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Hydrogen production during fermentation of acetoin and acetylene by *Pelobacter acetylenicus*

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1. SUMMARY

Pelobacter acetylenicus accumulated only small amounts of H_2 (< 3.5 kPa) during fermentation of acetoin or acetylene to acetate and ethanol. Formate was also produced in small amounts (< 0.5 mM). Growth on acetoin was retarded by addition of ethanol, but not by addition of H_2 or formate. However, addition of H_2 and/or formate resulted in increased production of 2,3-butanediol, whereas addition of H_2-scavenging *Methanospirillum hungatei* resulted in production of acetate plus H_2 (as CH_4) instead of acetate plus ethanol. Growth yields were consistent with acetate kinase as the sole ATP-generating reaction. The results are discussed with respect to thermodynamics and ATP synthesis during substrate conversion.

2. INTRODUCTION

Pelobacter acetylenicus ferments acetoin and acetylene to acetate and ethanol [1]. Fermentation of acetoin or acetylene is an exergonic reaction under standard conditions:

$$CH_3COCHOHCH_3 + H_2O \rightarrow CH_3CH_2OH + CH_3COO^- + H^+;$$
$$\Delta G^{\circ\prime} = -74.1 \text{ kJ mol}^{-1} \text{ acetoin} \quad (1)$$

$$2C_2H_2 + 3H_2O \rightarrow CH_3CH_2OH + CH_3COO^- + H^+;$$
$$\Delta G^{\circ\prime} = -148.9 \text{ kJ mol}^{-1} \text{ acetylene} \quad (2)$$

Pelobacter can also ferment ethanol to acetate and H_2. Since this reaction is endergonic under standard conditions, H_2 must not accumulate to partial pressures higher than 3–5 kPa [2]:

$$CH_3CH_2OH + H_2O \rightarrow CH_3COO^- + H^+ + 2H_2;$$
$$\Delta G^{\circ\prime} = +9.6 \text{ kJ mol}^{-1} \text{ ethanol} \quad (3)$$

H_2 is also formed in small amounts during fermentation of acetoin to acetate and ethanol [3]. In cocultures with H_2-utilizing *Acetobacterium woodii* or methanogenic bacteria, acetoin was fermented exclusively to acetate and H_2 [3,4]. The formation of acetate plus H_2 instead of ethanol allows generation of an additional ATP. Growth yields with acetoin or acetylene indicate that the acetate kinase reaction is the only ATP-forming step in acetoin

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or acetylene fermentation [1,4]. Formation of H_2 plus acetate thus should be more advantageous than formation of ethanol plus acetate.

In fact, there is no thermodynamic reason why H_2 should not be a major product of acetylene or acetoin fermentation. The fermentation of acetylene allows H_2 production in an exergonic reaction:

$$C_2H_2 + 2H_2O \rightarrow CH_3COO^- + H^+ + H_2;$$
$$\Delta G^{\circ\prime} = -144.1 \text{ kJ mol}^{-1} \text{ acetylene} \quad (4)$$

Similarly, acetoin could be converted to acetate, H_2, and 2,3-butanediol:

$$2CH_3COCHOHCH_3 + 2H_2O \rightarrow 2CH_3COO^- + 2H^+ + H_2 + CH_3CHOHCHOHCH_3;$$
$$\Delta G^{\circ\prime} = -53.3 \text{ kJ mol}^{-1} \text{ acetoin} \quad (5)$$

or acetate and H_2 only:

$$CH_3COCHOHCH_3 + 2H_2O \rightarrow 2CH_3COO^- + 2H^+ + 2H_2;$$
$$\Delta G^{\circ\prime} = -64.2 \text{ kJ mol}^{-1} \text{ acetoin} \quad (6)$$

In this study we show that *P. acetylenicus* nevertheless did not accumulate H_2 during fermentation of acetoin or acetylene to larger amounts than during fermentation of ethanol, and that addition of H_2 or formate shifted the fermentation pattern of acetoin to increased 2,3-butanediol production.

3. MATERIALS AND METHODS

Pelobacter acetylenicus strain WoAcyl (DSM 2348) and *Methanospirillum hungatei* strain Mlh were from our culture collection. They were grown at 28°C in a mineral medium [5] in serum bottles (120 ml) or Balch tubes (25 ml) with a N_2/CO_2 (8:2; v/v) or H_2/CO_2 (8:2; v/v) gas atmosphere.

Growth was followed by measuring the optical density (OD_{650}) at 650 nm and 1 cm light path in a spectrophotometer (Bausch and Lomb, Rochester, NY, U.S.A.). The dry cell mass was determined gravimetrically by drying washed cell suspensions at 80°C until constant weight was reached. At $OD_{650} = 1.0$, the suspensions contained 402 mg dry wt. l^{-1} of *P. acetylenicus* and 392 mg dry wt. l^{-1} of *M. hungatei*, respectively.

Samples (1–10 ml) were taken from the headspace of the cultures and analyzed for H_2 and CH_4 by gas chromatography [2]. H_2 partial pressures lower than 100 Pa were detected with a RGD2 detector (Techmation, Düsseldorf, F.R.G.) based on the HgO-to-Hg vapour conversion technique. Acetoin, butanediol, ethanol, and acetate were assayed by standard gas chromatographic procedures [6]. Formate was analyzed by HPLC (Sykam, Gauting, F.R.G.) using an Aminex HPX-87H column and 0.5 mM H_2SO_4 as eluent.

Cell-free extracts were prepared by French press treatment of cell suspensions at 140 MPa under a N_2 gas atmosphere. Formate dehydrogenase was measured analogous to hydrogenase [1] with formate as electron donor and benzyl viologen as electron acceptor ($\epsilon_{500} = 7.4$ cm^2 μmol^{-1}) in strictly anoxic cuvettes under N_2 gas.

The standard Gibbs free energy of a reaction ($\Delta G^{\circ\prime}$) was calculated from the standard Gibbs free energies of formation (ΔG_f°) of the reactants and products. The values were taken from ref. 7, except those for acetoin ($\Delta G_f^\circ = -279.7$ kJ mol^{-1}) and 2,3-butanediol (-321.8 kJ mol^{-1}) which were from ref. 4.

Table 1

Maximum concentrations of H_2 and formate produced by *P. acetylenicus* during growth with various substrates, and in coculture with *M. hungatei*

nm, not measured.

Substrate	H_2 (kPa)	dissolved H_2 (μM)	formate (μM)
No substrate	0.36	2.5	< 30
Ethanol (10 mM)	3.32	22.7	250
Acetylene (9 kPa)	2.37	16.2	375
Acetylene (40 kPa)	2.66	18.2	nm
Acetoin (4 mM)	2.79	19.1	230
Acetoin (10 mM)	3.42	23.4	380
Acetoin (10 mM) + H_2 (80 kPa)	80	547	930
Acetoin (10 mM) + *M. hungatei*	0.003	0.02	n.m.

4. RESULTS

Pelobacter acetylenicus grown on ethanol, acetylene, or acetoin produced H_2 and formate until a particular concentration was reached (Table 1, Fig. 1). The final concentrations of H_2 and

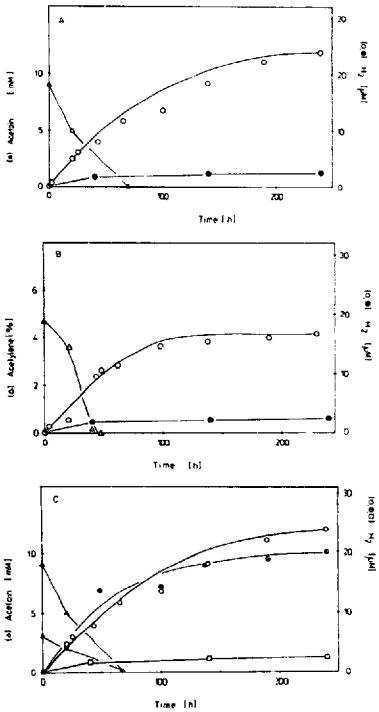

Fig. 1. H_2 production during fermentation of acetoin (A) or acetylene (B); consumption of acetoin or acetylene (Δ); production of H_2 in the presence (○) or absence (●) of substrate. (C) H_2 production (○, ●) during consumption of acetoin (▵, ▲) at low (closed symbols) and high (open symbols) concentrations, and without substrate (□).

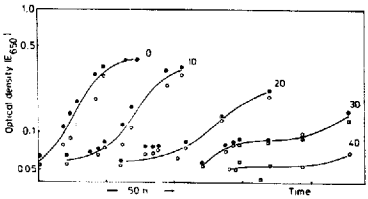

Fig. 2. Growth of *P. acetylenicus* with 10 mM acetoin in the presence of various (0 to 40 mM) ethanol concentrations. Replicate experiments are depicted by different symbols.

formate were similar with all three substrates. In cocultures with H_2-utilizing *Methanospirillum hungatei*, H_2 accumulated only intermediately to partial pressures of < 1 kPa, but finally reached values of about 3 Pa (Table 1) that were identical with the lower H_2 threshold concentrations of *M. hungatei* [2].

H_2 accumulation started simultaneously with consumption of acetoin or acetylene, but continued for some time after the substrate was depleted (Fig. 1) and converted completely to ethanol and acetate (not shown). The maximum concentrations of H_2 and formate that were finally reached were not proportional to the amounts of acetoin and acetylene fermented (Fig. 1, Table 1). However, the formate concentration increased if acetoin was fermented under an atmosphere of H_2/CO_2 instead of N_2/CO_2 (Table 1).

P. acetylenicus did not grow on ethanol due to inhibition by accumulating H_2 unless it was cocultured with a H_2-scavenging bacterium, e.g. *M. hungatei* [2]. Growth rates with acetoin, on the other hand, were not significantly affected by addition of either H_2 or formate (Table 2). However, addition of increasing amounts of ethanol resulted in increasing retardation of growth with acetoin (Fig. 2).

Acetoin was fermented to acetate and ethanol as main products and H_2 as a minor product (Table 2) in similar ratios and with similar growth yields as observed earlier [1,3]. 2,3-Butanediol was only a minor product. However, production of 2,3-butanediol increased relatively to that of ethanol and acetate, and the growth yield de-

Table 2

Fermentation balance, growth rates, and molar growth yields of *P. acetylenicus* on acetoin (100 μmol) in the presence or absence of H_2, formate, and/or *M. hungatei*

nm, not measured

Additions	μ_{max} (h^{-1})	Y ($g\ mol^{-1}$)	products formed (μmol)				recovery (%)	
			acetate	ethanol	H_2	butanediol	C	H
N_2/CO_2	0.107	9.0	104	72	12	0.6	97	94
N_2/CO_2 *M. hungatei*	0.080	10.1	170	nm	340	nm	117	110
H_2/CO_2	0.095	7.4	57	47	−28	28	87	86
N_2/CO_2 + formate	0.099	7.2	74	72	26	17	99	103
H_2/CO_2 + formate	0.045	5.1	62	58	−38	50	115	115

creased if acetoin was fermented in the presence of H_2 and/or at high formate concentrations (Table 2). On the other hand, the relative production of acetate and the growth yield increased if H_2 was scavenged in cocultures with *M. hungatei* (Table 2).

In crude extracts of acetylene- and of acetoin-grown cells, a benzyl viologen-dependent formate dehydrogenase activity of about 240 and 270 μmol min^{-1} g^{-1} protein, respectively, was detected. NAD^+ or $NADP^+$ was not reduced by this enzyme.

5. DISCUSSION

Although thermodynamics allow stoichiometric H_2 production during fermentation of acetoin or acetylene, H_2 was produced only in small amounts. The maximum H_2 partial pressures reached with acetoin or acetylene (< 3.5 kPa) were similar to those observed with substrates that allow only limited H_2 formation for thermodynamic reasons, e.g. ethanol or lactate [2,8], or alanine [9].

Fermentation of acetoin to acetate plus ethanol according to Eqn. 1 provides −74 kJ mol^{-1}. Irreversible ATP synthesis under physiological conditions in a fully charged living cell requires a Gibbs free energy change of about −70 kJ mol^{-1} [7]; Eqn. 1 releases nearly exactly this amount. H_2 formation from acetoin at stoichiometric amounts according to Eqns. 5 or 6 would have to be coupled to substrate-level synthesis of 2 mol ATP in both cases via acetate kinase reaction; it is obvious that the Gibbs free energy changes of Eqns. 5 and 6 cannot cover the corresponding energy demand (about −140 kJ per reaction). Therefore, ethanol has to be formed instead of acetate plus H_2, and H_2 can accumulate only to trace concentrations. These H_2 concentrations are identical to those of syntrophic ethanol oxidation [2] and thus, may explain why high background concentrations of ethanol impair acetoin fermentation as shown in Fig. 2.

It becomes obvious from these considerations that a fermentation reaction coupled to stoichiometric ATP formation does not only need to be thermodynamically permissive ($\Delta G^{\circ\prime} < 0$), but needs a $\Delta G^{\circ\prime}$ that is negative enough for concomitant ATP formation (at least if ATP is exclusively formed by substrate-level phosphorylation).

Fermentation of acetoin to acetate and H_2 according to Eqn. 4 seems to be sufficiently exergonic to allow concomitant ATP synthesis. However, most of the Gibbs free energy change of the fermentation reaction is due to the primary conversion of acetylene to acetaldehyde ($\Delta G^{\circ\prime}$ = −112 kJ mol^{-1} acetylene). There is no evidence that the energy of this reaction might be conserved by ATP synthesis or any other form useful for the cells [1]. Rather, this reaction leads to accumulation of toxic acetaldehyde whose further fermentation creates the same problem as discussed for acetoin fermentation. Whereas the Gibbs free en-

ergy change of oxidation of acetaldehyde to acetate plus H_2 is insufficient ($\Delta G^{\circ\prime} = -32.1$ kJ mol^{-1} acetaldehyde), dismutation to ethanol and acetate provides sufficient energy ($\Delta G^{\circ\prime} = -73.8$ kJ mol^{-1} acetaldehyde) for stoichiometric ATP synthesis via the acetate kinase reaction.

Formate was also produced in small amounts, probably due to a reversible formate dehydrogenase. Formate production concomitant with H_2 production has also been reported for fermentation of amino acids by *Acidaminobacter hydrogenoformans* [10] and *Eubacterium acidaminophilum* [9], as well as for methanogenic degradation of ethanol in anaerobic digestor flocs [11]. Both H_2 and formate may play a role in interspecies electron transfer to electron scavenging anaerobes. Since both reached only trace concentration levels, they may be in equilibrium with the same low-potential electron carrier, e.g. ferredoxin. Therefore, the energetic considerations above may apply to both H_2 and formate equally well.

High or low concentrations of H_2 or formate did not result in inhibition or stimulation of growth, but influenced the fermentation pattern and the growth yield on acetoin. Decrease of the H_2 partial pressure in cocultures with *M. hungatei* resulted in production of acetate plus H_2 (as CH_4) instead of ethanol, while an increase of H_2 or formate resulted in partial reduction of acetoin to butanediol. Production of butanediol has previously been observed with *P. carbinolicus* [3]. Growth yields with acetoin were highest in the presence of H_2-utilizing *M. hungatei* and lowest in the presence of high amounts of H_2 added.

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