Energetics of syntrophic ethanol oxidation in defined chemostat cocultures

2. Energy sharing in biomass production

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Abstract. The ethanol-oxidizing, proton-reducing *Pelobacter acetylenicus* was grown in chemostat cocultures with either *Acetobacterium woodii, Methanobacterium bryantii*, or *Desulfovibrio desulfuricans*. Y_{max} and m_e were determined from the total molar growth yields determined at growth (dilution) rates between 0.02 and 0.14 h^{-1}. The individual growth yields of the partner organisms were determined from their numbers and cellular mass in the chemostat cocultures. The Gibbs free energy ($\Delta G = -16.3$ kJ/mol ethanol) available to *P. acetylenicus* as well as its Y_{max} (1.7–2.2 g/mol ethanol) were almost constant in the different cocultures. *P. acetylenicus* shared 44–67% of the total biomass produced, whereas it shared only 19, 23, and 37% of the total Gibbs free energy (ΔG) available from ethanol oxidation coupled to sulfate reduction, methanogenesis, and homoacetogenesis, respectively. The residual 63–81% of the total available ΔG were shared by the H_2 oxidizers which exhibited Y_{max} values being highest for *A. woodii* (6.6 g/mol acetate) > *D. desulfuricans* (3.8 g/mol sulfide) > *M. bryantii* (2.2 g/mol CH_4). The results are discussed with respect to ATP generation and coupling of catabolism with cell production.

Key words: *Pelobacter acetylenicus* – *Acetobacterium woodii* – *Methanobacterium bryantii* – *Desulfovibrio desulfuricans* – Growth yield – Maintenance coefficient – Gibbs free energy – Y_{ATP}

Syntrophic oxidation of ethanol to acetate is accomplished in cocultures of ethanol-fermenting H_2-producing and H_2-consuming bacteria. The energy available from ethanol oxidation depends on the type of H_2-oxidizing species and on the terminal electron acceptor used; e.g. whether H_2 is oxidized by homoacetogenic, methanogenic, or sulfate-reducing bacteria ($\Delta G^{0\prime}$ per reaction):

$$2CH_3CH_2OH + 2HCO_3^- \rightarrow 3CH_3COO^- + 3H^+ + 2H_2O; (-85.6\ kJ)$$

$$2CH_3CH_2OH + HCO_3^- \rightarrow 2CH_3COO^- + 2H^+ + CH_4 + H_2O; (-116.8\ kJ)$$

$$2CH_3CH_2OH + SO_4^{2-} \rightarrow 2CH_3COO^- + HS^- + H^+ + 2H_2O; (-133.2\ kJ).$$

The energy of these reactions must be shared by three partial reactions, twice the oxidation of 1 mol of ethanol and once the oxidation of the resulting 4 mol of H_2 to form either acetate, CH_4, or HS^-. The reversible synthesis of 1 ATP requires under the conditions in a living cell about + 50 kJ mol^{-1} (Thauer et al. 1977). As part of the energy budget of a cell is always lost as heat, about + 70 kJ mol^{-1} is required for irreversible ATP biosynthesis (Schink 1990). Hence, syntrophic degradation of ethanol may yield fractions of one ATP per partial reaction. So far it is unknown how the energy is really shared among syntrophic partners. However, this share of energy must be sufficient to sustain cell integrity and drive biomass production for growth of each syntrophic partner.

In an accompanying paper (Seitz et al. 1990a), we determined the Gibbs free energy available to both ethanol oxidation and H_2 oxidation at steady state conditions in chemostat cocultures. Here, we determine the cell production rates and growth yields of each of the syntrophic partners during ethanol degradation in homoacetogenic, methanogenic, and sulfate-reducing chemostat cocultures.

Materials and methods

Organisms, cultivation, and analytical determinations

Pelobacter acetylenicus WoAcyl was grown in ethanol-limited chemostat cocultures with either *Methanobacterium bryantii* MoH, *Acetobacterium woodii* NZva16, or *Desulfovibrio desulfuricans* Essex

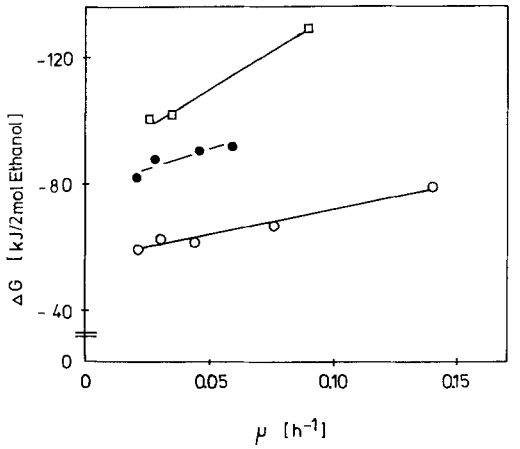

Fig. 1. Gibbs free energies (ΔG) of syntrophic ethanol oxidation determined in ethanol-limited chemostat cocultures of *Pelobacter acetylenicus* plus either *Acetobacterium woodii* (○), *Methanobacterium bryantii* (●), or *Desulfovibrio desulfuricans* (□) as partner organisms

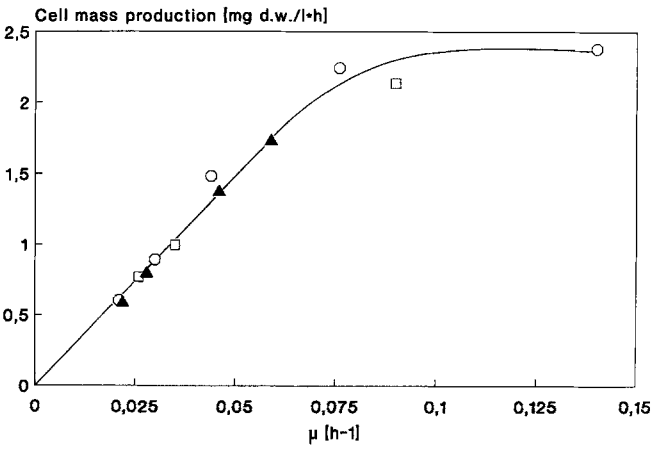

Fig. 2. Rates of total cell mass production in ethanol-limited chemostat cocultures of *P. acetylenicus* plus either *A. woodii* (○), *M. bryantii* (▲), or *D. desulfuricans* (□)

6 as H_2-oxidizing syntrophic partner. The origin of bacterial strains, the cultivation techniques, and other analytical determinations are described in the accompanying paper (Seitz et al. 1990a).

Growth yields

Growth was followed by measuring the optical density (OD_{650}) at 650 nm and 1 cm light path in a spectrophotometer (Bausch & Lomb, Rochester, NY, USA). Cell numbers were counted by phase contrast or fluorescence microscopy after fixation with 0.7% formaldehyde using a Helber counting chamber. Bacterial dry mass was determined gravimetrically in samples (800 ml) of pure bacterial cultures. The samples were gassed with CO_2 to remove H_2S, centrifuged and washed with 50 mM ammonium acetate buffer (pH 6.0). The washed suspension was transferred into glass tubes and dried at 80°C to constant weight. Cell suspensions of $OD_{650} = 1.0$ contained per liter: 402; 338; 392; 400 mg d.w. cells for *P. acetylenicus, A. woodii, M. bryantii, D. desulfuricans*, respectively. The corresponding cell number was ($\times 10^8$ cells/ml): 23.2; 8.4; 4.6; 22.0. The corresponding specific cell mass (x_i) was (pg d.w./cell): 0.17; 0.40; 0.85; 0.18.

The dry mass (X_i) of each of the cocultured bacteria was calculated from the total cell mass (X), the relative cell numbers (N_i) in the culture and the specific cell masses (x_i):

$$X_1 = X\, x_1\, N_1 / (x_1\, N_1 + x_2\, N_2)$$

$$X = X_1 + X_2$$

$$1 = N_1 + N_2.$$

Results

The Gibbs free energies of ethanol conversion under steady state conditions in chemostat cocultures increased linearly with the growth rate, and were higher (more negative) with sulfate reducers > methanogens > homoacetogens (Fig. 1). The Gibbs free energies available under steady state conditions in the chemostat were in a range of −60 to −128 kJ per 2 mol of ethanol as compared to −86 to −133 kJ per 2 mol of ethanol under standard conditions.

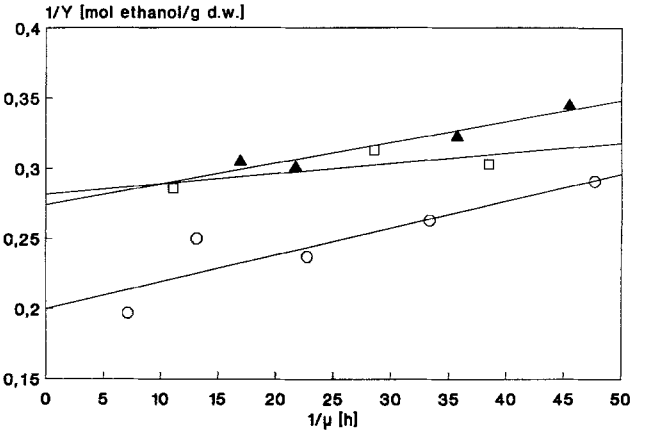

Fig. 3. Reciprocal plot of growth yields (1/Y) versus growth (dilution) rates (1/μ) in ethanol-limited chemostat cocultures of *P. acetlyenicus* plus either *A. woodii* (○), *M. bryantii* (▲), or *D. desulfuricans* (□)

Rates of total cell mass production increased linearly with growth rates and were almost identical in the cocultures with homoacetogens, methanogens, and sulfate reducers (Fig. 2). Slight differences in cell production and slight differences in ethanol consumption resulted in only slight but significant differences in total molar growth yields (Y). As theoretically implied by the Pirt equation (Pirt 1965), 1/Y increased linearly with 1/μ (Fig. 3). The maximum growth yields (Y_{max}) calculated from these plots resulted in higher values with the homoacetogenic *Acetobacterium woodii* than with the methanogenic *Methanobacterium bryantii* or the sulfate-reducing *Desulfovibrio desulfuricans* as partner organisms (Table 1). The maintenance coefficients (m_e) were higher with *A. woodii* > *M. bryantii* > *D. desulfuricans* (Table 1).

Growth yields of each syntrophic partner were determined individually via biomass. The cell shapes were sufficiently distinctive to separately count the cell titers

Table 1. Maximum growth yields (Y_{max}) and maintenance coefficients (m_e) of *Pelobacter acetylenicus* cocultured with different anaerobic H_2 oxidizers on ethanol

H_2-oxidizing partner	Y_{max} [g/mol ethanol]	m_e [mmol ethanol h^{-1} g^{-1}]
Acetobacterium woodii	5.0	1.93
Methanobacterium bryantii	3.3	1.22
Desulfovibrio desulfuricans	3.6	0.73

of *P. acetylenicus* and either *A. woodii, M. bryantii*, or *D. desulfuricans* in the cocultures (Fig. 4). The relative contribution of cells of the H_2 producers and H_2 oxidizers to the total cell number in the chemostat was independent of the growth rate, but changed with the type of coculture (Table 2). However, when these numbers were corrected for the cell mass of the individual species, the percentage contribution of *P. acetylenicus* to the total cell biomass was similar in the cocultures with the homoacetogens and the sulfate reducers, and was somewhat higher in cocultures with *M. bryantii* (Table 2).

The individual growth yields of the H_2 producers and H_2 consumers in the different cocultures were calculated from the relative amounts of their individual biomass and the total maximum growth yield (Table 3). The maximum growth yields of the H_2-producing *P. acetylenicus* were similar in all three different cocultures. However, the Y_{max} of the H_2 oxidizers were highest for *A. woodii* > *D. desulfuricans* > *M. bryantii* (Table 3). A similar trend was observed for $Y_{\Delta G}$, which was calculated from Y_{max} and the Gibbs free energy that was available to each of the syntrophic partners (Seitz et al. 1990a) assuming that the ΔG value extrapolated for a growth rate of $\mu = 0.2\ h^{-1}$ approximately represents the energy available for generation of Y_{max}.

Discussion

Syntrophic partners must grow together sharing the energy available from substrate degradation and sharing a common growth yield. Our results show for the first time that the energy content of a syntrophic degradation reaction is not equally shared between the contributing partners. Since ethanol conversion to acetate plus either "homoacetate", methane, or sulfide involves three stoichiometric partial reactions, a sharing fo 67% of the H_2-producing ethanol fermenter and 33% for the H_2 oxidizer would be expected.

However, ethanol-fermenting *Pelobacter acetylenicus* shared only 19–37% of the available Gibbs free energy of ethanol oxidation, but formed 47–67% of the total biomass produced. The H_2-oxidizing syntrophic partners, on the other hand, had a larger share of the available energy, but obtained a relatively smaller growth yield.

Sharing of substrate conversion energy and growth yields depended strongly on the type of H_2-oxidizing reaction. The homoacetogenic *Acetobacterium woodii*

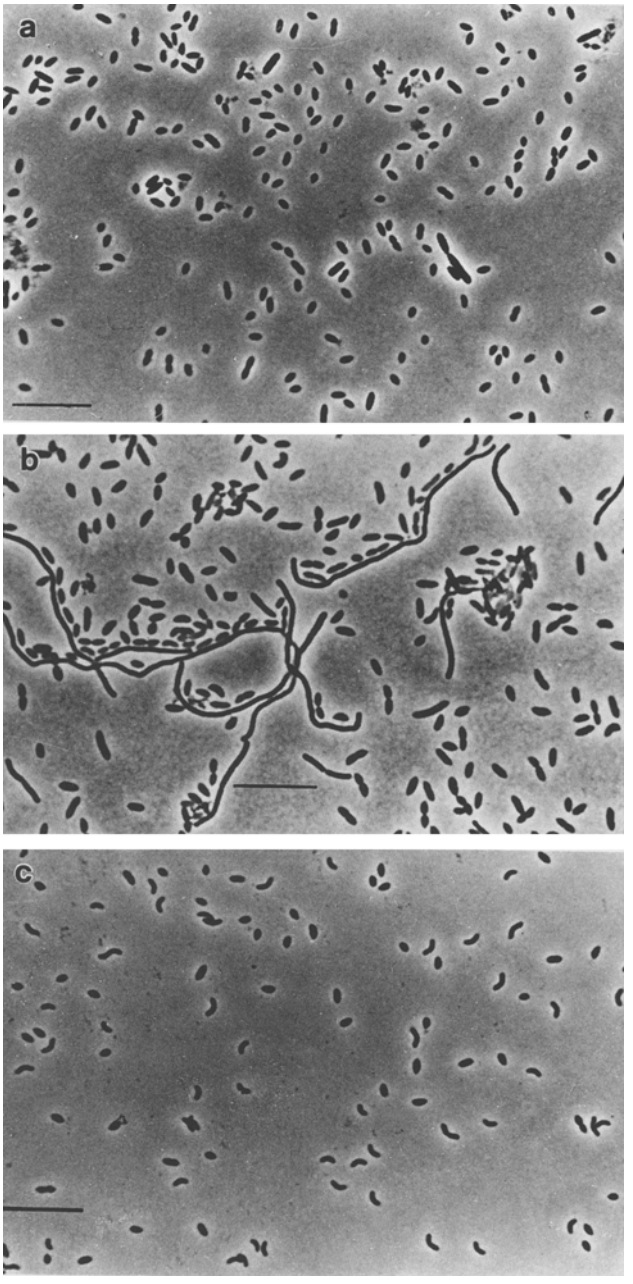

Fig. 4a–c. Phase-contrast photomicrographs of the bacteria in ethanol-limited chemostat cocultures of *P. acetylenicus* plus either *A. woodii* (**a**), *M. bryantii* (**b**), or *D. desulfuricans* (**c**). *Bar* = 10 μm

had the largest Y_{max} sharing 63% of the available energy and 52% of the total growth yield. The methanogenic *Methanobacterium bryantii*, on the other hand, had the smallest Y_{max} sharing 77% of the available energy and 33% of the total growth yield. The maintenance coefficients for syntrophic ethanol conversion were higher in homoacetogenic > methanogenic > sulfate-reducing cocultures.

The individual Y_{max} of *A. woodii, M. bryantii*, and *Desulfovibrio desulfuricans* in syntrophic coculture with ethanol-fermenting *P. acetylenicus* are due to growth on H_2 obtained by interspecies H_2 transfer. The Y_{max} determined for *A. woodii* (6.6 g/4 mol H_2) and *M bryantii*

Table 2. Partitioning of cell titer and cell mass between H_2-producing *P. acetylenicus* and different H_2-oxidizing anaerobes in chemostat cocultures with ethanol[a]

Coculture	*P. acetylenicus* + *A. woodii*	*P. acetylenicus* + *M. bryantii*	*P. acetylenicus* + *D. desulfuricans*
Titer [10^7 cells ml^{-1}]	11 ± 2	12 ± 1	16 ± 2
H_2 producer [%]	65 ± 3	91 ± 1	48 ± 6
H_2 oxidizer [%]	35 ± 3	9 ± 1	52 ± 6
Total cell mass [mg l^{-1}]	27.7 ± 6.3	28.6 ± 1.4	27.3 ± 3.2
H_2 producer [%]	44	67	47
H_2 oxidizer [%]	56	33	53

[a] Average values ± SD from determinations at various dilution rates between 0.02 and 0.14 h^{-1}

Table 3. Sharing of available Gibbs free energies (ΔG) and maximum molar growth yields (Y_{max}) between H_2-producing *P. acetylenicus* and different H_2-oxidizing anaerobes, and calculation of the individual $Y_{\Delta G}$

Coculture	*P. acetylenicus* + *A. woodii*	*P. acetylenicus* + *M. bryantii*	*P. acetylenicus* + *D. desulfuricans*
$-\Delta G$ [kJ/2 mol ethanol][a]			
H_2 producer	32.6 (37%)	32.6 (23%)	32.6 (19%)
H_2 oxidizer	54.8 (63%)	111.2 (77%)	136.0 (81%)
Y_{max} [g/2 mol ethanol][b]			
H_2 producer	4.4 (44%)	4.4 (67%)	3.4 (47%)
H_2 oxidizer	5.6 (56%)	2.2 (33%)	3.8 (53%)
$Y_{\Delta G}$ [g/70 kJ][c]			
H_2 producer	9.4	9.4	7.3
H_2 oxidizer	7.1	1.4	2.0

[a] Calculated from the chemostat data derived from Fig. 4 (Seitz et al. 1990a) extrapolated to $\mu = 0.2\ h^{-1}$
[b] Calculated from Y_{max} given in Table 1 and the percentage cell mass of H_2 producers and oxidizers given in Table 2
[c] $Y_{\Delta G} = 70\ Y_{max}/-\Delta G$; normalized to an energy quantum of 70 kJ

(2.2 g/4 mol H_2) compare well with growth yields reported in the literature for chemolithotrophic growth on H_2. E.g., the homoacetogenic *Butyribacterium methylotrophicum* had a Y = 6.4 g/4 mol H_2 (Lynd and Zeikus 1983) and varius hydrogenotrophic methanogens had Y values of 1.4 to 3.3 g/4 mol H_2 (Roberton and Wolfe 1970; Schönheit et al. 1980; Fardeau and Belaich 1986; Morii et al. 1987). The Y_{max} of methanogens depends on the supply with H_2: If H_2 is supplied in ample amounts, methanogens apparently uncouple cell biosynthesis from H_2 oxidation and Y decreases to values < 0.6 g/4 mol H_2 (Schönheit et al. 1980; Fardeau and Belaich 1986). The relatively high growth yield in our coculture experiments shows that growth of *M. bryantii* was indeed limited by the supply of H_2 from its syntrophic partner, and that these are the conditions to which such a bacterium is optimally adapted.

The Y_{max} determined for *D. desulfuricans* (3.8 g/4 mol H_2) in our coculture experiments was significantly lower than the values of 9.7 to 12.7 g/4 mol H_2 reported for chemolithotrophically grown monocultures of *D. vulgaris* (Badziong and Thauer 1978; Nethe-Jaenchen and Thauer 1984), *D. desulfuricans* (Seitz and Cypionka 1986) or *Desulfotomaculum orientis* (Cypionka and Pfennig 1986). We have no conclusive explanation for this discrepancy, but suggest that cell biosynthesis may have been partially uncoupled from H_2 oxidation, in favour of the syntrophic H_2-producer.

Interestingly, Y_{max} of the H_2-producing *P. acetylenicus* (1.7–2.2 g/mol ethanol) was relatively constant in the various syntrophic cocultures, making up about half (44–67%) of the total growth yield (3.3–5.0 g/mol ethanol). The total growth yields were comparable to those reported for *P. carbinolicus* (4.1–4.3 g/mol ethanol) growing on ethanol syntrophically with either *A. woodii* or *Methanospirillum hungatei* as partners (Schink 1984). The constant Y_{max} is probably the result of energetic homeostasis in the H_2-producing *P. acetylenicus* exhibiting a constant ΔG (−16.3 kJ/mol ethanol) at various growth rates and with different syntrophic partners (Seitz et al. 1990a).

The energy shared by *Pelobacter* was just sufficient for 0.32 mol reversible or 0.23 mol irreversible ATP synthesis per mol of ethanol oxidized (Thauer and Morris 1984; Schink 1990). ATP apparently is synthesized in these bacteria exclusively via acetate kinase (Schink 1984, 1985; Seitz et al. 1990b). A final explanation for synthesis of less than one ATP per fermentation reaction by substrate level phosphorylation is presently not available. It has been suggested that part of the ATP may be invested in acetate excretion (Schink 1991).

The Gibbs free energies of ethanol oxidation shared by the H_2-oxidizing syntrophs strongly depended on the growth rate (Seitz et al. 1990a). At zero growth, the minimum energy ("critical" Gibbs free energy) required for homoacetogenesis, methanogenesis, and sulfate reduction was about −20.4, −30.0, and −41.2 kJ per mol product, respectively (Seitz et al. 1990a). This energy allows irreversible synthesis of 0.3 to 0.6 mol ATP. The H_2-oxidizing syntrophs are able to generate a proton motive force and probably use the resulting membrane potential to synthesize ATP in fractions of 1/3 ATP per stoichiometric reaction (Thauer and Morris 1984). At high growth rates at which Y_{max} is generated, the H_2-oxidizing syntrophic partners apparently required more energy (−55 to −136 kJ per mol product; Table 3). This

energy allows irreversible synthesis of 0.8 to 1.9 mol ATP.

Comparison of the individual Y_{max} with the individual Gibbs free energies available at high maximum rates allow the formal calculation of $Y_{\Delta G}$. $Y_{\Delta G}$ is comparable with Y_{ATP} if ATP synthesis requires exactly 70 kJ/mol. The results indicate that both the ethanol-fermenting H_2-producing *P. acetylenicus* and the H_2-oxidizing homoacetogen *A. woodii* had a $Y_{\Delta G}$ which agrees with the Y_{ATP} observed in other bacteria (Stouthamer 1979). The H_2-oxidizing methanogenic *M. bryantii* and sulfate-reducing *D. desulfuricans*, on the other hand, exhibited a much lower $Y_{\Delta G}$. These differences may be due to various effects (for theoretical interpretation, compare Westerhoff et al. 1982; Van Dam et al. 1988):

(1) Efficiency of energy conversion may change with the bacterial species and the syntrophic status. E.g., *M. bryantii* and *D. desulfuricans* may produce less ATP than suggested from the available ΔG. Hence, they may uncouple ATP synthesis from substrate conversion, whereas *A. woodii* does not.

(2) The amount of energy required for ATP synthesis may be less than 70 kJ mol^{-1}, e.g. if the ratio of ATP/ADP is smaller than 10 (Schink 1990). In this case, $Y_{\Delta G}$ calculated from Y_{max} and ΔG values would be lower than expected in comparison with usual Y_{ATP} values, e.g. in syntrophically grown *M. bryantii* and *D. desulfuricans*.

(3) ATP spillage reactions, e.g. ion leakage and energy-dependent transport, may be higher in syntrophically grown *M. bryantii* and *D. desulfuricans* than in *P. acetylenicus* and *A. woodii*.

(4) The energy demand for cell production in syntrophic coculture may be higher in *M. bryantii* and *D. desulfuricans* compared to *P. acetylenicus* and *A. woodii*.

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