# Growth kinetics of *Acetobacterium* sp. on methanol-formate in continuous culture

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**Running headline:** GROWTH KINETICS OF *ACETOBACTERIUM* SP.

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A.E. BAINOTTI AND N. NISHIO. 1999. The fermentative metabolism of *Acetobacterium* sp. grown on methanol-formate in continuous culture is described. The reaction stoichiometry of methanol-formate, including cells, resulted as follows: $CH_3OH$ + 1·13 HCOOH ⟶ 0·87 $CH_3COOH$ + 0·47 cell C. Formate enhanced growth yields by *ca.* 60% compared to methanol-$CO_2$-grown cultures. Comparison of yields on methanol-formate allowed calculation of an energy yield of 1·3 mol ATP per mol acetate formed during homoacetate fermentation. The magnitudes of $Y_{EG}$, the theoretical maximum yield of $Y_E$, and $m$, the maintenance coefficient have been determined by growing the organism in methanol-formate and resulted in 16·5 g cell (mol methanol catabolized)$^{-1}$ and 0·674 mmol methanol catabolized (g cell)$^{-1}$ $h^{-1}$, respectively. Finally we conclude that formate might replace $CO_2$ as a source of carboxyl donor.

## INTRODUCTION

Anaerobic acetate-producing bacteria are generally referred to as acetogens (Ljungdahl and Wood 1982). Besides using saccharides, most of the known acetogens also ferment single-carbon compounds to acetate, and under certain conditions, some produce butyrate and caproate (Kerby *et al.* 1983). Bacterial growth on single-carbon substrates has been termed unicarbonotrophy or methylotrophy (Zeikus 1983). Methylotrophic growth is possible only if a more oxidized co-substrate, *i.e.* $CO_2$ is presented.

Most of the information on acetogen single-carbon transformations derive from analysis of the glucose catabolism of *Clostridium thermoaceticum* (Ljungdahl and Wood 1982). Later *Eubacterium limosum* has been shown to utilize $H_2$-$CO_2$, methanol-$CO_2$, and CO for growth (Lindley *et al.* 1987). *Acetobacterium woodii* possesses a single-carbon substrate range similar to

that of *B. methylotrophicum* and *E. limosum* but does not produce butyric acid (Balch *et al.* 1977). All three organisms contain high levels of corrinoids (Zeikus 1983). The reason of this fact is that these micro-organisms perform in their energy metabolism the transformation of unicarbon groups.

Evidence indicates that *Acetobacterium* can oxidize substrates other than hydrogen, but the substrate range is rather narrow, being limited to fructose, glucose, lactate, methanol, and glycerate (Zeikus 1983). The only major product detected is acetate as a sole fermentation product and for that reason this organism is thus referred to as homoacetogen. An acetogen recently isolated from sea sediments and classified as *Acetobacterium* sp. (Inoue *et al.* 1992) has been studied in its nutritional requirements using methanol-$CO_2$ (Bainotti *et al.* 1998), but other unicarbon mixtures might be proposed (Lebloas *et al.* 1994). In view of this fact, is reasonable think that formate may be another possible carbon substrate for *Acetobacterium* sp. since it is an important intermediate in the Acetyl CoA pathway (Loubière *et al.* 1987; Diekert and Wohlfarth 1994). The catabolic and anabolic pathways are closely linked in methanol-utilising anaerobes and the presence of a catabolic intermediate such as formate might be expected to create energetic effects other than that of acting as an extra source of energy, as reported for certain aerobic (Babel *et al.* 1983) and anaerobic micro-organisms (Lebloas *et al.* 1994). In this study the growth of *Acetobacterium* sp. in continuous culture has been studied in order to ascertain the possibility of replacement of $CO_2$ by formate as co-substrate to methanol.

## MATERIALS AND METHODS

### Microorganism and culture medium

*Acetobacterium* sp. strain 69, isolated by Inoue *et al.* (1992) as a high producer of intracellular vitamin $B_{12}$ was obtained from Tsukuba Research Center, Daicel Chemical Industries, Ltd. (Tsukuba, Ibaragi, Japan).

All manipulations of medium and cultures were carried out under the $O_2$-free atmosphere of $N_2$ gas (100% v/v). Medium preparation, culture techniques and culture conditions were followed according to the same protocol previously reported (Bainotti *et al.* 1996). The medium was slightly modified (referred as MF medium) from the previous one for methanol-$CO_2$ studies and contained the following materials (per liter of deionised water): methanol, 4 g; sodium formate, 8·5 g unless otherwise specified in the text; $NH_4Cl$, 1·0 g; $MgSO_4 \cdot 7H_2O$, 0·1 g; $CoCl_2 \cdot 6H_2O$, 0·02 g; 5,6-dimethylbenzimidazole, 0·02 g; yeast extract, 2·0 g; L-cysteine-HCl·$H_2O$, 0·5 g; trace element solution, 80 ml (Bainotti *et al.* 1996); vitamin solution, 40 ml (Bainotti *et al.* 1998); and titanium (III) citrate, 0·015 mmol $l^{-1}$. Instead of $NaHCO_3$, 15·0 g, a phosphate buffer ($NaH_2PO_4 \cdot 2H_2O$, 5·25 g; $Na_2HPO_4 \cdot 12H_2O$, 23·75 g) to maintain pH at 7·2 was used.

The medium without methanol, cysteine, titanium citrate (reductants), and the trace element solution was boiled for 20 min, cooled in ice water through which $N_2$ gas (100% v/v) was bubbled, and then sterilized (20 min, 121°C). Methanol and concentrated aqueous solutions of cysteine, titanium citrate, and trace elements anoxically prepared and filter-sterilized were then injected into the medium using a hypodermic syringe (final pH 7·2). Possible pH changes were corrected with oxygen-free HCl or NaOH (1 mol $l^{-1}$ solution) before inoculation.

## Precultures

Batch cultures for precultures were carried out in 125-ml vials containing 50 ml of medium, inoculated with 10% (v/v) of a late exponential phase culture grown in the same medium. Cultures were incubated at 30°C without shaking.

## Continuous culture

Continuous culture was performed using a chemostat culture in a 1000-ml flask (working volume, 500 ml) equipped with a pH controller (pH = 7·2 ± 0·1), a thermoregulator (30°C), a magnetic stirrer (50-100 rev $min^{-1}$), and a gas inlet. Before the start of each experiment the reactor system was made anaerobic by flushing for 2-3 h with $N_2$ gas (100% v/v). When the batch culture reached the late exponential phase, the continuous culture was started using a constant flow of $N_2$ gas and a steady-state was established at a dilution rate of 0·04 $h^{-1}$, unless otherwise indicated. Following each change in dilution rate the cultures were allowed to adapt for 3-4 residence times. After this period the cultures were assumed to be in a steady-state, and a number of parameters, *e.g.,* the cell concentration, the methanol and formate consumptions, and the acetate production were determined at regular time intervals. The pH was controlled at 7·2 with 1 mol $l^{-1}$ NaOH or HCl solution.

## Analytical methods

Cell concentration was measured by optical density at 660 nm ($O.D._{660}$) and then evaluated on dry weight (1 $O.D._{660}$ unit = 0·35 g DCW $l^{-1}$). Methanol was analysed by gas chromatography (Shimadzu GC-8A; column, Porapak QS, 2·6 mm x 1·5 m; detector, FID). Formate was analysed enzymatically (Boehringer GmbH, Mannheim, Germany) and measured spectrophotometrically at 340 nm. Acetate was also analysed by gas chromatography (Shimadzu GC-14A; column, PEG 6000, 3·2 mm x 3·1 m;

detector, FID). Vitamin $B_{12}$ formed intracellularly was extracted as the cyano-form and measured according to the previously reported method (Bainotti *et al.* 1996). Gas flow rates (0·01 vol $vol^{-1}$ $min^{-1}$) were controlled in all cases by a flow meter.

## Fermentation balances

Substrate-product balances were assessed at the steady-state of each fermentation for the recovery of carbon and reducing equivalents. To assess the cell carbon composition it was assumed that *Acetobacterium* sp. and *Butyribacterium methylotrophicum* have the same cell composition, that is, elemental analyses of glucose-, methanol- and CO-grown cells of *B. methylotrophicum* gave a carbon content of 45·1 ±0·4% and a cell formula of $C_{4·86}H_{8·85}O_{2·41}N_1$, plus 7·4 % ash (Lynd *et al.* 1982), and the overall degree of reduction was taken to be 4·22 per carbon atom (Erickson 1980). Cell yield based on ATP was calculated from the production of ATP in the known biochemical pathway proposed by Dieckert and Wohfarth (1994) and the stoichiometric balance equations.

## Chemicals

Yeast extract was produced by Difco Laboratories. HPLC solvent and other chemical reagents were purchased as the special grade from Katayama Chemical, Osaka, Japan. L-Cysteine and vitamins were obtained from Sigma Chemical Co., Ltd. $N_2$ (100% v/v) of more than 99·9999% purity (Chugoku Teisan Co., Hiroshima, Japan) was used without any treatment. All other chemicals of reagent grade were purchased from commercial sources.

## RESULTS

### Effect of dilution rate on a fixed methanol-formate concentration

Previous studies carried out in batch mode let us assumed an optimal concentration of 125 mmol $l^{-1}$ equimolar for the acetogenic fermentation. To more precisely envisage the methylotrophic behaviour of *Acetobacterium* sp. from monocarbon mixtures, the observations made during batch cultures were re-examined using a continuous culture.

The continuous culture under the steady-state for the growth of *Acetobacterium* on methanol-formate 125 mmol $l^{-1}$ are presented in Figure 1. The changes in cell concentration have been similar to those found in previous studies for the methanol-$CO_2$ mixture fermentation (Bainotti *et al.* 1998). However, when using formate 125 mmol $l^{-1}$ the acetate production rate ($q_P$) is lower (2·2 mmol $l^{-1}$ $h^{-1}$, $S_0$ = 125 mmol $l^{-1}$) than that in the former system (3·0 mmol $l^{-1}$ $h^{-1}$ and 4·2 mmol $l^{-1}$ $h^{-1}$ for $S_0$ = 100 and 150 mmol $l^{-1}$) at a dilution rate of *ca.* 0·05 $h^{-1}$. Both remaining substrates increased with the dilution rate, being always methanol in higher concentration than formate. Both molar yield coefficients of acetic acid from each substrate ($Y_{PM}$, $Y_{PF}$) were slightly increasing with the dilution rate, with an average value of 0·9 mol acetate produced (mol substrate consumed)$^{-1}$.

Profiles of cell concentration and substrate consumption in Fig. 1 deviated from those to be expected under carbon limitation and were not in accordance to Monod-based kinetics which predicts a constant value over a wide range of dilution rates, decreasing sharply as they approached washout. Additional experiments have been carried out to investigate the possibility of an extra limiting nutrient, but higher concentrations of trace element solution, vitamin solution, and yeast extract always resulted in no alteration of the steady-state characteristics (data not shown).

**Effect of inflow substrate concentration of methanol-formate mixtures**

*Acetobacterium* sp. was grown in continuous culture under different equimolar concentrations of the unicarbon methanol-formate mixture, fixing the dilution rate at a value of 0·046 $h^{-1}$.

Each set of experiments was started under batch conditions using the optimum substrate concentration of 125 mmol $l^{-1}$ equimolar in methanol and formate (MF medium). When the concentration of the carbon source became negligible the continuous culture was initiated by input of fresh medium with different increasing concentrations of methanol-formate mixture such as 25, 50, 75, 100, and 125 mmol $l^{-1}$ in both co-substrates.

The effect of different amounts of methanol-formate in the medium on the growth of *Acetobacterium* sp. and the product formed is shown in Figure 2. Good substrate consumption was obtained at both methanol-formate concentrations up to 50 mmol $l^{-1}$, demonstrating that the organism grew under both methanol and formate limitations during the lowest substrate concentration (25 mmol $l^{-1}$), and under formate limiting conditions in the neighbourhood of 50 mmol $l^{-1}$. Higher concentrations of substrates always resulted in remaining both carbon sources in the medium, thereafter increasing as a linear function of the initial substrate concentration over the range interpreted. Volumetric productivities ($q_M$, $q_F$ and $q_P$) enhanced with increasing the initial substrate concentration. On the other hand, yield coefficients of acetate and biomass from substrate ($Y_{PS}$, $Y_{XS}$) slightly decreased when the initial concentration of substrate was enhanced.

Figure 3 shows another parameters in the continuous culture where the substrate concentrations of the medium were changed. As it can be seen, increments in initial concentration of formate, resulted in a higher substrate consumption. On the other hand, the specific rates of substrate consumption [$q_{MX}$ and $q_{FX}$, defined as mmol substrate consumed (g dry cell)$^{-1}$ $h^{-1}$],

increased with initial concentration of single-carbon sources (especially in the case of formate), until a maximum obtained for an initial concentration of substrate of 75 mmol $l^{-1}$ equimolar. Thus, both formate and methanol served as co-substrates and were simultaneously consumed.

**Energetics and stoichiometry of growth**

A set of steady-state data for a 125 mmol $l^{-1}$ equimolar methanol-formate fermentation (Fig. 1) is summarized in Table 1 to elucidate the stoichiometry, yields, and energetics of the unicarbonotrophic metabolism. On the basis of the assumption that $Y_E$ ($Y_E = X / \Delta S_E$) is the actual yield of bacteria (grams of cell per mol of substrate catabolized) and $Y_{EG}$ is the theoretical maximum yield of $Y_E$ (if there were no maintenance), Neijssel and Tempest (1976) proposed eq. (1):

$$q_E = m + \frac{1}{Y_{EG}} \mu \qquad (1)$$

where $q_E$ = specific consumption rate of energy source [mmol substrate catabolized (g cell)$^{-1}$ $h^{-1}$], $m$ = maintenance coefficient [mmol substrate catabolized (g cell)$^{-1}$ $h^{-1}$], and $Y_{EG}$ = true growth yield for catabolized substrate [g cell (mol substrate)$^{-1}$]. From the data in Table 1, the plot of $q_E$ versus $D$ (= $\mu$) using eq. (1) is shown in Fig. 4. A linear relationship was obtained between the specific growth rate and the specific rate of substrate catabolized, demonstrating the validity of the eq. (1). Applying the least square regression results $m$ = 0·674 mmol substrate (g cell)$^{-1}$ $h^{-1}$ and $Y_{EG}$ = 16·5 g cell (mol substrate)$^{-1}$ with a linear regression coefficient $r$ = 0·997. The constant value of the maintenance coefficient implies that no growth-rate-related maintenance (Pirt 1982) took place, and this value is almost the

same as reported earlier by other authors (Pirt 1975; Loubière and Lindley 1991) for another homoacetogens.

From data shown in Table 2, a representative average fermentation balance for growth of *Acetobacterium* sp. on methanol-formate equimolar, including cells, may be as follows: $CH_3OH$ + 1·13 HCOOH ⟶ 0·87 $CH_3COOH$ + 0·47 cell C. The stoichiometry was in close agreement with the theoretical stoichiometry, since in all cases acetate was the sole product observed from the methanol-formate fermentation, and the stoichiometry of acetate formed per unit of carbon source consumed approximated the theoretical homoacetogenic ratio of **1:1:1**, especially at low concentrations of methanol as stated in Table 2.

These reactions also displayed a balanced carbon and available electron recovery, all the values obtained being within 104 ± 14% and 109 ± 15% of carbon- and electron-recovery, respectively (Table 2). Besides, *Acetobacterium* sp. fermentation displayed typically substrate / cell synthesis conversion ratios (both in carbon and electrons) for an anaerobe, as high as 30% of the carbon was assimilated into cells during growth on methanol-formate (Table 2).

Also the product yield coefficient from substrate ($Y_{PM}$, expressed in mol acetic acid produced per mol methanol consumed) and the cell mass yield from substrate ($Y_{XM}$, expressed in g dry cell per mol methanol consumed) in the range of initial substrate concentration studied were estimated based on the data of the table, being as follows: $Y_{PM}$ = 0·87 ± 0·12 and $Y_{XM}$ = 12·5 ± 1·8. The value of $Y_{XP}$ was nearly constant for all the initial concentrations assayed [$Y_{XP}$ = 14·32 ± 0·63 (g cell) (moles acetate produced)$^{-1}$], a similar finding reached in a previous paper (Bainotti, *et al.* 1998) for methanol-$CO_2$ fermentation [$Y_{XP}$ = 9·85 ± 0·55 (g cell) (moles acetate produced)$^{-1}$]. Then, the production of the organic acid, was seen to be related on the substrate consumed (or the growth rate).

Vitamin $B_{12}$ yields presented in Table 3 show that even though methanol-$CO_2$-grown cells yielded a better growth for *Acetobacterium* sp., both the specific concentration of vitamin $B_{12}$ [$C_{vit/X}$ expressed in (mg vitamin $B_{12}$) (g cell)$^{-1}$] and the specific vitamin production rate [$q_{vit}$ expressed in (mg vitamin $B_{12}$) (g cell)$^{-1}$ d$^{-1}$] were highly dependent on the unicarbon mixture used for growth. High levels of both values of vitamin [5·60 (mg vitamin $B_{12}$) (g cell)$^{-1}$ and 4·17 (mg vitamin $B_{12}$) (g cell)$^{-1}$ d$^{-1}$, respectively, at $D$ = 0·031 h$^{-1}$] were only seen in methanol-formate-grown cells.

## DISCUSSION

In heterotrophic growth the microorganism converted one mol glucose completely to near three moles of acetate as end product. This value corresponds to formation of at least 4·5 mol ATP per hexose: 2 mol ATP are derived from substrate level phosphorylation, 2 mol ATP result from acetate-kinase reaction (Andreesen *et al.* 1973) and at least one half mol of ATP may result from acetate synthesis via Wood Ljundahl pathway (see Figure 5). In our calculations, the Gibbs free energy change during the reduction of methylene tetrahydrofolate to methyl tetrahydrofolate was assumed to produce one third mol of ATP by a chemiosmotic mechanism (Diekert & Wohlfarth 1994), the energy change produced when the methyl tetrahydrofolate was transferred to a corrinoid/Fe-S-protein was assumed to produce one half mol of ATP by a chemiosmotic mechanism (see Fig. 5, methyl branch), and the activation of $CO_2$ to bound to CO could require one third mol of ATP (see Fig. 5, carbonyl branch). Thus, assuming a net synthesis of 4·5 ATP per mol of glucose the calculated $Y_{ATP/P}$ from our data would be 1·5 mol ATP (mol acetate)$^{-1}$, in agreement with the bibliography (Andreesen *et al.* 1973) (see Table 4).

Homoacetogenic bacteria are able to utilize a variety of one-carbon substrates that have the redox level of methanol. Besides methanol, methoxylated aromatic compounds and methyl chloride also can serve as homoacetogenic substrates (Fig. 6). Until now it is not yet known how these compounds are channeled into the methyl-group synthesis. One carbon-units at the level of methanol (*i.e.,* a methyl) could be converted either to methyl tetrahydrofolate or to the enzyme-bound methyl corrinoid, or they could be oxidized to formaldehyde or formate prior to being fed into the pool of tetrahydrofolate-bound $C_1$ intermediates. For the energetics of acetate synthesis from these compounds, it is important to know to which intermediate in methyl-group formation the substrates are converted. The conversion of methyl substrates can be expressed by the following reaction:

$$4\ CH_3\text{-}R + 2\ CO_2 + 2\ H_2O \longrightarrow 3\ CH_3COO^- + 7\ H^+ + 4\ R^- \quad (2)$$

In the assimilation of halocompounds or methanol-$CO_2$ mixtures, one methyl group has to be oxidized to $CO_2$ (Fig. 6, oxidative branch) to provide the 6 [H] required for the reduction of 3 $CO_2$ to 3 carbon monoxide (in a bound form) (Fig. 6, carbonyl branch). The carbonyl groups are then combined with three methyl groups (Fig. 6, reductive branch) to yield acetate. As the methyltransferase reaction is considered as a major site in energy conservation, an incorporation of the methyl group into the methyl tetrahydrofolate or the methyl corrinoid pool would imply different energetics of the methyl group conversion. If the methyl group is bound directly to tetrahydrofolate, the methyl-transferase would not be involved in the oxidation of the methyl group to $CO_2$ (Fig. 6, oxidative branch). Instead, the methyltransferase would mediate the methyl transfer in the exergonic direction, thus being involved in energy conservation (Fig. 6, reductive branch). If the target of the methyl incorporation is the enzyme-bound corrinoid, one methyl group has to be oxidized (Fig. 6, oxidative branch) via

the methyl-transferase reaction in the oxidative, *i.e.,* the endergonic direction, and three methyl groups have to be directly incorporated into C-2 of acetyl-CoA without involvement of the methyltransferase. This implies that the methyl group conversion to methyl tetrahydrofolate would be energetically more favorable than the conversion to an enzyme-bound methyl corrinoid. The values estimated for $Y_{ATP/P}$ in methylhalides and methanol-$CO_2$ mixtures agreed with those obtained experimentally (see Table 4).

The possible utilisation of formate as co-substrate with methanol was reported (Kerby *et al.* 1983; Loubière *et al.* 1987), but not thorough analysis of this compound's effect on growth and organic acid production by *Acetobacterium* was presented. If it is assumed that formate is oxidized to $CO_2$ in the carbonyl branch, implying a generation of energy of about two thirds mols of ATP by a chemiosmotic mechanism, and that the methyl group is bound directly to the methyl-transferase (Fig. 6, reductive branch), the $Y_{ATP/P}$ furnished according to this path would become similar to that obtained experimentally (see Table 4).

During growth on one-carbon compounds, the cell yield coefficient was correlated with the degree of reduction of the carbon substrate, *e.g.,* the cell yield on methanol-formate where formate was oxidized (2 available electrons per mol of C) was higher than on methanol-$CO_2$ (0 available electrons per mol of C) (see Table 4), that is $Y_{XS}$ = 10·8 g mol$^{-1}$ ($D$ = 0·046 h$^{-1}$, $S_0$ = 125 mmol l$^{-1}$) from Table 2 and $Y_{XS}$ = 4·0 g mol$^{-1}$ ($D$ = 0·05 h$^{-1}$, $S_0$ = 150 mmol l$^{-1}$) (Bainotti *et al.* 1998), respectively. The latter value is comparable to that obtained for *Methanosarcina barkeri* ($Y_{XS}$ = 3·9 g mol$^{-1}$, $\mu$ = 0·039 h$^{-1}$) on methanol (Krzycki *et al.* 1982).

The high cell yield during growth on methanol-formate may be due to direct assimilation of substrate into cell carbon, as has been demonstrated in *M. barkeri* (Kenealy and Zeikus 1982). Therefore, the cell yield coefficient (g mol$^{-1}$) and specific growth rate of *Acetobacterium* sp. ($Y_{XS}$ = 10·8, $\mu$ =

0·046 $h^{-1}$, on methanol-formate) are considerably higher than those reported for *M. barkeri* ($Y_{XS}$ = 3·9, $\mu$ = 0·039 $h^{-1}$, on methanol) (Krzycki *et al.* 1982).

In the absence of formate, one fourth of the consumed methanol must have been assimilated into acetyl-CoA as the carboxyl group *i.e.* direct fixation of $CO_2$ could only account for half of the carboxyl donors required. According to the present status of Acetyl-CoA pathway (Diekert & Wohlfarth 1994), the methyl-transferase (which has the vitamin $B_{12}$ as cofactor) is induced during the growth on methanol and plays an important role in the condensation of methyl donors with the carboxyl donors to produce the acetyl-CoA (Fig. 6). The concentration of vitamin $B_{12}$ is a function of the carbon flux between the methanol and the methyl group of the acetyl-CoA. Since a variable amount of methanol is oxidized to furnish the carboxyl, only a part of this substrate would be assimilated through the pathway in which vitamin $B_{12}$ is intermediate. If it were possible to enhance the latest fraction, also the concentration of corrinoid must be increased. This is what exactly happens when replacing the co-substrate $CO_2$ by formate as shown in Table 3. The addition of formate decrease progressively the amount of methanol oxidized to $CO_2$ (Fig. 6, oxidative branch), and at a certain concentration of formate almost all the methanol would become donor for the methyl (right branch of the pathway) (Loubière *et al.* 1987). Formate must replace both methanol and $CO_2$ as a source of carboxyl donor until the entire carboxyl group is furnished by formate (carbonyl branch).

Methanol-formate-grown cells differed significantly from methanol-$CO_2$-grown cells: $Y_{EG}$ for methanol-formate is nearly doubled and $m$ undergoes 40% increment compared to methanol-$CO_2$ cultures [$Y_{EG}$ = 16·5 g cell (mol substrate)$^{-1}$ and $m$ = 0·674 mmol substrate (g cell)$^{-1}$ $h^{-1}$, for methanol-formate and $Y_{EG}$ = 8·1 g cell (mol substrate)$^{-1}$ and $m$ = 0·479 mmol substrate (g cell)$^{-1}$ $h^{-1}$, for methanol-$CO_2$, Bainotti *et al.* 1998]. The high value of $Y_{EG}$ for methanol-formate-fermentation means that under a similar consumption of

substrate, *Acetobacterium* sp. produces cell more easily than in methanol-$CO_2$-system. The lower value of *m* in methanol-$CO_2$ indicates that the expenditure of energy for maintenance purposes in methanol-$CO_2$ culture is less than in methanol-formate-one.

Since corrinoids are involved as prosthetic groups in the enzymatic transfer of $CH_3$-groups and in carbon rearrangement reactions, among others (Diekert 1992), and considering that acetogenic bacteria perform in their energy metabolism the transformation of one-carbon groups, it is intelligible that they contain rather high levels of corrinoids (van der Meijden *et al.* 1984). In fact, bacteria with operative Acetyl-CoA pathway contain high levels of corrinoids, as reported by Dangel *et al.* (1987). Also, as stated by Tanner *et al.* (1978), whole cells of *Acetobacterium woodii* contain levels of corrinoids about 100 times higher than those found in the non-homo-acetate-fermentative organisms. The same bacterium produces considerably higher specific levels of $B_{12}$ than *P. shermanii* in hollow fiber reactors used to produce high biomass yields, with the additional advantage that the latter produce true vitamin $B_{12}$ (Hatanaka *et al.* 1988). Furthermore the complex media essential for growth of the propionic acid bacterium were not necessary for the methylotroph which is able to develop on a simple salts medium, hence simplifying the upstream preparation of medium. These reasons made *Acetobacterium* a very interesting microorganism for the production of cyanocobalamin.

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**Fig. 1** Chemostat steady-state values for growth of *Acetobacterium* sp. at various dilution rates on methanol-formate (125 mmol $l^{-1}$ equimolar) mixture. Symbols: ●, cells; ■, methanol; ▲, formate; ○, acetate; □, yield coefficient of acetate from methanol, $Y_{PM}$; △, yield coefficient of acetate from formate, $Y_{PF}$; ⊡, yield coefficient of cell mass from methanol, $Y_{XM}$; ◬, yield coefficient of cell mass from formate, $Y_{XF}$; ▽, yield coefficient of acetate from cell mass, $Y_{PX}$; ◨, methanol consumption rate, $q_M$; ◭, formate consumption rate, $q_F$; ◑, acetate production rate, $q_P$.

**Fig. 2** Influence of methanol-formate concentration on growth of *Acetobacterium* sp. in continuous culture at a fixed dilution rate ($D$ = 0·046 $h^{-1}$). Measurements were performed after steady-state was reached. Symbols as stated in Fig. 1.

**Fig. 3** Influence of methanol-formate concentration on kinetic parameters and growth of *Acetobacterium* sp. in continuous culture at a fixed dilution rate ($D$ = 0·05 $h^{-1}$). Measurements were performed after steady-state was reached. Symbols: ■, methanol; ▲, formate; ○, acetate; ◧, specific methanol consumption rate, $q_{MX}$; ◭, specific formate consumption rate, $q_{FX}$.

**Fig. 4** Tempest plot for specific rate of methanol consumption as a function of dilution rate in chemostat cultures of *Acetobacterium* sp. growing on methanol-formate 125 mM equimolar. $Y_{EG}$ = 16·5 g cell (mol methanol)$^{-1}$, $m$ = 0·674 mmol methanol (g cell)$^{-1}$ $h^{-1}$, linear regression coefficient ($r$) = 0·997.

**Fig. 5** Tentative scheme of acetate formation from $C_1$-compounds. A scheme proposed by Diekert and Wohlfarth (1994) slightly modified according to the energetic consideration shown in Table 4.
Symbols: ⬅ and ➔ indicate a reaction requiring the input of energy and producing the output of energy via a chemiosmotic mechanism, respectively.
$FH_4$ = tetrahydrofolate. $CH_3$-Co/E = methylated corrinoid / Fe-S protein.
[CO-Ni-E] = enzyme bound carbonyl.

**Fig. 6** Acetate formation from $C_1$ substrates at the redox level of methanol. Left side, oxidative part; right side, reductive part of the pathway.
Symbols: ⬅ and ➔ indicate a reaction requiring the input of energy and producing the output of energy via a chemiosmotic mechanism, respectively.
$FH_4$ = tetrahydrofolate. $CH_3$-Co/E = methylated corrinoid / Fe-S protein.
[CO-Ni-E] = enzyme bound carbonyl.

**Table 1** Steady-state values of methanol-formate fermentation for *Acetobacterium* sp. in continuous culture at different dilution rates using an initial concentration of both substrates of 125 mmol $l^{-1}$ equimolar.

| $D$ | $X$ | $S_M$ | $S_F$ | $P$ | $\Delta S_M$ | $\Delta S_F$ | $\Delta S_E$ | $Y_{XM}$ | $Y_{PM}$ | $Y_E$ | $q_E$ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 0·031 | 0·72 | 54 | 52 | 61 | 71 | 73 | 61 | 10·1 | 0·86 | 11·8 | 2·63 |
| 0·046 | 0·63 | 64 | 60 | 47 | 61 | 65 | 47 | 10·3 | 0·77 | 13·4 | 3·43 |
| 0·073 | 0·43 | 94 | 92 | 29 | 31 | 33 | 29 | 13·9 | 0·94 | 14·8 | 4·93 |
| 0·089 | 0·39 | 98 | 95 | 27 | 27 | 30 | 27 | 14·4 | 1·00 | 14·4 | 6·18 |

All the experiments are presented as means of two replicate determinations. Symbols: $S_0$, $S$, substrate concentration in inlet and outlet flow stream, expressed in mmol $l^{-1}$ of methanol-formate; $\Delta S$, substrate consumed = $S_0 - S$, in mmol $l^{-1}$; $P$, acetate produced in mmol $l^{-1}$; $D$, dilution rate in $h^{-1}$; $X$, cell concentration expressed in g $l^{-1}$; $\Delta S_E$, substrate (methanol or formate) catabolized = acetic acid produced x 1 / 1, according to the stoichiometry: $CH_3OH + HCOOH \longrightarrow CH_3COOH + H_2O$, in mmol $l^{-1}$; $Y_{XM}$, growth yield coefficient from consumed methanol ($Y_{XM} = X/\Delta S_M$) in g cell (mol of formate consumed)$^{-1}$; $Y_{PM}$, product yield coefficient ($Y_{PM} = P / \Delta S_M$), in mmol of acetic produced (mmol of methanol consumed)$^{-1}$; $Y_E$, true growth yield on substrate catabolized ($Y_E = X / \Delta S_E$), in (g dry cell) (mol substrate catabolized)$^{-1}$; $q_E$, specific substrate catabolizing rate ($q_E = D / Y_E$), in (mmol of substrate catabolized) (g of dry cell)$^{-1}$ $h^{-1}$. Subscript $M$ refers to methanol and $F$ to formate.

**Table 2** Steady-state values of *Acetobacterium* sp. growing in different concentrations of methanol-formate at dilution rate of 0·05 $h^{-1}$.

| $S_0$ | $\Delta S_M$ | $\Delta S_F$ | $P$ | Ratio $M:F:A:C$ * | $X$ | $Y_{XM}$ | $Y_{XF}$ | $R_c$ † (%) | $R_e$ § (%) | Substrate / cell synthesis conversion rate (%) | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | carbon ¶ | electrons ¢ |
| 25 | 23 | 25 | 24 | 1:1·09:1·04:0·57 | 0·35 | 15·2 | 14·0 | 127 | 132 | 27·3 | 29·4 |
| 50 | 30 | 34 | 27 | 1:1·13:0·90:0·49 | 0·39 | 13·0 | 11·5 | 107 | 112 | 23·0 | 25·0 |
| 75 | 37 | 46 | 32 | 1:1·24:0·86:0·45 | 0·45 | 12·2 | 9·8 | 98 | 104 | 20·0 | 22·4 |
| 100 | 45 | 50 | 38 | 1:1·11:0·84:0·43 | 0·51 | 11·3 | 10·2 | 100 | 104 | 20·4 | 22·0 |
| 125 | 60 | 65 | 43 | 1:1·08:0·71:0·41 | 0·65 | 10·8 | 10·0 | 88 | 91 | 19·7 | 21·2 |

All the experiments are presented as means of two replicate determinations. For symbols refer to footnote in Table 1.

* Molar ratio: methanol consumed (*M*) : formate consumed (*F*) : acetic acid produced (*A*) : cell carbon produced (*C*), expressed in mmol $l^{-1}$, considering methanol = 1.00. A cell formula of $C_{4·86}H_{8·85}O_{2·41}N_1$, plus ash 7·4% with a a carbon content of 45·1 ±0·4%, was assumed (Lynd *et al.* 1982). The amount (moles) of cell carbon was calculated as follows: $C$ (mol cell carbon $l^{-1}$) = $X$ (g cell $l^{-1}$) x [0·451 g C (g cell)$^{-1}$] x (mol C / 12 g C).

† Carbon recovery (%) was calculated as: $\{[A\ (2) + C\ ] / [M + F\ ]\}$ x 100.

§ Available electron recovery (%) was calculated as: $\{ [A\ (8) + C\ (4{\cdot}22)] / [M\ (6) + F\ (2)] \} \times 100$. The number of electron equivalents (4·22) per mole of cell C was calculated from the cell formula by the method of Harris and Adams (1979).

¶ Substrate carbon incorporated to cell synthesis (% mol cell C / mol substrate C) $= C / (M + F) \times 100$.

¢ Substrate electron incorporated to cell synthesis (% electron equivalent per mol cell C / electron equivalent per mol substrate C) $= C \cdot 4{\cdot}22 / M\ (6) \times 100$.

**Table 3** Comparison of cell mass and vitamin $B_{12}$ production by *Acetobacterium* sp. cultivated on different unicarbonotrophic mixtures in continuous culture.

| Substrate | $S_0$ (mmol $l^{-1}$) | $D$ ($h^{-1}$) | $X$ (g cell $l^{-1}$) | $C_{vit}$ (mg $l^{-1}$) | $C_{vit/X}$ (mg g $cell^{-1}$) | $q_{vit}$ (mg g $cell^{-1}$ $d^{-1}$) | Reference |
|---|---|---|---|---|---|---|---|
| MeOH + Formate | 125/125 | 0.031 | 0.72 | 4.04 | 5.60 | 4.17 | |
| MeOH + Formate | 125/125 | 0.089 | 0.39 | 2.21 | 5.66 | 12.11 | (this paper) |
| MeOH + $CO_2$ | 100 | 0.031 | 0.73 | 3.22 | 4.41 | 3.28 | |
| MeOH + $CO_2$ | 150 | 0.031 | 0.86 | 3.14 | 3.65 | 3.36 | |
| MeOH + $CO_2$ | 100 | 0.094 | 0.64 | 4.51 | 4.47 | 10.08 | |
| MeOH + $CO_2$ | 150 | 0.094 | 0.65 | 2.87 | 4.36 | 9.84 | (Bainotti *et al.* 1998) |

**Table 4** Energetics of homoacetogenic reactions on different substrates.

| Reaction | $\Delta G'_0$ * | No. of electron pairs | $Y_{XP}$ † | $Y_{ATP/P}$ obtained § | $Y_{ATP/P}$ estimated ¶ | Efficiency % ¢ | Acetogen (Reference) |
|---|---|---|---|---|---|---|---|
| Heterotrophic | | | | | | | |
| $C_6H_{12}O_6 \longrightarrow 3\ CH_3COOH$ | - 104 | 24 | 16·7 | 1·59 | 1·5 | 48·6 | *Cl. thermoaceticum* (Andreesen *et al.* 1973) |
| Autotrophic | | | | | | | |
| $4\ H_2 + 2\ CO_2 \longrightarrow CH_3COOH + 2\ H_2O$ | - 105 | 0 | 5·4 | 0·51 | 0·5 | 15·4 | *A. woodii* (Tschech and Penning 1984) |
| Halocompounds | | | | | | | |
| $4\ CH_3Cl + 2\ CO_2 + 2\ H_2O \longrightarrow 3\ CH_3COOH + 4\ HCl$ | - 140 | | 11·3 | 1·07 | 1·4 | 24·3 | Strain MC (Traunecker *et al.* 1991) |
| Methylotrophic | | | | | | | |
| $4\ CH_3OH + 2\ CO_2 \longrightarrow 3\ CH_3COOH + 2\ H_2O$ | - 73 | 0 | 9·8 | 0·94 | 0·89 | 40·9 | *Acetobacterium* sp. (Bainotti *et al.* 1998) |
| $3\ CH_3OH + 3\ HCOOH \longrightarrow 3\ CH_3COOH + 3\ H_2O$ | - 80 | 2 | 14·3 | 1·36 | 1·33 | 54·6 | *Acetobacterium* sp. (this paper, Table 2) |

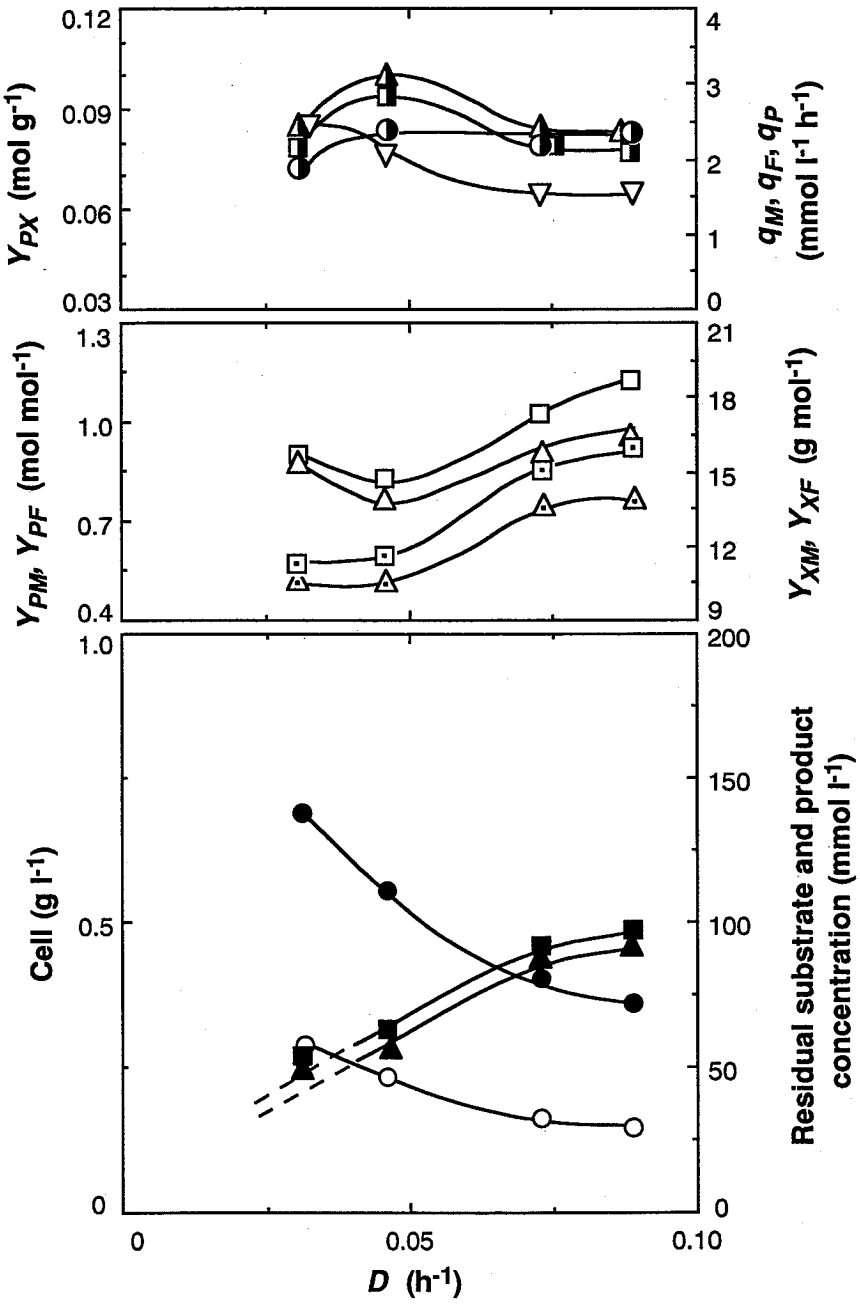
* Gibbs free energy under standard conditions in kJ (mol acetate)$^{-1}$, estimated using the proposed pathway shown in Fig. 5 and 6.

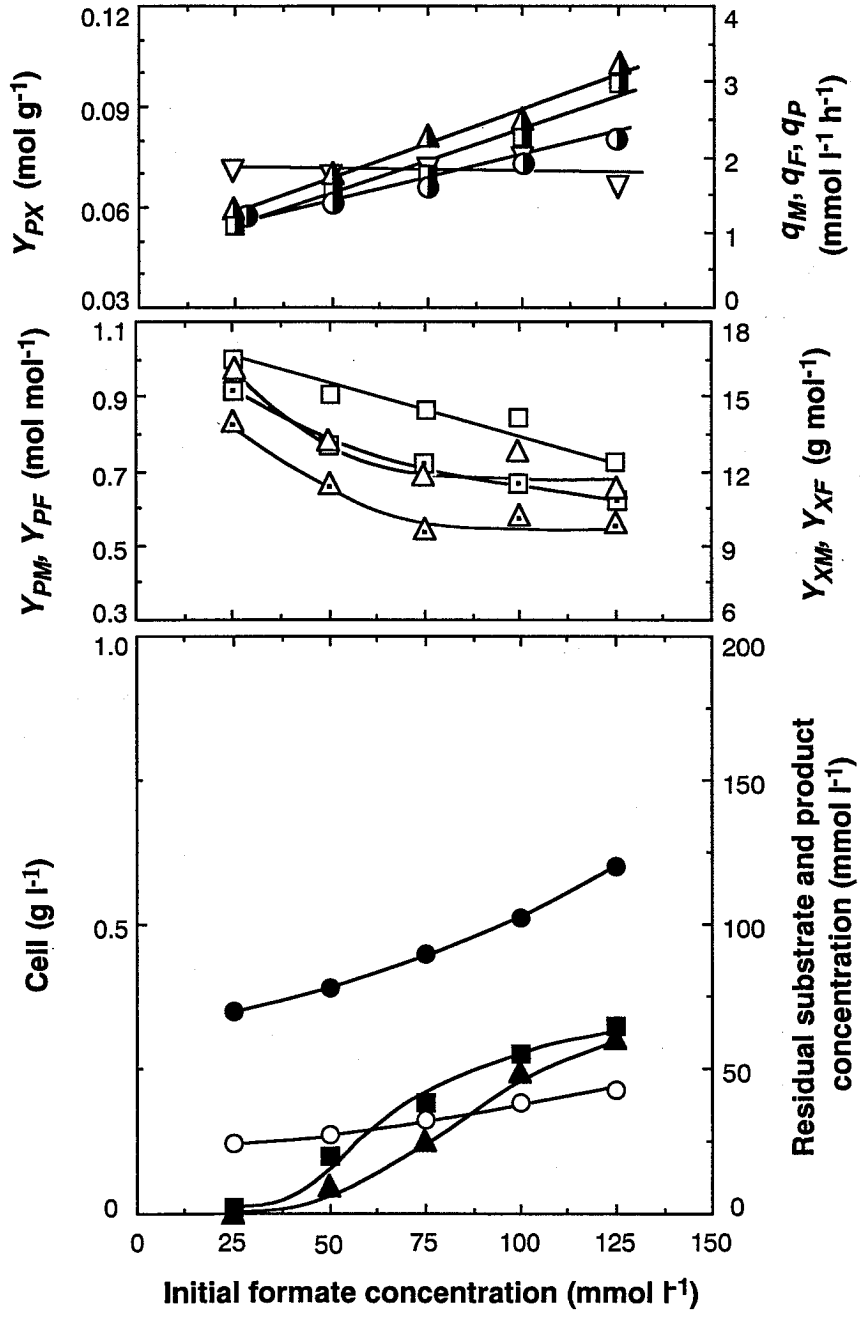
† Cell yield coefficient on acetate produced, in g dry cell (mol acetate)$^{-1}$ according to the references.

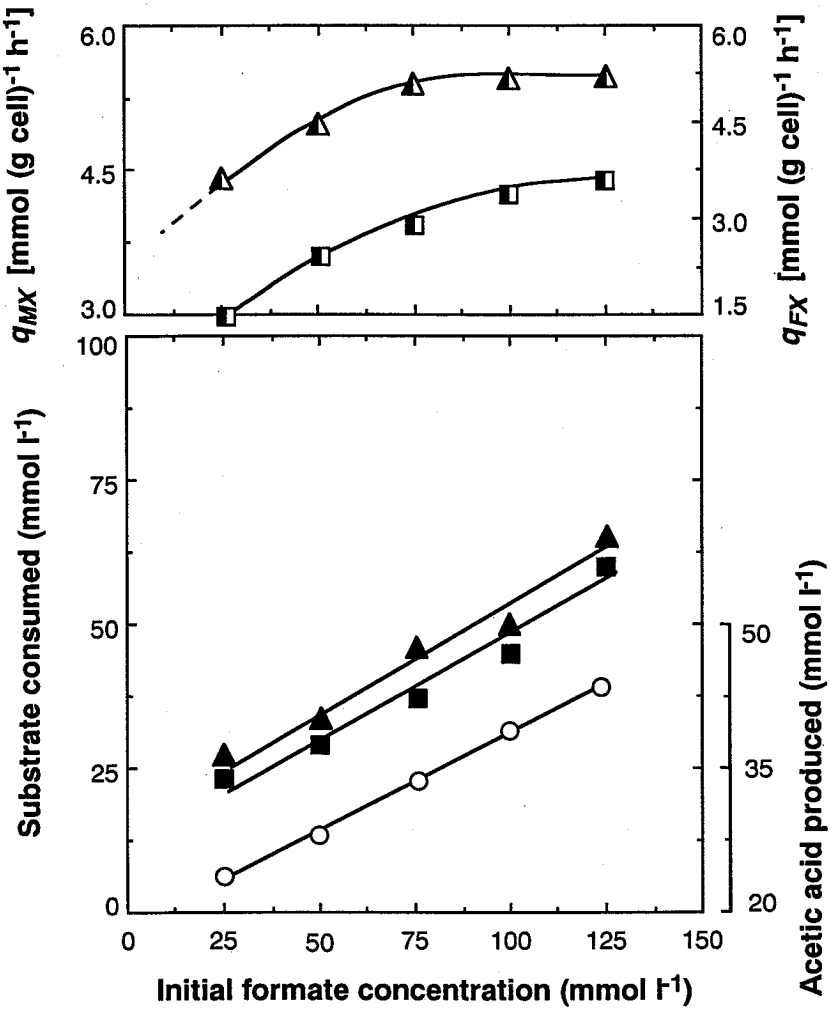
§ ATP yield coefficient in mol ATP (mol acetate)$^{-1}$, obtained by $Y_{XP}$ values in the references and $Y_{X/ATP}$ value assumed as 10·5 g dry cell (mol ATP)$^{-1}$.

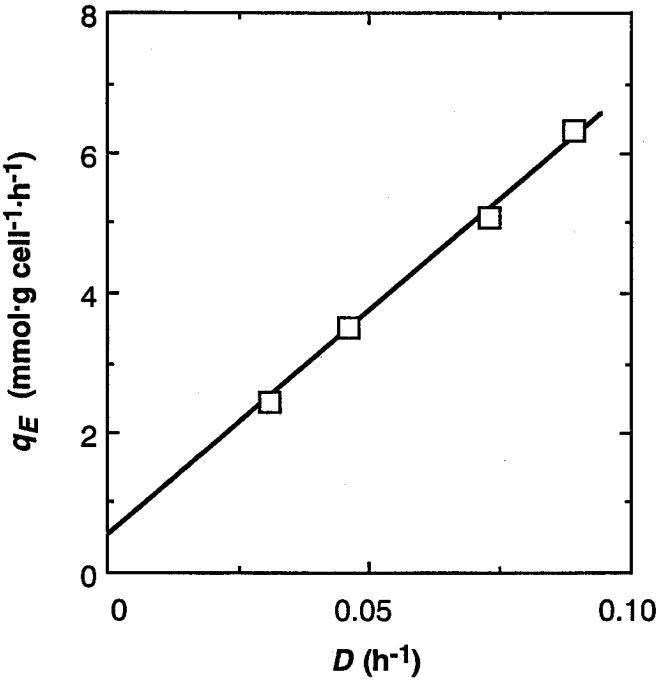
¶ ATP yield coefficient in mol ATP (mol acetate)$^{-1}$, estimated using the proposed pathway shown in Fig. 5 and 6. The $\Delta G'_0$ change during the reduction of methylene tetrahydrofolate to methyl tetrahydrofolate was assumed to produce 1/3 ATP by a chemiosmotic mechanism (Diekert & Wohlfarth 1994). The $\Delta G'_0$ change when the methyl tetrahydrofolate was transferred to a corrinoid/Fe-S-protein was assumed to produce 1/2 ATP by a chemiosmotic mechanism.

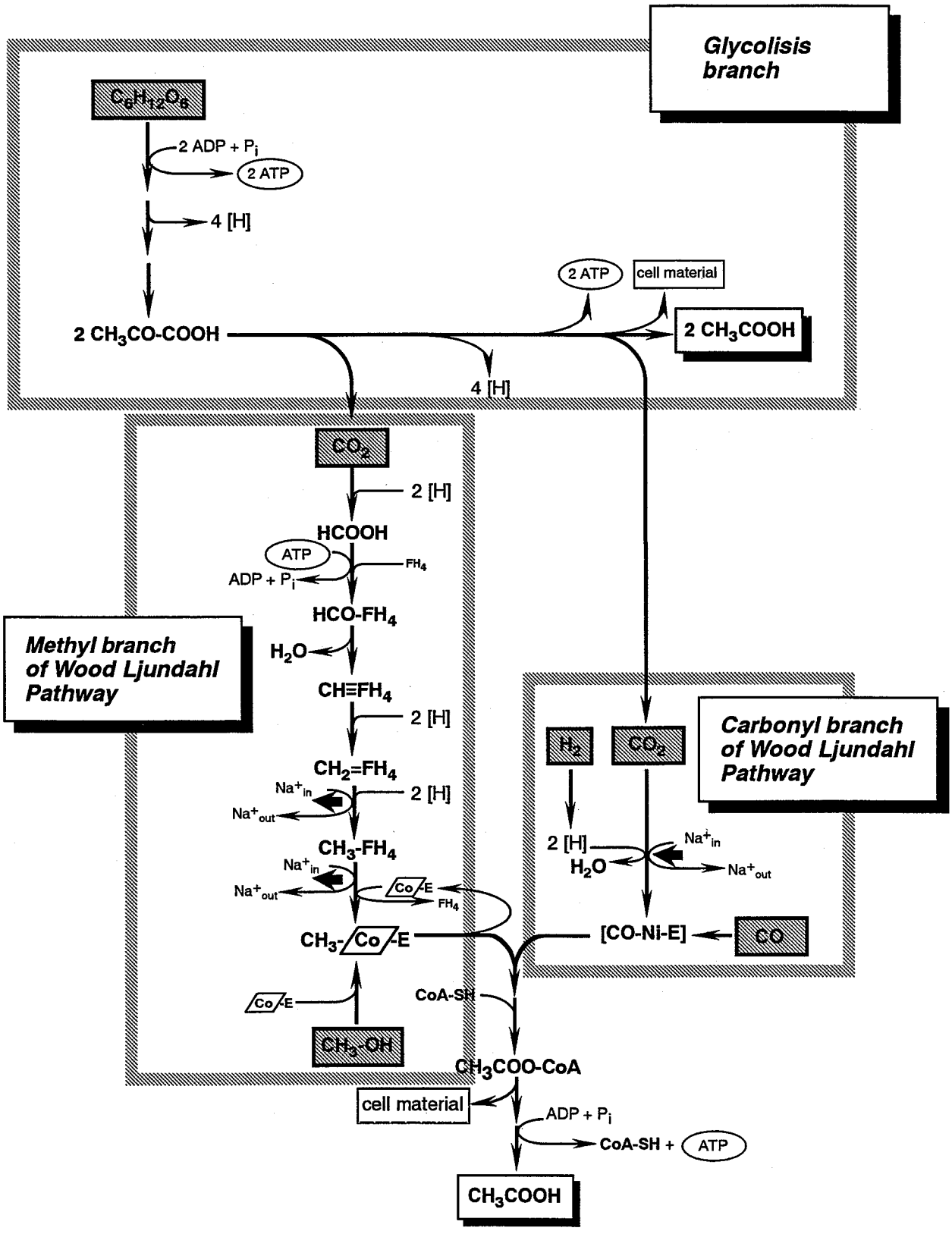
¢ Based on the free energy change of ATP hydrolysis ($\Delta G'_0$ = 31·8 kJ (mol ATP)$^{-1}$). The efficiency was calculated as follows: $Y_{ATP/P\ obt}$ [mol ATP (mol acetate)$^{-1}$] x [31·8 kJ (mol ATP)$^{-1}$] / $\Delta G'_0$ [kJ (mol acetate)$^{-1}$].

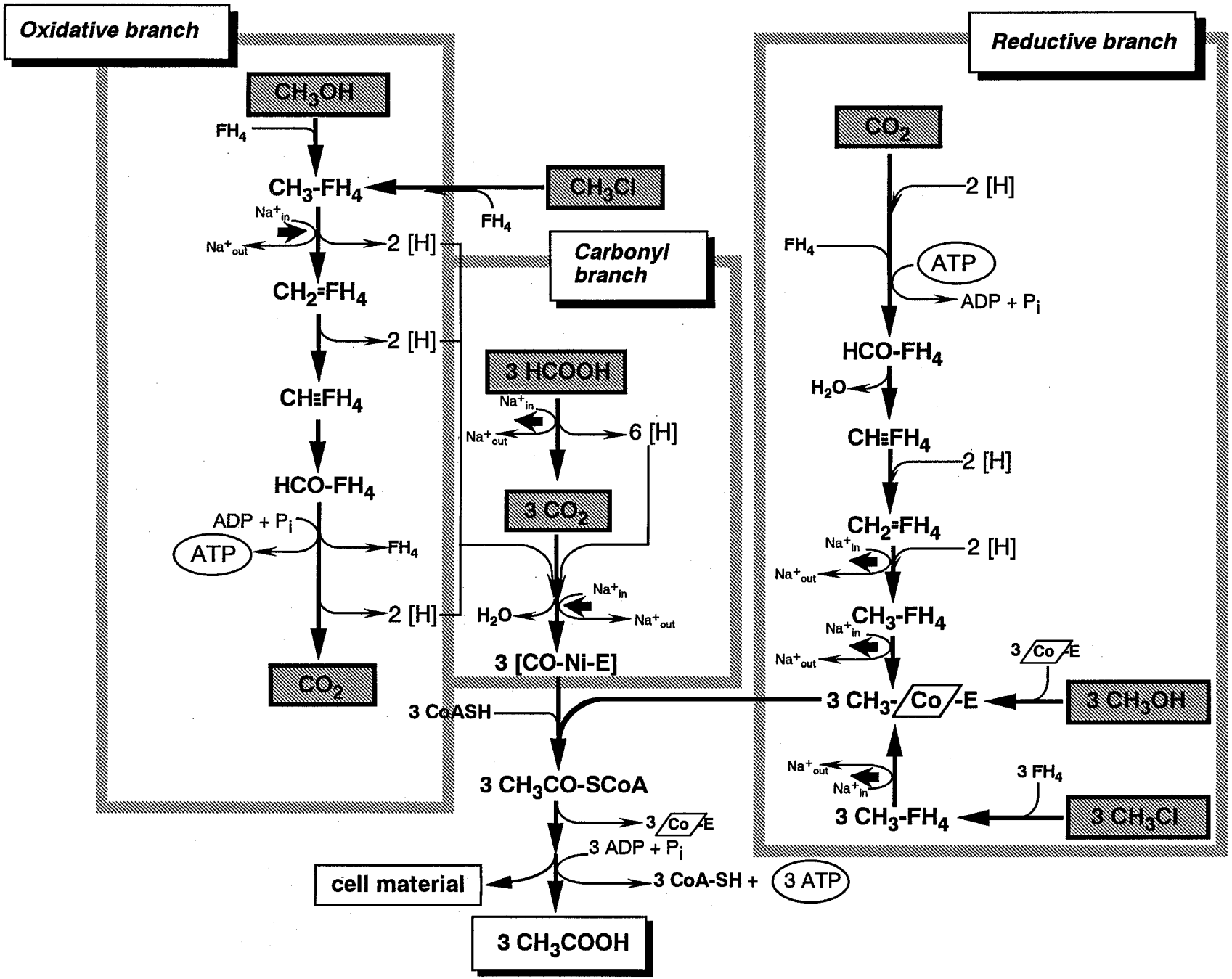

0.12
0.09
0.06
0.03
$Y_{PX}$ (mol g$^{-1}$)
4
3
2
1
0
$q_M$, $q_F$, $q_P$ (mmol l$^{-1}$ h$^{-1}$)
1.3
1.0
0.7
0.4
$Y_{PM}$, $Y_{PF}$ (mol mol$^{-1}$)
21
18
15
12
9
$Y_{XM}$, $Y_{XF}$ (g mol$^{-1}$)
1.0
0.5
0
Cell (g l$^{-1}$)
200
150
100
50
0
Residual substrate and product concentration (mmol l$^{-1}$)
0
0.05
0.10
$D$ (h$^{-1}$)


0.12
0.09
0.06
0.03
$Y_{PX}$ (mol g$^{-1}$)
4
3
2
1
0
$q_M$, $q_F$, $q_P$ (mmol l$^{-1}$ h$^{-1}$)
1.1
0.9
0.7
0.5
0.3
$Y_{PM}$, $Y_{PF}$ (mol mol$^{-1}$)
18
15
12
9
6
$Y_{XM}$, $Y_{XF}$ (g mol$^{-1}$)
1.0
0.5
0
Cell (g l$^{-1}$)
200
150
100
50
0
Residual substrate and product concentration (mmol l$^{-1}$)
0
25
50
75
100
125
150
Initial formate concentration (mmol l$^{-1}$)


6.0
4.5
3.0
6.0
4.5
3.0
1.5
$q_{MX}$ [mmol (g cell)$^{-1}$ h$^{-1}$]
$q_{FX}$ [mmol (g cell)$^{-1}$ h$^{-1}$]
100
75
50
25
0
50
35
20
Substrate consumed (mmol l$^{-1}$)
Acetic acid produced (mmol l$^{-1}$)
0
25
50
75
100
125
150
Initial formate concentration (mmol l$^{-1}$)


8
6
4
2
0
0
0.05
0.10
$q_E$ (mmol·g cell$^{-1}$·h$^{-1}$)
$D$ (h$^{-1}$)


Glycolisis branch
$C_6H_{12}O_6$
2 ADP + $P_i$
2 ATP
4 [H]
2 $CH_3CO$-COOH
2 ATP
cell material
2 $CH_3COOH$
4 [H]
$CO_2$
2 [H]
HCOOH
ATP
ADP + $P_i$
$FH_4$
$HCO$-$FH_4$
$H_2O$
$CH{\equiv}FH_4$
2 [H]
$CH_2$=$FH_4$
$Na^+_{in}$
$Na^+_{out}$
2 [H]
$CH_3$-$FH_4$
$Na^+_{in}$
$Na^+_{out}$
Co/E
$FH_4$
$CH_3$-Co/E
Co/E
$CH_3$-OH
Methyl branch of Wood Ljundahl Pathway
$H_2$
$CO_2$
Carbonyl branch of Wood Ljundahl Pathway
2 [H]
$H_2O$
$Na^+_{in}$
$Na^+_{out}$
[CO-Ni-E]
CO
CoA-SH
$CH_3COO$-CoA
cell material
ADP + $P_i$
CoA-SH + ATP
$CH_3COOH$


Oxidative branch
Reductive branch
Carbonyl branch
CH3OH
FH4
CH3-FH4
CH3Cl
FH4
Na+in
Na+out
2 [H]
CH2=FH4
2 [H]
CH≡FH4
HCO-FH4
ADP + Pi
ATP
FH4
2 [H]
CO2
3 HCOOH
Na+in
Na+out
6 [H]
3 CO2
H2O
Na+in
Na+out
3 [CO-Ni-E]
3 CoASH
3 CH3CO-SCoA
3 Co E
3 ADP + Pi
cell material
3 CoA-SH +
3 ATP
3 CH3COOH
CO2
2 [H]
FH4
ATP
ADP + Pi
HCO-FH4
H2O
CH≡FH4
2 [H]
CH2=FH4
Na+in
Na+out
2 [H]
CH3-FH4
Na+in
Na+out
3 Co E
3 CH3-Co-E
3 CH3OH
Na+out
Na+in
3 FH4
3 CH3-FH4
3 CH3Cl