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Formate production and utilization by methanogens and by sewage sludge consortia – interference with the concept of interspecies formate transfer

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Abstract. Pure cultures of H_2/CO_2- and formate-utilizing methanogens or mixed consortia of sewage sludge generated some formate from H_2/CO_2 at H_2 partial pressure in the gas phase above 200 kPa. At decreasing H_2 partial pressure the formate was taken up again and converted to methane. If methanogenesis was inhibited by bromoethanesulphonic acid (BESA) or a high redox potential (−180 to −200 mV), formate-utilizing methanogens produced high amounts of formate from H_2/CO_2. No formate was excreted by the species, which could only utilize H_2/CO_2 for methanogenesis. In contrast, H_2 formation from formate was observed in cultures of *Methanobacterium thermoformicicum* and *M. formicicum*. Measurable amounts were, however, only formed if its immediate utilization for methane production was inhibited by BESA. In the light of the data on formate formation from H_2/CO_2 and its re-utilization by all formate-utilizing methanogens, the concept of interspecies formate transfer of Thiele and Zeikus should be reconsidered. In pure cultures of methanogens or complex ecosystems with excess H_2, formate formation seemed to serve more as a means of disposal of surplus reducing power than for H_2 transfer.

Introduction

The H_2 partial pressure seemed to be a key parameter of the anaerobic metabolism of carbohydrates, alcohols, fatty acids and xenobiotics in pure and defined mixed cultures and complex ecosystems (Bleicher and Winter 1991; Bleicher et al. 1989; Bryant et al. 1967, 1977; Boone and Bryant 1980; Chen and Wolin 1977; Ferry and Wolfe 1976; McInerney et al. 1979; Miller and Wolin 1973; Wolin and Miller 1982; Winter and Wolfe 1979, 1980; Winter 1980, 1984). Interspecies hydrogen transfer from the H_2-producing, fermentative or acetogenic bacteria to the H_2-consuming, sulphate-reducing or methanogenic bacteria was a prerequisite for quantitative substrate conversion to methane and CO_2. Only sulphate reducers outcompeted methanogens for reducing power in sulphate-containing sediments, due to their higher affinity for H_2 (Winfrey and Zeikus 1977).

The exclusive importance of interspecies hydrogen transfer for substrate conversion to methane and CO_2 was doubted by Thiele and Zeikus (1988), on the basis of their observation that in anaerobic micro-niches with floc formation formate concentrations ranging from <30 μM (methanogenesis not inhibited) to 300 μM (methanogenesis inhibited with 0.04% chloroform) were detected. The formate was, however, supposed to be generated from CO_2 by exclusively fermentative and acetogenic bacteria with reducing equivalents coming from ethanol oxidation or lactate decarboxylation. This conclusion was drawn from the fact that formate formation continued under conditions of a methanogenesis inhibited by chloroform (Thiele and Zeikus 1988).

In this paper we present data on the formation of formate from H_2/CO_2 by pure cultures of methanogens or a complex methanogenic consortium. The methanogens excreted even higher concentrations of formate if H_2 utilization for methane production was inhibited with chloroform, ethanol or bromoethanesulphonic acid (BESA).

Materials and methods

Organisms and growth conditions. The methanogens mentioned in Table 1 were obtained either from the Deutsche Sammlung für Mikroorganismen und Zellkulturen (Braunschweig, Germany) or isolated and cultivated in our laboratory. Sewage sludge was taken from the anaerobic reactors of the Klärwerk Regensburg by preventing heavy oxygenation and was stored under anaerobic conditions.

The basic medium for growth of the methanogens contained per litre: mineral solutions 1 and 2 (Balch et al. 1979), 38 ml of each; yeast extract (Merck no. 3753), 2 g; peptone (Merck no. 7213), 2 g; sodium acetate, 5 g; $FeSO_4 \cdot 7H_2O$, 2 mg; $Ni(NH_4)_2(SO_4)_2$, 2 mg; $NaHCO_3$, 6 g; resazurin, 1 mg; vitamins

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and trace minerals (Wolin et al. 1963), 10 ml of each; reducing agent (Balch et al. 1979), double concentrated, 40 ml. In the presence of an H_2/CO_2 or N_2/CO_2 gas phase (80:20%, v/v; 300 kPa) the pH was 7.0. For growth of *Methanogenium cariaci* and *M. marisnigri* 3% (w/v) NaCl was added to the basic medium. For growth of all strains on formate, 50 mmol/l of sodium formate was supplied and N_2/CO_2 (80:20%, v/v; 300 kPa) served as the gas phase.

Media were prepared and cultures grown anaerobically as described by Balch et al. (1979). Methane production was inhibited by addition of BESA from a 50 mM sterile, anaerobic stock solution to reach a final concentration of 2.5 mM. For other inhibition experiments the medium was supplemented with 0.05% $CHCl_3$ (v/v) or 8–20 mM ethanol. Sulphate reduction was prevented by addition of a sodium molybdate solution (40 mM) to give a final concentration of 2 mmol/l.

Analyses. Formate was analysed with a formate dehydrogenase test kit of Boehringer (Mannheim, Germany). The detection limit was 0.1 mmol/l. H_2 and methane were analysed by gas chromatography (Winter et al. 1984). The optical density of cultures was measured in an Ultrospec II LKB spectrophotometer (Pharmacia, Heidelberg, Germany) at 578 nm (E_{578}).

Chemicals. Chemicals were purchased from Merck (Darmstadt, Germany) or Sigma (Munich, Germany), and gases from Linde (Höllriegelskreuth, Germany).

Results

Formate generation from H_2/CO_2 by pure cultures of methanogens

When the H_2/CO_2 gas phase in cultures of *Methanobacterium formicicum* DSM 3636 at an E_{578} of 0.8 was renewed, 2.5 mM formate was excreted within the next 2 h. With decreasing H_2 concentration, most of the formate was utilized again for methane production (Fig. 1). Little formate (0.1–0.3 mM) remained in the medium until the H_2 was completely used up. If methane formation from H_2/CO_2 was inhibited by BESA, formate accumulated up to 17 mM (Fig. 1). The formate concentration remained at this high level even after prolonged incubation (not shown). Measurable amounts of formate (1–2 mM) were also formed from H_2/CO_2 if methanogenesis by the cultures was inhibited by 8–20 mM ethanol (data not shown).

When in grown-up cultures of *M. palustre* DSM 3108 ($E_{578}=0.9$) the methane was replaced by H_2/CO_2 (80:20%, v/v, 300 kPa), within the next 6 h of methanogenesis 10 mM formate was excreted into the medium. With decreasing H_2 concentration (<50% H_2 in the gas phase) the formate was re-utilized for methanogenesis (Fig. 2a). If methane formation from H_2/CO_2 by cultures of *M. palustre* was inhibited with BESA, 20–23 mM formate was formed from H_2/CO_2 within 8–19 h, respectively (Fig. 2b). Formate formation from H_2/CO_2 by *M. palustre* was also observed when methane formation was prevented by an unsuitable redox potential of −180 to −200 mV, which could be maintained in a mixed culture of *M. palustre* with a nitrate reducer (Fig. 2b).

Formate formation from H_2/CO_2 by pure cultures of methanogens was observed in almost all of the species tested that were known to grow on either H_2/CO_2 or formate (Table 1). Its concentration ranged from <0.1 (not detectable) to 13 mM, generated after 24 h of incubation. All formate-utilizing cultures excreted more formate (range 1.2–24 mM) if methane formation from H_2/CO_2 was prevented by BESA (Table 1), whereas methanogens that could not utilize formate for growth were not able to form formate from H_2/CO_2.

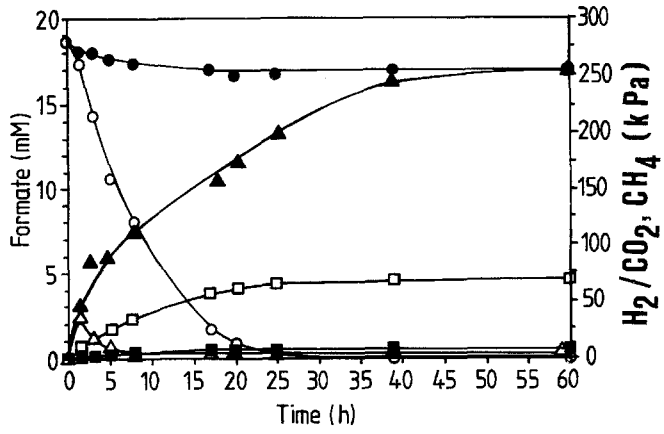

Fig. 1. Formate production from H_2/CO_2 by *Methanobacterium formicicum* in the absence or presence of 2.5 mM bromoethanesulphonic acid (BESA). *M. formicicum* was inoculated into 20 ml basic medium in serum bottles of 120-ml total volume, containing a H_2/CO_2 gas atmosphere (80:20%, v/v, 300 kPa) and grown to reach an optical density of $E_{578}=0.8$. Then the gas phase was renewed and methanogenesis in two of four parallel cultures was inhibited by addition of 2.5 mM BESA. H_2, methane and formate were analysed. The optical density of the inhibited cultures remained at 0.8, that of the non-inhibited cultures increased to 1.1 within the following 60 h of incubation: *open symbols,* no BESA added; *closed symbols,* 2.5 mM BESA added; O–O, ●–●, H_2; △–△, ▲–▲, formate; □–□, ■–■, methane

Dependence of formate formation from H_2/CO_2 by M. palustre on the incubation temperature

If parallel cultures of *M. palustre* were incubated at temperatures ranging from 22 to 45° C, formate formation proceeded most rapidly at 45° C, to reach a concentration of 4 mM after only 1 h. At lower temperatures less formate was produced within the first hour, with decreasing rates at decreasing temperature, respectively (Fig. 3). After 8 h, H_2 consumption for methane production was highest at the optimal growth temperature (30° C) and was considerably lower at 37, 45 and 22° C (Fig. 3).

H_2 production from formate by pure cultures of methanogens and by complex consortia from sewage sludge

For testing the ability of methanogens to generate H_2 from formate, the residual H_2 in cultures of *M. thermoformicicum* and *M. formicicum* that had reached

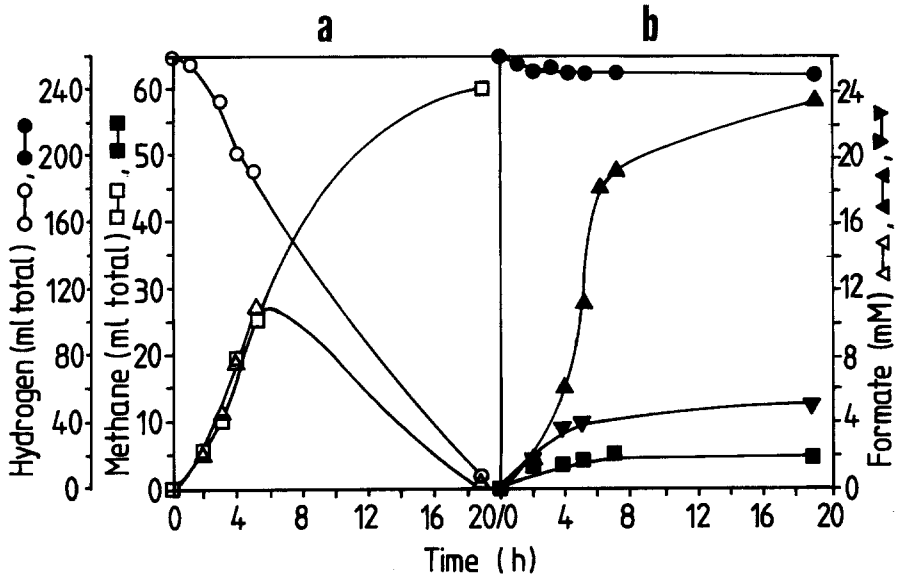

Fig. 2a,b. Formate production from H_2/CO_2 by cultures of *M. palustre* in the absence (**a**) or presence of 2.5 mM BESA (**b**) and in the presence of a nitrate reducer (**b**). The incubation conditions were as for Fig. 1. The optical density of BESA-inhibited cultures remained at $E_{578}=0.8$ and increased to $E_{578}=1.2$ in the non-inhibited cultures. Two cultures of *M. palustre* were supplemented with 3 ml of a log-phase culture of a nitrate reducer (strain LA B3, Bambauer 1992) grown on *n*-butyrate/nitrate. The redox potential in the mixed cultures remained constantly between −180 and −200 mV, and in the pure methanogenic cultures between −420 mV and −440 mV. The pure culture of strain LA B3 could not produce formate. H_2 utilization and methane production in the mixed culture was too little to be measurable, if it occurred at all: *open symbols,* no BESA added; *closed symbols* (except ▼-▼), 2.5 mM BESA added; ○-○, ●-●, H_2; □-□, ■-■, methane; △-△, ▲-▲ formate; ▼-▼, formate in mixed cultures of *M. palustre* and strain LA B3

Table 1. Formate production by methanogens from H_2/CO_2 with and without bromoethanesulphonic acid (BESA)

Organisms	DSM-no.	Growth temp (°C)	Formate utilizer	Formate concentration (mM)	
				No BESA	BESA (2.5 mM)
Methanobacterium spp.					
M. palustre	3108	37	Yes	13.0	24.0
M. formicicum	3636	37	Yes	0.3	13.0
M. thermoformicicum	3720	60	Yes	0.0	15.0
M. thermoautotrophicum	1053	60	No	0.0	0.0
M. wolfei	2970	60	No	0.0	0.0
M. ivanovii	2611	37	No	0.0	0.0
M. espaniolae		37	No	0.0	0.0
M. bryantii M.o.H.	863	37	No	0.0	n.d.
Methanogenium spp.					
M. tationis	2702	37	Yes	0.0	12.0
M. liminatans	4140	37	Yes	0.0	3.0
M. cariaci	1497	30	Yes	3.0	8.0
M. marisnigri	1498	30	Yes	0.0	2.0
Methanocorpusculum spp.					
M. parvum	3823	37	Yes	0.0	2.0
M. bavaricum	4179	37	Yes	0.0	3.5
M. sinense	4274	37	Yes	0.8	1.2
M. aggregans	3027	37	Yes	1.3	2.0
M. labreanum	4855	37	Yes	2.5	2.5
Methanosphaera spp.					
M. stadtmanae	3091	37	No	0.0	0.0

Cultures were grown in serum bottles in basic medium with an H_2/CO_2 gas phase (80:20%, v/v; 300 kPa). The medium for *Methanogenium marisnigri* and *M. cariaci* was supplemented with 0.5 ml of a 3 M NaCl/1 M $MgSO_4$ solution. Twenty millilitres of medium was inoculated with 2 ml of a fresh culture and incubated for 24 h. Methanogenesis in two of four cultures was inhibited by 2.5 mM BESA; n.d., not determined

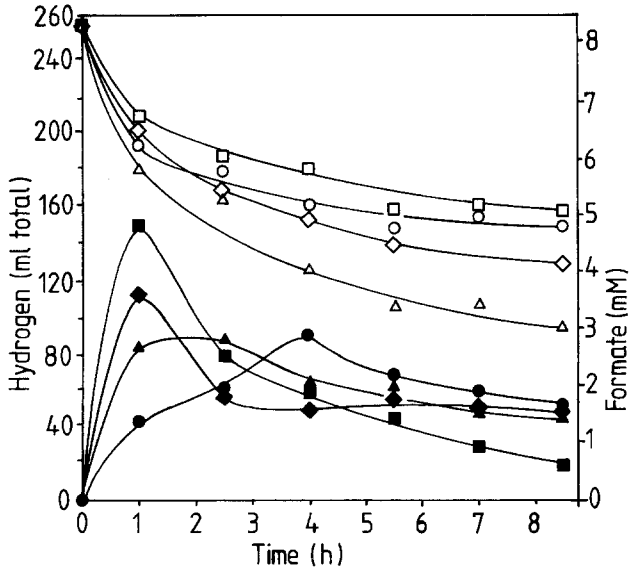

Fig. 3. Dependence of H_2 utilization and formate production by *M. palustre* on the incubation temperature. Parallel cultures of *M. palustre* (20 ml basic medium in 120-ml serum bottles; 300 kPa H_2/CO_2, 80:20% v/v) were grown to an optical density of $E_{578}=1.0$. The gas phase was renewed and hydrogen utilization as well as formate production were measured: *open symbols,* H_2; *closed symbols,* formate; ○–○, ●–●, 45° C; ◇–◇, ◆–◆, 37° C; △–△, ▲–▲, 30° C; □–□, ■–■, 22° C

$E_{578}=0.8$ was replaced by N_2/CO_2. The medium was supplemented with 100 mM formate (2 mmol absolutely) and with inhibitors of methanogenesis. If the immediate utilization of H_2 from formate cleavage for methanogenesis was prevented by the addition of 5 mM BESA, *M. thermoformicicum* formed 0.3 mol H_2 and some methane within 2 days of incubation. If all formate were converted to H_2+CO_2 without methane being produced, maximally 2 mmol H_2 could have been released into the gas phase. In cultures of *M. formicicum* in which methanogenesis was inhibited totally by 0.04% chloroform, 0.26 mmol H_2 was released from 0.25 mmol formate, which was cleaved within 6 days (Table 2).

In sewage sludge that had been incubated with H_2/CO_2 (80:20%, 300 kPa) for 2 days to restore methanogenesis after sludge transfer into the serum bottles and that was supplemented with formate and a N_2/CO_2 gas phase, formate was converted to H_2 and presumably CO_2 if methanogenesis was inhibited by either 5 mM BESA or 0.05% chloroform (Table 2).

Dependence of formate production in sewage sludge on temperature and substrate supply

Formation of methane and formate from H_2/CO_2 in sewage sludge was compared for different incubation conditions (Table 3). If digested sewage sludge was incubated under an N_2/CO_2 gas atmosphere, only little methane was produced and formate could not be detected (<0.1 mM), indicating substrate depletion.

If the sewage sludge was supplied with H_2/CO_2 and incubated at 37° C, little formate was formed, as long as H_2 was not depleted by methanogenesis. Much more formate was formed at 22 and 60° C (at a reduced methane productivity compared to 37° C) or when methanogenesis of the sludge cultures was almost completely inhibited by BESA. No significantly increased formate levels were detected in sludge cultures in which sulphate reduction was prevented by molybdate, indicating that sulphate reducers were apparently not involved in formate metabolism. If both methanogenesis and sulphate reduction were inhibited by BESA and molybdate, some formate was excreted.

Discussion

Interspecies H_2 transfer was required for a complete methanogenic degradation of sugars, fatty acids and aromatic compounds. With mixed cultures of *Bifidobacterium bifidum* and methanogens a hydrid transfer was excluded (Winter 1984). As an alternative, interspecies formate transfer was proposed by Thiele and Zeikus (1988) to contribute a large portion of electron transfer during methanogenesis in complex ecosys-

Table 2. Hydrogen production from formate by two *Methanobacterium* spp. and by sewage sludge consortia

Organisms/inhibitor/substrate	H_2 (mM) produced from formate after:		
	2 days	4 days	6 days
M. thermoformicicum/BESA/none	0.0	0.0	0.0
M. thermoformicicum/BESA/formate	0.3	n.d.	n.d.
M. formicicum/$CHCl_3$/none	0.0	0.0	0.0
M. formicicum/$CHCl_3$/formate	0.14	0.18	0.3
Sewage sludge/$CHCl_3$/none	0.0	0.0	0.0
Sewage sludge/$CHCl_3$/formate	0.5	0.6	0.7
Sewage sludge/BESA/formate	0.3	1.0	1.5

Serum bottles of 120-ml total volume, containing 20 ml cultures of *M. thermoformicicum*, *M. formicicum* ($E_{578}=0.8$) or sewage sludge plus either 0.05% $CHCl_3$ or 2.5 mM BESA with or without 2 mmol formate (totally), gas phase N_2/CO_2 (300 kPa, 80:20%) were incubated at 37° C for the indicated times

Table 3. Methane and formate production by a mixed population of sewage sludge

Incubation conditions	Methane production (ml/culture) after (h)			Formate production (mmol/ml) after (h)		
	24	48	96	24	48	96
22° C, H_2/CO_2	4.0	7.5	42.0	4.5	2.3	0.0
37° C, H_2/CO_2	16.4	27.0	54.0	0.5	0.4	0.0
60° C, H_2/CO_2	0.8	1.5	42.0	4.2	0.5	0.0
37° C, H_2/CO_2, MoO_4^{2-}	11.5	21.0	51.0	0.9	0.3	0.0
37° C, H_2/CO_2, BESA	4.0	5.6	9.0	8.1	10.3	13.5
37° C, H_2/CO_2, MoO_4^{2-}, BESA	2.7	4.0	5.5	2.5	4.1	0.5
37° C, N_2/CO_2	1.6	4.2	8.0	0.0	0.0	0.0

Sewage sludge (10 ml) was inoculated into serum bottles of 120-ml volume, containing 10 ml basic medium (without yeast extract and peptone) and incubated at the stated temperature. The gas phase was either H_2/CO_2 or N_2/CO_2 (80:20%, v/v) at 300 kPa pressure. BESA was supplemented at 2.5 mmol/l, sodium molybdate at 2.0 mmol/l and sodium formate at 50 mmol/l final concentration

tems. It was argued that the characterization of mainly H_2/CO_2- and formate-utilizing methanogens from these ecosystems would corroborate this assumption. However, only weak indirect evidence was presented to show that formate was not a product of CO_2 reduction by H_2 gas, either by fermentative or methanogenic bacteria.

As shown in this paper for many pure cultures of methanogens and for a complex sewage sludge culture, some formate was formed intermediately during non-inhibited growth on H_2/CO_2. High concentrations of formate were formed from H_2/CO_2 when conditions for methanogenesis were not optimal, e.g. through inhibition by BESA, chloroform and ethanol or an elevated redox potential in co-cultures with nitrate reducers. Since nitrate was a frequent component of, for example, dairy waste-water, the formate in anaerobic reactors of dairy companies may not only be produced by the fermentative or acetogenic population, but also by the methanogenic bacteria. Under conditions of varying H_2 partial pressure, formate and H_2/CO_2 might be interconvertible by a reversible enzyme-catalysed reaction.

Interspecies formate transfer was deduced from formate formation by a complex population in a whey anaerobic reactor, in which methane production was completely inhibited with chloroform (Thiele and Zeikus 1988). However, as shown in this paper, many methanogens were able to form formate from H_2/CO_2 under conditions of completely inhibited methanogenesis, either by BESA, chloroform or ethanol. Therefore, methanogens may not only benefit from formate as a methanogenic substrate, supplied by other bacteria in a complex ecosystem, but also contribute to its formation during growth on surplus H_2/CO_2. Vice versa, molecular H_2 was formed from formate as the only carbon source if its immediate utilization for methanogenesis was inhibited by BESA.

One might argue that the concentration of H_2 in the gas phase of methanogenic pure and mixed cultures, used for our experiments to demonstrate formate formation, was higher than the maximal H_2 concentration that occurred in ecosystems such as waste-water digestors. However, not the H_2 concentration in the gas phase, but that in the close vicinity of the micro-organisms was crucial for formate generation. In methanogenic cultures, formate was excreted at $PH_2 > 200$ kPa (*M. formicicum*) or $PH_2 > 220$ kPa (*M. palustre*). Since the H_2 solubility at 37° C and 100 kPa was approximately 19 ml/l of water (Weast 1979) or medium, formate production started in the cultures of *M. formicicum* when more than 38 ml H_2 (=200 kPa in gas phase) and in the cultures of *M. palustre* when more than 42 ml H_2 (=220 kPa in gas phase) were solubilized per litre of culture suspension.

In log-phase cultures of *M. palustre*, *M. formicicum* or *M. bryantii* strain M.o.H., the H_2 utilization rate was 14.9, 22.4 and 22.4 ml/l per minute, calculated from methane data of Zellner et al. (1989) with H_2 consumption estimated by Eq. 1:

$$4 \text{ mol } H_2 + 1 \text{ mol } CO_2 \rightarrow 1 \text{ mol } CH_4 + 2 \text{ mol } H_2O \quad (1)$$

At this H_2 utilization rate and at 200 kPa pressure, resaturation of the medium with H_2 would be necessary roughly once per 2 min. For resaturation at 58° C, a shaking frequency of >40 strokes/min was sufficient (Zabel et al. 1985) to meet this requirement.

For estimation of the average H_2 concentration in highly loaded waste-water fermentors with floc or biofilm formation, H_2 transport from the gas phase via the aqueous phase to the micro-organisms was not essential. The H_2 was produced by the bacteria of the fermentative phase, e.g. by conversion of carbohydrates according to the following simplified Eq. 2:

$$1 \text{ mol glucose} \rightarrow 2 \text{ mol acetate} + 2 \text{ mol } CO_2 + 4 \text{ mol } H_2 \quad (2)$$

Thus, in a whey fermentor with a chemical oxygen demand (COD) load (mainly lactose) of 15 g per day, that was completely degraded (Wildenauer and Winter 1985), 332 mmol/l per day of H_2 (=5.2 ml/l per minute) must have been formed according to Eq. 2. This amount of H_2 was released by the fermentative bacteria. Since fermentative, acetogenic and methanogenic

bacteria in the flocs or in a biofilm were closely associated, an H_2 gradient was marginal, if it occurred at all. If the active sludge volume in a whey reactor responsible for methane production was assumed to be restricted to approximately 20% of the total waste-water volume (de facto it may be even less), then the H_2 production rate of the fermentative bacteria in the flocs or biofilm was in the order of 26 ml/l per minute. The production rate of H_2 by fermentative bacteria in whey was thus almost exactly identical with the H_2 consumption rate of densely grown pure cultures of *M. palustre* or *M. formicicum.* Under these conditions in both log-phase cultures of methanogens growing on H_2/CO_2 and complex consortia in waste-water growing by fermentative COD removal, formate would be produced by the respective methanogens.

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