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Thermoanaerobacter ethanolicus in a Comparison of the Growth Efficiencies of Thermophilic and Mesophilic Anaerobes

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Maintenance coefficients and theoretical maximum growth yields, with respect to both substrate and ATP, were estimated for *Thermoanaerobacter ethanolicus* growing in a glucose-limited, continuous culture. A comparison of these values with those for other bacteria showed that, contrary to predictions by others, anaerobic thermophiles had neither low observed growth yields nor high maintenance energy coefficients.

The chemostat culture method permits a separate assessment, as follows, of growth-dependent and growth-independent substrate consumption, as described by Pirt (15): rate of substrate consumption equals rate of substrate consumption for growth plus rate of substrate consumption for maintenance functions, which is expressed by the equation $Q_s = \mu/Y_s = \mu/Y_s^{max} + m_s$ in which Q_s is the specific rate of consumption of the growth-limiting substrate; μ is the specific growth rate; Y_s is the observed growth yield; Y_s^{max} is the growth yield corrected for maintenance energy consumption; and m_s is the maintenance energy coefficient, representing the specific rate of substrate consumption at zero growth rate. The value for μ is established by the rate of flow (F) of nutrient medium through the chemostat of constant volume (V), since in a steady state, the specific growth rate (μ) is equal to the dilution rate (D) where $D = F/V$.

It has been proposed that the cellular growth yields of thermophiles are less than those of mesophiles (14, 23, 25). Furthermore, it has been suggested that the lower yields result from higher maintenance energy requirements (23, 25). This supposition is based on the fact that maintenance energy requirements have been shown to increase with temperature for a given species (5, 9, 11, 13) and on the general observation that the temperature dependency of Y_s in mesophilic yeast and bacteria is mainly determined by the temperature dependency of m_s (3). m_s values for three aerobically grown thermophilic *Bacillus* species (4, 9, 12) were high in comparison with values determined for aerobically grown mesophiles (6, 7, 8, 20). Wiegel (23) has proposed that the lower growth yields of thermophiles are a potential advantage to be considered in industrial fermentation processes. This would certainly be the case in a system in which the major constraint on productivity is the biomass load, such as in a continuous system with cell recycling.

The studies of McKay et al. (13) and Farrand et al. (4) with thermophilic aerobes have indicated that the molecular basis for lower growth yields in thermophiles may be an increased proton permeability of the cell membrane. Anaerobes, like aerobes, depend on transmembrane proton gradients for many energy-coupled cell functions (16). Therefore, an increased proton permeability would also be expected to affect the energetics of thermophilic anaerobes.

We are currently investigating the ethanologenic thermophile *Thermoanaerobacter ethanolicus* (24) to evaluate its potential for industrial alcohol production. As a part of these studies, we have estimated m_s, maintenance coefficient with respect to ATP (m_{ATP}), Y_s^{max}, and theoretical maximum ATP yield (Y_{ATP}^{max}) for wild-type *T. ethanolicus* (ATCC 31550) by the following methods. A single-colony isolate stock was used in all of the experiments. The medium was the same as that described previously (24) with the following alterations: $Na_2S \cdot 9H_2O$ and $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ were omitted, and cysteine · HCl · H_2O (0.32 g liter^{-1}), $FeCl_3 \cdot 9H_2O$ (0.027 g liter^{-1}), citric acid (0.21 g liter^{-1}), and yeast extract (Difco Laboratories, Detroit, Mich.) (4.0 g liter^{-1}) were added. The vitamins were sometimes omitted, as noted below. The media was autoclaved, kept anaerobic under nitrogen gas, and delivered to the fermentor through stainless steel tubing and a small section of silicon tubing which passed through the peristaltic pump. Cells were grown at pH 7.0 and 69°C in a chemostat (model C30; New Brunswick Scientific Co., Inc., Edison, N.J.) with a 370-ml

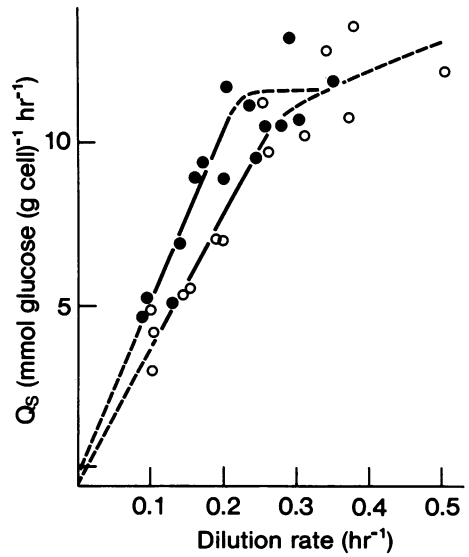

FIG. 1. Effect of growth rate on the specific rate of glucose consumption of *T. ethanolicus* growing in glucose-limited, continuous culture. ●, Media without vitamin supplementation. Data were taken from three experiments. Because points level off, only points up to $D = 0.2$ h^{-1} were included in linear regression ($r = 0.91$). ○, Media with vitamin supplementation. Data were taken from two experiments. Points up to $D = 0.25$ h^{-1} were included in linear regression ($r = 0.96$). Correlation coefficients indicated that the two sets of data should be considered separately.

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TABLE 1. Growth parameters for anaerobically grown bacteria

Organism	Medium[a]	Growth temp (°C)	m_s[b]	m_{ATP}	Y_s^{max}	Y_{ATP}^{max}	Reference
Mesophiles							
Bacteroides ruminicola	R	39	0.8	—[c]	90	—	17
Butyrivibrio fibrisolvens	R	39	0.3	—	72	—	17
Enterobacter aerogenes	M	—	2.4	6.8 (5.4–8.0)[d]	31	12 [14][e] (±1)[d]	20
Enterobacter aerogenes	E	—	—	2.3	—	[18] (±2)	21
Escherichia coli	M	37	5.8	19	20	10	
Lactobacillus casei	R	37	0.1	1.5	62[f]	24	1
Microbacterium thermosphactum	E	25	0.4	0.8	46	20	8
Streptococcus mutans	E	—	−0.2	—	32	—	2
Zymomonas mobilis	M	30	9.4	9	9.0	9	[g]
	E	30	8.3	8	11	11	
Thermophiles							
Clostridium thermocellum	E	60	1.5	3.2	39	16	10
Thermoanaerobacter ethanolicus							
Minus vitamins	E	69	−0.3 (±3.7)[h]	−0.5 (±3.7)	19 (13–35)	11 (9–14)	
Plus vitamins	E	69	−0.5 (±3.7)	−0.5 (±3.7)	31 (19–32)	11 (9–14)	
Thermoanaerobium brockii	E	70	3	6.7	50	23	18

[a] R, Rich, complex medium with crude amino acid and lipid sources: E, enriched medium with crude or synthetic amino acids, concentration by weight of the same order as the sugar source; M. minimal medium.

[b] m_S is expressed as millimoles of glucose equivalent gram (dry weight) of cells^{-1} hour^{-1}; m_{ATP} is expressed as millimoles of ATP gram (dry weight) of cells^{-1} hour^{-1}; Y_S is expressed as grams (dry weight) of cells mole of glucose equivalent^{-1}; Y_{ATP} is expressed as grams (dry weight) of cells mole of ATP^{-1}.

[c] —, data not available.

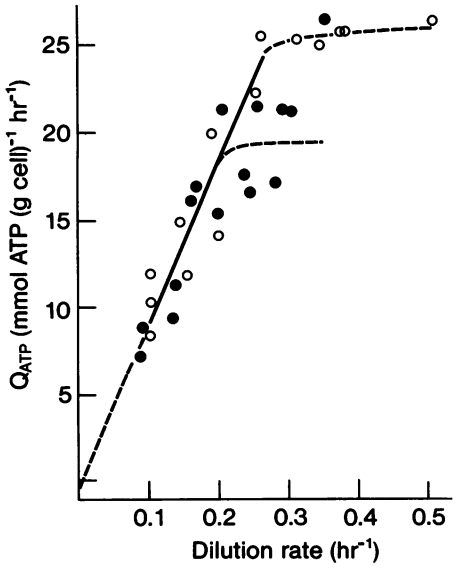
[d] Values in parentheses indicate a 95% confidence interval.

[e] Numbers in brackets are the Y_{ATP}^{max} values determined by the authors; unlike other researchers cited, these authors applied a correction factor for carbon substrate used as a cell structural component and not as an energy source.

[f] Estimated from linear low-D section of nonlinear graph.

[g] H. G. Lawford, unpublished data.

[h] Negative m_S is a physiological impossibility and reflects the inaccuracy of the method.



working volume. The agitation rate was 200 rpm, and the nitrogen gas flow was 20 ml min^{-1}. At least 5 fermentor volume changes occurred between samplings, enabling the system to achieve steady state. Glucose was measured with a model 27 glucose analyzer (Yellow Springs Instrument Co., Yellow Springs, Ohio). The dry cell weight was determined by filtering the culture broth through preweighed membrane filters (pore size, 0.45 μm; Millipore Corp., Bedford, Mass.), rinsing the cells with distilled water, and drying them to a constant weight under an infrared lamp. Cell-free fermentor broth samples were analyzed for lactate, acetate, and ethanol by high-pressure liquid chromatography with an HPX-87H column (Bio-Rad Laboratories; Richmond, Calif.).

FIG. 2. Effect of growth rate on the specific rate of ATP production in *T. ethanolicus* growing in a glucose-limited, continuous culture. ATP production was calculated from product analyses. ●, Media without vitamin supplementation. Data were taken from three experiments. Points up to $D = 0.2$ h^{-1} were included in linear regression. ○, Media with vitamin supplementation. Data were taken from two experiments. Points up to $D = 0.25$ h^{-1} were included in linear regression. Correlation coefficients indicated that the two sets of data should be considered together. Combined correlation coefficients, $r = 0.92$.

Fermentation product analysis allowed estimation of specific rates of ATP production. In cases where product distribution varies with growth rate so that the energy yield from the substrate can also vary, the calculated energy yield (Y_{ATP}) is the more appropriate comparative parameter (1). *T. ethanolicus* has been reported to use the Embden-Meyerhoff pathway for glucose catabolism (24). Consequently, it was assumed that the molar ATP yield from glucose associated with either lactate or ethanol production was 1, whereas that associated with acetate production was 2.

The data used to estimate maintenance coefficients and theoretical maximum growth yields with respect to glucose equivalents and ATP are represented in Fig. 1 and 2, respectively.

Kundy et al. (10) and Sonnleitner et al. (18) provide two more examples of thermophilic growth in continuous culture, where m_s and Y_s^{max} were estimated (10, 18). However, the implications of these results on generalizations about thermophilic growth are not discussed. From the product formation data included in their studies, we also estimated m_{ATP} and Y_{ATP}^{max}.

The growth parameters determined in studies with thermophiles were compared with data or values calculated from data obtained with mesophilic obligate and facultative anaerobes (Table 1). A comparison of these parameters from continuous culture experiments was preferable to a comparison of observed growth yields determined from batch studies because the growth rate variable was not controlled in the latter case. Although it is acknowledged that growth parameters can be manipulated by altering the growth conditions (see reviews by Stouthamer [19] and Tempest and Neijssel [22]), the data presented were thought to be obtained under conditions of enough similarity (glucose-limited growth in all cases except *Clostridium thermocellum*, for which the carbon source was cellulose; low glucose concentration [≤3% glucose]; low salt concentration; optimal temperature and pH conditions) to allow a comparison and the detection of any dramatic differences.

The growth-yield parameters for the thermophiles easily fell within the range of those for mesophiles. A comparison of the mean values for thermophiles with those for mesophiles with the Student t test ($\alpha = 0.05$) provided neither evidence that the m_s for thermophiles is greater than that for nor evidence that the Y_s for thermophiles is less, than those values for mesophiles. (For these statistical analyses, m_s and Y_s^{max} for *Zymomonas mobilis* were halved and doubled, respectively, to allow an appropriate comparison of this organism, which uses the Entner-Doudoroff pathway for glucose catabolism and thereby derives only 1 molecule of ATP per mole of glucose). One can predict observed growth yields for a given dilution rate with Y_s^{max} and m_s, allowing a hypothetical comparison among organisms with different maximum growth rate (μ_{max}). Small-sample statistical analysis ($\alpha = 0.05$) of growth yields predicted for $D = 0.2\ h^{-1}$ and $D = 1.0\ h^{-1}$ provided no evidence that the observed growth yields of mesophiles are greater than those of thermophiles. The Mann-Whitney U test, a nonparametric test which does not assume that the parameter data of Table 1 were taken from a normal distribution, was also applied with the same conclusions as above. Thus, the predicted effect of temperature on growth yield was not observed.

In conclusion, the evidence presented above did not support the generalization that thermophiles have lower growth yields than do mesophiles, nor did it support the supposition that anaerobic thermophiles have higher maintenance coefficients than anaerobically growing mesophiles. We therefore consider it premature to make such generalizations and to expect that lower growth yields will be an advantage of thermophilic fermentations over mesophilic fermentations.

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