European J Appl Microbiol Biotechnol (1981) 13:242–247

European Journal of Applied Microbiology and Biotechnology

End Product Inhibition in Methane Fermentations: Effects of Carbon Dioxide on Fermentative and Acetogenic Bacteria

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Summary. The rates of glucose utilization by fermentative bacteria and propionate and butyrate utilization by acetogenic bacteria were studied and their dependence of pCO_2 in the interval 0–1 bar was determined. A batch fermentation method was used permitting good control of fermentation parameters and rapid experiments.

The rate of glucose fermentation to acids, CO_2 and H_2 was in the order of 12,000 mg glucose/l · day which was about two orders of magnitude faster than the utilization of propionic and butyric acid by acetogenic bacteria. The rate of glucose utilization was about 30% greater at low values of pCO_2 compared with 1 bar CO_2.

Propionate degradation was strongly affected by pCO_2; rates were 60 mg/l · day at pCO_2 = 1 bar and 200 mg/l · day at pCO_2 = 0.2 bar. Some CO_2 was required since the rate of propionate utilization dropped rapidly below pCO_2 = 0.2 bar. The rate of butyric acid utilization was constant at 170 mg/l · day; slightly lower at pCO_2 = 1 bar.

Yields of methane from glucose or acids were close to the theoretical value 50% of degraded substrate-carbon. Yields were 20–30% higher at low values of pCO_2 compared with 1 bar CO_2.

The redox potential was usually between −200 and −250 mV, slowly increasing to between −150 and −200 mV during fermentation. No clear connection between rates of substrate utilization, pCO_2 and E_h was detected.

Introduction

End product inhibition is well-known in many fermentation processes and inhibition by CO_2 and/or CH_4 may be expected in methane fermentations. Three main groups of bacteria producing CO_2 or CH_4 are involved in the final stages of methane fermentations according to the following equations (Bryant 1979):

I. Acetic-, propionic- and butyric acid production from carbohydrates such as glucose by fermentative bacteria:

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 4H_2 + 2CO_2 \quad (1)$$

$$C_6H_{12}O_6 + 2H_2 \rightarrow 2CH_3CH_2COOH + 2H_2O \quad (2)$$

$$C_6H_{12}O_6 \rightarrow CH_3CH_2CH_2COOH + 2CO_2 + 2H_2 \quad (3)$$

II. Oxidation of propionic acid and butyric acid by acetogenic bacteria:

$$CH_3CH_2COOH + 2H_2O \rightarrow CH_3COOH + CO_2 + 3H_2 \quad (4)$$

$$CH_3CH_2CH_2COOH + 2H_2O \rightarrow 2CH_3COOH + 2H_2 \quad (5)$$

III. Methane production from acetic acid and H_2/CO_2 by methanogens:

$$CH_3COOH \rightarrow CH_4 + CO_2 \quad (6)$$

$$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O \quad (7)$$

Studies on end product inhibition may be performed by comparing fermentation kinetics and yields in fermentations at different levels of pCO_2 or pCH_4. This may be achieved by carrying out fermentations at reduced or elevated pressures (Bloodgood and Anderson 1963; Mangel et al. 1980; Wise et al. 1978; Finney and Evans 1975). Another method is to control the gas phase by introducing CO_2, CH_4 or inert gas in the fermenters (Keefer and Kratz 1932; Hartz and Kountz 1966; Hansson 1979). The

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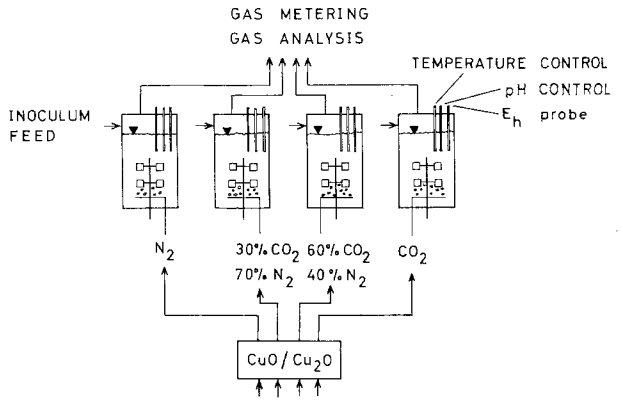

Fig. 1. Schematic diagram of fermentor panel for batch fermentations at different pCO_2

results in the literature regarding end product inhibition are conflicting. In some cases the noted inhibiting effects are due to insufficient control of the experiments rather than CO_2- or CH_4-inhibition. However, it seems clear that CO_2 has inhibiting effects on some methanogens, except when grown on $H_2 + CO_2$, although the degree of inhibition at different pCO_2 is not known. No reports regarding inhibition by CH_4 have been found.

Effects of pCO_2 on fermentative and acetogenic bacteria are reported in this paper while effects of pCO_2 and pCH_4 on methanogens are reported separately (Hansson and Molin, submitted for publication).

Material and Methods

Fermentor Cultivation Technique. To determine the rate of substrate utilization at different pCO_2, a batch cultivation system was used (Fig. 1). Three or four fermentors (New Brunswick Multigen 2 l) were run in parallel, being sparged with mixtures of N_2 and CO_2 to obtain certain values of pCO_2. One of the fermentors in each set of fermentations was sparged with pure N_2 as a standard to be able to compare rates of substrate utilization between different sets of fermentations. All gases passed through a CuO/Cu_2O-column to remove traces of oxygen and were then sparged into the fermentors at approx. 1 l/h. The fermentations were performed at 35 °C and the cultures mixed using impellers at 600–700 rpm to ensure good contact between gas and liquid phase. pH was measured continuously in the fermentor with glass-calomel electrodes and controlled at 7.0 by automatic addition of 4 M NaOH or 4 M HCl. Redox potentials were measured continuously with platinum-calomel electrodes. Fermentations were started by blowing oxygen-free gas through the empty fermentor to remove all air, then inoculating with 1,000 ml culture and, after equilibration, adding of 200 ml substrate.

Microbial Culture. As inoculum in the fermentations a mixed culture adapted to methane production from glucose was used. This culture was originally obtained from a sewage sludge digestor and had been cultivated continuously for 3 years on a glucose-based medium in a stainless steel digestor. The methane-forming component in this mixed culture is probably a *Methanosarcina* since an increased number of *Methanosarcina*-clumps are observed under the microscope following cultivation on acetate. The total cell density in the inoculum culture was 3–4 g/l.

Cultivation Media. Glucose medium (g/l): glucose, 16; yeast extract, 4.0; NH_4Cl, 4.0; $Na_2HPO_4 \cdot 2H_2O$, 1.6; KH_2PO_4, 0.15; $MgSO_4 \cdot 7H_2O$, 0.4. Trace elements (mg/l): $FeCl_3 \cdot 6H_2O$, 0.60; $CaCl_2 \cdot 2H_2O$, 0.66; $ZnSO_4 \cdot 7H_2O$, 0.18; $CoCl_2 \cdot 6H_2O$, 0.18; $CuSO_4 \cdot 5H_2O$, 0.14; $MnSO_4 \cdot 4H_2O$, 0.14. The substances were dissolved in distilled water and the medium was boiled and allowed to cool in a N_2-atmosphere.

Fatty acid media: The glucose was replaced by acetic-, propionic- or butyric acid or combinations of acids. Otherwise the composition and preparation were the same as for glucose medium. After substrate addition to the fermentors the total glucose/fatty acid concentration was around 3 g/l to avoid inhibition by high substrate concentrations.

Analytical Procedures. Fatty acids were analyzed by gas chromatography using a Varian model 940 chromatograph with flame ionization detector, autosampler and CDS 101 integrator. *Column:* 8 ft teflon column 1/16 inch by 1/8 inch with a 100 mm empty glass column at the injection end. *Support:* Chromosorb 101. *Carrier gas:* N_2 30 ml/min, saturated with distilled formic acid. *Temperatures:* Injector 190 °C, Column 175 °C, Detector 190 °C. *Preparation of samples:* Fermentation liquids are acidified, centrifuged and then injected (0.5 µl) into the glass column. Most contaminants in the fermentation liquid will adhere to the walls of the glass column which is regularly changed to a clean column.

Gas analyses (N_2, CH_4, CO_2) were carried out by gas chromatography according to van Huyssteen (1967). Glucose was measured by the hexokinase method (Schmidt 1961).

Results and Discussion

Effects of pCO_2 on Fermentative Bacteria

The fermentation of glucose to fatty acids was studied at six different levels of pCO_2 in the interval 0-1 bar. Data on the rate of glucose degradation and the distribution of glucose-carbon in products after completed fermentation of glucose (12–20 h) is compiled in Table 1.

It is quite clear that the production of acids from glucose is much more rapid than the degradation of acids. This is in agreement with several investigations (e.g. Ghosh and Pohland 1974; Lawrence and McCarty 1969). The rate of glucose decomposition is affected by pCO_2; the rate is about 30% greater in N_2-atmosphere compared with CO_2-atmosphere. However, since the rate of glucose fermentation is about 50–100 times faster than acid degradation this should not be of any practical significance.

The main fatty acid products were acetic and propionic acid with only small amounts of butyric acid. The proportions of acetic and propionic acid were not significantly affected by pCO_2 while the amount of butyric acid increased slightly with increasing pCO_2. According to theories on interspecies hydrogen transfer (Wolin 1975; Zeikus 1977; McInerney and Bryant 1978) an increased production of reduced acids by fermentative bacteria should be

Table 1. Rate of glucose degradation and distribution of fermentation products as a function of gas composition

	N_2	20% CO_2	40% CO_2	60% CO_2	70% CO_2	CO_2
Glucose degradation mg/l · h	552	504	503	508	504	415
% of glucose carbon in products						
HAc	15	14	26	18	25	20
HPr	33	33	19	31	33	24
HBut	0.5	1.8	0	6	3.2	8
CH_4	18	18	13	9	10	6
CO_2	34	–	–	–	–	–
Total	100					

Table 2. Rates of propionic and butyric acid degradation in 17 fermentations on glucose mixed acids

	Expt no	N_2	20% CO_2	30% CO_2	40% CO_2	60% CO_2	70% CO_2	CO_2
HPr (mg/l · d)	I	47		150		113		59
	II	77				157		94
	III	54	205				114	
	IV	55			190			63
HBut (mg/l · d)	I			166		170		138

expected when methanogenic bacteria are disturbed in a complex methanogenic system due to their impaired capacity to keep a sufficiently low pH_2. However, in this system pCO_2 affects the utilization of acetic acid by methanogens rather than their utilization of $H_2 + CO_2$ (Hansson 1979). Therefore, pCO_2 should not affect pH_2 or the product pattern of fermentative bacteria.

The amount of methane produced during the fermentative step was greatly affected by pCO_2 and was about 3 times greater in N_2 compared with CO_2- atmosphere. If, according to equations (1) and (2) above, propionic acid is found in equal or somewhat higher amount than acetic acid, not much hydrogen will be left for methane production from $H_2 + CO_2$ and therefore acetic acid will be the main precursor to methane. The differences in methane production during the fermentative step were therefore mainly due to the effects of pCO_2 on the rate of methane production from acetate.

During N_2-sparging the CO_2-production was 2–3 times higher than CH_4-production during the initial phase of the fermentative step as was expected from the equations (1)–(3). Later on these gases were produced in almost equal amounts. Nearly all glucose-carbon in the substrate was found in products after the fermentative step during N_2-sparging thus indicating little or no cell growth. However, some carbon from the yeast extract in the substrate may have been used. Carbon balances could be done only for N_2-sparged fermentations since CO_2-production could not be measured during CO_2-sparging.

Effects of pCO_2 on Acetogenic Bacteria

The activity of acetogenic bacteria utilizing propionic and butyric acid according Eqs. (4) and (5) was measured as the rate of utilization of these acids at different values of pCO_2. In one fermentation (Fig. 2) a mixture of acetic, propionic and butyric acid was used as substrate. Another method was to use a glucose-substrate where, after the initial fermentation of glucose to acids (Table 1), the acids were utilized by acetogenic bacteria and methanogens and rates of acid utilization were calculated. Altogether, four sets of fermentations were carried out at different pCO_2 and data on propionic and butyric acid utilization are compiled in Table 2. Mean values, corrected for differences in inoculum quality, are shown in Fig. 3.

The rate of propionic and butyric acid utilization was in the order of 100–200 mg/l · d, while the decomposition of glucose to acids was in the order of 12,000 mg/l · d. The utilization of acetic acid by methanogens was also faster than acetogenic activities.

Low rates of propionic and butyric acid utilization are expected since the thermodynamics of these reactions are unfavourable. $\Delta G_0'$ for buyrate-degradation (Eq. (5)) and

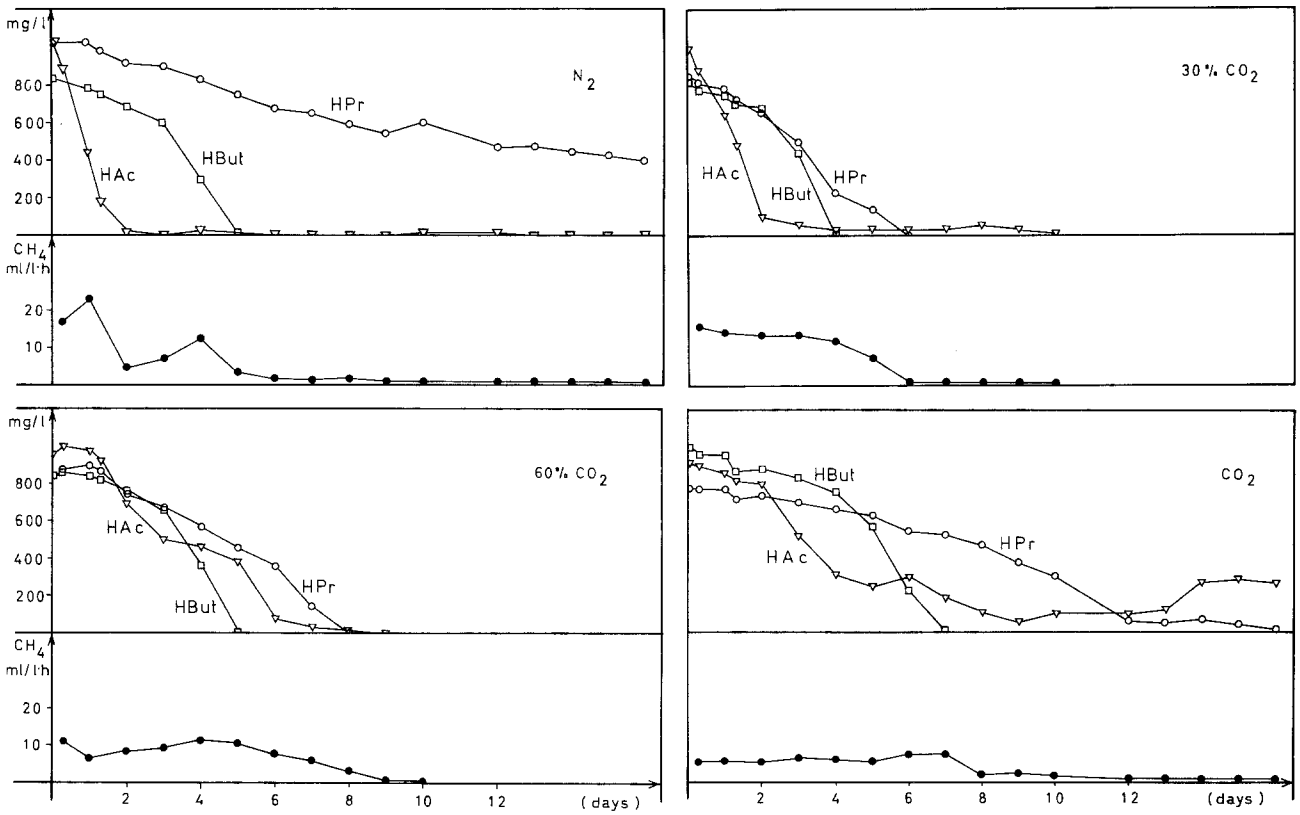

Fig. 2. Fermentation of a mixture of acetic, propionic and butyric acid in N_2, 30% CO_2, 60% CO_2 and CO_2

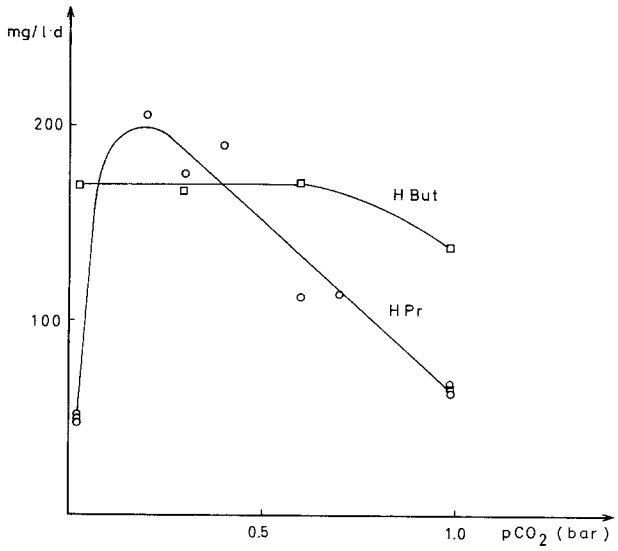

Fig. 3. Rates of acetogenic reactions as a function of pCO_2

propionate-degradation (Eq. (4)) are + 11.5 kcal/mole and +18.5 kcal/mole, respectively, which means that very efficient hydrogen-consumption by methanogens or Desulfovibrio is necessary for acid-degradation to proceed (Thauer et al. 1977). Consequently, the known propionate and butyrate-degrading organisms are very slow-growing with generation times of 105 h and 160 h, respectively (McInerney et al. 1979; Boone and Bryant 1980). Kaspar and Wuhrmann (1978) reported propionate degradation rates of 50 mg/l · d in digested sludge and 340 mg/l · d when the sludge was saturated with propionate.

Butyrate-degradation was not much affected by pCO_2, and only a slight inhibition occurred at 1 bar CO_2. This was expected since no CO_2 is formed according to Eq. (5). In the propionate-degradation however, CO_2 is formed and reaction rates are also significantly affected by pCO_2. Propionic acid utilization was 3–4 times faster in 20% CO_2 than in 100% CO_2. Evidently some CO_2 was required since the rate dropped rapidly below 20% CO_2 and was as low in N_2-atmosphere as in CO_2-atmosphere.

These results suggest that in methane production from glucose the degradation of propionic and butyric acid are the slowest steps. At normally occurring values on pCO_2 (0.4–0.5 bar) the rates of propionate and butyrate utilization are about the same but at high values of pCO_2 the rate of propionate utilization drops rapidly. The rate also drops below pCO_2 = 0.2 bar but this situation should not occur in practical applications.

In a well-working digestor the fermentation is pulled towards acetate-production due to the mechanism of inter-

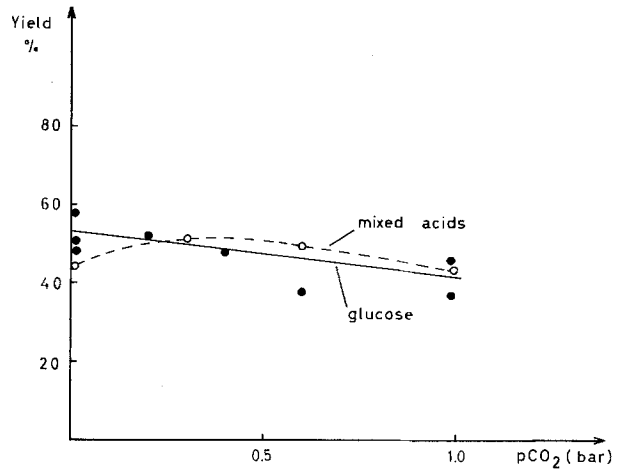

Fig. 4. Yields of methane on glucose and mixed acid substrates at different pCO_2

species hydrogen transfer. The problem of propionate and butyrate degradation would therefore be most important in a fermentation where a disturbance has led to increased concentrations of these acids or in situations where the normal decomposition of substrates proceeds with reduced acids as intermediates. However, in methane-production from complex materials the initial hydrolytic and fermentative steps may be rate-limiting rather than acid degradation (Hobson et al. 1974).

Yields of Methane

Total gas yields (CH_4 + CO_2) can be calculated only in N_2-sparged fermentors since otherwise external CO_2 is introduced into the fermentors. Therefore methane yields have been calculated for all fermentations on glucose and mixed acid substrates. Yields are expressed as percentage of substrate-carbon degraded to methane. Yield data from 13 fermentations are shown in Fig. 4. In most fermentations complete degradation of acids was accomplished within 5–12 days.

Theoretically, CO_2- and CH_4-yields from glucose are 50% each (Buswell and Mueller 1952). This is in agreement with our results at low values of pCO_2. Yields were even slightly higher than 50% but some methane formation may be due to decomposition of cell mass and yeast extract. Methane yields were decreased with increasing pCO_2 and at 1 bar CO_2 yields were around 40–45%, i.e. the difference in methane yields at high and low pCO_2 were 20–30%.

On mixed acid substrates the theoretical methane yield is also 50%. In this study the yields and their dependence on pCO_2 were the same or slightly higher compared with yields on glucose substrate. In N_2-atmosphere, however, the methane yield dropped as the rate of propionate utilization dropped as discussed above.

Redox Potential Measurements

The redox potential (E_h) was measured continuously in all batch fermentations. The objective was partly to detect any connections between redox potential and gas phase composition or substrate consumption rate, partly to monitor the general condition of the culture and detect sudden changes due to disturbances.

In general, after inoculation and substrate addition, the redox potential rapidly dropped to between –200 and –250 mV (actual readings were around 238 mV less due to the potential of the reference calomel electrode). This was the case in all gas atmospheres: N_2, CO_2 or N_2/CO_2-mixtures. The redox potential then usually increased slowly to between –150 and –200 mV but in some cases it remained rather constant, in other cases, especially at high pCO_2, it increased rapidly to –100mV towards the end of the fermentation. Since there was no clear connection between rates of substrate utilization, pCO_2 and E_h, the mechanism by which CO_2 affects methane-producing systems does not seem to involve the redox potential. The observed redox potentials in this work, –150 to –250 mV correspond to electrode potentials of –388 to –488 mV. According to the literature methane fermentations work well at electrode potentials below –360 mV (Smith and Hungate 1958).

Acknowledgements. This work was supported by the Swedish Board for Technical Development. Thanks are due to Ann Barthel, Katarina Åkesson and Karl Mohlin for skilful technical assistance.

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Received July 13, 1981