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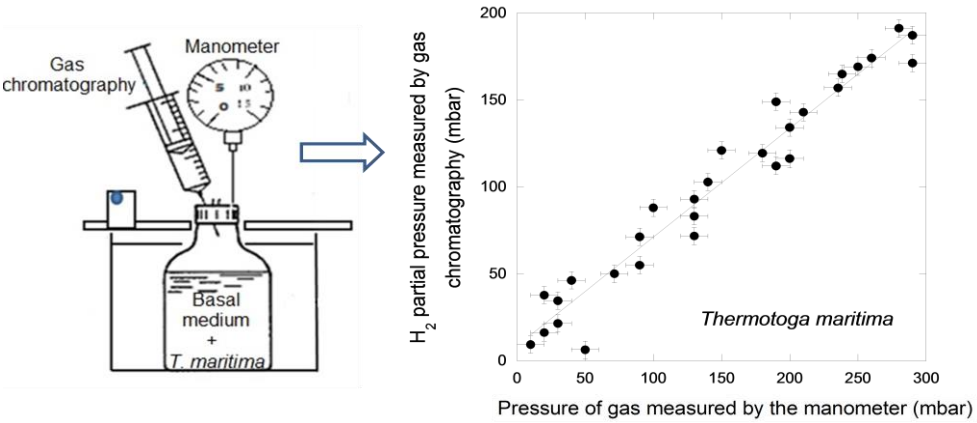
Graphical abstract



HIGHLIGHTS

- The hydrogen producing capacities of *Thermotoga maritima*, *Thermococcus* k*odakarensis and Enterobacter cloacae* were studied in 116 mL-serum-bottle.
- A linear relationship was established between the partial pressure of H_2 and the sum of the partial pressures of H_2 and CO_2 of each microorganisms.
- Under some assumptions, determination of H_2 partial pressure was achievable using a simple manometer.

Abstract

This study investigated the determination of hydrogen production by three H_2-producing microorganisms (*Thermotoga maritima*, *Thermococcus kodakarensis* KOD1 and *Enterobacter cloacae)* cultured in 116-mL serum bottles. A gas pressure manometer was used to measure total pressure in the serum-bottle headspace. It was demonstrated that total pressure is the sum of the saturation pressure of water, the pressure expansion of gases, and the partial pressures of H_2 (P_{gH_2}) and CO_2 (P_{gCO_2}). A linear relationship was established between the partial pressure of H_2 measured by gas chromatography and the sum of the partial pressures of H_2 and CO_2 measured by the manometer. When pH of culture medium was not controlled (pH decreased from 7 to 5), the P_{gH_2}/P_{gCO_2} ratio was close to stoichiometric H_2/CO_2 yield ratio of the most plausible metabolic pathways of each strain. P_{gH_2}/P_{gCO_2} values were 1.7, 1.9 and 0.9 for *T. maritima*, *T. kodakarensis* and *E. cloacae*, respectively. In these experimental conditions, P_{gH_2} can be deduced from total pressure measured by manometer.

Keywords: hydrogen, total pressure, manometer, hydrogenogenic microorganisms

Introduction

Rising worldwide energy consumption needs, diminishing fossil fuel reserves, and environmentally damaging carbon dioxide emissions from the combustion of hydrocarbon fuels are driving efforts to develop alternative sustainable energy sources. Hydrogen is considered as viable alternative fuel of the future, it is clean and renewable with no CO_2 emissions, and can be produced through different methods (biophotolysis, photofermentation, or dark fermentation) from organic biomass using aerobic anaerobic bacteria [1–3]. Dark fermentation (DF) is currently considered the most promising technology as it is an economically advantageous process capable of achieving high hydrogen output rates with low energy input requirements [4]. During fermentation, different anaerobic groups such as *Enterobacter*, *Clostridium*, *Bacillus* and *Thermotoga* convert several organic and complex carbohydrate rich-substrates (sugary wastewater, cellulose, municipal solid waste and corn pulp) to H_2, CO_2, carboxylic acids and organic solvents [5].

Different methods have been used to measure the hydrogen produced. In general, the choice of appropriate technique depends on the size of the anaerobic reactor and the sensitivity and capacity of the gas-measuring apparatus. The techniques proposed generally range from the low-cost intermittent pressure release method (Owen method, [6]) where yields are usually reduced due to high partial pressures of hydrogen, to the more expensive continuous gas release method using a bubble measurement device (respirometric method, [6]). Note that calibrated pressure manometers can be also used to measure the production of specific gas in anaerobic lab-scale reactors. However, the method requires careful calibration of gas volume versus pressure, and accurate measurement of the production of specific gases is made difficult by the fact that calibration is related to gas composition and solubility, temperature, headspace volume, and ratio of liquid to gas volume. Other techniques can also

be used, such as wet-test or wet-tip meters, lubricated syringes, and manometer-assisted syringes, but these methods are unfortunately labour-intensive, time-consuming and/or subject to numerous sources of error.

Anaerobic respirometers could help overcome these deficiencies and save considerable test time. These instruments were purpose-designed to automatically give precise instantaneous measurements of gas production on a continuous basis in increments as small as 0.1 mL, and record the data by counters or computers. The technique is not just accurate but also advantageous as it does not allow significant pressure buildup.

Previous biohydrogen production studies conducted in two types of batch tests (Owen and respirometer) showed that under otherwise identical conditions, the respirometric method resulted in the production of 43% more hydrogen gas from glucose than the Owen method [6]. In other studies, the composition and total mass flow rate of outlet biogas mixture (O_2, H_2 and CO_2) produced by fermentation were measured on-line with a mass flow-meter associated with a mass spectrometer [7,8]. These instruments enabled to follow the instantaneous kinetics of gases released or consumed by the culture. The use of mass spectrometry was described as advantageous as it is a sensitive, high-resolution separation technique with wide applicability. Specific electrodes such as Pt electrode or Clark-type electrode used to determine H_2 production rate in the gas phase as well as in aqueous solution for different cultures were considered sensitive instruments but still only efficient at low hydrogen concentrations [9,10].

A multi-channel analyzer called the Automatic Methane Potential Test System (AMPTS) has been used as a laboratory instrument to measure the biohydrogen produced from cheese whey by dark fermentation [11]. This instrument was developed for automatic real-time measurement of biogas production during anaerobic digestion from any organic biomass [12]. It gives reproducible results due to the relatively high number of parallel

experiments and the possibility to directly compare different process configurations. Recently, Donval & Guyader [13] developed a specific analytical device based on the headspace method for quantifying H_2 and CH_4 in seawater at trace level. The aim was to have a compact, portable and automated system composed of independent and heavy instruments such as valves (selection, sampling, open/close), small oven, controlled micropump in a time program via an electronic interface. No gas chromatograph was used to keep analytical system weight and dimensions to a minimum. Jones et al. [14] also proposed an original technique based on measuring percentages of gas (methane, carbon dioxide, hydrogen) removed from the headspace with specific sensors placed in a gas loop connected to the fermenter. Among all these instruments, gas chromatography (GC) employing a thermal conductivity detector (TCD) remains the classic widely-used technique for measuring widely varying hydrogen concentrations [15]. This analytical technique is characterized by a good resolution and sensitivity, a short analysis time, a high separation power and an easy recording data. The main problem related to this instrument remains its high cost compared to other simple devices.

The aim of this study was to propose a simple and indirect technique to determine partial pressure of hydrogen in serum bottles during anaerobic fermentation. Mesophilic and hyperthermophilic hydrogenogenic microorganisms were tested, and the limits of the technique were discussed.

Material and Methods

Strains and growth media

Four strains were used in this study: *Thermotoga maritima* MSB8 (DSMZ 3109) (TM), *Thermococcus kodakarensis* KOD1 (JCM 12380) (TK), *Enterobacter cloacae* (ATCC 35929) (EC), and *Caldicellulosiruptor saccharolyticus* (CS) belonging to the orders

Thermotogales, Thermococcales, Enterobacteriales and Thermoanaerobacteriales, respectively. Optimal growth temperature for each strain was 80 °C for TM and TK, 70 °C for CS and 35 °C for EC.

Three strains (TM, TK and CS) were cultured using a common basal medium (BM) containing (per liter) NH_4Cl 0.5 g, KH_2PO_4 0.3 g, NaCl 20 g and Na_2HPO_4 0.3 g to which Balch's trace-mineral-element solution (10 mL) was added [16]. The medium was pH-adjusted to 7.0 with 1 mol L^{-1} NaOH, then autoclaved at 120°C for 20 min and stored at room temperature. The *Enterobacter cloacae* (EC) strain was cultivated in a BM containing (per liter) NH_4Cl 1 g, KH_2PO_4 0.3 g, K_2HPO_4 0.3 g, $CaCl_2$ 0.2 g, NaCl 20 g and KCl 1 g to which Balch's trace-mineral-element solution (10 mL) was added.

A second BM (BM1) was used with the same ingredient composition as BM but with the concentrations of KH_2PO_4 and Na_2HPO_4 increased to 0.6 g/L and 5 g/L, respectively.

Thermotoga Maritima. Before inoculation, the BM was supplemented with glucose (15 mM), yeast extract 1 g/L, $CaCl_2$ 0.1 g/L, $MgCl_2$ 0.3 g/L, cysteine-HCl 0.3 g/L and Na_2S 0.4g/L. *T. maritima* cultures performed in serum bottles were incubated at 80°C.

Thermococcus kodakarensis. After sterilization, the BM was supplemented with glucose (15 mM) and elemental sulfur (5 g/L). TK was cultivated under strictly anaerobic conditions at 80°C [17].

Caldicellulosiruptor saccharolyticus. Before inoculation, the BM was supplemented with yeast extract 1 g/L, $CaCl_2$ $2H_2O$ 0.10 g/L, $MgCl_2$ $6H_2O$ 0.2 g/L, cysteine-HCl 0.5 g/L, cellobiose 1M (0.5 mL) and Na_2S 2% (0.5 mL) and the CS culture was incubated at 70°C.

Enterobacter cloacae. After sterilization, the BM was supplemented with $MgCl_2$, $6H_2O$ 0.2 g/L, yeast extract 2 g/L, peptone 2 g/L and maltose (0.5M). EC cultures performed in serum bottles were incubated at 35°C.

Preparation of media and stock solutions

The medium was boiled then cooled down to room temperature under a stream of O_2-free N_2, then distributed into 116-mL serum bottles (25 mL of medium). After sealing the serum bottles, the gaseous phase was flushed with a stream of O_2-free N_2 for 30 min. The medium was then autoclaved at 120°C for 20 min and stored at room temperature. All stock solutions were prepared under anoxic conditions as described by Miller & Wolin [18], and stored under O_2-free N_2. Glucose (2M), fructose (2M), maltose (1M) and cellobiose solutions, yeast extract and peptone were sterilized by filtration. Na_2S (2%), cysteine-HCl (5%), $MgCl_2$ (3%) and $CaCl_2$ (2%) solutions were sterilized by autoclaving (120°C for 20 min).

Experimental systems and operating conditions

All strains were grown in 116 mL serum bottles with 30 mL of culture medium after inoculation. Before culture, the serum bottle was sealed with rubber stoppers, and anoxia was obtained by flushing the bottle headspaces with an O_2-free N_2 gas stream for 20 minutes. All the bottles were placed in a temperature-controlled oven (± 0.1 °C).

The measurements of total pressure and H_2 concentration in the headspace were made after transferring the bottles to a temperature-controlled (± 0.1°C) heating water bath at the optimal temperature for each strain (Fig. 1).

Analytical methods

During fermentation, hydrogen contents were periodically measured by withdrawing 250 µL gas samples from the serum bottle headspace in gas-tight syringes and injecting the samples into a GC-TCD system (Perichrom, France) equipped with a concentric CTR1 column (Alltech, USA). Operating temperatures of the detector, injector and oven were 100°C, 100°C and 40°C, respectively. Argon was used as carrier gas at a flow rate of 20

$mL.min^{-1}$. This system was connected to a computer running WINILAB III software (Perichrom, France). A GC-TCD calibration curve was generated by running various dilutions of the H_2 and CO_2 and then plotting response times against concentration.

Total pressure in the headspace of the serum bottles was measured using a manometer (Wika, France) with a fine needle robust enough to pass through the rubber stopper with any deformation. The manometer gave full-scale readings of 2 bars at an accuracy of ± 10 mbars. For some experiments, to determine pH, the entire culture medium contained in the serum bottles was withdrawn at different times. pH was also determined from the culture medium at the end of all experiments.

At the end of fermentation, concentrations of the main soluble metabolite products (acetate, lactate, butyrate, ethanol, formate, etc.) and residual glucose were analyzed. Liquid samples harvested from the serum bottles were centrifuged at 14000 *g* for 5 min, and the supernatants were filtered through a 0.45-µm cellulose acetate minisart® syringe filters (Sartorius Stedim). They were analyzed by HPLC (Agilent 1200 series, USA) on a system equipped with a quaternary pump coupled to a refractometer index detector and 300 x 7.8 mm Aminex HPX-87 H ion-exchange columns (Bio-Rad). The HPLC system was connected to a computer running WINILAB III software (Perichrom, France). Sulfuric acid 5 mM (in milliQ water) was used as mobile phase at a flow rate of 0.5 mL/min. All analyses were performed in duplicate.

Theory

Total pressure (P_t) in the headspace of the serum bottle is defined as follows:

$$P_t = P_{vs\,(H_2O)}(T) + P_{exp}\,(T) + P_{prod} \qquad (1)$$

where $P_{vs\,(H_2O)}(T)$ is the saturation pressure of water and $P_{exp}(T)$ is the pressure expansion of gases, both depending on temperature (T), and P_{prod} is the sum of the partial pressures of the volatile compounds produced during the fermentation.

$P_{vs\,(H_2O)}(T)$ was calculated using Antoine's equation [19]

$$P_{vs\,(H_2O)}(T) = \exp\left[A-\left(\frac{B}{C+T}\right)\right] \qquad (2)$$

with A = 16.39, B = 3885.7 and C= 230.17. T is in °C and $P_{vs\,(H_2O)}(T)$ is in Pa.

$P_{exp}(T)$ was determined as follows:

$$P_{exp}(T) = P(T_0)\,\frac{T}{T_0} \qquad (3)$$

with $P_{exp}(T)$ in Pa, T_0 = 293°K and $P(T_0)$ = 101325 Pa.

$$P_{prod} = \sum_{\alpha} P_{g\alpha} \qquad (4)$$

with α = H_2, CO_2 and volatile-compound end-products.

The thermodynamic equilibrium of the dissolved compounds α is described by Henry's law:

$$[C_\alpha] = K_h(T)\,P_{g\alpha} \qquad (5)$$

Here, $[C_\alpha]$ is concentration of the α compound in the aqueous phase, $P_{g\alpha}$ is partial pressure of the α compound in the gas phase under equilibrium conditions, and $K_h(T)$ is Henry's constant (mol/L/Pa) for the α compound at temperature T (°K) [20].

$$K_h(T) = K_h^{\theta} \exp\left(\frac{-\Delta_{soln} H}{R}\left(\frac{1}{T}-\frac{1}{T^{\theta}}\right)\right) \qquad (6)$$

K_h^{θ} refers to standard conditions (T^{θ}= 298.15 °K). $\Delta_{soln}H$ is the enthalpy of solution (Pa L/mol), and R is the ideal gas constant (Pa L/mol °K).

CO_2 does dissolve in water. The CO_2 in the aqueous phase is only in equilibrium with the hydrogen carbonate HCO_3^- as the dissociation of HCO_3^- into CO_3^{2-} can be considered

negligible at pH ≤ 7. The conversion reaction between CO_2 and HCO_3^- and the corresponding dissociation constant are as follows:

$$[CO_2] + [H_2O] \rightleftarrows [H^+] + [HCO_3^-] \qquad (7)$$

$$K_1 = \frac{[H^+][HCO_3^-]}{[CO_2]} \qquad (8)$$

with $[H^+] = 10^{-pH}$ (9)

Total dissolved CO_2 ($[C_T]$) is the sum of the concentrations of CO_2 ($[C_{CO_2}]$) and HCO_3^- ($[HCO_3^-]$) in the aqueous phase:

$$[C_T] = [C_{CO_2}] + [HCO_3^-] \qquad (10)$$

Using equations 5, 6, 8 and 9, $[C_T]$ is:

$$[C_T] = P_{gCO_2} K_h(T) \left(1 + \frac{K_1}{10^{-pH}}\right) \qquad (11)$$

$K_h(T)$was obtained from Sander and K_1 was obtained from Amend and Shock [20,21]. For a temperature of 80°C, K_h and K_1 were 0.958 10^{-7} mol/L/Pa and 0.493 10^{-6} mol/L, respectively. For a temperature of 35°C, K_h and K_1 were 2.584 10^{-7} mol/L/Pa and 0.463 10^{-6} mol/L, respectively.

Among the wide range of byproducts from microbial metabolism, the two metabolic pathways producing hydrogen from carbohydrates are essentially the "acetate" and "butyrate" pathways well known in many species of *Thermotogae*, *Thermococcaceae*, *Thermoanaerobacteraceae* (eq. 12) and *Enterobacteriaceae* (eq. 13). The production of acetate gives a theoretical stoichiometric yield of 2 moles of H_2 per mole of CO_2, while in the butyrate pathway; molar hydrogen yield is lower, at 1 mole of H_2 per mole of CO_2.

$$C_6H_{12}O_6 + 2\,H_2O \rightarrow 2\,C_2H_4O_2 + 2\,CO_2 + 4\,H_2 \qquad (12)$$

$$C_6H_{12}O_6 \rightarrow 2\,C_4H_8O_2 + 2\,CO_2 + 2\,H_2 \qquad (13)$$

Results and Discussion

Determination of the total pressure for abiotic experiments

First we used empty 116-mL serum-bottles to determine whether total pressure measured in the bottles remained constant after several punctures with the manometer needle. Overpressure (800 mbar) in the closed bottle was obtained by introducing a constant N_2 flow rate. After 6 successive manometer-needle punctures in the bottle-cap septum, total pressure was kept quasi-constant at 790 ± 10 mbar. Six additional 250 µL samples of gas were withdrawn from the bottle using a gas syringe to simulate the successive samplings required for gas chromatography measurement of percentage H_2 in the headspace of the bottles. The decrease in total pressure due to the 6 punctures was 1.5%, i.e. equivalent to a pressure of less than 12 mbar. These results show that (i) gas leakage was very low, (ii) dead volume of the manometer was negligible, and (iii) successive samples made with the gas syringe lead to very little pressure drop in the serum-bottle headspace.

Experiments were performed to measure total pressure (P_t) in the headspace of serum bottles at three temperatures (35, 70 and 80°C) corresponding to the optimum culture temperatures of *E. cloacae, C. saccharolyticus,* and *T. maritima* and *T. kodakarensis,*

respectively. All serum bottles contained 30 mL of pure water. Bottle transfer from oven to water bath was quick, and consequently the equilibrium of total pressure in the headspace was reached in less than one minute. In this case, P_t is the sum of $P_{vs\ (H_2O)}$ deduced from equation 2 and P_{exp} (eq. 1, P_{prod} = 0) deduced from equation 3 (Table 1). The sum of $P_{vs\ (H_2O)}$ and P_{exp} was compared to P_t, measured by the manometer. For these three temperatures, P_t measured by the manometer and P_t determined from theoretical calculation were near-identical (Table 1). The effect of the volatile compounds released at 80 °C by the culture medium in the headspace of the serum bottle was evaluated. No overpressure was measured showing that the volatility of the culture medium compounds was negligible (data not shown).

Determination of total pressure and H_2 partial pressure during microbial growth

Experiments were performed with two hyperthermophilic (*T. maritima* and *T. kodakarensis*) and one mesophilic (*E. cloacae*) microorganisms by measuring P_t and P_{gH_2} by gas manometer and gas chromatography, respectively, 6 times for each bottle. This experimental protocol was shown to not reduce the total pressure in the headspace (see above). Whatever the microorganism used in these experiments, the pressure (P_t) measured in the headspace is the contribution of physical ($P_{vs\ (H_2O)}$ + P_{exp}) (eqs. 2 and 3) and biological (P_{prod}) phenomena (eq. 4). The term $P_{vs\ (H_2O)}$ + P_{exp} depends on the temperature and is constant with time (Table 1). P_{prod} is the sum of the partial pressures of H_2, CO_2 and volatile-compound end-products. Among the wide range of byproducts, various metabolic pathways producing hydrogen from carbohydrates are essentially the "acetate" pathway (eq. 12) or the "butyrate" pathway (eq. 13) well known in many species of *Thermotogae* and *Enterobacteriacae*, respectively. Others end-products such as lactate, formate and ethanol are also observed but at lower concentrations. The K_h (T) values [20] for each end-product (α = acetate, butyrate, lactate, etc.) were determined using equation 6. $P_{g\alpha}$ was calculated from

equation 5, considering a concentration of the α compound in the aqueous phase [C_α] of 40 mmol/L [16]. All of these compounds had a $P_{g\alpha}$ of less than 1 mbar for temperatures between 35 et 80°C, showing that their partial pressures are negligible compared to the partial pressures of H_2 and CO_2. Subsequently, for this study we consider that P_{prod} is only the sum of P_{gH_2} and P_{gCO_2}. P_{prod} was then deduced from the manometer measurement of P_t by subtracting $P_{vs\,(H_2O)}(T) + P_{exp}(T)$ (eq. 1, Table 1).

Figures 2, 3 and 4 represent the relation between the partial pressure of H_2 measured by gas chromatography (P_{gH_2}) and P_{prod} deduced from the total pressure measured by the manometer gauge for the three microorganisms *T. maritima*, *T. kodakarensis* and *E. cloacae*. For all strains, a linear relation between P_{gH_2} and P_{prod} was obtained with a relatively good correlation coefficient ($0.975 < R < 0.995$). The slope of regression line (β) was almost the same for the two hyperthermophilic strains *T. maritima* (0.623, Fig. 2) and *T. kodakarensis* (0.653, Fig. 3) but lower for the mesophilic bacteria *E. cloacae* (0.46, Fig. 4). Slope of regression line (β) is the ratio between P_{gH_2} and P_{prod}.

$$\frac{P_{gH_2}}{P_{prod}} = \left(\frac{P_{gH_2}}{P_{gH_2} + P_{gCO_2}}\right) = \beta \qquad (14)$$

$$\frac{P_{gH_2}}{P_{gCO_2}} = \frac{\beta}{1-\beta} \qquad (15)$$

with $P_{prod} = P_{gH_2} + P_{gCO_2}$

Using equation 15 with the β values determined for each strain (Fig. 2, 3 and 4), P_{gH_2}/P_{gCO_2} ratios were 1.7, 1.9 and 0.9 for *T. maritima*, *T. kodakarensis* and *E. cloacae*, respectively. The main end-products measured at the end of experiments for *T. maritima* and *T. kodakarensis* and *E. cloacae* were acetate and butyrate (data not shown), respectively, as reported by different authors [16,22]. The P_{gH_2}/P_{gCO_2} ratios were comparable to the stoichiometric

parameters obtained from equations 12 and 13, essentially associated with the "acetate" (H_2/CO_2=2) and "butyrate" (H_2/CO_2=1) pathways. Five experiments were performed using *C. saccharolyticus*, an extreme thermophilic strain, in which P_{gH_2} and P_{prod} were measured by gas chromatography and manometer gauge, respectively, only at the end of experiment when H_2 production was maximum. P_{gH_2} was 116.5 ± 15 mbar and P_{prod} was 180 ± 10 mbar. *C. saccharolyticus* uses the "acetate" pathway (acetate was main end-product; data not shown). In this case, when the stoichiometric parameter (eq. 12 (H_2/CO_2=2)) was applied, P_{gH_2} was 120 mbar, i.e. very close to that measured by gas chromatography (116.5 ± 15 mbar). Considering the similarities between these techniques, the correct use of a manometer gauge could be considered as a simple instrument, efficient enough to measure the partial pressure of hydrogen produced during anaerobic fermentation, with maximum accuracy at minimal cost, in terms of both time and cost.

Partial pressure of H_2 (P_{gH_2}) is an important factor for continuous H_2 synthesis. Boileau et al [16] showed that the cellular-production rate and the glucose-consumption rate of *T. maritima* were not afected when P_{gH_2} was maintained in a range of 7–178 mbar. This result is consistent with some authors' conclusion that a P_{gH_2} lower than 200 mbar was required for an optimal growth [23–25]. Moreover, when P_{gH_2} increases, metabolic pathways shift to production of more reduced substrates such as lactate, ethanol, acetone, etc.... Van Niel *et al.* [24] reported that for P_{gH_2} higher than 200 mbar, lactate becomes the dominant fermentation product during *C. saccharolyticus* growth. To our knowledge, no information on the effect of P_{gH_2} on *T. kodakarensis* and *E. cloacae* growth cultured in serum bottles is available.

Effect of pH on the P_{gH_2}/P_{gCO_2} ratio during H_2 production

Analysis found a linear relationship between P_{gH_2} and $P_{gH_2} + P_{gCO_2}$ for *T. maritima, T. kodakarensis* and *E. cloacae*. Moreover, the P_{gH_2}/P_{gCO_2} ratios obtained from the experiments are close to the stoichiometric parameters of the most plausible metabolic pathways of each strain (eqs. 12 and 13). Initial pH was 7.0 ± 0.1 for all three microorganisms. Final pH was 5 ± 0.2 for *T. maritima* and *T. kodakarensis* and 4.4 ± 0.2 for *E. cloacae*. Therefore, as the dissociation of $[HCO_3^-]$ into $[CO_3^{2-}]$ can be considered negligible at pH ≤ 7, total dissolved CO_2 ($[C_T]$) (eq. 10) is only the sum of the concentration of CO_2 ($[C_{CO_2}]$) and the concentration of HCO_3^- ($[HCO_3^-]$). At the end of the experiments, pH was low (≤ 5) for all three microorganisms. Consequently, dissolved CO_2 ($[C_T]$) in the aqueous phase is low, and most of the CO_2 is present in the headspace of the bottles.

CO_2 ($[C_{CO_2}]$) and HCO_3^- ($[HCO_3^-]$) concentrations in the aqueous phase were calculated using equations 5, 6, 10 and 11, taking into account a final P_{gCO_2} of 100 mbar for *T. maritima* and *T. kodakarensis* and 130 mbar for *E. cloacae* (Fig. 2, 3 and 4). For the two hyperthermophilic microorganisms, dissolved CO_2 ($[C_T]$) in the aqueous phase represented 10% of total CO_2 produced at the end of the experiment. These 10% are distributed as follows: 95% of C_{CO_2} and 5% of HCO_3^- (eq. 10). For *E. cloacae*, 24% of total CO_2 was dissolved in the aqueous phase (C_{CO_2}: 95.4% and HCO_3^-: 4.6%) at the end of experiment. Then, taking into account these values of total dissolved CO_2 ($[C_T]$) in the aqueous phase, the corresponding P_{gH_2}/P_{gCO_2} ratios (calculated from equations 5, 6, 8, 10 and 11) were 2.2 for *T. maritima* and *T. kodakarensis* and 1.24 for *E. cloacae*. These values are comparable to those obtained experimentally (1.8 and 1.17). The effect of the medium culture acidification on P_{gH_2}/P_{gCO_2} ratio was also confirmed for *C. saccharolyticus*, for which final pH was 4 and the corresponding P_{gH_2}/P_{gCO_2} ratio was 1.83, which is close to the theoretical stoichiometric yield of 2 moles of H_2 per mole of CO_2 (eq. 11).

To illustrate the influence of pH, experiments were performed using BM1 for *T. maritima* growth. The increase of KH_2PO_4 and Na_2HPO_4 concentrations from 0.3 to 0.6 g/L and 0.3 to 5 g/L was expected to bring better pH control at around 7. Figure 5 charts the variation in partial pressures of H_2 and CO_2 with in pH versus time during the growth of *T. maritima* for BM1 and BM, respectively. With BM1, pH held quasi-constant during 40 hours (initial pH =7 ± 0.1 and final pH = 6.9 ± 0.1), whereas with BM it dropped from 7 ± 0.1 to 5.5 ± 0.1. In both cases, the increase of H_2 partial pressure was comparable at hour 40 of the experiment. Peak P_{gH_2} was 110 mbar (310 mL H_2/L medium). On 48 hours of cultivation of *T. maritima* with a similar culture medium, Nguyen et al. [26] obtained a maximum cumulative H_2 production of about 180 mL H_2/L medium in batch experiments (120-mL serum bottles), and showed an effect of different initial pH values on bacterial growth and hydrogen production. When initial pH level decreased from 6.5-7.0 to 5.5, there was a 30% decrease in cumulative H_2 production. Here, the same decrease in pH did not inhibit H_2 production by *T. maritima*: in contrast with Nguyen et al. [26], pH decreased slowly over 40 hours, allowing *T. maritima* to gradually adapt. On 40 hours of the experiment, P_{gCO_2} (Fig. 5) was slightly lower (about 20%) using BM1. Due to the better control of pH (7 ± 0.2) during *T. maritima* growth, more CO_2 ($[C_T]$) got dissolved in the liquid phase, and thus P_{gCO_2} in the serum-bottle headspace was lower. The difference between the P_{gCO_2} obtained from BM *vs* BM1 seems to be due to the equilibria (CO_2gas ↔ CO_2aqueous ↔ HCO_3^- (eq. 10 and 11)) more than the inhibition of *T. maritima* growth due to the decrease in pH (fig. 5). In a closed serum-bottle without shaking, hydrogen transfer from liquid phase to headspace is limiting. Here, the volumetric mass transfer coefficient (Kla) of hydrogen is several orders of magnitude less than that obtained for a reactor continuously flushed with nitrogen [25,27]. The concentration of dissolved hydrogen will therefore increase rapidly, thus inhibiting the

growth of *T. maritima*. In this case, the effect of the decrease in pH from 7 to 5.5 (Fig. 5) on hydrogen production could be low compared to the effect of hydrogen inhibition.

To illustrate this point and better establish the effect of pH on P_{gCO_2} and P_{gH_2}, experiments were conducted using BM and BM1 for *T. maritima* growth. P_{gCO_2} and P_{gH_2} were measured by gas chromatography. Figure 6 plots P_{gH_2} versus P_{gCO_2} for BM and BM1. For both these basal media, we observed a linear regression between P_{gH_2} and P_{gCO_2}. For BM and BM1, the final pHs were consistently within the range of 5–5.5 and 6.6–6.9, respectively. The experimental P_{gH_2}/P_{gCO_2} ratios were 2.65 and 2.09 with "controlled" (BM) pH and "uncontrolled" (BM1) pH, respectively (Fig. 6). The difference between these ratios shows that when pH remains almost constant (i.e. "controlled"), an amount of CO_2 gets solubilized in the liquid phase, whereas when pH decreases (i.e. "uncontrolled"), a great fraction of CO_2 gets transferred to the headspace. Then, the P_{gH_2}/P_{gCO_2} ratio (2.65) measured for the "controlled" pH experiment is therefore logically high compared to the value of the stoichiometric parameter (eq.12, $P_{gH_2}/P_{gCO_2} = 2$). In this case, 58% of the total CO_2 was dissolved in the aqueous phase ($[C_T]$), as calculated from equations 5, 6 and 11, for P_{gCO_2} ranging from 10 to 60 mbars (Fig. 6). The corresponding P_{gH_2}/P_{gCO_2} value is 3.16, which is higher than the P_{gH_2}/P_{gCO_2} ratio (2.65) obtained experimentally. This higher ratio could be due to over/underestimation of parameters such as the K_h (T) and K_1 coefficients. *T. maritima* is a hyperthermophilic halophile that grows at 20 g/L (see Material and Methods). Effects of salt were not taken into account for K_h (T) and K_1 because there is no data available at this salt concentration for a temperature of 80°C. However, for a temperature of 45°C, when salinity increased from 2 to 20 g/L, K_h (T) decreased by about 10% [28] while at the same time K_1 increased by 30% [29]. The decrease of K_h (T) releases CO_2 from the aqueous phase to the headspace, which consequently decreases the value of the P_{gH_2}/P_{gCO_2}

ratio. At the same time, the increases of K_1 will have the effect of shifting the equilibrium ($CO_{2\ aqueous} \leftrightarrow HCO_3^-$) towards HCO_3^- and thus decrease the CO_2 concentration in the aqueous phase. Many authors have studied CO_2 transport across the air-sea interface using the stagnant film model theory [30–32], and shown that the exchange mechanism for CO_2 gas may indeed vary with the environmental conditions. The rate of CO_2 exchange near the air-sea interface is influenced by chemical processes (i.e. hydration/dehydration reactions) [32]. In particular, the rate of CO_2 exchange for a solution pH in the 6.5 region (where CO_2 can react with water and hydroxyl ions to a significant extent) was found to be greater than the rate in the pH < 4 region (where CO_2 effectively acts as an inert gas) [31]. More studies are needed to improve our understanding of these mechanisms and more accurately determine the K_h (T) and K_1 coefficients at high temperature in the presence of salt.

Conclusion

Here we demonstrated that under certain assumptions, the hydrogen partial pressure of *T. maritima, T. kodakarensis and E. cloacae* cultures in closed serum bottles can feasibly be determined using a simple manometer. However, the use of this technique requires (i) that the main volatile compounds in the serum-bottle headspace are hydrogen, carbon dioxide and water vapor, (ii) that the metabolic pathway of the hydrogen-producing microorganisms is known, which makes it possible to use the stoichiometric H_2/CO_2 yield ratio, and (iii) that pH decreases during the fermentation, releasing a maximum of dissolved CO_2 from the culture medium into the serum-bottle headspace. Further studies are needed to better understand the mechanisms of H_2 and CO_2 transfer from the liquid to the gaseous phase of the serum bottle, and the effects of salinity and high temperature on Henry's law constant for CO_2 and the dissociation constant for CO_2 into the bicarbonate ion, respectively.

Competing interests

The authors declare that they have no competing interests.

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List of figures

Figure 1: Experimental system for the measurements in serum-bottle of the total pressure using a manometer gauge and the hydrogen content by gas chromatography. 1: water bath, 2: syringe, 3: manometer, 4: anaerobic culture.

Figure 2: Partial pressure of H_2 measured by gas chromatography versus total pressure of gas measured by the manometer for *T. maritima*.

Figure 3: Partial pressure of H_2 measured by gas chromatography versus total pressure of gas measured by the manometer for *T. kodakarensis.*

Figure 4: Partial pressure of H_2 measured by gas chromatography versus total pressure of gas measured by the manometer for *E. cloacae*.

Figure 5: Partial pressures of H_2 and CO_2 and, pH versus time: partial pressure of H_2 (○), partial pressure of CO_2 (∇) and pH (□) with BM1; partial pressure of H_2 (•), partial pressure of CO_2 (▼) and pH (▮) with BM.

Figure 6: Partial pressure of H_2 versus partial pressure of CO_2 for *T. maritima.* (●) BM (○) BM1.

1. $H_2 = - 0.84 + 2.09\ CO_2$ (R = 0.994); **2.** $H_2 = 0.33 + 2.65\ CO_2$ (R = 0.984)

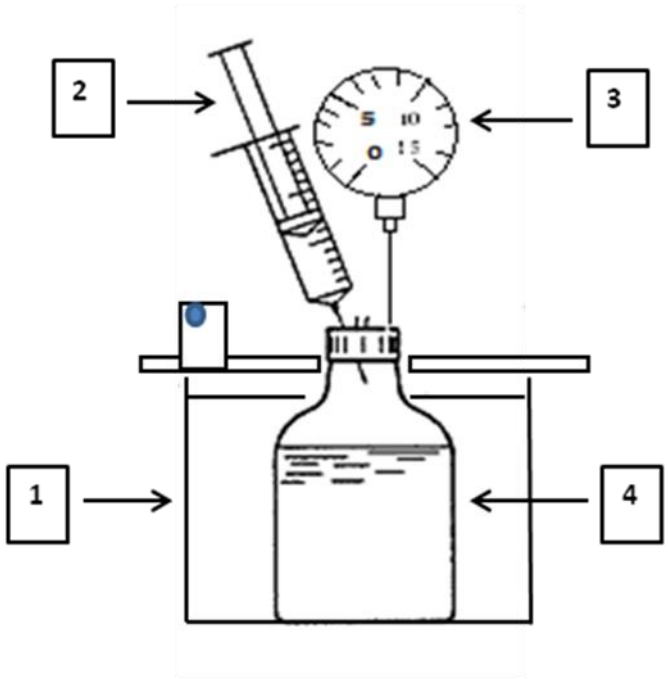

Figure 1

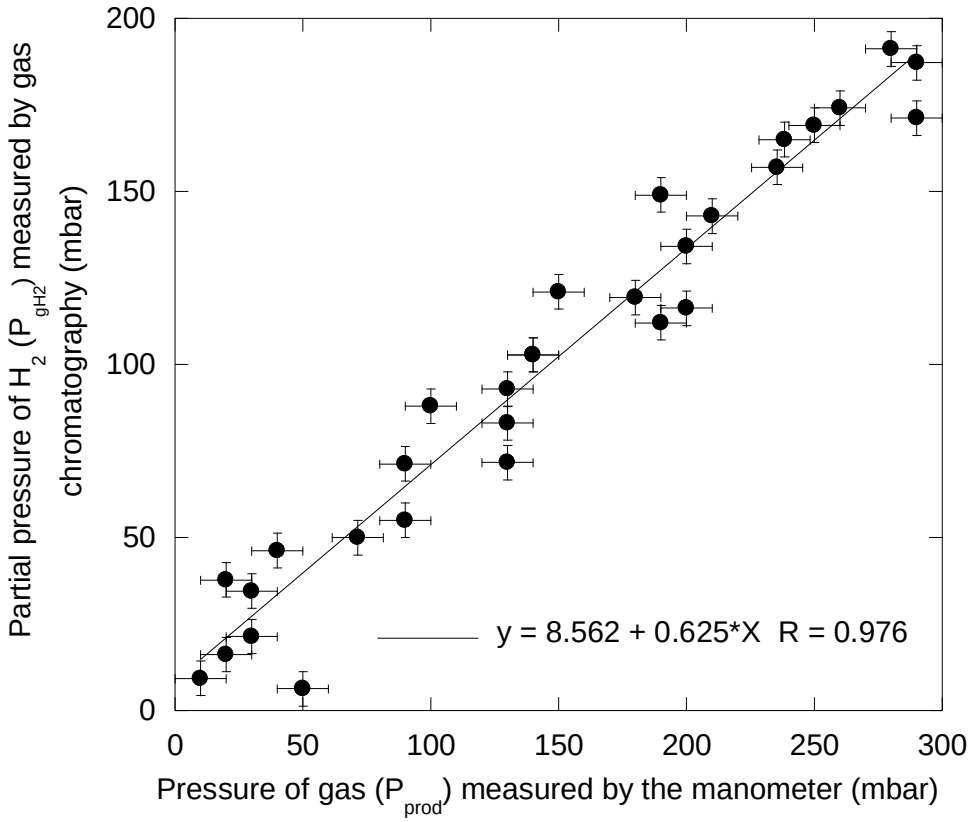

Figure 2

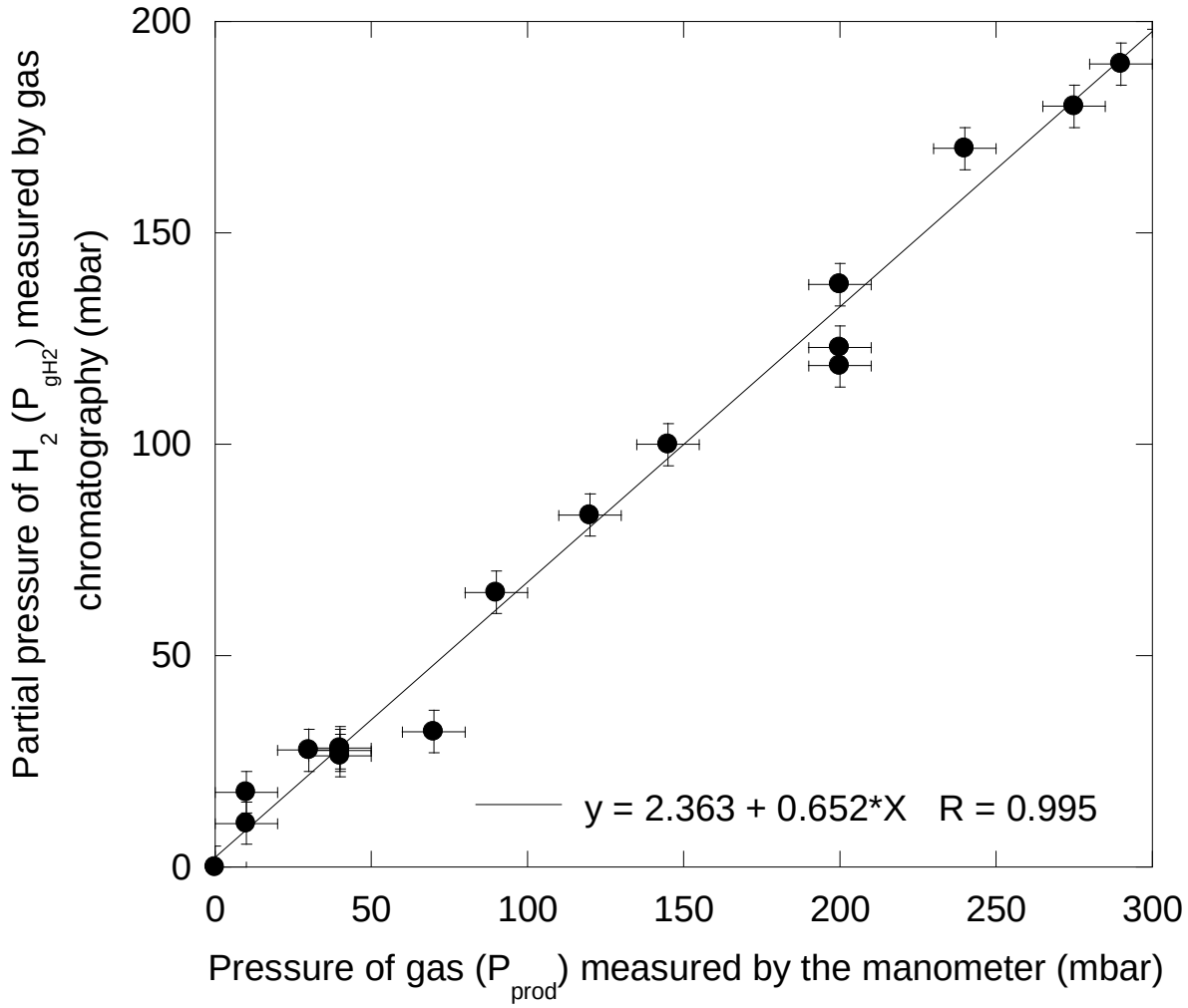

Figure 3

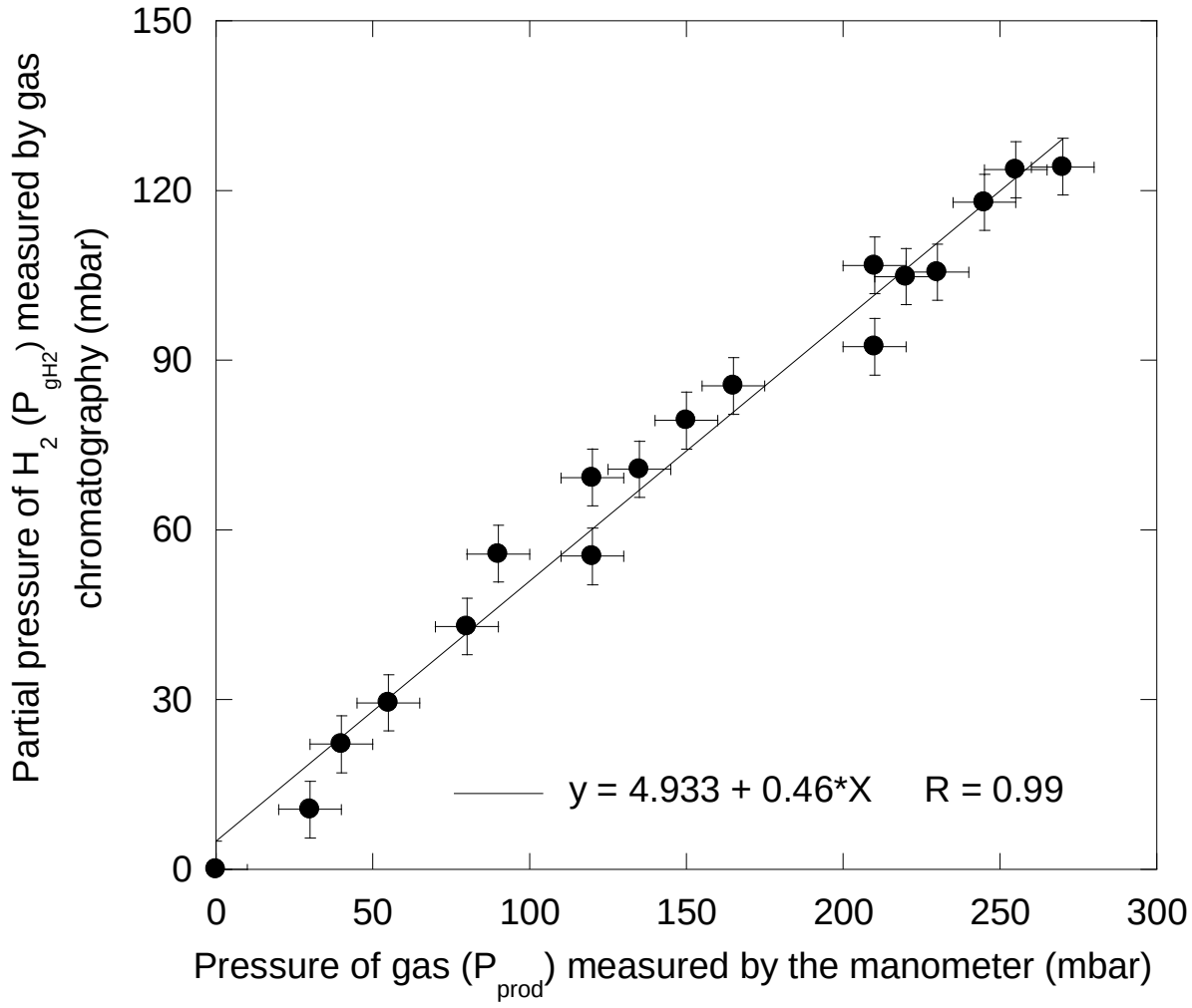

Figure 4

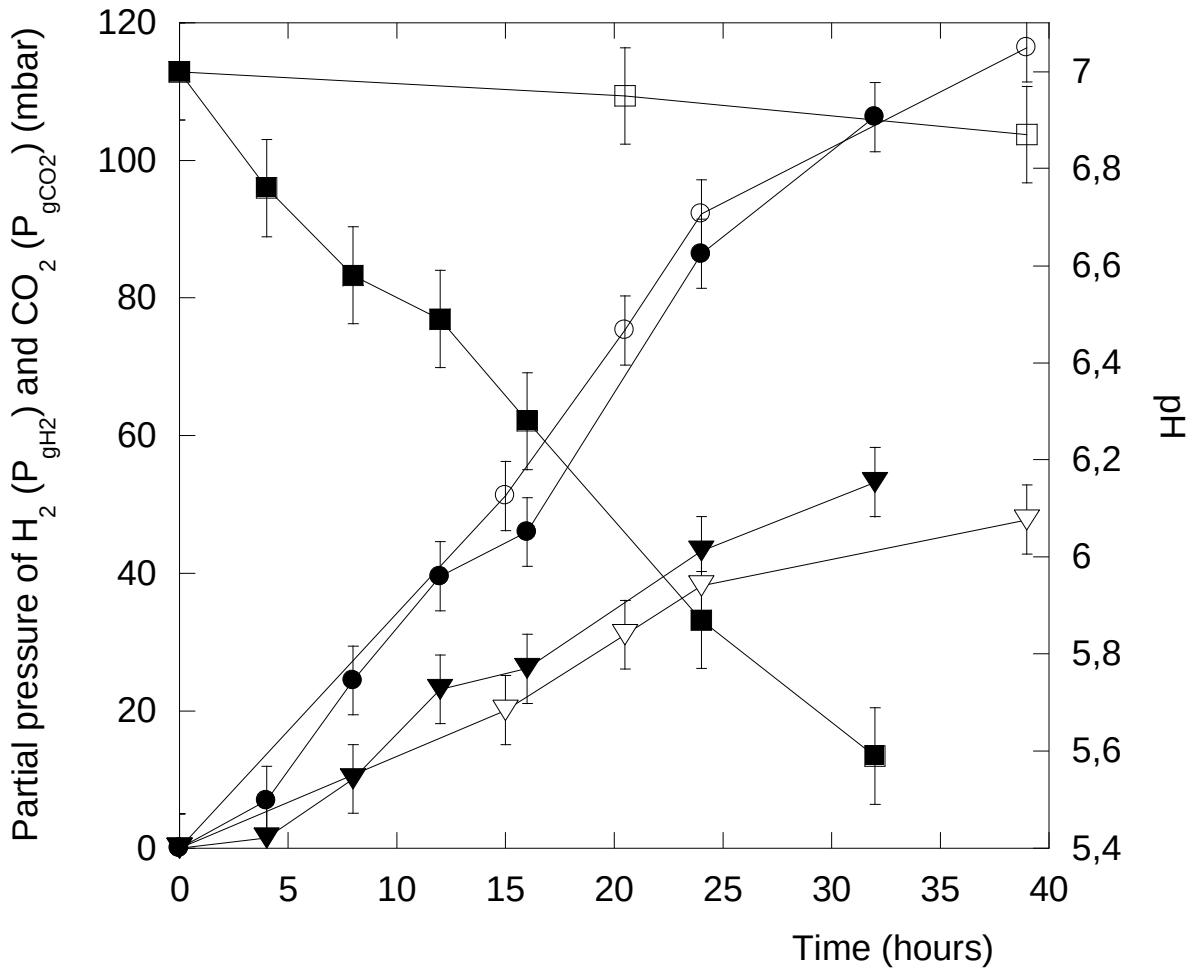

Figure 5

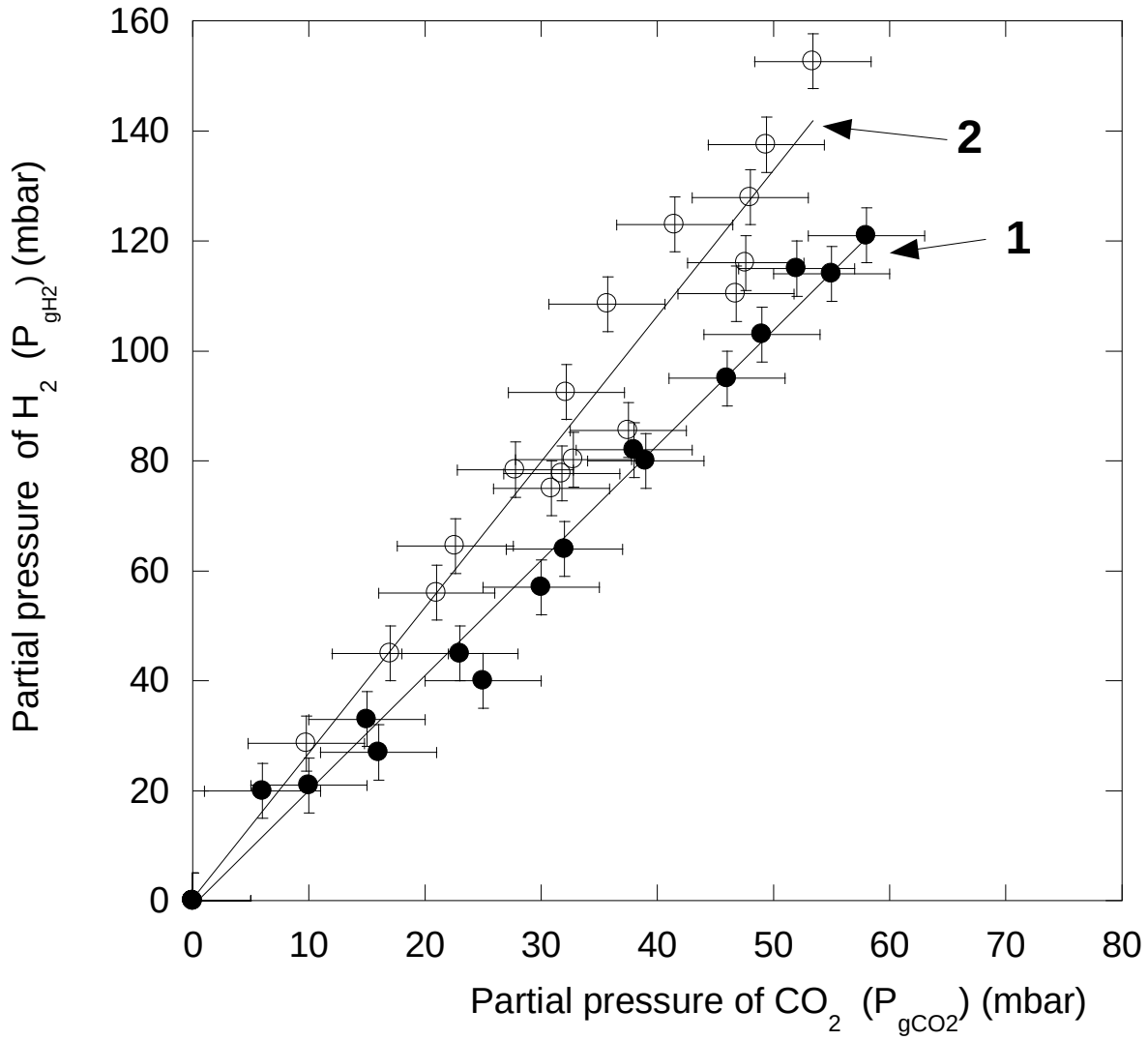

Figure 6

List of table

Table 1: Saturation pressure of water ($P_{vs\,(H_2O)}$) and pressure expansion of gases (P_{exp}) versus temperature. (*) calculated, (**) measured.

Table 1

T (°C)	$P_{vs\,(H_2O)}$ (mbar) (*)	P_{exp} (mbar) (*)	$P_{vs\,(H_2O)} + P_{exp}$ (mbar) (*)	$P_{vs\,(H_2O)} + P_{exp}$ (mbar) (**)
35	55.9	51.8	107.7	100 ± 10
70	308.5	185.1	493.6	500 ± 10
80	468.4	207.3	675.7	680 ± 10