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The role of hydrogen mass transfer for the growth kinetics of *Methanobacterium thermoautotrophicum* in batch and chemostat cultures

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Hydrogen concentration was determined in batch and chemostat cultures of *Methanobacterium thermoautotrophicum*, both in the headspace and in the medium using mass spectrometry. The calculated dissolved hydrogen concentration in the medium as derived from the headspace hydrogen concentration when equilibrium conditions between gas and liquid phase were assumed, was ten times higher than the experimentally determined hydrogen concentration. Variation of the partial pressure of hydrogen resulted in different values for substrate affinity for hydrogen (K_s) and yield (Y) of the cells. Upon hydrogen limitation, K_s decreased while the yield coefficient for hydrogen increased, indicating a change in the affinity of the cells towards hydrogen.

Keywords: dissolved hydrogen; gas-to-liquid mass transfer; growth yield; substrate affinity constant; Fe^{2+} limitation; *Methanobacterium thermoautotrophicum*

Abbreviations

vvm	=	volume per volume per minute	(min^{-1})
rpm	=	stirrer speed, revolutions per minute	(min^{-1})
k_La	=	mass transfer coefficient	(min^{-1})
K_s	=	half saturation substrate affinity constant	(μM)
Y_{H_2}	=	growth yield for hydrogen	(g/μM)
Q_{H_2}	=	hydrogen uptake rate	(mM/(L*h))
$S_{(H_2)}$	=	liquid-phase hydrogen concentration	(mM)
$S^*_{(H_2)}$	=	liquid-phase hydrogen concentration in equilibrium with the bulk gas phase	(mM)

Introduction

Hydrogen is an important electron donor for methanogenic bacteria. Its low solubility in aqueous systems often makes hydrogen the growth-limiting factor in cultures as well as in natural systems [14]. Hydrogen uptake and interspecies hydrogen transfer has been studied mostly in batch cultures maintained in closed vessels. Mass balances have been obtained for such systems, but it is difficult to get information on the actual chemical environment concerning eg the hydrogen concentration in the vicinity of the cells. It is thus not surprising that values of the apparent substrate affinity constant for hydrogen, K_s, in the wide range of 0.05–80 μmol L^{-1} have been reported for methanogens [15,16,21]. Many of these values exceed by far the actual hydrogen concentration observed in natural habitats (0.03–6 μM), as reviewed by Keltjens and van der Drift [13]. This suggests that the K_s value has been systematically overestimated and that experimental difficulties do not allow exact determination of *in situ* hydrogen concentrations in growing cultures. Similar conclusions were reached by Boone *et al* [3]. Compared to the large amount of free energy available from the complete oxidation of organic substrates (eg 2822 kJ $mole^{-1}$ for glucose) by aerobic heterotrophs [4], methanogenic bacteria gain only between 7.5 kJ and 33 kJ (= $\Delta G^{\circ\prime}$) per mole of hydrogen depending on the hydrogen concentration [24]. Thus growth of methanogenic cultures on hydrogen and carbon dioxide as sole energy and carbon sources respectively, need by far larger quantities of gas to produce equal amounts of biomass. Therefore growth of methanogens is usually limited by the supply of hydrogen and controlled by the mass transfer of hydrogen. When the concentration of hydrogen dissolved in the medium of growing cultures is calculated from the head space concentration, the values are far higher than the ones measured in natural habitats [6,20,23] This implies that hydrogen-dependent methanogenic bacteria will only grow at very low rates under natural conditions.

In the present study hydrogen concentrations were determined in the gas phase as well as well as in the liquid phase and the latter compared to the calculated liquid phase concentration which is expected from gas-liquid equilibirum using *Methanobacterium thermoautotrophicum* during growth on hydrogen and carbon dioxide.

Materials and methods

Organism, growth medium and growth conditions

Methanobacterium thermoautotrophicum, strain Hveragerdi (DSM 359) was previously isolated in our laboratory [5]. Stock cultures were kept at –80°C. The medium was based on Schonheit *et al* [21] containing: NH_4Cl (60 mM); $MgCl_2 \cdot 6H_2O$ (1.5 mM); NTA (nitrilo-triacetate, 1.2 mM);

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NaCl (10 mM); KH_2PO_4 (10 mM); $CoCl_2 \cdot 6H_2O$ (4 μM); $Na_2MoO_4 \cdot 2H_2O$ (4 μM); $NiCl_2 \cdot 6H_2O$ (4 μM); $FeCl_2 \cdot 4H_2O$ (0.1 or 0.2 mM, see legends to figures). The cells were grown in a 2-L bioreactor equipped with a conventional blade stirrer and controls for temperature, pH and redox potential (Mini, MBR Wetzikon, Switzerland). In all experiments the temperature was held at 60°C, and the pH at 6.9. The culture volume was 1.8 L, leaving a headspace of 0.2 L. The bioreactor was supplied continuously with Na_2S (stock concentration 640 mM), leading to a final concentration of 0.5 mM sulfide. The gassing rate and the composition of gas supplied to the bioreactor were controlled electronically and varied depending on the experiment. These data are given separately for each experiment. The bioreactor was sterilized *in situ* for 20 min at 121°C. The sterile stock media were kept under nitrogen.

The concentration of hydrogen solubilized in the culture medium of *Methanobacterium thermoautotrophicum* at 60°C is very low, thus the soluble hydrogen lost from the fermenter through the medium under chemostat conditions can be neglected. The difference in the amount of hydrogen at the gas inlet compared to the gas outlet of the fermenter in the steady-state of a chemostat culture is therefore about equal to the amount of hydrogen transported from the gas phase into the liquid phase and eventually into the cells. The amount of hydrogen taken up per time unit can thus be used to determine the mass transfer coefficient, k_L [1]. In contrast to physical methods which are carried out in a system lacking cells, this biological method is to be preferred since effects of the cells on the mass transfer, eg by a change in viscosity of the medium, which would alter the k_La value, are corrected directly.

The k_La value for hydrogen was thus determined in the steady-state of chemostat conditions using the formula

$$Q_{(H_2)} = k_La \cdot (s^*_{(H_2)} - s_{(H_2)}) \quad (1)$$

which gives, after rearrangement:

$$k_La = \frac{Q_{(H_2)}}{(s^*_{(H_2)} - s_{(H_2)})} \quad (2)$$

where k_L is the mass transfer coefficient, a is the gas-liquid interface area per unit liquid volume, $s^*_{(H_2)}$ is the hydrogen liquid-phase concentration which in equilibrium with the bulk gas phase and $(s^*_{(H_2)} - s_{(H_2)})$ is the overall concentration driving force for hydrogen. The Henry constant for hydrogen at 60°C is 0.0129 [8].

The half saturation constant for hydrogen, k_s, was determined during steady-state chemostat conditions, where the gas-to-liquid H_2 mass transfer rate of hydrogen was equal to the rate of H_2 consumption by the microorganisms:

$$Q_{(H_2)} = \frac{1}{Y_{(H_2)}} \cdot \mu_{max} \cdot \frac{s_{(H_2)}}{K_{s,(H_2)} + s_{(H_2)}} \cdot x \quad (3)$$

$Y_{(H_2)}$ is the yield coefficient for *Methanobacterium thermoautotrophicum* for hydrogen, $s_{(H_2)}$ is the real concentration of hydrogen in the liquid phase of the bioreactor, μ_{max} is the maximum growth rate and x the biomass concentration in the bioreactor. μ_{max} was determined to be 0.35 (h^{-1}) in chemostat cultures by washout experiments [18] and was used for all calculations.

Rearrangement of Eqn (3) gives:

$$K_{s,(H_2)} = \frac{s_{(H_2)} \cdot \mu_{max} \cdot x}{Q_{(H_2)} \cdot Y_{(H_2)}} - s_{(H_2)} \quad (4)$$

The gas supplied to the bioreactor was a mixture of hydrogen, carbon dioxide and argon. To prevent growth limitation by carbon dioxide, the proportion of hydrogen to carbon dioxide was changed to 3:1 instead of 4:1 as suggested by the stoichiometry of methane formation (unpublished results). The mixtures were made up continuously from pure gases (Carbagas, Zurich, Switzerland) with flowmeters and a gas flow controller (MKS, Munchen, Germany). Traces of oxygen were removed in a Hungate oven [11].

Analytical techniques

Outlet gases were analyzed in a quadrupole mass spectrometer (PGA100, Leybold, Koln, Germany). Argon in the gas mixture (1–5%) served as internal standard for calibration to determine the mass flow of the outlet gases. The gas composition was determined by mass spectroscopy at the gas outlet of the reactor after passing over a cool surface (cooled by a Peltier element) with a capillary inlet. Gases dissolved in the growth medium were sampled with a home-made membrane probe which was coupled to the quadrupole mass spectrometer. A hydrophobic 0.25-mm Teflon FEP membrane suited best for the hydrogen measurements. Membrane probes have been used before to analyse on-line microbial products [2,19].

Valves alternately allowed the gas flow from the different sampling sites to the mass spectrometer. The signal was linear over a range of hydrogen concentrations from 20 μM to 580 μM in the medium, obtained by dilution of calibration gas (Carba, Zurich) by electronic mass flow meters (MKS, Munich, relative accuracy ± 2%). The biomass concentration was measured online from the backscattering signal of an AS 82 probe (Aquasant, Bubendorf, Switzerland) and correlated to the absorption of the culture at 660 nm. There was a linear correlation between backscattering and dry cell weight concentration in the range from 0 to 15 g L^{-1} dry weight. The sulfide concentration was determined colorimetrically [9].

Results

Effect of the hydrogen mass transfer on growth parameters in batch cultures

Figure 1a shows a basic patch experiment from which defined changes to vary the availability of hydrogen to the cells were made. All experiments presented were carried out at 60°C and pH 6.9. Further experimental conditions are given in the legends to the figures. After a lag phase, growth was exponential for 12 h under the conditions given in Figure 1a. Then it switched to a linear increase of the biomass indicating limitation of bacterial growth. As the biomass concentration increased the H_2 consumption of the

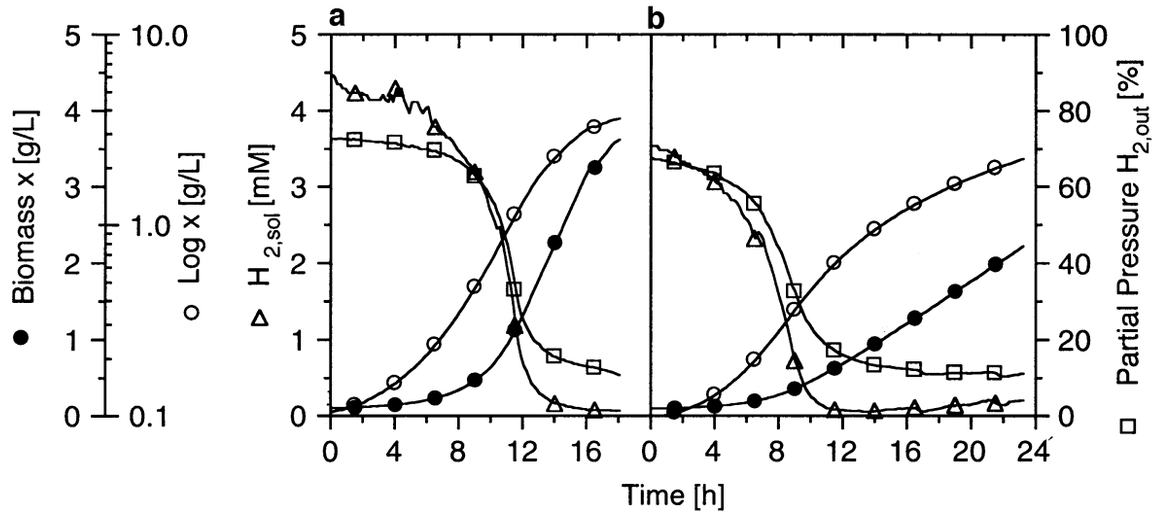

Figure 1 Time course of growth of *Methanobacterium thermoautotrophicum* in batch cultures at different mass transfer rates for hydrogen. Conditions (a): T = 60°C; rpm = 800; pH = 6.9; $H_{2,in}$ = 0.294 (vvm); $CO_{2,in}$ = 0.094 (vvm); k_La = 1363 (h^{-1}); Fe^{2+} = 0.1 (mM). Conditions (b): T, rpm, pH, Fe^{2+} as in (a); $H_{2,in}$ = 0.0941 (vvm); $Co_{2,in}$ = 0.0235 (vvm); k_La = 220 (h^{-1}).

cells increased accordingly until the H_2 consumption rate was equal to the maximal H_2 mass transfer rate under the experimental conditions. Then the H_2 mass transfer rate was governing the H_2 consumption rate leading to linear growth kinetics. Under the experimental conditions the mass transfer of hydrogen and thus $Q_{(H_2)}$ was small, similar, to the conditions present in a simple system with inefficient mixing or a low gas flow rate, eg in closed bottles.

In the experiment represented in Figure 1b the gas flow rate of hydrogen was reduced to 1/3. The effect of mass transfer limitation became visible even more dramatically. Linear growth started earlier and continued over 24 h, and no stationary phase was reached within this time.

In a bioreactor the mass transfer rate is determined by the shape of the stirrer, its rotation velocity and by the gas flow rate.

Increasing the latter two parameters will result in increased availability of hydrogen for the cells and thus the culture will grow to higher cell densities (Figure 2a). After 16 h, the culture arrived at a biomass concentration of approximately 4.5 g L^{-1} dry weight, whereas at the conditions in Figure 1a only about 3.2 g L^{-1} dry weight was obtained after the same cultivation time. Due to the lack of bioavailable iron in the medium, growth ceased after 16 h and the culture switched to the stationary phase. Limitations were tested by the 'pulse and shift' technique [10]. A variety of macro- and microelements not present in the original medium were analysed for growth limitation. A metabolic stimulation was observed only after iron addition.

When the concentration of iron in the medium was doubled but the supply of hydrogen (rotation of the stirrer and the hydrogen gas flow rate) and thus the k_La value were kept the same, the transition of exponential to linear growth occurred again after about 14 h of cultivation time (Figure 2b). However, growth limitation by a component of the medium started only after 24 h and the culture reached the stationary state with a biomass of nearly twice the one reached in Figure 2a. At this point, uptake of H_2 by the cells was drastically reduced and the concentration of H_2 in the medium increased to levels seen at the beginning of the experiment at much lower cell densities.

Effect of hydrogen mass transfer on growth parameters in chemostat cultures

Chemostat cultures were analyzed at similar biomass concentrations as in batch cultures under conditions when the culture was limited either by a component in the medium or by the hydrogen provided in the gas phase.

In the experiment represented in Figures 3a and b the concentration of ammonia was lowered from 60 to 20 mM, becoming the limiting component which controlled growth after the exponential phase. A steady-state was reached after 26 h. The concentration of hydrogen at the outlet as well as the hydrogen dissolved in the medium decreased during the first part of the experiment due to a rapid increase in cell mass during batch growth. After 25 h a significant increase in the concentration of hydrogen in both the gas and the liquid phase was observed until the steady-state was reached. Figure 3b shows the hydrogen concentration calculated to be dissolved from the headspace concentration at equilibrium conditions with no consumption as compared to the dissolved hydrogen which actually has been measured with the membrane probe in the medium. The large difference between the two concentrations represents the concentration gradient built up by the high uptake rate for hydrogen by the organisms.

Growth in the chemostat culture at restored NH_4^+ concentrations of 60 mM (Figure 4a) became controlled by the mass transfer of hydrogen when the gas flow rate was reduced by 50%. Although the biomass concentration at the beginning of the experiment was the same as in Figure 3a, the steady state was only reached after 36 h, ie 10 h later than in the culture in which ammonium was the limiting component for growth. Due to a two-fold decrease in the H_2 gassing rate the concentration of soluble hydrogen dropped early in the exponential growth phase and stayed

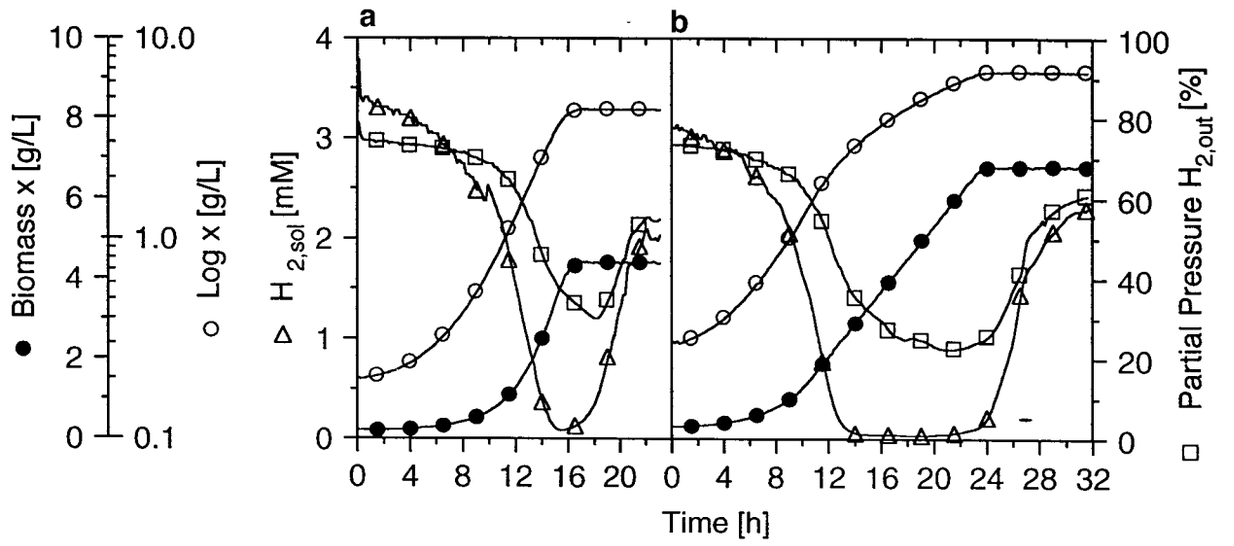

Figure 2 Time course of growth of *Methanobacterium thermoautotrophicum* in batch culture at increased mass transfer rates for hydrogen and varying Fe^{2+}-concentration. Conditions (a): T, pH, Fe^{2+} as in Figure 1; rpm = 1600; $H_{2,in}$ = 0.833 (vvm); $CO_{2,in}$ = 0.300 (vvm); k_La = 1470 (h^{-1}). Conditions (b): T, rpm, pH, $H_{2,in}$, $CO_{2,in}$, k_La as in (a); Fe^{2+} = 0.2 (mM).

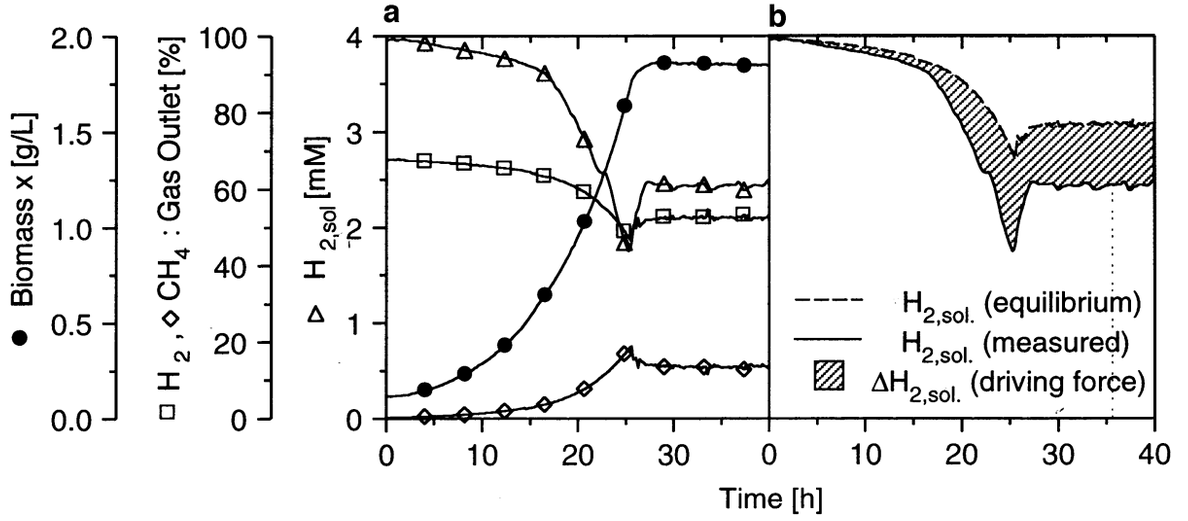

Figure 3 Time course of the transition from batch to chemostat culture of *Methanobacterium thermoautotrophicum* under conditions limited by ammonium ions. Conditions (a): T, rpm, pH as in Figure 1a; $H_{2,in}$ = 0.67 (vvm); k_La = 1540 (h^{-1}); D = 0.30 (h^{-1}); NH_4^+ in inflow = 20 mM. (b) Differences in the gradient between calculated and experimentally determined concentrations of H_2 during an ammonia-limited chemostat culture of *Methanobacterium thermoautotrophicum.*

very low until the end of the experiment. Figure 4b again compares the calculated and the measured concentrations of dissolved hydrogen in the medium of the bioreactor as in Figure 3b before.

The yield coefficient and the saturation constant for hydrogen were calculated under chemostat conditions and varying gas flow rates for hydrogen. The total flow rate of the gas mixtures was kept constant by replacing hydrogen with nitrogen. As seen in Figure 5a, the biomass yield coefficient of hydrogen increased at low hydrogen partial pressure in the medium. A variable stoichiometry indicating uncoupling of growth from methane production has been described previously [7,12,21], however, no physiological explanation has been given so far [25].

As the yield coefficient of hydrogen was not constasnt, the question was raised whether the saturation constant K_s could also be sensitive to different concentrations of hydrogen in a chemostat culture. Figure 5b clearly shows that the apparent K_s varied with varying H_2 pressure. It decreased linearly with lowered hydrogen concentrations. This demonstrates that the affinity of the cells for hydrogen increased with decreasing concentrations of available hydrogen.

Discussion

The experiments presented with varied concentrations of molecular hydrogen in the medium and thus different bioavailability of the electron source suggest that the H_2

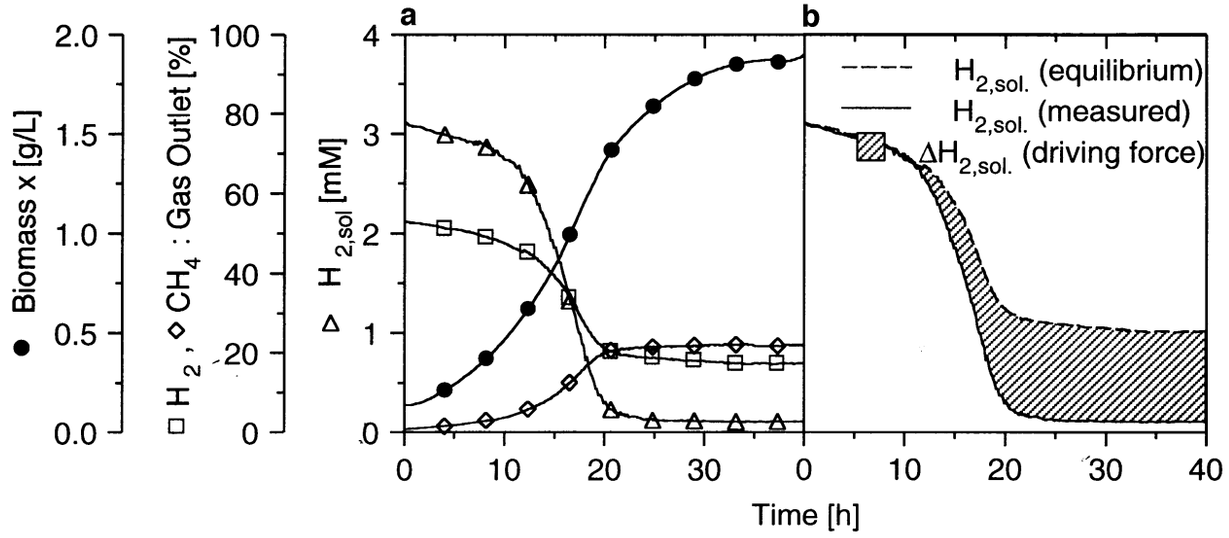

Figure 4 Time course of the transition from batch growth to a chemostat culture of *Methanobacterium thermoautotrophicum* under conditions limited by hydrogen. Conditions (a): T, rpm, pH, D as in Figure 3; $H_{2,in}$ = 0.33 (vvm); $CO_{2,in}$ = 0.13 (vvm); k_La = 940 (h^{-1}). (b) Differences in the gradient between calculated and experimentally determined concentrations of H_2 during a hydrogen-limited chemostat culture of *Methanobacterium thermoautotrophicum.*

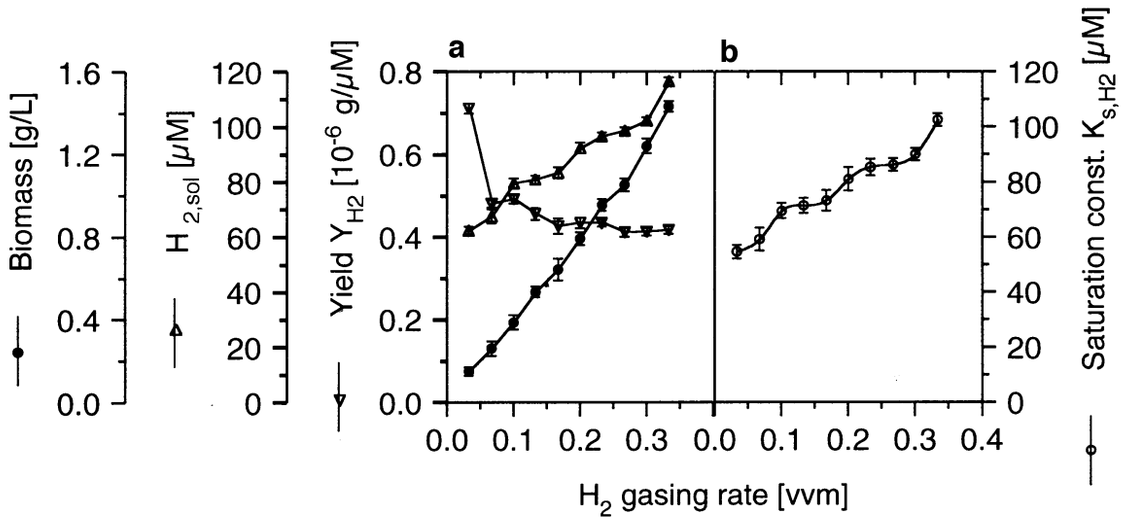

Figure 5 (a) Dependence of biomass, hydrogen yield coefficient and soluble hydrogen on hydrogen gassing rates in chemostat cultures of *Methanobacterium thermoautotrophicum*. Each data point is the mean of five determinations; standard deviation is given by error bars. Conditions: T, pH, rpm, D as in Figure 4; $H_{2,in}$ = variable, as indicated; $CO_{2,in}$ = 0.167 (vvm); total gas flow rate (sum of H_2, CO_2 and N_2) = 0.542 (vvm). (b) Dependence of half-saturation constant $K_{s,H2}$ upon hydrogen gassing rates in chemostat cultures of *Methanobacterium thermoautotrophicum*. Each data point is the mean of five determinations; standard deviation is given by error bars. Values for Y are taken from Figure 5a. Conditions: as in Figure 5a.

concentration determined in the headspace of the bioreactor bears no simple relation to the conditions in the vicinity of the cells. It is concluded that the overall mass transfer rate from the gas phase of the bioreactor into the bulk liquid phase is the growth-limiting process.

The concentration of hydrogen as measured experimentally in the steady-state of cultures under conditions of limitation of the mass transfer of hydrogen was ten times lower than the values calculated from the hydrogen partial pressure in the head space. This phenomenon is well described for oxygen-limited systems. However, it has often been neglected in anaerobic systems where for thermodynamic reasons it is even more pronounced than in oxic systems. Under conditions with poor or little agitation such as closed bottles with magnetic stirrers, the actual concentration of hydrogen in the liquid phase can be up to 70 times lower than the one calculated from the headspace concentration in systems where growth was followed under hydrogen [16,17]. In contrast in mixed cultures or natural habitats where H_2 is produced within the liquid system and transferred from the oversaturated liquid to the gas phase, H_2 concentration in the liquid is many times higher than in the gas phase [16,17]

When growth becomes limited in a chemostast culture by a component in the medium, the hydrogen concentration in the medium as well as at the gas outlet increases during

the transition to the steady state. This contrasts to cultures limited by the mass transfer of hydrogen where the difference between the measured and the calculated concentration of hydrogen stays constantly large after the stationary phase is reached. Only under conditions when medium compounds are growth-limiting, is the shift from growth to sole maintenance metabolism experimentally seen in the respective energy requirement.

It has been suggested previously that when culture conditions change in general, cells may alter their affinity towards substrates [22]. Our results clearly indicate that *Methanobacterium thermoautotrophicum* changes its affinity towards hydrogen by at least a factor of two when the gas concentration drops from 120 to about 60 μM in the medium. Thus different culture conditions result in different apparent K_s values.

The increase of biomass in a batch culture is usually represented in a semi-logarithmic plot. In such diagrams a short phase of linear growth before the culture reaches the stationary conditions is often overlooked. The presence of a linear phase in growth curves of cultures which depend on a gaseous compound indicate limitation of growth by the transfer of this compound from the headspace to the cells. Hydrogen limitation in cultures of methanogens is thus easily detected without knowing the actual concentration of hydrogen in the medium.

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