---|---|---|
| *Thermotoga neapolitana* | 10 | 2 | 70 | ATCC 1977 |
| *Thermotoga elfii* | 10 | 2 | 65 | ATCC 1977 |
| *Petrotoga miotherma* | 0 | 0[b] | 55 | DSM 718 |
| *Thermosipho africanus* | 20 | 2[b] | 75 | ATCC 1977 |
| *Fervidobacterium pennavorans* | 0 | 0.5[b] | 65 | DSM 740 |

All species were grown under microaerobic conditions at a preset incubation temperature in an appropriate medium.

[a]The ATCC medium used for growth of *Tga. neapolitana* and *Tga. elfii* was optimized for its NaCl and NH$_4$Cl content as indicated.

[b]The NH$_4$Cl content for *P. miotherma*, *Tso. africanus* and *F. pennavorans* was kept at a preset concentration, as suggested by DSM, but might not reflect optimum conditions.

evaporation. The microplates were incubated for 20–24 h at 70 °C and subsequently analyzed using the Biolog microplate reader at 590 nm, software and protocols to establish a database for the carbohydrate utilization profile of *T. neapolitana*, and later, confirm strain identification. The 95 carbon sources of the AN microplate were divided into substrate 'types' for data analysis as suggested previously (Dobranic & Zak 1999, see Table 2).

## Results and discussion

### H$_2$ production

The H$_2$ and CO$_2$ production of *P. miotherma*, *Tso. africanus*, *Tga. elfii*, *F. pennavorans*, and *Tga. neapolitana* were determined under microaerobic conditions. To exclude the possibility that H$_2$ production by other strains resulted from contamination with *Tga. neapolitana*, their identity was confirmed by 16S rRNA gene sequencing at the end of the experiment. Figure 1 shows plots of the average H$_2$ and CO$_2$ concentrations in the head space produced as a function of time. All strains were found to produce H$_2$ but three strains, *Tga. elfii*, *F. pennavorans* and *Tga. neapolitana*, showed 20% (v/v) or higher H$_2$ levels. These data confirm earlier observations (Van Ooteghem *et al.* 2002) that *Tga. neapolitana* and other members of the order Thermotogales, which were believed to be strict anaerobes, were able to produce significant amounts of H$_2$ gas when the cultures were inoculated under microaerobic conditions (6–8% v/v O$_2$, see Figure 1). Thus, it appears that none of the organisms tested are obligate anaerobes as claimed in earlier studies (Childers *et al.* 1992, Huber *et al.* 1986). Baughn &

Malamy (2004) recently made a similar observation that the strict anaerobe *Bacteroides fragilis* grows in and even benefits from nanomolar concentrations of O$_2$. With species belonging to the order Thermatogales, we have observed that the O$_2$ tolerance range could be extended to the micromolar level.

For four out of the five strains tested, equimolar amounts of H$_2$ and CO$_2$ were produced in the headspace. Only *Tga. neapolitana* repeatedly yielded a H$_2$/CO$_2$ ratio of approx. 2:1 suggesting that this species uses a different metabolic pathway for H$_2$ production compared to other tested strains. It is, however, possible that other examined strains were not grown under optimal conditions, or did not have the optimal substrate for their growth, and thus did not show the 2:1 ratio observed with *Tga. neapolitana*. In addition, with all five strains, the O$_2$ concentration at the beginning of the experiment rapidly decreased over time as more CO$_2$ was produced. Interestingly, none of the tested cultures produced detectable amounts of carbon monoxide (CO) or methane (CH$_4$) based on the sensitivity of our GC measurements. The high-purity H$_2$ produced (CO < 50 ppm) contrasts commercial H$_2$ production from fossil fuels using chemical catalysis, in which the occurrence of CO contamination adversely affects the platinum catalyst in the proton exchange membrane (PEM) fuel cell (Larminie & Dicks 2003). H$_2$ purification technologies to remove CO to < 50 ppm including one based on a single-site catalyst are the subject of a recent patent from our group (Mahajan 2003). Our present result is noteworthy in that it signifies the applicability of biologically produced CO-free H$_2$ as a direct feed to PEM fuel cell.

*Table 2.* Carbon source utilization spectrum of *Thermogata neapolitana*.

| Type | Substrate | % Positive Bio-Bag | $N_2$ | Type | Substrate | % Positive Bio-Bag | $N_2$ |
|---|---|---|---|---|---|---|---|
| Amine/amide | Alaninamide | 20 | 20 | | D-Raffinose | 75 | 80 |
| | Succinamic acid | 0 | 0 | | L-Rhamnose | 100 | 100 |
| Amino acid | L-Alanine | 60 | 53 | | D-Sorbitol | 15 | 20 |
| | L-Alanyl-L-glutamine | 25 | 27 | | Stachyose | 6 | 7 |
| | L-Alanyl-L-histidine | 25 | 33 | | Sucrose | 60 | 60 |
| | L-Alanyl-L-threonine | 45 | 53 | | D-Trehalose | 70 | 73 |
| | L-Asparagine | 25 | 33 | | Turanose | 95 | 100 |
| | L-Glutamic acid | 10 | 13 | Carboxylic acid | D-Galacturonic acid | 95 | 100 |
| | L-Glutamine | 10 | 17 | | D-Gluconic acid | 75 | 80 |
| | Glycyl-L-aspartic acid | 25 | 33 | | D-Glucosaminic acid | 60 | 67 |
| | Glycyl-L-glutamine | 5 | 7 | | Acetic acid | 0 | 0 |
| | Glycyl-L-methionine | 20 | 27 | | Formic acid | 0 | 0 |
| | Glycyl-L-proline | 0 | 0 | | Fumaric acid | 0 | 20 |
| | L-Methionine | 35 | 33 | | Glyoxylic acid | 20 | 27 |
| | L-Phenylalanine | 45 | 33 | | $\alpha$-Hydroxybutyric acid | 0 | 0 |
| | L-Serine | 60 | 67 | | $\beta$-Hydroxybutyric acid | 25 | 20 |
| | L-Threonine | 30 | 40 | | Itaconic acid | 10 | 13 |
| | L-Valine | 30 | 27 | | $\alpha$-Ketobutyric acid | 90 | 93 |
| | L-Valine + L-aspartic acid | 60 | 67 | | $\alpha$-Ketovaleric acid | 95 | 100 |
| Carbohydrate | N-Acetyl-D-galactosamine | 60 | 67 | | DL-Lactic acid | 0 | 0 |
| | N-Acetyl-D-glucosamine | 90 | 100 | | L-Lactic acid | 0 | 0 |
| | N-Acetyl-D-mannosamine | 60 | 67 | | D-Malic acid | 10 | 13 |
| | Adonitol | 0 | 0 | | L-Malic acid | 50 | 53 |
| | D-Arabitol | 75 | 80 | | Propionic acid | 0 | 7 |
| | Arbutin | 70 | 67 | | Pyruvic acid | 100 | 100 |
| | D-Cellobiose | 65 | 73 | | D-Saccharic acid | 0 | 0 |
| | Dulcitol | 20 | 27 | | Succinic acid | 0 | 0 |
| | Erythritol | 50 | 67 | | *meso*-Tartaric acid | 0 | 0 |
| | D-Fructose | 100 | 100 | Miscellaneous | Amygdalin | 80 | 87 |
| | L-Fucose | 100 | 100 | | Glycerol | 95 | 100 |
| | D-Galactose | 100 | 100 | | DL-$\alpha$-Glycerol phosphate | 85 | 100 |
| | Gentiobiose | 75 | 80 | | Glucose 1-phosphate | 65 | 73 |
| | $\alpha$-D-Glucose | 85 | 80 | | Glucose 6-phosphate | 70 | 80 |
| | *m*-Inositol | 60 | 53 | | Salicin | 85 | 80 |
| | $\alpha$-D-Lactose | 80 | 93 | | DL-Lactic acid methyl ester | 0 | 0 |
| | Lactulose | 90 | 100 | | Pyruvic acid methyl ester | 80 | 80 |
| | Maltose | 80 | 67 | | Succinic acid mono-methyl ester | 0 | 0 |
| | Maltotriose | 65 | 67 | | Urocanic acid | 0 | 0 |
| | D-Mannitol | 75 | 80 | | 2'-Deoxy adenosine | 20 | 27 |
| | D-Mannose | 100 | 100 | | Inosine | 75 | 80 |
| | D-Melezitose | 40 | 40 | | Thymidine | 35 | 67 |
| | D-Melibiose | 75 | 80 | | Uridine | 70 | 73 |
| | 3-Methyl-D-glucose | 75 | 87 | | Thymidine-5'-monophosphate | 25 | 33 |
| | $\alpha$-Methyl-D-galactoside | 30 | 33 | | Uridine-5'-monophosphate | 0 | 13 |
| | $\beta$-Methyl-D-galactoside | 50 | 40 | Polymers | $\alpha$-Cyclodextrin | 55 | 53 |
| | $\alpha$-Methyl-D-glucoside | 85 | 93 | | $\beta$-Cyclodextrin | 75 | 80 |
| | $\beta$-Methyl-D-glucoside | 80 | 87 | | Dextrin | 90 | 100 |
| | Palatinose | 95 | 93 | | | $n=20$ | $n=43$ |

The carbon source utilization spectrum of *Tga. neapolitana* was determined using the Biolog AN microtiterplate system The substrates were grouped per type of carbon source. Per substrate the percentage of positive events in function of incubation conditions (Biolog bag with $H_2$ atmosphere or $N_2$ atmosphere) is presented. The data for the Biolog Bag are based on 20 experiments, while the results of the incubation under a $N_2$ atmosphere are the average of 40 experiments.
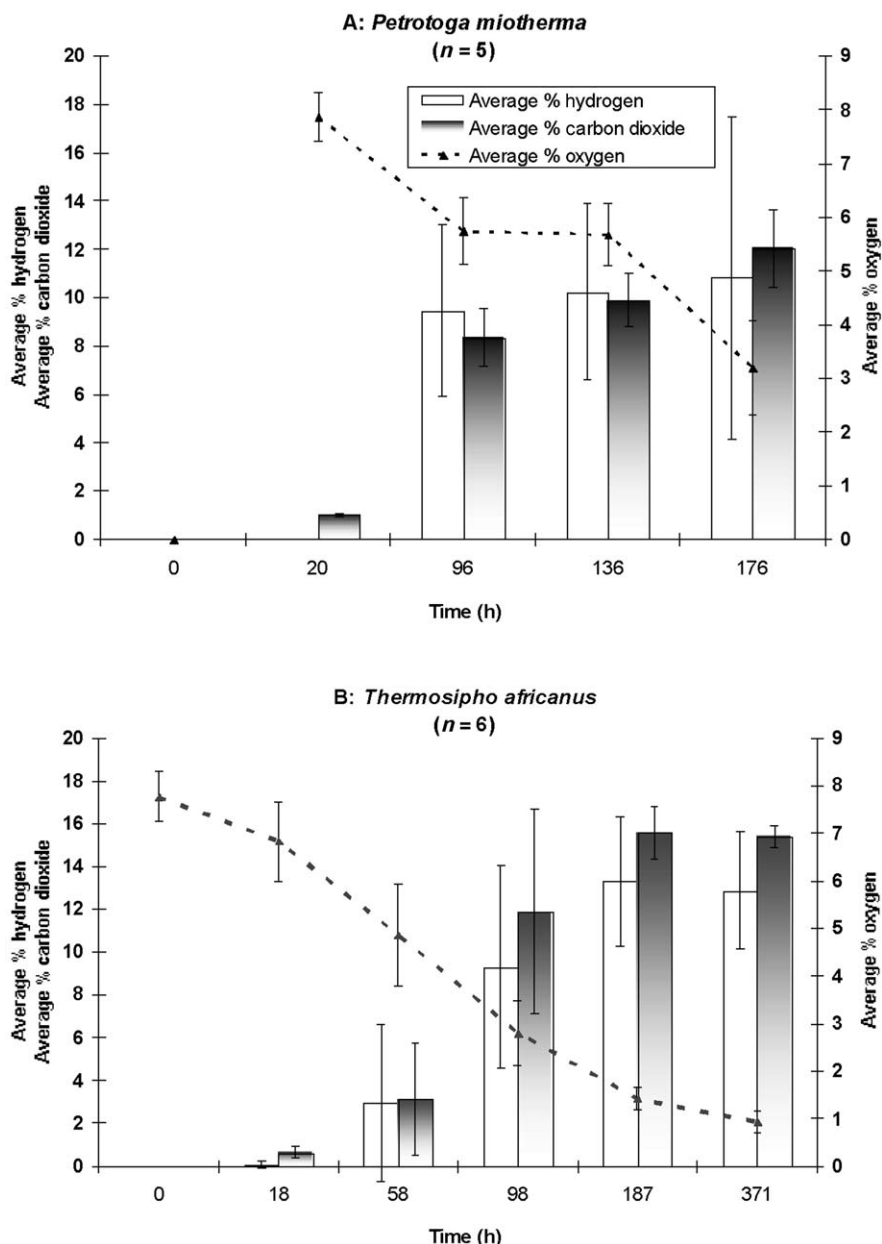
Fig. 1. $H_2$ and $CO_2$ production by *P. miotherma*, *Tso. africanus*, *Tga. elfii*, *F. pennavorans*, and *Tga. neapolitana* under microaerobic conditions. Concentrations of $H_2$, $CO_2$ and $O_2$ in the headspace of the cultures were measured as function of time by GC. Standard deviations, as well as the number of repeats (*n*) are indicated.

*Cell growth and density*

Attempts to grow members of the order Thermotogales on solid media have been unsuccessful. In order to link physiological data such as $H_2$ production to cell numbers, we determined the relation between cell counting data and the optical density of a *Tga. neapolitana* culture as a function of time. The res-

ults are presented in Figure 2. In agreement with the previously reported data (Childers *et al.* 1992), we observed that *Tga. neapolitana* could vary in morphology and, depending on the growth phase, two different stages were noted. The bacteria appeared as small, single rods in the early hours of the experiment. After approximately 6 h, the culture entered the log growth phase with accompanying morphological
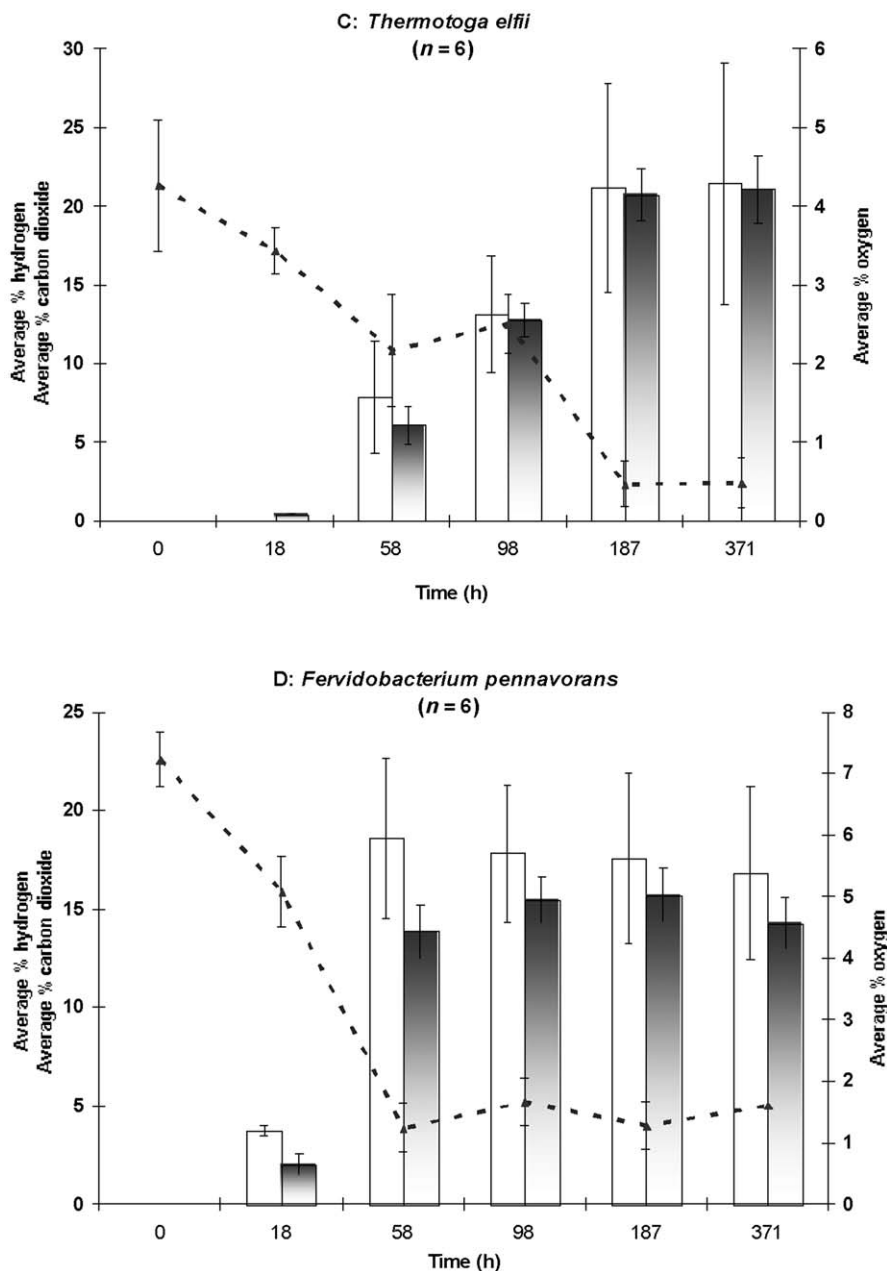
*Fig. 1.* Continued.

changes wherein the cells became larger and were sometimes present in pairs. The culture achieved a maximum cell density of approx. $3.0 \times 10^9$ cells after 14 h at 70 °C. Thereafter, the absorbance continued to increase with no increase in the corresponding cell numbers.

*Carbon source utilization*

The Biolog system has proven to be very accurate for identifying specific mesophilic bacteria, and a Biolog database is available to enable rapid identification of 1449 mesophiles (Preston-Mafham *et al.* 2002). However, Biolog microplates have not previously been used to characterize extremophilic bacteria. We successfully used the Biolog microplates to es-
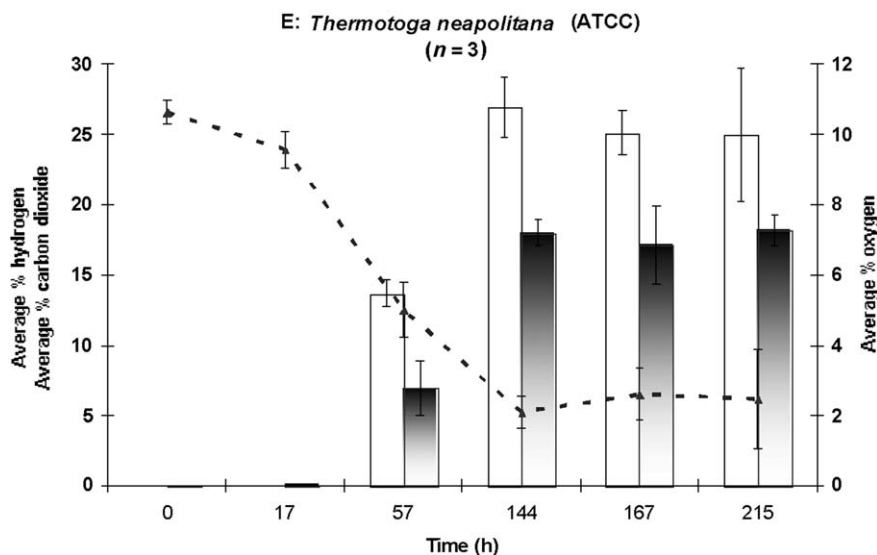
*Fig. 1.* Continued.
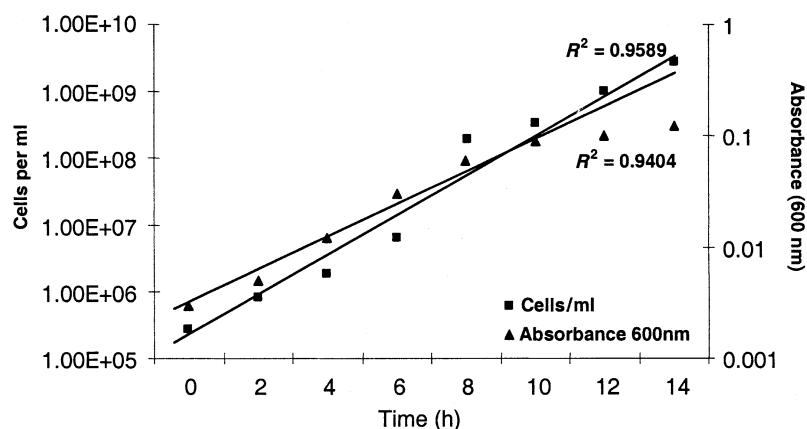


*Fig. 2.* Cell counts and optical density for *Tga. neapolitana*. Measurements were made every 2 h, until the culture had reached the stationary growth phase. Cell numbers were determined by counting using a light microscope. The O.D. of the culture was determined at 660 nm.

tablish the carbon utilization pattern of *T. neapolitana* (see Table 2) as a function of composition of the anaerobic gas phase. The data were categorized to show that 57 carbon sources consistently gave higher scores when zip-lock bags with a $N_2$ gas atmosphere were used, while 14 carbon sources gave a higher score for Bio-Bags under a $H_2$ atmosphere. D-Fructose, L-fucose, L-galactose, D-mannose, L-rhamnose, and pyruvic acid were always positive (positive 100% of the time at the end point) with both types of incubation. The wells that gave a 100% positive score in Bio-Bag-incubated plates were also 100% positive in zip-lock bag-incubated plates. However, incubation in zip-lock bags yielded eight more 100% positive scores for *N*-acetyl-D-glucosamine, lactulose, turanose, D-

galacturonic acid, $\alpha$-ketovaleric acid, DL-$\alpha$-glycerol phosphate, glycerol, and dextrin.

The 14 carbon sources that were not utilized by *Tga. neapolitana* were succinamic acid, glycyl-L-proline, adonitol, acetic acid, formic acid, $\alpha$-hydroxybutyric acid, $\beta$-hydroxybutyric acid, DL-lactic acid, L-lactic acid, D-saccharic acid, succinic acid, *meso*-tartaric acid, DL-lactic acid methyl ester, and urocanic acid. In addition, a number of additional carbon sources such as itaconic acid were only poorly catabolized by *Tga. neapolitana*.

While the gases present during incubation affected the results, the density of the inoculum at either 32 ± 3% transmittance or 63 ± 3% transmittance did not change the outcome of the test and thus bacterial iden-
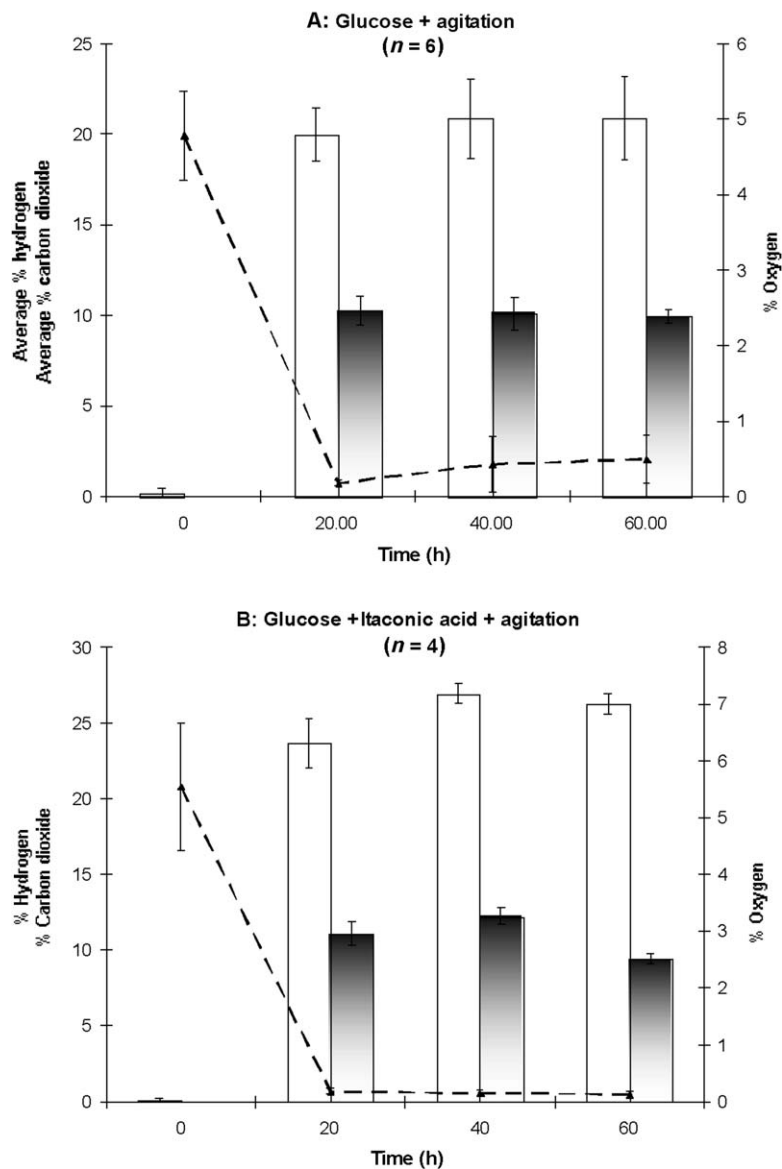
*Fig. 3.* Improved $H_2$ production by *T. neapolitana* in the presence of itaconic acid as buffering substance. Cultures were grown under agitation (75 rpm) at 70 °C using glucose as an easily metabolized carbon source. As a control, a culture without glucose was prepared. The concentrations of $H_2$, $CO_2$ and $O_2$ in the headspace of the cultures were measured over time. Standard deviations, as well as the number of repeats (*n*) are indicated.

tification. Once we established a database of substrate utilization and verified that accurate identification of *Tga. neapolitana* could be obtained, this database was used to rapidly verify that bacterial samples for future experiments were pure and uncontaminated. This was subsequently confirmed by sequencing the 16S rRNA gene of the strain used.

The most commonly observed problem with the use of BBL Bags for testing anaerobes is that $H_2$,

which is produced to remove the $O_2$, interacts with the tetrazoleum dye in the Biolog plate, resulting in many false positive readings (Barry Bochner, private communication). We did not encounter this problem as our results showed a reduction in the number of positive reactions when using BBL bags. A logical explanation for the presence of a greater number of positive reactions when the microplates were incubated in zip-lock bags under a $N_2$ atmosphere is that the
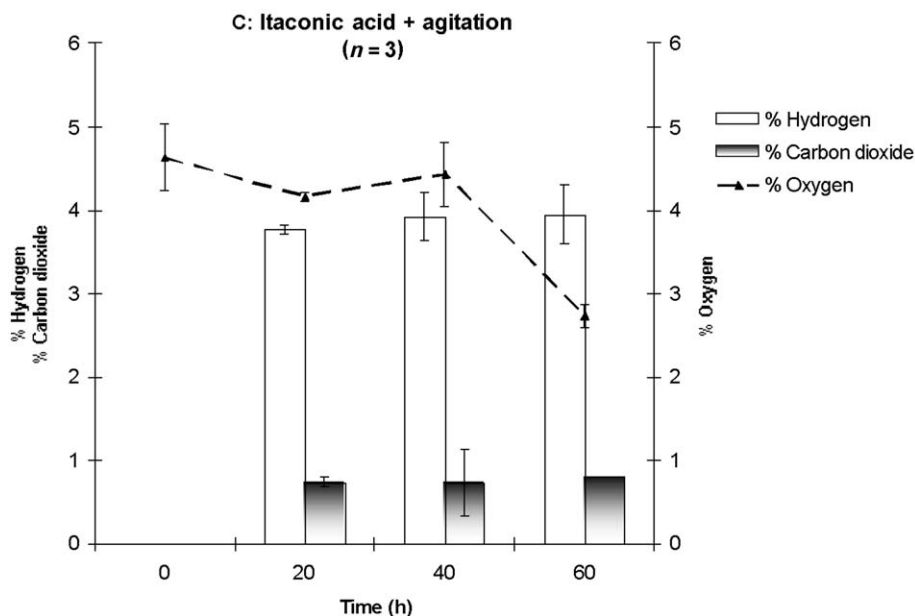
*Fig. 3.* Continued.

organisms were not really obligate anaerobes. Rather, they can survive, grow in, and utilize a greater range of substrates in the presence of low levels of $O_2$ such as might be found in the zip-lock bags.

*Improved growth and H₂ production*

The amount of growth and $H_2$ production by *Tga. neapolitana* in a small batch reactor is often limited by a rapid decrease of pH. Based on the Biolog results, it was possible to determine carbon sources that could serve as simple, non-toxic physiological buffers to overcome this pH limitation. Figure 3 compares the $H_2$ production results with glucose as carbon source in the absence and presence of itaconic acid as a buffering substrate. Residual growth in the absence of glucose was also determined. The pH of the medium that only contained glucose dropped within 20 h from 7.4 to 4.5. However, in the presence of itaconic acid, the pH decreased more slowly, from 7.4 to 5.9, after 20 h with $H_2$ production reaching a maximum plateau value of 27% (v/v). In addition, the agitation of cultures at 75 rpm greatly improved $H_2$ production: 20 h after the beginning of growth, an agitated *Tga. neapolitana* culture had approx. 24% $H_2$ (v/v) gas in its head-space, while a non-agitated culture after 57 h under similar conditions produced about 15% $H_2$ (compare Figures 1E and 3A,B). Thus, pH control and moderate agitation are critical factors for stimulated growth and

an enhanced $H_2$ production by *T. neapolitana* under microaerobic conditions.

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