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# Growth Kinetics and Yield Coefficients of the Extreme Thermophile *Thermothrix thiopara* in Continuous Culture

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*Thermothrix thiopara* did not appear to be stressed at high temperature (72°C). Both the actual and theoretical yields were higher than those of analogous mesophilic sulfur bacteria, and the specific growth rate ($\mu_{max}$) was more rapid than that of most autotrophs. The specific growth rate (0.58 $h^{-1}$), specific maintenance rate (0.11 $h^{-1}$), actual molar growth yield at $\mu_{max}$ ($Y_{max}$ = 16 g $mol^{-1}$), and theoretical molar growth yield ($Y_G$ = 24 g $mol^{-1}$) were all higher for *T. thiopara* (72°C) than for mesophilic (25 to 30°C) *Thiobacillus* spp. The growth efficiencies for *T. thiopara* at 70 and 75°C (0.84 and 0.78) were significantly higher than at 65°C (0.47). Corresponding specific maintenance rates were highest at 65°C (0.41 $h^{-1}$) and lowest at 70 and 75°C (0.11 and 0.15 $h^{-1}$, respectively). Growth efficiencies of metabolically similar mesophiles were generally higher than for *T. thiopara*. However, the actual yields at $\mu_{max}$ were higher for *T. thiopara* because its theoretical yield was higher. Thus, at 70°C, *T. thiopara* was capable of deriving more metabolically useful energy from thiosulfate than were mesophilic sulfur bacteria at 25 and 30°C. The low growth efficiency of *T. thiopara* reflected higher maintenance expenditures. *T. thiopara* had higher maintenance rates than *Thiobacillus ferroxidans* or *Thiobacillus denitrificans*, but also attained higher molar growth yields. It is concluded that sulfur metabolism may be more efficient overall at extremely high temperatures due to increased theoretical yields despite increased maintenance requirements.

Thermobiosis has intrigued biologists for over two centuries (27). Since the initial discovery of a thermophilic bacillus by Miguel in 1888 (19), biologists have speculated about the origin, physiology, and growth of thermophilic bacteria. Origin hypotheses range from exotic Venusian beginnings (1) to the more likely assumption that bacterial life began when the earth's environment was hot and reducing (4, 8). The latter hypothesis indicates the common origin of mesophiles and thermophiles. The physiology and biochemistry of thermophiles further emphasize their common origin with mesophiles. Metabolic pathways are similar, and thermophilic enzymes differ from their mesophilic counterparts by as little as a single amino acid substitution which confers the necessary thermal stability on the protein (17, 33).

*Thermothrix thiopara* is a sulfur-oxidizing, extreme thermophile found in sulfide-containing hot springs (3, 5). Its optimum growth temperature is 72°C. The growth rate of *T. thiopara* is rapid compared with mesophilic sulfur-oxidizing *Thiobacillus* species (3). Chemostat studies were used to determine the growth efficiency ($E_g$) and molar growth yields of *T. thiopara* to define the role high temperature plays in affecting efficiencies and maintenance expenditures in sulfur-oxidizing thermophiles.

In contrast to most bacteria, thermophiles generally attain maximal yields at temperatures below their optimum for growth rate (6, 20, 23, 26). This is due to high maintenance requirements in thermophiles resulting in lower yields compared with metabolically similar mesophiles (29, 33). Final yields, however, are not only a function of maintenance, but also depend on energy available from substrate and how well the organism conserves substrate energy. Low yields might be compensated in a thermophile if its metabolism was thermodynamically more favorable at high temperature or if it had evolved a more efficient mechanism of energy conservation. This possibility was examined with *T. thiopara*. Despite higher maintenance requirements, its growth yields exceeded those of mesophilic sulfur oxidizers.

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## MATERIALS AND METHODS

**Organism and culture conditions.** *T. thiopara* (UNM B-142, Museum of Southwestern Biology, Microbiology Collection; ATCC 33745) has previously been described (3, 5). *T. thiopara* was grown on a modified TXB medium (3) composed (in grams per liter of distilled, deionized water) of the following: $Na_2S_2O_3 \cdot 5H_2O$, 1.0; $NaH_2CO_3$, 2.0; $NH_4Cl$, 1.0; $KNO_3$, 1.0; $MgCl_2 \cdot 6H_2O$, 0.5; $KH_2PO_4$, 2.0; and $FeSO_4 \cdot 7H_2O$–EDTA chelate, 0.02. The trace elements and pH indicator have been described before (3). Batch cultures (100 ml) were grown at 70°C in 250-ml screw-cap flasks at 150 rpm. Early-stationary-phase cultures (200 ml) were then used to inoculate the continuous cultures.

**Continuous cultures.** Continuous culture studies were carried out at three temperatures (65, 70, and 75°C) in a Bioflo model C30 bench-top chemostat (New Brunswick Scientific Co., New Brunswick, N.J.) with a culture volume of 350 ml, provided with agitation (200 rpm). Temperature was accurately regulated with rheostat-controlled heat tapes and a thermostatically controlled heating element. Aeration was provided by controlling the flow rate (350 $cm^3$/min) of air enriched with 5% (vol/vol) $CO_2$, using a Manostat flow meter (Manostat, New York). The culture pH remained constant (6.7 ± 0.2) during steady states. Wall growth was minimized by coating the culture vessel with 5% (vol/vol) dichlorosilane (Dow Corning, Midland, Mich.) in chloroform or by coating the vessel with silicone high-vacuum grease (Dow Corning), baking the vessel at 80°C for 2 h, and then removing the excess grease with hot 10 N NaOH. The dilution rate was varied from 0.1 to 0.34 $h^{-1}$ at 65°C. At 70 and 75°C the dilution rate was varied from 0.08 to 0.6 $h^{-1}$. Steady states at each dilution rate were reached after three volume changes.

**Determination of $\mu_{max}$ by washout kinetics.** The maximum specific growth rate ($\mu_{max}$) was determined by washout kinetics (13). During washout, cells were counted at 0.5-h intervals over 4 h, using a Petroff-Hauser bacterial counter. Cell numbers were also determined by absorbance at 460 nm ($A_{460}$), using a cell number-absorbance calibration curve ($A_{460}$ of 0.1 = 10.2 × $10^6$ cells per ml).

**Analyses of steady-state cultures.** Biomass was estimated from $A_{460}$, using a dry weight-absorbance calibration curve ($A_{460}$ of 0.1 = 37.6 mg [dry weight]/liter). Filtrate from the culture samples was analyzed for thiosulfate, sulfite, and polythionates by the titrimetric method of Koh and Taniguchi (14). Residue collected on the filters (0.2 μm; Amicon Corp., Lexington, Mass.) was analyzed for sulfur and none was present.

**Graphical determination of $Y_G$ and $m$.** Double-reciprocal plots of $Y^{-1}$ against $D^{-1}$ were linear, with theoretical growth yield ($Y_G$) equal to the reciprocal of yield on the ordinate intercept and maintenance coefficient ($m$) equal to the slope. Also, plots of specific rate of substrate utilization ($q$) against dilution rate were used, with $Y_G$ equal to the reciprocal of the slope and $m$ equal to $q$ on the ordinate intercept. Best-fit lines were plotted by regression analysis ($r > 0.9$ for all plots).

**Statistical analyses.** Three separate chemostat runs were made at each temperature. All kinetic data are reported as the mean with 95% confidence limits. Significant differences ($P = 0.05$) between the three temperatures were determined by Bartlett's test, ANOVA, and Student-Newman-Keuls analyses (32).

**Determination of growth efficiency ($E_g$).** The equations of Marr et al. and Pirt were used to account for substrate used for growth and maintenance, where maintenance is the consumption of potential biomass (18, 24):

$$\mu X/Y = \mu X/Y_G + aX/Y_G \quad (1)$$

where $\mu$ = specific growth rate, $X$ = biomass, $Y$ = actual or observed yield, $Y_G$ = theoretical growth yield (yield corrected for maintenance), $a$ = specific maintenance rate, and $m$ = maintenance coefficient = $a/Y_G$.

When $\mu = \mu_{max}$, the equation becomes:

$$\mu_{max}X/Y = \mu_{max}X/Y_G + aX/Y_G \quad (2)$$

Thus, the total rate of substrate utilization at $\mu_{max}$ ($\mu_{max}X/Y_T$) equals the rate used for growth ($\mu_{max}X/Y_G$) plus that used for maintenance ($aX/Y_G$). The fraction ($F$) of substrate used for growth at $\mu_{max}$ is:

$$F = (\mu_{max}X/Y_G)/(\mu_{max}X/Y) \quad (3)$$

Substituting for $\mu_{max}X/Y$ from equation 2 results in the following ratio:

$$F = (\mu_{max}X/Y_G)/[(\mu_{max}X/Y_G) + (aX/Y_G)] \quad (4)$$

By canceling terms and defining $F$ as the growth efficiency, the following equation is obtained:

$$E_g = \mu_{max}/(\mu_{max} + a) \quad (5)$$

Growth efficiency ($E_g$) can then be determined from $\mu_{max}$ and the specific maintenance rate ($a$). Alternatively, growth efficiency can be represented as $Y_{max}/Y_G$ by canceling the $\mu_{max}X$ terms from the ratio in equation 3.

In considering the energetics of thermophiles, maintenance can be represented as the consumption of biomass (18) which results in a lowered growth efficiency ($E_g$), defined as the fraction of potential growth obtained. This is equal to $\mu/(\mu + a)$, where $a$ is the specific maintenance rate and $\mu$ is the growth rate. A low $E_g$ indicates poor growth efficiency and high maintenance energy requirements. Despite poor growth efficiency, actual yields ($Y_{max}$) can still be high if the potential theoretical yield in the absence of maintenance ($Y_G$) is high. Thus, poor growth efficiency due to high maintenance rates does not necessarily result in low yields. Therefore, the overall metabolic efficiency (reflected by $Y_{max}$) of a thermophile may be higher than its mesophilic counterpart despite high maintenance energy requirements (low growth efficiency).

## RESULTS AND DISCUSSION

The $E_g$ for *T. thiopara* at 70°C indicates that 84% of the theoretical growth yield was attained (Table 1). Literature values were used to obtain $a$, $Y_{max}$, $Y_G$, and $E_g$ for other organisms grown under a variety of conditions (Table 2). The growth efficiency of *T. thiopara* at $\mu_{max}$ (0.84 $h^{-1}$) was lower than those of *Thiobacillus ferroxidans* (0.94 $h^{-1}$) and *Thiobacillus dentrificans* (0.94 $h^{-1}$) but greater than that of *Thioba-*

TABLE 1. Kinetic parameters of *T. thiopara* grown in continuous culture at 65, 70, and 75°C[a]

| Temp (°C) | $a$ ($h^{-1}$) | $\mu_{max}$ ($h^{-1}$) | $Y_{max}$ (g $mol^{-1}$)[b] | $Y_G$ (g $mol^{-1}$) | $E_g$[c] |
|---|---|---|---|---|---|
| 65 | 0.42 ± 0.14 | 0.36 ± 0.004 | 19.03 ± 2.2 | 41.4 ± 12 | 0.47 ± 0.08 |
| 70 | 0.11 ± 0.04 | 0.57 ± 0.009 | 20.27 ± 2.7 | 24.3 ± 4 | 0.84 ± 0.04 |
| 75 | 0.15 ± 0.09 | 0.54 ± 0.01 | 14.29 ± 1.1 | 18.8 ± 4 | 0.78 ± 0.1 |

[a] Values are reported as the mean with 95% confidence limits ($n$ = 6).

[b] $Y_{max}$ is the actual yield at $\mu_{max}$ obtained by reading the values of $Y$ at $\mu_{max}$ from linear regression equations of plots of $1/Y$ versus $1/D$ and of $q$ versus $D$. This value can be calculated with the formula $Y_{max} = (E_g)(Y_G)$.

[c] Calculated with the formula $E_g = \mu_{max}/(\mu_{max} + a)$. The growth efficiency, $E_g$, is the fraction of potential biomass ($Y_G$) conserved at $\mu_{max}$.

*cillus neapolitanus* (0.60 $h^{-1}$). The lower growth efficiency of *T. thiopara* was due to its higher specific maintenance rate ($a$). The high maintenance expenditures of *Thiobacillus neapolitanus* are due to a growth-dependent "slip" due to intracellular limitation of reducing power (15). The substrate (thiosulfate) supplies both energy and electrons for generation of reducing power. The growth-dependent "slip" mechanism occurs when cultures are limited by reducing power rather than energy (21, 22). Such conditions are evident in nonlinear plots of $Y^{-1}$ versus $D^{-1}$ (15). In contrast to *Thiobacillus neapolitanus*, *T. thiopara* showed linear plots.

The high maintenance rates of *T. thiopara* did not prevent high yields. Maintenance costs are losses resulting from the consumption of potential biomass. Such losses can be due to motility, production of extracellular enzymes and chelating agents, DNA repair, maintenance of concentration gradients, preservation of correct ionic strength and intracellular pH, repair of DNA, and replacement of denatured protein (18, 28, 33). Although the losses are severe in thermophiles, they can be compensated for if the potential biomass ($Y_G$) is higher. Thus, although *T. thiopara* had higher maintenance expenditures than *Thiobacillus denitrificans*, it still had a higher overall metabolic (energy) efficiency.

Yields for *T. thiopara* at 65 or 70°C were not significantly different. In general, however, thermophiles show higher yields at temperatures below their thermal optimum for growth (6). The effect is due to increased maintenance requirements at the higher temperatures (33). However, the higher maintenance requirement for *T. thiopara* at 65°C (0.42 $h^{-1}$), compared with the requirement at 70 or 75°C (0.11 or 0.15 $h^{-1}$, respectively), contradicts this generalization.

In the case of *T. thiopara*, metabolic efficien-

TABLE 2. Kinetic parameters of five bacteria grown in continuous culture under various conditions of temperature and limiting substrate

| Organism | Reference | Growth conditions | $a$ ($h^{-1}$) | $\mu_{max}$ ($h^{-1}$) | $Y_{max}$ (g $mol^{-1}$)[a] | $Y_G$ (g $mol^{-1}$) | $E_g$[b] |
|---|---|---|---|---|---|---|---|
| *Thiobacillus ferrooxidans* | 7 | Aerobic, thiosulfate limiting; 30°C | 0.0073 | 0.129 | 7.0 | 7.48 | 0.94 |
| *Thiobacillus denitrificans* | 12 | Aerobic, thiosulfate limiting; 25°C | 0.007 | 0.13 | 13.8 | 14.69 | 0.94 |
| | 12 | Anaerobic, thiosulfate limiting, 25°C | 0.015 | 0.08 | 9.6 | 11.37 | 0.84 |
| *Thiobacillus neapolitanus* | 9 | Aerobic, thiosulfate limiting; 25°C | 0.303 | 0.48 | 8.3 | 13.9 | 0.60 |
| *Thiomicrospira denitrificans* | 11 | Anaerobic, thiosulfate limiting; 25°C | 0.0079 | 0.06 | 1.9 | 5.65 | 0.88 |
| *Microbacterium thermosphactum* (psychrotroph) | 10 | Aerobic, glucose limiting, 25°C | 0.015 | 0.495 | 70.8 | 73 | 0.97 |
| | | Anaerobic, glucose limiting; 25°C | 0.018 | 0.46 | 44.2 | 46 | 0.96 |
| *Thermoactinomyces* sp. | 16 | Aerobic, glucose limiting; 55°C | 0.01 | 0.36 | 73.7 | 76 | 0.97 |

[a] $Y_{max}$ is the actual yield at $\mu_{max}$, calculated with the formula $Y_{max} = (E_g)(Y_G)$. This value could also be obtained by reading the values of $Y$ at $\mu_{max}$ from a plot of $1/Y$ versus $1/\mu$.

[b] See footnote *c*, Table 1.

cy is higher than in other mesophilic sulfur oxidizers. The $Y_{max}$ $S_2O_3^{2-}$ for *T. thiopara* at 65, 70, or 75°C was greater than that of any known mesophilic sulfur chemoautotroph despite its higher maintenance requirements. The high yield despite high maintenance is likely a result of a different, and more efficient, mechanism of energy conservation that more than compensates for the losses due to maintenance. This is also seen in other thermophiles. For example, growth yields of *Methanobacterium thermoautotrophicum* grown at 55°C on methane are of the same order as those of the mesophile *Methanobacterium barkeri* (25, 30, 31). A *Thermoactinomyces* sp. (16) grown at 55°C on glucose has higher molar growth yields than *Microbacterium thermosphactum* (10) grown at 25°C with limiting glucose (Table 2). Therefore, thermophiles are not necessarily limited to lower growth yields simply because they have a higher maintenance requirement. In fact, some thermophiles appear to have evolved more efficient mechanisms of energy conservation to compensate for the increased maintenance requirements at high temperature. This has not occurred in all thermophiles, however. For example, some thermophilic fermenters have lower yields than their mesophilic counterparts (2).

Reasons for optimization of sulfur autotrophy at higher temperature might include not only more efficient mechanisms of energy conservation but also increased energy yields from sulfur compounds, a relative reduction in the oxygenase function of RuBP carboxylase, and increased solubility of sulfur at the high temperatures.

Additionally, higher yields despite high maintenance may be a result of the metabolic process being more thermodynamically favorable at the high temperature. Determination of $Y_{max}$, $\mu_{max}$, $Y_G$, and $a$ for additional thermophiles could reveal that some metabolic processes are more favorable at higher temperatures. The implication for industrial microbiology is that many of the chemical processes that use microbial catalysts are not necessarily optimized at the conditions required for the organism to grow. It may thus be possible to adapt or select organisms for optimum growth under conditions that are most thermodynamically favorable for the chemical process being catalyzed.

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