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Fermentative degradation of acetone by an enrichment culture in membrane-separated culture devices and in cell suspensions

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Abstract: A mixed culture, WoAct, growing on acetone, consisted of two dominant morphotypes: a rod-shaped acetone-fermenting bacterium producing acetate, and an acetate-utilizing *Methanosaeta* species. Dense cell suspensions, largely free of the aceticlastic methanogen and supplemented with bromoethanesulfonate, were able to degrade acetone and grow in small volumes in membrane-separated culture devices in which the acetate produced could diffuse into a large volume of medium. Acetone degradation and growth halted when the acetate concentration reached about 10 to 12 mM. Cell suspensions were able to degrade acetone in the absence of active methanogenesis, but the addition of 10 mM acetate inhibited acetone metabolism. Addition of an active culture of *Methanosaeta* sp. greatly stimulated the rate of acetone degradation. The results show that acetate removal in the mixed culture is not a prerequisite for growth and acetone degradation by the acetone-fermenting bacterium.

Key words: Acetone; Acetate; Interspecies acetate transfer; Anaerobic degradation; *Methansaeta* sp.

Introduction

Under anoxic conditions in the absence of electron acceptors, acetone can be degraded completely to methane and carbon dioxide [1–4]. In the enrichment culture WoAct [4], which had been transferred for more than 20 transfers with acetone, two organisms predominated: a rod-shaped fermenting bacterium forming two acetate from acetone and carbon dioxide, and a *Methansaeta* sp. converting acetate to methane and carbon dioxide [4]. Attempts to purify the acetone-fermenting bacterium failed, and it was hypothesized that growth of this bacterium depends on low acetate concentrations (around 100 μM) maintained by the aceticlastic methanogen. In this communication we show that acetone fermentation and growth do not necessarily depend on efficient acetate removal.

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Materials and Methods

All experiments were carried out with the enrichment culture WoAct [4] using carbonate-buffered (30 mM) mineral salts medium with acetone as substrate [4]. Enrichment culture WoAct was routinely cultivated in 120 ml serum bottles with 60 ml medium supplemented with 10 mM acetone (without BES). *Methanosaeta concilii* GP6 (DSM 3671, [5]) was cultivated in the same mineral medium containing trace element solution SL-9 [6], a vitamin solution ([7], plus 50 μg DL-6,8-thioctic acid, 50 μg riboflavin and 20 μg folic acid per litre, final concentrations in medium), and 20 to 50 mM sodium acetate. The agar shake dilution technique [7] and dilution series in liquid medium with *M. concilii* as partner organism (10% inoculum of an outgrown culture) were applied to attempt the preparation of a pure or a defined mixed culture. The membrane-separated culture devices used (1 and 10 l) are shown in Fig. 1. Optical density was measured in cuvettes of 1 cm light path at a wavelength of 578 nm. Cells were harvested by centrifugation under anoxic and sterile conditions as previously described [4]. Sodium bromoethanesulfonate (BES) was added from a filter-sterilized 1 M stock solution.

Acetone, acetate and methane were determined by gas chromatography [4]. Acetate was also measured by HPLC [8], and acetone colorimetrically in 1.5 ml plastic reaction vessels as follows. Samples of 100 μl were added to 100 μl 0.1% 2,4-dinitrophenylhydrazine in 2 M HCl, care being taken to avoid loss of acetone by introducing the sample below the reagent surface. After addition of 500 μl water and 100 μl 10 M NaOH, these were thoroughly mixed and then centrifuged (5 min) in a bench-top microcentrifuge at 5000 rpm. The absorbance was measured at 540 nm, and the concentration calculated from a standard curve after correcting for a medium blank.

Results

The enrichment culture WoAct (Fig. 2a) was diluted in liquid medium with *Methanosaeta concilii* GP6 added as partner organism. After two months a mixed culture of *M. concilii* and mainly one cell type, a non-motile short rod, was obtained (Fig. 2b). In contrast to the original enrichment culture, in which both bacteria formed closely associated microcolonies up to 300 μm in diameter (Fig. 2a), the culture with *M. concilii* as partner organism consisited of well suspended single cells. Nevertheless, it was not possible to obtain a defined mixed culture of only two bacteria; a highly-motile vibrio-shaped bacterium was always present in low numbers (approximately 1:20 compared to the acetone-fermenting bacterium). This bacterium could not be removed by liquid culture dilution techniques, but was isolated with 10 mM acetate as substrate in the presence of 20 mM fumarate as electron acceptor. This organism could not grow with acetone (10 mM) in pure culture, nor in co-culture with *M. concilii*, but grew with acetate plus flower of sulfur.

Cells of an outgrown 'WoAct plus *M. concilii*' culture were harvested anoxically. A sticky pellet was formed, and consisted of almost only one cell type, the short rod-shaped bacterium. *M. concilii*

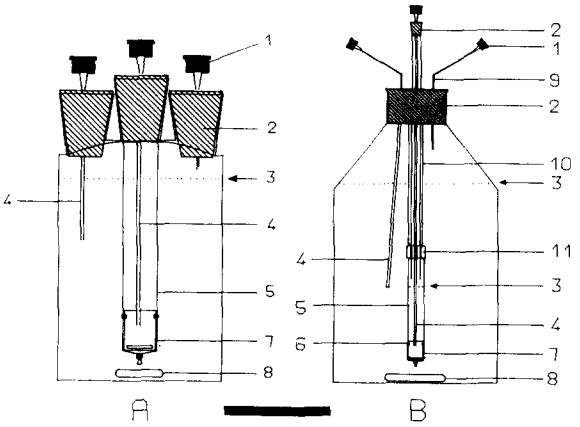

Fig. 1. Membrane-separated culture devices of (**A**) 1 litre and (**B**) 10 litre volume. The volume of the inner compartment was 20 ml in both cases. Bar equals 70 mm for (**A**) and 137 mm for (**B**). Key: 1, sampling port; 2, butyl rubber stopper; 3, medium surface; 4, teflon tubing; 5, dialysis tubing (Ø= 21 mm, exclusion limit 14 kDa, Serva, Heidelberg, FRG); 6, O-ring; 7, glass beaker (Ø= 18 mm); 8, teflon-coated magnetic stirring bar; 9, stainless steel canula; 10, glass tubing (Ø= 12 mm); 11, silicon rubber ring.

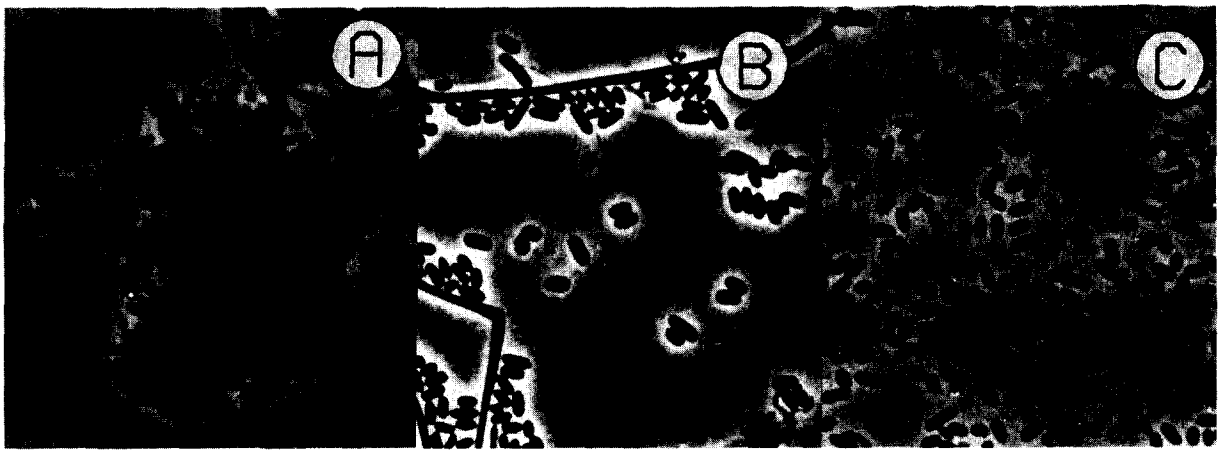

Fig. 2. Phase contrast photomicrographs of acetone-degrading cultures. Bar equals 10 μm for all panels. (**A**) Original enrichment culture WoAct. (**B**) Enrichment culture WoAct after cultivation in liquid dilution series with *M. concilii* GP6. (**C**) Culture grown for 4–5 generations in a membrane-separated culture device in the presence of BES.

did not sediment well, and formed a layer above the pellet which could be removed easily. The pellet from 160 ml liquid culture was resuspended in 2 ml medium and transferred into the inner comparment of a one litre membrane-separated culture device with 10 mM acetone and 20 mM BES. After more than 60 days of cultivation, turbidity increased, and acetate was formed. In this culture, no *Methanosaeta*-like cells and no methane formation were detected. Most cells were non-motile and similar to those of the inoculum; a few highly-motile vibrio-shaped cells were also present (Fig. 2c). From this culture, several one litre and 10 litre membrane-separated cultures were inoculated with 1 ml inoculum in the 20 ml inner compartment (see Fig. 1). The

Table 1

Growth of the acetone-fermenting enrichment culture in the absence of *Methanosaeta* sp. in membrane-separated culture devices. The medium contained 20 mM BES in all cases.

Acetone (mM)		Acetate (mM)		pH		Culture density (OD_{578})	
Initial	Final	Initial	Final	Initial	Final	Initial	Final
Cultures grown in one-litre membrane-separated culture devices							
< 0.05	< 0.05	< 0.05	0.2	7.30	7.07	0.20	0.17
4.6	0.3	< 0.05	10.1	7.23	6.79	0.34	3.40
9.8	3.4	< 0.05	10.2	7.38	6.60	0.23	3.26
10.1	4.1	< 0.05	11.8	7.22	6.72	0.54	2.66
Cultures grown in ten-litre membrane-separated culture devices							
< 0.05	< 0.05	< 0.05	0.2	7.21	7.10	0.30	1.56
2.2	0.5	< 0.05	3.9	7.24	6.94	0.33	8.24
5.0	3.5	< 0.05	4.1	7.22	6.98	0.35	6.30
9.8	7.5	< 0.05	3.4	7.24	6.83	0.34	11.60
Cultures grown in 120 ml serum bottles							
10.0	nd [a]	< 0.05	7.2	7.20	6.87	0.23	0.23
10.0	nd	< 0.05	4.3	7.20	6.92	0.27	0.23

[a] nd, not determined.

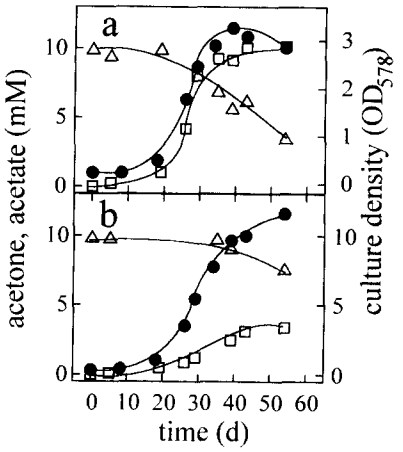

Fig. 3. Growth (●, OD_{578}), acetate formation (□), and acetone consumption (△) in membrane-separated culture devices of (**a**) 1 litre, and (**b**) 10 litre volume in the presence of 20 mM BES. An optical density of 1.0 corresponds to 5.5×10^9 cells per ml.

results are given in Table 1 and Fig. 3. Cultures supplemented with BES were able to grow without active methanogenesis, and acetate accumulated. The acetate concentration increased to over 10 mM in the one-litre membrane-separated culture devices before growth ceased. The increase in culture density was paralled by acetate accumulation. In the 10-litre membrane-separated devices, the acetone-fermenting bacterium was able to grow to very high culture densities ($OD_{578} >$ 11), and the acetate concentration reached about 4 mM before the growth rate decreased. No methane or hydrogen was detected in outgrown cultures. In serum bottle cultures inoculated from the same membrane-separated cultures, acetate formation was detectable, but no growth occurred. Acetate formation was always associated with a pH decrease. In the 10 l culture device without acetone, a slight increase in optical density was detected; however, the predominant bacterium in this culture was the vibrio-shaped bacterium rather than the acetone-fermenting short rod.

Cell suspensions were prepared from anoxically-harvested cultures of 'WoAct plus *M. concilii*' which had been cultivated on acetone for a further 20 transfers. The sticky cell pellet consisting largely of the short rod-shaped bacterium was resuspended in mineral medium containing 10 mM acetone and variously supplemented with 10 mM Na acetate, 10 mM NaCl, 20 mM BES, and heavy inocula (anoxically harvested cells of an outgrown 50 ml culture grown with 50 mM acetate) of pure cultures of *M. concilii*. Cell suspensions not inhibited by BES and supplemented with *M. concilii* rapidly degraded acetone (Fig. 4). The presence of 10 mM NaCl did not affect the degradation of acetone. Acetate did not accumulate in these cultures (< 100 μM), and methane was formed. In BES-inhibited cultures, only traces of methane were detected after nearly 1000 h. The kinetics of acetone degradation the presence or absence of 10 mM NaCl were identical (Fig. 4), being slower than in the cultures supplemented with *M. concilii*, and stopping when the acetate concentration reached 12 mM. Incubation of BES-inhibited cell suspensions with 10 mM Na acetate resulted in no acetone degradation (Fig. 4). In all cases, the accumulation of acetate was accompanied by a pH decrease. In these experiments, there was no significant increase in culture density, indicating no growth.

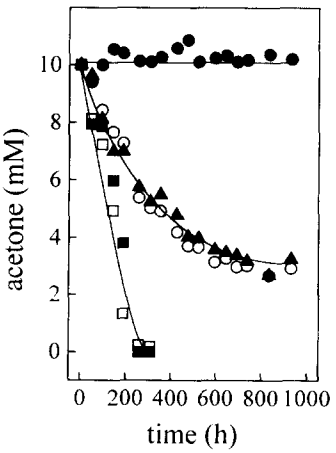

Fig. 4. Acetone degradation by dense suspensions of essentially methanogen-free preparations of the mixed culture WoAct. The suspensions were supplemented with (▲) 20 mM BES, (●) 20 mM BES plus 10 mM Na acetate, (○) 20 mM BES plus 10 mM NaCl, (□) *M. concilii* GP6, (■) *M concilii* GP6 plus 10 mM NaCl.

Discussion

In the mixed culture WoAct, acetone is fermented to two acetate by a strictly anaerobic bacterium via CO_2-dependent carboxylation to an acetoacetate (or acetoacetyl-CoA) residue. Our results indicate that the short rod-shaped bacterium is the acetone-fermenting bacterium because it is the predominant microorganism in all cultures. The original enrichment culture formed aggregates, but the use of *M. concilii* strain GP6 as the partner organism resulted in a culture of more uniformly suspended cells. This was attributed to the characteristics of the methanogenic partner bacterium; evidently the original culture [4] contained a *Methanosaeta* strain which readily formed aggregates. The highly motile vibrio-shaped bacterium is probably not directly involved in acetone degradation. Such organisms have been previously observed to occur in other slow-growing cultures (e.g. [9]). Since the vibrio-shaped bacterium was always present in all positive tubes of dilution series, we assume that this organism produces some growth factor essential for growth of the acetone-fermenting bacterium. Removal of this organism in higher dilutions therefore means the (more numerous) acetone-fermenting bacterium is not able to grow.

Fermentative acetone degradation is an exergonic process ($\Delta G'_0$ calculated after Thauer et al. [10]):

$$CH_3COCH_3 + CO_{2(aq)} \rightarrow 2CH_3COO^- + 2H^+$$

$$\Delta G'_0 = -34.2\ kJ \cdot mol^{-1}$$

The change in Gibb's free energy appears to be sufficient for growth of a microorganism in pure culture without a syntrophic partner organism; several bacteria have been isolated which carry out reactions with a $\Delta G'_0$ of only -17 to -27 $kJ \cdot mol^{-1}$ and do not depend on syntrophic removal of end products [11–14]. The observation that the acetone-fermenting bacterium in our mixed culture WoAct could not be cultivated in pure culture led to the hypothesis that growth of this bacterium depends on removal of acetate by an aceticlastic methanogen [4]. Bacteria of the genus *Methanosaeta* display a high affinity for acetate, and can keep acetate concentrations below 100 μM [15]. After acetone carboxylation to acetoacetate ($\Delta G'_0 = +17.1\ kJ \cdot mol^{-1}$ [4,10]), and activation at the expense of one acetyl-CoA, the thiolytic cleavage of acetoacetyl-CoA yields only one acetyl-CoA which can be used for ATP synthesis via substrate level phosphorylation. However, it is not known if the carboxylation of acetone is coupled mechanistically to the energetic equivalent of $\frac{1}{3}$ ATP (e.g. via an ion-driven carboxylase [16]), or to one ATP (analogous to pyruvate carboxylation via phospho*enol*pyruvate [17]). In the latter case, the net ATP yield would be zero, and acetate excretion coupled to ion-symport [18] could contribute to the energy metabolism of this bacterium.

Our results show that the acetate concentration can exceed 10 mM in dialysis cultures and cell suspensions before acetone degradation and growth cease. From the growth curves in membrane-separated culture devices, a doubling time of 3.8 to 4.0 days was calculated for the acetone-fermenting bacterium; this value agrees with the doubling times calculated from acetone consumption in the original enrichment culture WoAct [4]. The parallel increases of culture density and acetate concentration indicated that acetone metabolism was closely linked to growth. In the membrane-separated cultures, it is unclear if the inhibition of acetone catabolism was caused primarily by acetate accumulation, or secondarily by pH decrease. In the cell suspension experiments, however, the presence of 10 mM acetate resulted in total inhibition of acetone fermentation (under conditions where no growth was observed) at pH 7.2. Thus it appears that growth is not strictly dependent on acetate removal, but at high (10 mM) concentrations, acetone degradation, and thus growth, are inhibited.

The enhancement of growth of some fermentative bacteria in the presence of acetate-utilizing methanogens has been shown previously [19–21], but in these cases degradation was primarily via hydrogen syntrophy, and acetate utilization enhanced the degradation rates. In the mixed culture WoAct, acetate utilization appears not to be obligately required for acetone degradation and growth, and at acetate concentrations found in

natural freshwater environments (0.1 to 660 μM, [22–24]), acetone fermentation will proceed at maximal rates. Further investigations on the mechanisms of acetone carboxylation and acetate excretion in this bacterium are required to clarify the growth energetics of the acetone-fermenting bacterium.

Acknowledgement

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