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Temperature-Dependent Growth Kinetics of *Escherichia coli* ML 30 in Glucose-Limited Continuous Culture

K. KOVÁŘOVÁ, A. J. B. ZEHNDER, AND T. EGLI*

Swiss Federal Institute for Environmental Science and Technology, Swiss Federal Institute of Technology, CH-8600 Dübendorf, Switzerland

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Detailed comparison of growth kinetics at temperatures below and above the optimal temperature was carried out with *Escherichia coli* ML 30 (DSM 1329) in continuous culture. The culture was grown with glucose as the sole limiting source of carbon and energy (100 mg liter^{-1} in feed medium), and the resulting steady-state concentrations of glucose were measured as a function of the dilution rate at 17.4, 28.4, 37, and 40°C. The experimental data could not be described by the conventional Monod equation over the entire temperature range, but an extended form of the Monod model [$\mu = \mu_{max} \cdot (s - s_{min})/(K_s + s - s_{min})$], which predicts a finite substrate concentration at 0 growth rate (s_{min}), provided a good fit. The two parameters μ_{max} and s_{min} were temperature dependent, whereas, surprisingly, fitting the model to the experimental data yielded virtually identical K_s values (approximately 33 μg liter^{-1}) at all temperatures. A model that describes steady-state glucose concentrations as a function of temperature at constant growth rates is presented. In similar experiments with mixtures of glucose and galactose (1:1 mixture), the two sugars were utilized simultaneously at all temperatures examined, and their steady-state concentrations were reduced compared with to growth with either glucose or galactose alone. The results of laboratory-scale kinetic experiments are discussed with respect to the concentrations observed in natural environments.

Knowledge concerning the influence of environmental factors such as temperature, pH, salinity, etc., on microbial growth is of crucial practical importance in the control of bioprocesses, for the safe handling of food (1, 2, 12, 50, 51), in wastewater treatment (7), and in bioremediation (2). In addition, in taxonomy, cardinal temperatures for growth are key characteristics of microbial strains (37).

In recent years, several models for predicting the growth rate of microorganisms as a function of either temperature alone (11, 13, 22, 31, 32, 52, 53) or of temperature in combination with other factors have been proposed (1, 5, 20–22, 36, 50). Surprisingly, few attempts at a better basic understanding have been made to relate the rate of growth and actual substrate concentration. This relationship is traditionally termed growth kinetics (23, 30). (However, note that the same expression has also been used for the description of the time courses of population densities [5].) The current lack of systematic data on the influence of temperature on the kinetics of growth makes the prediction of this effect difficult. Temperature modulation of growth kinetics is to be expected, because both metabolism and cellular composition are affected by cultivation temperature, as was demonstrated by the cellular fatty acid composition (16, 26, 41), the synthesis or degradation of certain proteins (14, 15, 17, 20, 25), changes in protein activity (15, 35), changes in maintenance requirements of cells (19, 27, 39, 42, 47), changes in end products of metabolism (17), and increases in pigment formation (27).

Bacterial metabolism represents a network of reactions. Although these individual biochemical reactions are temperature dependent, the fundamental question of whether the parameters used in growth kinetic models are temperature dependent must be asked. To determine this, a detailed comparison of growth kinetics at temperatures below and above the optimal temperature was carried out for *Escherichia coli* ML 30 cultivated in continuous culture with glucose and/or galactose. Such investigations are possible only by using an extremely sensitive method for measuring low concentrations of sugars (in micrograms per liter) in culture media (40). The objective of the present study was to compare the experimentally established relationships between growth rate and steady-state substrate concentrations at different constant temperatures and to find out whether the whole set of relationships can be described by a simple mathematical model (Table 1 summarizes the nomenclature used throughout). Additionally, the effect of temperature on steady-state substrate concentrations at constant growth rates (dilution rates in continuous culture) was studied.

Compendium of the models proposed in the literature. (i) Conventional growth kinetics and models containing an s_{min} term. Various mathematical models have been proposed to quantitatively describe microbial growth kinetics. The Monod model (equation 1) is considered the basic equation (23), which has since been improved by including expressions for, e.g., maintenance, diffusion, or transport limitation (29, 30, 45; for detailed comparison, see reference 40). Microbial growth kinetics in both batch and continuous culture have been investigated. Earlier experiments carried out in batch cultures mostly relied on indirect methods, i.e., growth was measured, whereas the substrate concentrations were not directly determined but were estimated by calculation. In contrast, when growth kinetics in continuous culture were investigated, the actual steady-state concentrations of the growth-limiting substrate were determined as a function of the dilution rate. For such an experimental setup, the $s = f(D)$ form of the kinetic model (equation 1) correctly expresses the variable dependence, and not the $\mu = f(s)$ form, in which the models were originally reported (discussed in reference 40).

$$s = K_s \frac{D}{\mu_{max} - D} \quad (1)$$

Monod's original kinetic equation (equation 1) implies a sub-

* Corresponding author. Phone: 41-1-823 5158. Fax: 41-1-823 5547. Electronic mail address: egli@eawag.ch.

TABLE 1. Nomenclature

Symbol	Definition	Unit
a	Specific maintenance rate (equations 3 and 5)	h^{-1}
A	Parameter in Esener model (equation 9)	h^{-1}
b	Parameter in Ratkowsky model (equation 10)	$K^{-1}\ s^{-0.5}$
B	Model parameter (equation 11)	$K^2\ \mu g\ liter^{-1}$
c	Parameter in Ratkowsky model (equation 10)	K^{-1}
C	Model parameter (equation 11)	K^{-1}
D	Dilution rate (specific growth rate in chemostat)	h^{-1}
k_i, k_j, and k_h	Rate constants	$\mu g\ liter^{-1}\ h^{-1}$ or $\mu g^2\ liter^{-2}\ h^{-1}$
K	Parameter in Esener model (equation 9)	
K_m	Michaelis-Menten substrate saturation constant	$\mu g\ liter^{-1}$
K_s	Substrate saturation constant	$\mu g\ liter^{-1}$
n	Number of steady states analyzed (here, number of experiments, not number of datum points collected from a particular steady state)	
$(n - p)$	Number of degrees of freedom	
R	Gas constant	$kJ\ kg^{-1}\ mol^{-1}$
RR	Relative residuals (equation 14)	%
$RSS\ (s)$	Residual sum of squares with respect to s (equation 13)	$\mu g^2\ liter^{-2}$
s	Steady-state substrate concn	$\mu g\ liter^{-1}$
s_{min}, s^*_{min}, s^{**}_{min}, and s^{***}_{min}	Predicted substrate concentration at $D = 0\ h^{-1}$ for different growth models	$\mu g\ liter^{-1}$
s_{obs}, μ_{obs}	Experimentally established value (for s or μ)	$\mu g\ liter^{-1}$ or h^{-1}
s_{pred}	Value predicted by model equation (s or μ)	$\mu g\ liter^{-1}$ or h^{-1}
T	Cultivation temperature	K or °C
T_{min}, T_{opt}, and T_{max}	Minimum, optimum, and maximum temperatures, respectively	K or °C
x	Parameter in Westerhoff model (equation 7)	h^{-1}
y	Parameter in Westerhoff model (equation 7)	h^{-1}
ΔH_1, ΔH_2	Enthalpy changes (in Esener model [equation 9])	$kJ\ mol^{-1}$
μ	Specific growth rate	h^{-1}
μ_{max}	Maximum specific growth rate	h^{-1}

strate concentration of 0 at a growth rate of 0 h^{-1} (23). This model represents a special case of a more general kinetic expression (equation 2; Fig. 1), in which a term for a finite substrate concentration, $s = s_{min}$ at D = 0 h^{-1}, is incorporated. When $s_{min} << s$, s_{min} becomes negligible, and equation 2 reduces to equation 1:

$$s = K_s \frac{D}{\mu_{max} - D} + s_{min} \tag{2}$$

Expressions having a meaning similar to that of s_{min} (and noted

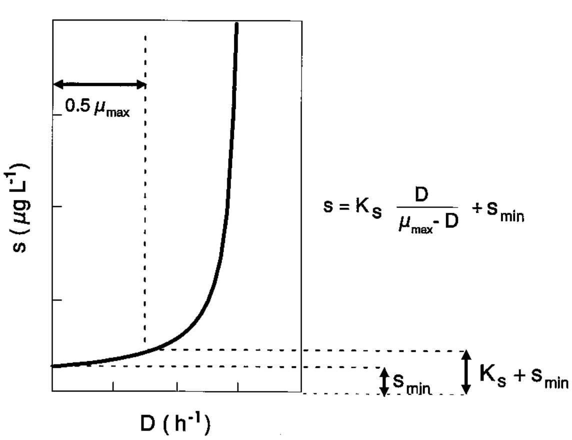

FIG. 1. Example of Monod model with apparent substrate term (equation 2) indicating the model parameters.

here as s^*_{min}, s^{**}_{min}, and s^{***}_{min}) are implicitly also present in other kinetic models (equations 3, 5, and 7). For instance, the original relationship proposed in 1967 by Powell (30) can be easily converted into an $s = f(D)$ form (equation 3). The first part of this expression is identical to the Monod model (equation 1), and to this a dilution rate-dependent s^*_{min} term (equation 4) is added:

$$s = K_s \frac{D}{\mu_{max} - D} + K_s \frac{a}{\mu_{max} - D} \tag{3}$$

$$s^*_{min} = K_s \frac{a}{\mu_{max} - D},$$

$$\text{hence at } D = 0\ h^{-1}, s^*_{min} = K_s \frac{a}{\mu_{max}} \tag{4}$$

In contrast to equation 2, s^*_{min} in equation 4 is dependent on D. In this model, the contribution of s^*_{min} to the steady-state substrate concentrations at high dilution rates is usually negligible, and s^*_{min} becomes important at growth rates near 0. Essentially, it can be also assumed that the s^*_{min} term is not dependent on the dilution rate (equation 2).

In another analysis of microbial growth, van Uden (45) in 1967 followed up Pirt's original 1965 proposal (28, 29) that only the overall growth rate is reduced by the maintenance rate a (in its s form [equation 5]). The resulting substrate concentration at $D = 0\ h^{-1}$ is shown in equation 6:

$$s = \frac{K_s (a + D)}{(\mu_{max} - D - a)} \tag{5}$$

$$s_{\min}^{**} = \frac{a}{\mu_{\max} - a} K_s \qquad (6)$$

While all of the previous relationships are extensions of the Monod-type kinetics, a fundamentally different model, based on nonequilibrium thermodynamics, was proposed by Westerhoff and coworkers in 1982 (48). This logarithmic expression [exponential in $s = f(D)$ form; equation 7] also predicts at $D = 0\ h^{-1}$ a positive substrate concentration (equation 8):

$$s = \exp\left(\frac{D - x}{y}\right) \qquad (7)$$

$$s_{\min}^{***} = \exp\left(-\frac{x}{y}\right) \qquad (8)$$

(ii) Steady-state substrate concentrations versus temperature at various growth rates. Because the effect of temperature on steady-state substrate concentrations has not yet been studied systematically, there is no mathematical description of this relationship. Assuming a link between μ and s, we here suggest using a modification of a model which was developed previously to describe the temperature dependence of the maximum specific growth rate. This suggestion follows the approach taken by McMeekin et al. (22), who proposed that the simple Ratkowsky model [i.e., $\mu = f(T)$ (equation 10)] might be extended for any set of restricting conditions involving temperature as one of the factors.

Several unsegregated and unstructured phenomenological models (11, 12, 31, 32, 37) have been proposed both to predict the cardinal temperatures and to describe bacterial growth in batch cultures within the growth-permissible temperature range. It has been shown (see, e.g., reference 31) that within this temperature range μ_{max} follows a bell-shaped curve, which has a maximum at the optimum growth temperature (T_{opt}). In the master reaction model proposed by Esener (11) (equation 9), μ_{max} asymptotically approaches the abscissa at both the maximum (T_{max}) and minimum (T_{min}) growth temperatures:

$$\mu_{\max} = \frac{A \exp(-\Delta H_1/RT)}{1 + K \exp(-\Delta H_2/RT)} \qquad (9)$$

In contrast, the square root model proposed by Ratkowsky et al. (31) (equation 10) is defined for $T_{min} < T > T_{max}$, out of this range μ_{max} is 0:

$$\sqrt{\mu_{\max}} = b(T - T_{\min})\{1 - \exp[c(T - T_{\max})]\} \qquad (10)$$

In continuous culture, T_{min} and T_{max} can be determined for each particular dilution rate, e.g., by washout experiments or from the $T = f(\mu_{max})$ relationship. In this contribution, we were primarily interested in how well the minimum and maximum temperatures for growth at particular dilution rates can be predicted, not in the model structure.

For the $s = f(T)$ model, we assumed that steady-state substrate concentrations are reciprocally proportional to the square of the temperature, i.e., the square root of the concentration is reciprocally proportional to the temperature. This assumption can be justified in the following way. Under optimum conditions, a microbial cell grows with the highest possible μ and, therefore, a cell's overall metabolic efficacy should also be at its optimum. This implies that close to T_{opt}, the lowest steady-state substrate concentrations should be expected at a particular growth rate, in the same way as the highest μ_{max} is reached at T_{opt}. This assumption provides a link between the square root model (equation 10) and the steady-state substrate concentration model (equation 11). For these reasons, we propose to describe the s versus T relationship (equation 11) by a reciprocal form of the maximum specific growth rate versus temperature relationship (equation 10):

$$s = \frac{B}{(T - T_{\min})^2 \{1 - \exp[C(T - T_{\max})]\}^2} \qquad (11)$$

where the units of C are identical to those of c from the Ratkowsky model (equation 10) and the parameter B is related to b from the Ratkowsky model according to equation 12:

$$B \cong \frac{1}{b^2} \qquad (12)$$

Since the two regression coefficients (B and C) vary with the dilution rate, it is possible to simply extend equation 11 by introducing terms to describe variations in B (or b) and C with the growth rate in the chemostat, as determined from experimental data (e.g., see Fig. 7). Additionally, it should be pointed out that in theory, T_{min} and T_{max} used in equation 11 are equivocally defined because they can be understood as either constant properties of a given strain and medium composition (e.g., its cardinal temperatures) or as variables changing with the dilution rate.

MATERIALS AND METHODS

Organism, medium, and culture conditions. *E. coli* ML 30 (DSM 1329) was grown in mineral medium (40) supplemented either with glucose (100 mg liter^{-1} in chemostat; 500 mg liter^{-1} in batch culture) or with a mixture of glucose and galactose (each 50 mg liter^{-1} in chemostat) as the only sources of carbon and energy. The bioreactor (MBR, Wetzikon, Switzerland), provided with both pH (7.00 ± 0.05) and temperature (±0.1°C) control, was operated in chemostatic mode with a working volume of 1.5 liters. The impeller speed control was set at 1,000 min^{-1}, and the oxygen saturation was >90% air saturation. The bioreactors were regularly checked for wall growth to avoid artifacts as reported by Pirt (29).

Maximum specific growth rates (μ_{max}). μ_{max} rates were determined in batch cultures at different temperatures. The cells used as an inoculum were pregrown exponentially for more than 50 generations at the particular temperature. At least duplicate measurements were made at each temperature. The standard deviation obtained for μ_{max} was reproducibly ± 0.05 h^{-1}.

Steady-state substrate concentrations. Steady-state concentrations of glucose and/or galactose were determined in continuous culture during independent chemostat runs, either as a function of the dilution rate at constant temperatures (17.4, 28.4, 37, and 40°C) or as a function of temperature at constant dilution rates (0.2, 0.3, 0.4, and 0.5 h^{-1}). At each temperature, the continuous cultivation was restricted either by the critical dilution rate below which the organism was able to grow without washing out or by wall growth. The entire temperature range within which the organism was able to grow in the chemostat at a particular D (i.e., $\mu_{max} \geq D$) was extrapolated from batch data (for an explanation, see Fig. 2).

Individual steady-state concentrations of glucose or galactose represent the means of approximately 10 measurements determined over a time period of more than 40 generations after the culture had reached steady state with respect to glucose (galactose) concentration. The standard deviations of these values were between ±5% to ±10%, and for clarity they are not given in all figures presented. The sugar analysis has been described in detail elsewhere (40).

Data processing. The models were fitted to the experimental data by nonlinear regression (33, 34). The minimum of the least-squares criterion (residual sum of squares [*RSS*]) (equation 13) was computed with a Simplex algorithm:

$$RSS(s) = \Sigma(s_{obs} - s_{pred})^2 \qquad (13)$$

Initial estimates of the model parameters were required, because the structural correlation between parameters made their estimation otherwise difficult. Starting values were chosen from the best extrapolations of the experimental data by exponential and/or polynomial functions.

The quality of the fits was evaluated by standard tools, such as comparing the variance, applying the χ^2 criterion, or, for the discrimination of competing models, the F test (4), or by analyzing the linear regression between the measured and predicted substrate concentrations. Statistical validation was best visible in plots of relative residuals (equation 14, e.g., Fig. 3):

$$RR = \frac{s_{obs} - s_{pred}}{s_{obs}} \times 100 \qquad (14)$$

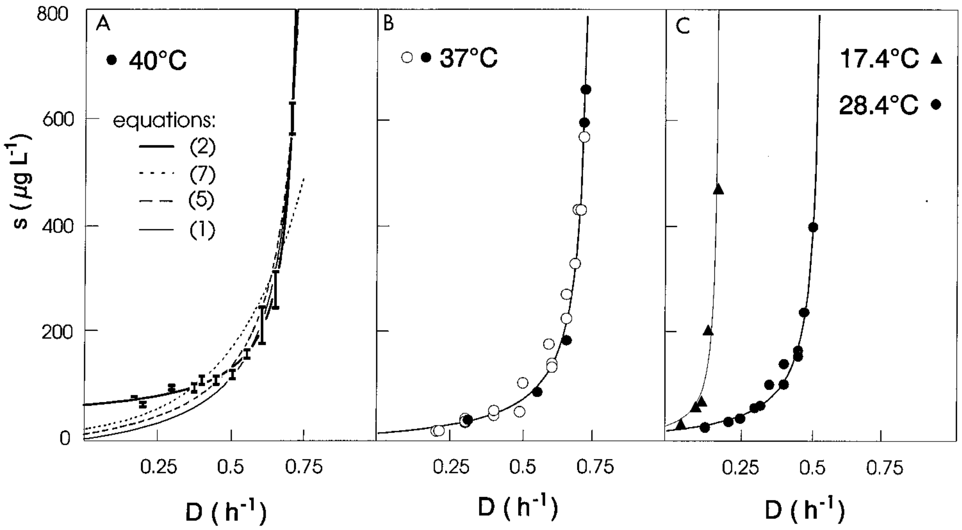

FIG. 2. Experimentally determined and predicted steady-state glucose concentrations (with the extended Monod model [equation 2]) for growth of *E. coli* ML 30 in glucose-limited chemostat cultures at 17.4, 28.4, 37, and 40°C, as a function of dilution (growth) rate. (A) Bars, steady-state substrate concentrations (height indicates standard deviation [approximately 10%] of the steady-state glucose concentrations estimated as an average of approximately 10 measurements; the horizontal extensions of the bars give the approximate variations in *D*); lines, predictions of steady-state glucose concentrations by different models. (B and C) open circles, data from Senn et al. (40); closed circles and triangles, own steady-state glucose measurements; lines, best fit of equation 2.

RESULTS

Temperature dependence of growth kinetics. The kinetics of growth of *E. coli* ML 30 [i.e., the $s = f(D)$ relationship] was investigated at four different temperatures (17.4, 28.4, 37, and 40°C, respectively) both above and below the optimum growth temperature (Fig. 2).

For growth at 40°C, the steady-state glucose concentrations were measured as a function of *D* during three independent glucose-limited chemostat runs. This cultivation temperature was slightly higher than the calculated optimum growth temperature (T_{opt} = 38.7°C) of *E. coli* ML 30 growing unrestricted in this medium in batch culture (Table 2). The glucose concentrations measured at low and moderate dilution rates (up to approximately 0.35 h^{-1}) were distinctly enhanced compared with the previously reported growth kinetic data at 37°C (40) (Fig. 2). Nevertheless, as judged by Student's *t* test, the two μ_{max} values estimated by fitting equation 2 to the data at 37°C (0.76 ± 0.01) and 40°C (0.74 ± 0.01) were not significantly different (Fig. 2).

TABLE 2. Comparison of the Ratkowsky and Esener models describing dependence of maximum specific growth rate of *E. coli* on temperature with data experimentally obtained in batch cultures[a]

Variable	U	Ratkowsky model value	Esener model value
A	h^{-1}		$(4.22 \pm 0.35)\ 10^{11}$
K	h^{-1}		$(8.07 \pm 0.11)\ 10^{49}$
ΔH_1	kJ mol^{-1}		68.9 ± 6.2
ΔH_2	kJ mol^{-1}		300.6 ± 25.4
b	$K^{-1}\ h^{-0.5}$	$(2.76 \pm 0.25)\ 10^{-2}$	
c	K^{-1}	1.12 ± 0.10	
T_{min}	K	275.7 ± 0.3	
T_{max}	K	315.2 ± 0.2	
T_{opt}	K	311.9 ± 0.2*	311.4 ± 0.2*
μ_{max}	h^{-1}	0.95 ± 0.05*	0.92 ± 0.05*
$RSS/(n - p)$	h^{-2}	$2.5\ 10^{-4}$*	$4.7\ 10^{-2}$*

[a] Values of the model parameters (equations 9 and 10), optimum growth temperatures, and statistical parameters were computed for the batch culture data. *, value calculated from the best-fit parameters.

In contrast to the predictions made by the Monod model (equation 1), these results clearly demonstrate that the steady-state glucose concentrations of the limiting-substrate glucose did not approach 0. Therefore, the quality of the fit of various mathematical models (equations 2, 5, and 7), which contain implicitly a finite substrate concentration at $D = 0\ h^{-1}$, and of the original Monod model (equation 1) was compared with experimentally obtained data (Fig. 2). The quality of the fit of these models (expressed as means of relative residuals [equation 14]) was compared with the theoretical measurement error of ±10% (Fig. 3). It is obvious that three of the models (equations 2, 5, and 7) exhibited a systematic deviation from the glucose concentrations measured at low dilution rates. The model proposed by Westerhoff (equation 7) could not be successfully fitted to this type of experimental data by the applied minimizing procedure. Only the model, which contains an apparent substrate term (equation 2), exhibited a random distribution of the relative residuals. The mean estimation error for this model (6.7%) was in the same order of magnitude as the measurement errors (10%) and was considerably lower than the mean estimation errors obtained for the other three models (26.8, 20.9, and 23.2% for equations 2, 5, and 7, respectively).

The data at 37, 28.4, and 17.4°C were used to test the validity of equation 2. In Fig. 2, the fits to data from different temperatures are given and the measured steady-state glucose concentrations are compared with predicted curves. It should be pointed out that at 37°C, only data up to $D = 0.71\ h^{-1}$ were used because data at higher dilution rates can be easily affected by experimental artifacts (40). The excellent correlation between the measured steady-state glucose concentrations and predicted values at all experimental temperatures (Fig. 4) confirms the utility of the model containing s_{min}. The model parameters computed for equation 2 from experimental data measured at four different temperatures are collected in Table 3. Interestingly, the K_s values did not vary with temperature, whereas the two parameters μ_{max} and s_{min} were temperature

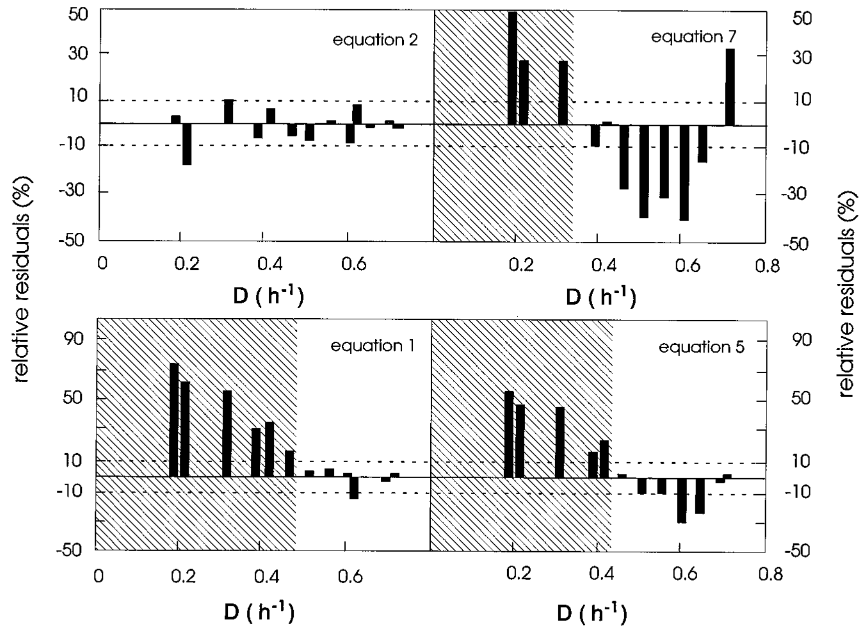

FIG. 3. Relative residuals for the fits of four different kinetic models to experimentally determined steady-state glucose concentrations during growth of *E. coli* at 40°C. Hatched areas, a systematic underestimation of steady-state glucose concentrations; dashed lines, 10% deviation from experimental steady-state glucose concentrations.

dependent. K_s remained constant at approximately 33 μg liter^{-1}, which is approximately 1/3 of the value obtained when the original Monod model (equation 1) was fitted to the data (40). The μ_{max} values estimated in chemostat culture were generally some 15% lower than those measured under substrate excess conditions in batch culture (Fig. 5a); this phenomenon has also been observed in another study (40). Interestingly, the increase in μ_{max} values at temperatures lower than the T_{opt} is more pronounced than their decrease at the superoptimal range. An inverse pattern was observed for s_{min}. The computed values (Table 3) indicate a slight increase in s_{min} with decreasing temperatures and a steep increase at temperatures higher than the T_{opt}.

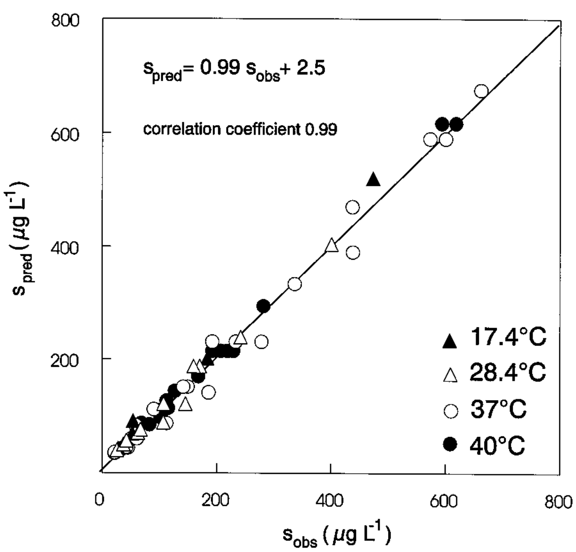

FIG. 4. Correlation between measured and predicted steady-state glucose concentrations by the extended Monod model (equation 2). Line, the calculated regression line for all pairs of s_{obs} and s_{pred} values for the temperatures of 17.4, 28.4, 37, and 40°C.

Modelling substrate concentration as a function of temperature. Glucose steady-state concentration as a function of cultivation temperature was measured for growth of *E. coli* at a constant dilution rate of 0.3 h^{-1} (Fig. 6). The observed parabola-like relationship can be described by equation 11, which is a modification of the Ratkowsky model (equation 10) commonly used to predict the temperature dependency of the maximum specific growth rate. Unfortunately, the original model contains four parameters, T_{min}, T_{max}, c, and b. However, the number of parameters in equation 11 could be reduced to two, assuming that T_{min} and T_{max} are either the cardinal temperatures for growth (predicted by equation 10) or that T_{min} and T_{max} represent the temperature boundaries within which growth is possible at a particular dilution rate. Therefore, the maximum specific growth rate was determined in batch culture at different temperatures, and the bell-shaped curve in Fig. 5 indicates the temperature boundaries within which growth is possible at a particular dilution rate in continuous culture. By using these temperature boundaries, the experimental data at D = 0.3 h^{-1} could be described by an almost symmetrical

TABLE 3. Values of the best-fit parameters of the apparent substrate model[a]

Temp (°C)	K_s (μg liter^{-1})	μ_{max} (h^{-1})	s_{min} (μg liter^{-1})	$\frac{RSS}{(n-p)}$	n
17.4	33.3 ± 4.2	0.185 ± 0.02	22 ± 2	7,503.2	4
28.4	33.3 ± 3.3	0.54 ± 0.01	18 ± 2	302.6	11
37	32.8 ± 3.2	0.76 ± 0.01	12 ± 2	8,122.8	23
40	33.6 ± 1.5	0.74 ± 0.01	64 ± 8	161.8	15

[a] Values were obtained from fitting equation 2 to steady-state glucose concentrations experimentally determined during growth of *E. coli* at the temperatures indicated as a function of the dilution rate.

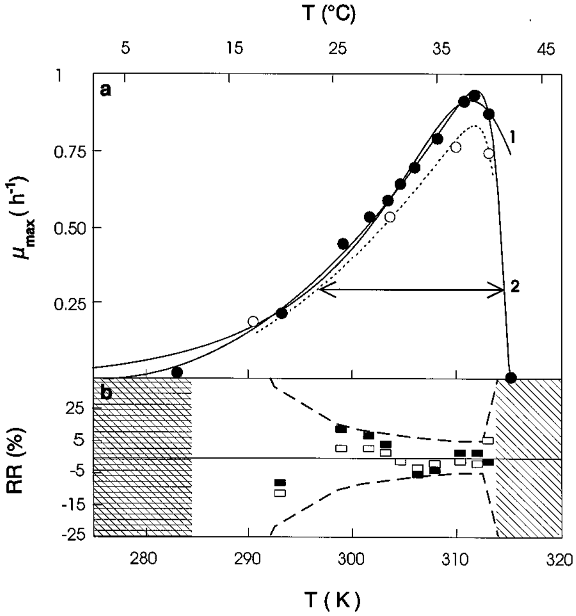

FIG. 5. Temperature dependency of specific growth rate of *E. coli* ML 30 grown in mineral medium with glucose. (a) μ_{max} as a function of temperature. Closed circles, experimental data from batch cultures; open circles, maximum specific growth rates calculated from chemostat data; curve 1, predictions by the Esener model (equation 9); curve 2, predictions by the Ratkowsky model (equation 10); dashed line, predictions of growth rates in continuous culture by the Ratkowsky model; arrow, the temperature boundary within which growth is possible at $D = 0.3$ h^{-1}. (b) Relative residuals (*RR* [equation 14]) for the two fits of batch culture data. Hatched areas, areas in which the model predictions of the Ratkowsky (right area) and the Esener (right and left areas) equations did not hold; open rectangles, relative residuals of the Ratkowsky model; closed rectangles, relative residuals of the Esener model; dashed line, isoline of 0.05 h^{-1} standard deviation of experimental measurements.

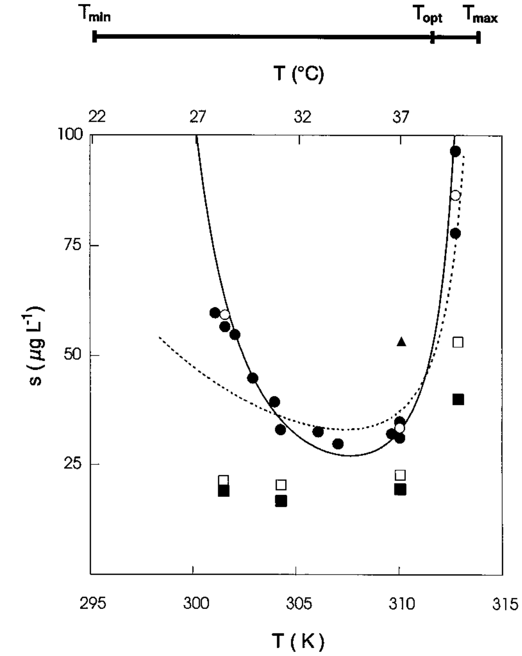

FIG. 6. Temperature dependency of steady-state substrate concentrations at a dilution rate of 0.3 h^{-1}. Closed circles (glucose) and closed triangles (galactose), experimentally determined steady-state sugar concentrations at 37°C when 100 mg $liter^{-1}$ of glucose (or galactose) was supplied in the inflowing medium as the only substrate; open circles, steady-state glucose concentrations predicted by equation 2 for different temperatures; open (closed) squares, steady-state glucose (galactose) concentrations when 1:1 mixture of glucose and galactose was supplied in the inflowing medium; solid line, model equation 11 fitted with T_{max} and T_{min} values, which represent experimental boundaries at $D = 0.3$ h^{-1}; dashed line, model equation 11 fitted with the cardinal temperature values of *E. coli* ML 30 instead of T_{min} and T_{max} parameters.

parabola-like curve (Fig. 6). In contrast, using the cardinal temperatures from Table 2 did not lead to a good prediction of the substrate concentrations at lower temperatures (Fig. 6).

As mentioned above, the temperature range at a particular growth rate within which growth in the chemostat is possible (the experimentally accessible range lies below the bell-shaped curve in Fig. 5a) had to be determined in order to obtain T_{min} and T_{max} for equation 11. The maximum growth rates measured in batch cultures at various temperatures (Fig. 5) were compared with the predictions of the Ratkowsky and the Esener model equations (equations 9 and 10). Neither model described the data well at very low growth rates. Additionally, the Esener model slightly overestimated the maximum growth temperature. Despite these inaccuracies in the extreme low and high temperature ranges, both models allowed a good extrapolation (mean estimation error, less than ±5%) of the experimental data (Fig. 5b). In comparison to the μ_{max} values measured under unrestricted growth conditions in batch cultures, those determined from the kinetic investigation made in glucose-limited chemostat cultures were consistently lower (Fig. 5a). However, the μ_{max} values estimated from continuous culture data were also well described by the Ratkowsky model. In this estimation procedure, the two parameters *b* and *c* were optimized, whereas T_{min} and T_{max} values were set to those obtained from batch culture data (Table 2). From this curve, the minimum and maximum growth temperatures at particular dilution rates were estimated.

When fitting equation 11 [$s = f(T)$], the two regression coefficients (*B* and *C*) were optimized with respect to the experimental data obtained at dilution rates of 0.2, 0.3, and 0.4 h^{-1} and with respect to predicted s_{min} values. Both T_{min} and T_{max} remained fixed during this fitting procedure. From the *B* versus *D* and *C* versus *D* plots (Fig. 7), it can be seen that for the range of 0 $h^{-1} \leq D \leq 0.4$ h^{-1} the dependency of the two parameters on *D* is approximately linear. Hence, steady-state substrate concentrations as a function of temperature and growth rate can be described by replacing these parameters in equation 11 with linear relationships. The resulting model is presented in Fig. 8b. The width of the parabola-like curve is

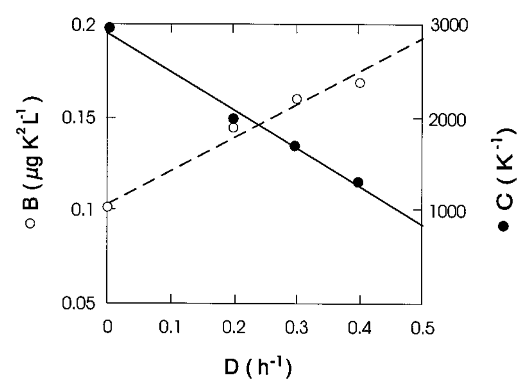

FIG. 7. Relationships between the two regression coefficients *B* and *C* and the dilution rate. *B* and *C* are the best-fit model parameters of the steady-state substrate concentration model (equation 11).

narrower with increasing dilution rates, i.e., the temperature range for growth becomes restricted with increasing dilution rates. Unfortunately, the minimum of this function changed at different growth rates, and this minimum is not always equivalent to the optimum temperature for growth.

Extrapolation of the simple relationships into more complex systems. The fact that relationships exist between model parameters and temperature (Table 3; Fig. 7) is of advantage for developing the complex $s = f(D,T)$ model. In addition, a consistent kinetic description, i.e., using only equation 2 for all temperatures examined, is an advantageous basis for developing additional models, since it is the only equation which accurately described the experimental data at 40°C and, at the same time, the data at lower temperatures. An equation with only two variables (T and D) can be proposed to describe the relationship between steady-state substrate concentration, growth rate/dilution rate, and temperature. However, two different experimental approaches can be used to establish this model. First, D can be varied and s can be measured at constant T, and second, T can be varied and s can be determined at constant D. A three-dimensional projection of the $s = f(D,T)$ relationship was computed by fitting the best surface to the experimental data measured by the first approach (Fig. 8a). The same relationship, but projected into two dimensions, is shown in Fig. 8b, representing data collected by the second experimental approach.

All of the previous results were obtained with a simple model system with glucose as the only carbon and energy substrate for growth. However, it has been shown recently that *E. coli* cells are able to utilize mixtures of sugars simultaneously when cultivated in carbon-limited continuous culture (10). Therefore, steady-state sugar concentrations were measured as a function of temperature in cultures growing with a 1:1 mixture of glucose and galactose. In Fig. 6, it is shown that at a constant growth rate the steady-state concentrations of glucose were reduced when an additional substrate (e.g., galactose) was utilized simultaneously compared with those during growth with glucose only. Except for the lowest temperature tested, steady-state glucose concentrations were reduced to approximately 50%. For galactose, a similar effect was observed, as judged from the results obtained at T = 37°C, where the concentration was reduced from 41 μg liter^{-1} (10) to 19 μg liter^{-1} at D = 0.3 h^{-1}. The data obtained at 28.4°C indicate that the reduction of individual steady-state sugar concentrations during mixed substrate growth might be even more pronounced at lower temperatures. Although this observation has to be confirmed, it might open a promising road to optimizing biodegradation processes in which pollutants have to be removed to low concentration levels.

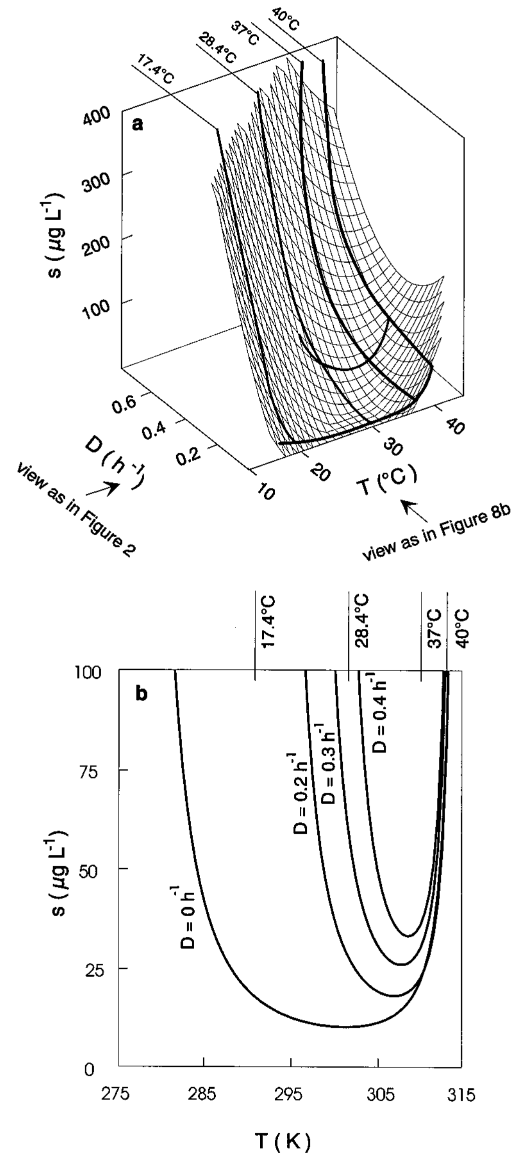

FIG. 8. Steady-state glucose concentrations as a function of temperature and dilution rate for the growth of *E. coli* in glucose-limited continuous culture. (a) Three-dimensional relationship describing $s = f(D,T)$. Thin-lined three-dimensional surface, the best fit to the experimental data; heavy lines, the particular growth kinetics and the $s = f(T)$ relationships at 0 and 0.3 h^{-1} used in model building. (b) The $s = f(D,T)$ relationship projected into the s versus T plane. Lines, isolines of the same dilution rate. Predictions of residual glucose concentrations as function of temperature at dilution rates of 0, 0.2, 0.3, and 0.4 h^{-1} were made by equation 11.

DISCUSSION

What is the physiological meaning of an s_{min} term? The conventional Monod equation (equation 1) did not hold for the description of the experimental data at all temperatures. This discrepancy was solved by using an extended form of the Monod model (equation 2), which predicts a finite substrate concentration at 0 growth rate. It must be pointed out that s_{min} is usually negligible compared with actual steady-state substrate concentrations (i.e., $s >> s_{min}$ and, therefore, $s + s_{min} \cong s$), and no statistically significant difference between the Monod model (equation 1) and the model including an apparent substrate term (equation 2) can be observed, for example, at 37°C. At T_{opt} (and perhaps also at lower temperatures), both models predict the experimental data well and, therefore, the original Monod model can be used when a less complex model is preferred. On the other hand, the data at 40°C cannot be predicted well by any of the alternative models, and, therefore, equation 2, in which the Monod relationship was extended with s_{min}, must be preferred for describing the $s = f(D)$ relationship at all temperatures.

The existence of s_{min} can be justified on the basis of the maintenance energy concept, and the existence of a cellular maintenance energy requirement can be easily explained by thermodynamic reasoning. A consequence of this concept is the existence of a finite concentration or flux of an energy (carbon) substrate at 0 growth rate. In a system open with respect to the supply of substrate, this results in a finite concentration of the energy (carbon) source at $D = 0\ h^{-1}$. For a culture of *E. coli* cells grown with glucose at 30°C, Schulze and Lipe (39) measured a small rate of substrate consumption, even when no growth was observed. This rate, 55 mg of glucose $(h \cdot g\ [dry\ weight])^{-1}$, which represents a specific maintenance rate of 0.0286 h^{-1} when the yield coefficient of 0.52 experimentally determined by these authors is used, was just enough to sustain cellular metabolism, but not enough to allow growth and reproduction. Similarly, Shehata and Marr (42) estimated that 18 μg liter^{-1} (which, for the reported K_s of 68 μg liter^{-1} and $\mu_{max} = 0.78\ h^{-1}$, represents a specific maintenance rate of 0.163 h^{-1}) was the lowest substrate concentration that allowed maintenance of growth of *E. coli* in batch culture at 30°C. Wallace and Holms (47) and Mainzer and Hempfling (19) have also found the maintenance requirements of *E. coli* strains affected by temperature.

To compare the s_{min} values estimated in this study at different temperatures with previously reported maintenance requirements (19, 39, 42, 47), s_{min} values were converted via equation 6 into specific maintenance rates. The resulting specific maintenance rates of 0.074 h^{-1} (17.4°C), 0.191 h^{-1} (28.4°C), 0.204 h^{-1} (37°C), and 0.485 h^{-1} (40°C) are in the same order of magnitude as that estimated by Shehata and Marr (42). All of them are at least 1 order of magnitude higher than the maintenance requirement measured by Schulze and Lipe (39).

Additionally, the s_{min} estimated from the experimental data (12 μg of glucose liter^{-1} at 37°C) can be compared with the threshold substrate concentration of 10.8 μg liter^{-1} computed according to the model proposed by Button (6), using the values of $K_s = 32.8$ μg liter^{-1} and $\mu_{max} = 0.76\ h^{-1}$ obtained in the present study and a rate of endogenous metabolism of 0.25 h^{-1} (reported in reference 6). The very complex model proposed by Schmidt and coworkers (38) predicts a threshold concentration for growth of 2.25 μg liter^{-1} glucose, which is slightly lower than the value of 12 μg liter^{-1} extrapolated from our experimental data. However, it should be stressed that such comparisons have to be made carefully, because many of the model parameters required for calculation of s_{min} in some of the more complex models were not measured in this study but had to be taken from other studies.

The reported threshold (s_{min}) concentrations are difficult to compare, because in each case different experimental systems were used. However, an analysis of the trends exhibited by the data should not be affected by the experimental setup. Interestingly, the trend for the estimates of specific maintenance rates (Fig. 9) from our own data as a function of temperature is comparable with those measured for *E. coli* by Wallace and Holms (47) and also with the effect of temperature on the rate of synthesis of β-galactosidase in *E. coli* (20). A dramatic increase in the specific maintenance rate was also observed in the superoptimal temperature range by Mainzer and Hempfling (19). The dependence of s_{min} on temperature did not exactly follow the pattern shown in Fig. 9. This can be due to the fact that temperature affects the maintenance rate at the level both of s_{min} and of μ_{max} (see equation 6).

Is the substrate saturation constant temperature dependent? Virtually identical K_s values for glucose (Table 3) were obtained from fitting the extended Monod model (equation 2) to the experimental data at 17.4, 28.4, 37, and 40°C. This is in agreement with data already reported by von Meyenburg (46), who found similar values for K_m for a mutant of *E. coli* growing with glucose at 30 and 37°C. However, in the few studies which are available on temperature modulation of saturation constants, both positive (7, 43, 49) and negative (7, 16, 18) modulations of K_s by temperature have been reported. In each of

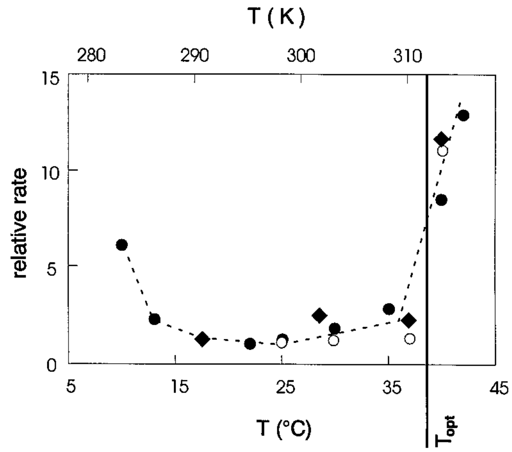

FIG. 9. Comparison of temperature dependence of maintenance rates and rate of synthesis of β-galactosidase in *E. coli* strains. (Rates are relative values standardized with respect to the minimal value.) Symbols: open circles, specific maintenance rates calculated from s_{min} values for *E. coli* growing with glucose (see Table 3, equation 6); diamonds, specific maintenance rates calculated from the experimental data of Wallace and Holms (47) for *E. coli* during growth with glucose; closed circles, differential rates of β-galactosidase synthesis in submaximally induced cryptic strain (20).

these studies, the Arrhenius equation was used to describe the temperature modulation of the K_s or K_m constant (7, 49), since it was assumed to be generally valid for defining the temperature dependency of chemical rate constants (8). However, it has never been confirmed that this thermodynamic concept can be applied to such a complex parameter as the Monod saturation constant.

Considering that even for one particular temperature the steady-state glucose concentrations supporting half-μ_{max} (K_s values) have been reported to vary by more than 3 orders of magnitude (40), one has to question the accuracy of such studies of temperature modulation of growth constants. Some investigations indicate (40, 46) that this enormous variability is due to insufficient adaptation of bacteria to low substrate concentrations. To overcome this, standardization of experiments is necessary in comparative studies. In our experimental study, we used cells fully and reproducibly adapted to growth-limiting concentrations in continuous culture. Interestingly, for *Methanosarcina barkeri* it was shown that the temperature dependency of the affinity constant for molecular hydrogen and acetate was reduced when the substrate concentration was lowered to subsaturating levels, i.e., toward concentrations found in balanced ecosystems (49). These data support the results reported here for *E. coli*. For growth at very low substrate concentrations, Button (6) proposed that the ability of bacteria to grow is better described by a specific affinity term which is defined as the ratio of μ_{max} and K_s. Since saturation constants are temperature independent and μ_{max} varies with temperature, specific affinity will also be temperature dependent.

These observations raise the issue of whether the reported temperature modulation of substrate affinity is an experimental artifact and what is the theoretical background to such behavior. In the appendix, we present a simplified example and suggest possible reasons for a temperature-independent K_s value. The main difference between K_s and the other model parameters (s_{min} and μ_{max}) is that because of the complexity of the Monod saturation constant, both the negative and the positive temperature effects may compensate each other. Despite the clear evidence obtained in our experiments that the K_s value for glucose was temperature independent, it would still be premature to generalize this finding. When comparing with other published results (7, 16, 18, 43, 46, 49), one has to keep in mind several factors such as the differences in experimental setup (batch versus continuous culture cultivation, concentration of the feed, etc.), different methods that were used in determining model parameters (linearization or nonlinear parameter estimation), and the specific limitations inherent in hyperbolic models, namely, that the parameters strongly influence each other.

What are the main factors influencing the steady-state substrate concentrations? In our experience (for comparison, see Fig. 2), steady-state concentrations of the growth-limiting substrate are reproducible under defined conditions in continuous culture. Similarly, in open systems the growth conditions will determine the limit down to which particular compounds can be degraded. Here, specific growth rate, temperature, and composition of the utilized C pool in the feed were shown to be important factors affecting a steady-state concentration of glucose during growth of *E. coli* in the chemostat. Under optimal growth conditions (defined as pH ≅ 7, T ≅ 37°C), the steady-state glucose concentration of 32 ± 4 μg/liter was measured at a dilution rate of 0.3 h^{-1}. This concentration increased when the cultivation temperature was out of the optimum range and at higher growth rates. Vice versa, the steady-state glucose concentration decreased with decreasing growth rates or when the cells were simultaneously utilizing an additional substrate.

We are aware of only two other reports in which parabola-like $s = f(T)$ dependencies similar to those for glucose described in this study were observed, both of them for nitrifiers in batch reactors (7, 18). The trend in steady-state glucose concentrations affected by temperature was sufficiently well described by the reciprocal Ratkowsky model (equation 11). Unfortunately, the minimum steady-state substrate concentration does not coincide with the optimum temperature for growth. At $D = 0.3$ h^{-1}, approximately the same glucose concentrations (i.e., 31 ± 4 μg/liter) are predicted between the range of 31 and 37°C (Fig. 6); however, according to batch data (Fig. 5), one would expect the minimum to be at approximately 38°C. As such, a simple parabolic function would describe the data similarly well. Such models have been proposed for the description of the effect of pH and temperature on the growth rate (36, 51). Fortunately, the reciprocal Ratkowsky model is only a modification of an already accepted model for temperature dependency of bacterial growth. This modification was also successfully applied for the description of the influence of temperature on lag time (50, 53).

Are the concentrations observed in real environments comparable to those of laboratory-scale experiments? In natural waters, threshold concentrations of certain compounds have been observed below which these compounds were not significantly utilized or at which the rate of their degradation slowed down enormously (summarized in reference 2). This phenomenon has been attributed to the fact either that a certain amount of substrate is needed to sustain necessary metabolic functions (i.e., maintenance energy [29]) or that the concentration of a particular compound is too low to provoke induction of the enzymes necessary for its degradation (2, 9). As discussed above, the former phenomenon is based on the kinetic properties of a particular strain. However, the efficiency with which a bacterium takes up substrate is influenced by a variety of environmental factors, an important one being the presence and simultaneous utilization of additional substrates (10). Therefore, one would expect that the concentrations of particular substrates under environmental conditions should be lower than those observed for laboratory-scale experiments in which these compounds are supplied as the only substrates for growth. The s_{min} would, therefore, be the highest expected substrate concentration under conditions of no growth. The fact that the observed maintenance energy requirements of indigenous soil microbial populations were some 3 orders of magnitude lower than those known from pure cultures supports this line of thinking (3, 24). It should be pointed out that such thresholds should not be observed in closed systems such as batch cultures (44), because the maintenance requirement of cells implies continued utilization until all available substrate is exhausted. This might not be the case when, e.g., toxic metabolites are accumulated, or when the culture is limited by an element other than carbon.

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APPENDIX

Some quite contrasting temperature dependencies of K_s have been reported in the literature (7, 46), although to our knowledge, this problem has not yet been treated theoretically. On the basis of the mathematical analogy between K_s and the Michaelis-Menten saturation constant (K_m), we will try here to explain theoretically our experimental data that, for *E. coli* growing with glucose, have demonstrated that K_s was independent of temperature. Analogously to K_m (for its definition, see reference 8), the K_s can be interpreted as a ratio of rate constants of a single enzymatic step (35) that limits the specific growth rate (equation 15):

$$K_s = \frac{(k_j + k_h)}{k_i} \tag{15}$$

For the special case in which all of the individual rate constants exhibit similar temperature dependencies (equations 16a, b, and c and 17), the apparent K_s parameter should also be independent of temperature (equation 18).

When

$$k_i(T_1) = q_1 \times k_i(T_2) \tag{16a}$$

$$k_j(T_1) = q_2 \times k_j(T_2) \tag{16b}$$

$$k_h(T_1) = q_3 \times k_h(T_2) \tag{16c}$$

and

$$q = q_1 = q_2 = q_3 \tag{17}$$

then K_s is independent of temperature (equation 18), i.e., K_s (T_1) and K_s (T_2) are identical:

$$K_s(T_1) = \frac{(k_j + k_h)}{k_i} \text{ and therefore,}$$

$$K_s(T_2) = \frac{q \times k_j + q \times k_h}{q \times k_i} = \frac{q \times (k_j + k_h)}{q \times i_i} = K_s(T_1) \tag{18}$$

Assuming that the rate constants are dependent on temperature according to the Arrhenius equation (equation 19), the parameter q is expressed as shown in equation 20. For the case in which q_1, q_2, and q_3 are identical, this equation implies that the activation energies are also the same:

$$\ln\frac{k(T_1)}{k(T_2)} = \frac{E_a(T_1 - T_2)}{R\,T_1\,T_2} \tag{19}$$

$$q = \exp\left(\frac{E_a(T_1 - T_2)}{R\,T_1\,T_2}\right) \tag{20}$$

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