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Thermodynamic efficiency of bacterial growth calculated from growth yield of *Pseudomonas oxalaticus* OX1 in the chemostat

Michiel Rutgers, Hanneke M.L. van der Gulden and Karel van Dam

Laboratory of Biochemistry and Biotechnology Centre, University of Amsterdam, Amsterdam (The Netherlands)

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In order to determine the thermodynamic efficiency of bacterial growth, *Pseudomonas oxalaticus* OX1 was grown in carbon-limited continuous cultures. 11 different carbon sources, ranging from oxalate (most oxidised component) to ethanol (most reduced component), were used as limiting substrate in these experiments. From the experimental yield values (expressed as C-mol dry weight produced per C-mol carbon substrate consumed) the thermodynamic efficiencies were calculated. On substrates more reduced than biomass (such as ethanol and glycerol) the thermodynamic efficiency of growth of *P. oxalaticus* was negative but it reached a maximum of 23 ± 3% with substrates with a degree of reduction of 3 (citrate) and lower. The actual concentrations of the components involved were incorporated into the calculations but this affected the overall thermodynamic efficiency only to a small extent. This result strengthens the conclusion of Westerhoff et al. (Westerhoff, H.V., Hellingwerf, K.J. and Van Dam, K. (1983) Proc. Natl. Acad. Sci. 80, 305–309) that bacteria have been optimised towards a theoretical thermodynamic efficiency of 24%, corresponding with maximisation of growth rate at optimal efficiency, with highly oxidised substrates.

Introduction

The oldest quantitative data on microbial growth are the so-called yield values [1], which are still used today [2]. Comparison of the theoretical yield, based on known metabolic pathways and the chemical composition of cells, with experimental values revealed large differences [3,4]. Incomplete coupling between anabolism and catabolism of a cell due to maintenance functions, overflow processes and futile cycling was suggested as a possible explanation for the relatively low yield values [3]. However, this explanation although essentially correct, was not very useful because of lack of knowledge about the precise processes involved in this incomplete coupling.

Westerhoff and coworkers [5–7] analysed the energetics of microbial growth in terms of non-equilibrium thermodynamics in order to tackle the problem of the relatively low yield values. They used the equation of thermodynamic efficiency of bacterial growth:

$$\eta = \frac{-J_a}{J_c}\frac{\Delta G_a}{\Delta G_c} \quad (1)$$

This model divides bacterial metabolism in two parts; anabolism (biosynthesis) and catabolism (energy-generating processes), denoted by subscripts a and c, respectively. For example, $-J_a$ is the flow of anabolism and corresponds to the symbol μ used in the microbiological literature as the specific growth rate.

In Eqn. 1, the free-energy production rate ($-J_a \cdot \Delta G_a$, ΔG_a is the free-energy difference of the anabolic reac-

List of symbols:

Symbol	Unit	Meaning
μ_i^0	kJ/mol	standard free energy of compound i
J	h^{-1}	flow or flux
ΔG	kJ/mol	Gibb's free-energy difference
ΔG^0	kJ/mol	standard Gibb's free-energy difference
η	%	thermodynamic efficiency
Y	C-mol/C-mol	yield
Y^{max}	C-mol/C-mol	yield corrected for maintenance
$Y^{0.2}$	C-mol/C-mol	yield at dilution rate 0.2 h^{-1}
μ	h^{-1}	specific growth rate
D	h^{-1}	dilution rate
subscript a		anabolism
subscript c		catabolism
subscript s		substrate

Correspondence: K. van Dam, Laboratory of Biochemistry and Biotechnology Centre, University of Amsterdam, P.O. Box 20151, 1000 HD Amsterdam, The Netherlands.

tions) is divided by the free-energy consumption rate ($J_c \cdot \Delta G_c$).

When all energy generated in catabolism is used for anabolism, the thermodynamic efficiency would be equal to 1 (or 100%). Zero efficiency can be reached when there is either no growth ($-J_a = 0$) or when the free-energy difference between substrates and biomass (ΔG_a) is zero. The thermodynamic efficiency becomes negative when the carbon substrate is considerably more reduced than the anabolic product, biomass ($\Delta G_a < 0$).

The thermodynamic equivalent of Y (expressed in moles carbon) is $-J_a/J_s$. The rate of total substrate consumption (J_s) can be subdivided into J_c and $-J_a$, when the catabolic and anabolic carbon source is the same. In that case Eqn. 1 can be transformed into:

$$\eta = \frac{Y}{1-Y} \frac{\Delta G_a}{\Delta G_c} \quad (2)$$

The $\Delta G_a/\Delta G_c$ ratio can be calculated using standard free energies of substrates, biomass and of catabolic products and taking into account the concentrations of these compounds [5,6]. Consequently, thermodynamic efficiency of bacterial growth can be derived from the experimental yield values. It should be noted that the anabolic reaction is defined in such a way that the carbon of the substrate is completely incorporated in biomass. Thus, all CO_2 production is formally assigned to catabolism [6].

Using compilations of literature data of Linton and Stephenson [8] and of Roels [9], Westerhoff and coworkers [6] calculated thermodynamic efficiencies of bacterial growth of about −80% for growth on reduced substrates (such as methane and methanol) increasing to a maximum of about +23% for growth on highly oxidised carboxylic acids (such as formate and oxalate).

They concluded from a number of theoretical optimisation calculations by Stucki [10] that growth on highly oxidised substrates corresponds to optimisation for maximal rate of biomass synthesis at optimal thermodynamic efficiency (24%), whereas growth on highly reduced substrates may correspond to optimisation only for maximal growth rate and/or maximal yield.

This analysis has provided a framework to explain how actual yield values can fall short of those predicted on the basis of biochemical pathways. However, the conclusions were based on results obtained with different experimental procedures to determine yield values (batch cultures and continuous cultures with and without corrections for maintenance) and with various organisms.

We report in this paper determinations of growth efficiencies of one species, *Pseudomonas oxalaticus* OX1 in a well-defined environment (continuous culture), and include some extensions and refinements of the calculations of free-energy differences of the anabolic and catabolic reactions.

Materials and Methods

Organism

Pseudomonas oxalaticus OX1 was kindly supplied by W. Harder and L. Dijkhuizen (University of Groningen, Groningen, The Netherlands) and was maintained on 0.8% yeast agar and subcultured and stored according to the standard procedures [11].

Growth conditions. The organism was grown in carbon-limited chemostat cultures (500 series fermentors, L & H Engineering Inc., U.K.) with a working volume of 600 ml (±50 ml), at dilution rates (D) from 0.04 h^{-1} up to 0.20 h^{-1}, a temperature of 30° ± 1°C and pH of 7.4 ± 0.1. The cultures were sparged with filtered air (30 ± 4 1/h) and stirred with a magnetic device at 1100 ± 50 r.p.m. Simple salts media as specified by Dijkhuizen et al. [12] were used supplemented with either ethanol (0.05 M), glycerol (0.025 M), fructose (0.01 M), acetic acid (0.05 M), succinic acid (0.03 M), citric acid (0.02 M), glyoxylic acid (0.1 M), sodium potassium tartrate (0.05 M), malonic acid (0.05 M), formic acid/sodium formate (0.2 M) or oxalic acid/potassium oxalate (0.2 M) as carbon and energy sources.

With some oxidised substrates it was rather difficult to start up the culture in the chemostat. Possibly *P. oxalaticus* is sensitive to the high salt concentration needed as neutralising agent for the carboxylic acids (W. Harder, personal communication). Indeed, growth could be initiated by diluting the culture with sterile water.

The pH was monitored constantly and maintained by automatic addition of 4 M NaOH or 1 M H_2SO_4 also containing 1 g/l $CaCl_2$ and 1 g/l $FeCl_2$.

The cultures were checked daily for contamination or phenotypic/genotypic adaptation by transferring culture to oxalate, formate and yeast extract agar plates as described by Quayle and Keech [13].

It was assumed that a steady state was established after five volume changes of the culture liquid.

Determinations. Bacterial dry weight was determined on two 40 ml samples according to the procedure of Herbert et al. [14]. Carbon dioxide production was measured with an IR gas analyser (Servomex PA404).

Concentrations of limiting substrates in culture fluid and media were determined by HPLC chromatography using a Biorad Aminex ion exclusion HPX 87 H column, a refractometer (Knauer, Berlin) and a Spectraphysics SP 4270 integrator (CA, U.S.A.). Oxalate was determined using the volumetric method described by Vogel [15].

Calculations. Yield values were derived from bacterial dry-weight determinations divided by the amount of carbon substrate used. The concentration of the limiting substrate in the culture was never more than 0.1% of the input. Plots of $1/D$ versus $1/Y$ revealed straight lines in accordance with the model of Pirt [16]. These plots

were used to calculate extrapolated yield values and mean deviations belonging to the highest experimental dilution rate of 0.2 h^{-1} ($Y^{0.2}$).

ΔG_a and ΔG_c were calculated according to the procedure described by Westerhoff et al. [5], by writing down the complete anabolic and catabolic reactions and summing up all free-energy differences multiplied by the stoichiometry factor of the components involved.

Results and Discussion

The strictly aerobic methylotroph *Pseudomonas oxalaticus* OX1 is able to grow on a wide range of highly oxidised carboxylic acids such as oxalate, formate and citrate but also on the more reduced components glycerol, fructose and ethanol as single carbon and energy source [17]. Harder, Dijkhuizen and Wiersma [11,12,18] already performed some continuous culture experiments with *P. oxalaticus*. For these reasons, *P. oxalaticus* seemed a very attractive organism to study thermodynamic efficiency of bacterial growth.

In Table I, results are presented of continuous culture experiments with 11 different carbon sources as the limiting substrate. In this study the most oxidised component was oxalate and the most reduced component was ethanol with degrees of reduction of 1 and 6, respectively (as defined in Ref. 9). The dilution rate was varied from 0.04 to 0.20 h^{-1}. Ammonia was the nitrogen source. 5–10 steady states of each growth limitation were analysed for dry weight (ranging from 0.6 to 1.4 g/l), carbon recovery (between 83 and 115%) and carbon dioxide production rates (up to 70 mmol CO_2/h per g dry weight). The dry weights of oxalate- and formate-limited cultures of *P. oxalaticus* reported by Dijkhuizen et al. [12] were slightly lower than the values reported here, but probably within the experimental error.

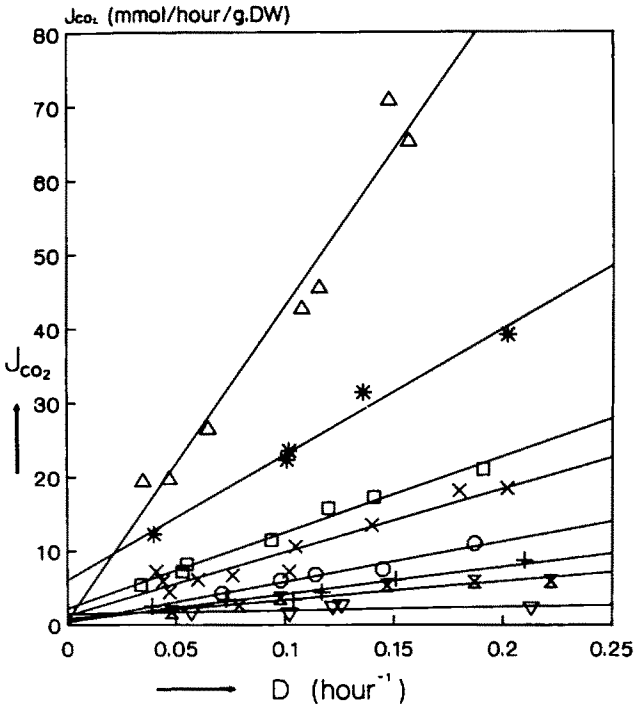

Fig. 1. Carbon dioxide production rates of cultures of *P. oxalaticus* as a function of the dilution rate for eight different carbon sources: △, oxalate; *, formate; □, glyoxylate; ×, tartrate; ○, citrate; +, acetate; ⧖, fructose; and ▽, ethanol.

During oxalate-limited growth the cultures showed some decrease in dry weight at dilution rates of 0.15 h^{-1} and higher and complete wash out was reached at 0.20 h^{-1}. Growth inhibitory effects of increasing oxalate and bicarbonate concentrations in the culture or lack of oxygen in our experimental set up at higher dilution rates could account for this phenomenon [11]. This is most prominent for oxalate-limited growth because these cultures produced the largest amount of carbon dioxide (see Fig. 1) and concomitantly showed the highest oxygen-consumption rates.

TABLE I

The thermodynamic efficiencies of growth (η) of P. oxalaticus on substrates with different degree of reduction

The values were derived from the standard chemical potential of the substrate (μ_i^0), the $\Delta G_a/\Delta G_c$ ratio and the experimental $Y^{0.2}$ (in C-mol/C-mol) according to the calculation method described in the text. n is the number of steady states.

Substrate	Degree of reduction	μ_i^0 (kJ/mol)	$\Delta G_a^0/\Delta G_c^0$	$\Delta G_a/\Delta G_c$	$Y^{0.2}$ (C-mol/C-mol)	n	η (%)
Oxalate	1	−676	2.62	2.57	0.086±0.003	7	24±1
Formate	2	−335	0.87	0.99	0.162±0.050	5	19±7
Glyoxylate	2	−459	0.86	0.89	0.220±0.016	6	25±3
Tartrate	2.5	−1010	0.60	0.57	0.280±0.001	10	22±1
Malonate	2.7	−700	0.64	0.63	0.238±0.041	7	20±5
Citrate	3	−1170	0.38	0.39	0.390±0.011	6	25±1
Succinate	3.5	−691	0.26	0.25	0.385±0.014	4	16±1
Acetate	4	−373	0.12	0.14	0.406±0.006	6	10±1
Fructose	4	−917	0.00	−0.02	0.505±0.049	5	−3±1
Glycerol	4.7	−489	−0.13	−0.13	0.569±0.019	5	−17±2
Ethanol	6	−182	−0.28	−0.28	0.558±0.013	5	−35±1

In all cases, linear relationships were found between the reciprocal of the yield ($1/Y$) and the reciprocal of the dilution rate ($1/D$) as predicted by the model of Pirt [16]. These plots were used to calculate extrapolated yield values and standard deviations belonging to a growth rate of 0.20 h^{-1} ($Y^{0.2}$). The results of these yield calculations are presented in Table I.

The rationale for this procedure needs some clarification. The $1/Y$-versus-$1/D$ plots are generally used to calculate a yield value (Y^{max}) corrected for maintenance via extrapolation to infinite growth rate. However, Y^{max} is a purely mathematical constant [2], while the thermodynamic model intends to describe actual growth behaviour of microbes [5]. Extrapolation of the yield to 0.20 h^{-1} produces the highest possible actual yield values and by doing so maximal obtainable thermodynamic efficiencies of growth. The lower experimental growth rates were of use to inspect the growth curve for unexpected phenomena such as substrate or product inhibition, and for calculating standard deviations of $Y^{0.2}$.

Nevertheless, the differences in $Y^{0.2}$ and Y^{max} remained fairly small relative to the total magnitude of the Y values, as a result of relatively constant dry weight and, consequently, nearly horizontal $1/D$-versus-$1/Y$ plots. Also the extrapolated HCO_3^- production rates at zero growth rate in Fig. 1 suggest a low maintenance requirement of carbon-limited *P. oxalaticus*. Differences in these intercepts roughly correlate with the degree of reduction of the substrates. However, small differences in ATP requirement at zero growth rate are to be expected, e.g., as a consequence of differences in transport energetics.

The degree of reduction and the standard chemical potential (μ_i^0; not to be confused with μ, the growth rate) of the substrates are presented in Table I. For some compounds (glyoxylic, malonic and tartaric acid) we could not find the data of the standard potential in the literature [19–21]. For example, the μ_i^0 of tartrate had to be estimated from the standard enthalpy and from extrapolations of μ_i^0 of malic and succinic acid.

Using a biomass composition of $CH_{1.8}O_{0.5}N_{0.2}$, as given by Roels [9] and standard free energies of substrates in Table I and of biomass (−67.1 kJ/C-mol [9]) we calculated the $\Delta G_a^0/\Delta G_c^0$ ratio for each substrate. This ratio refers to the standard-state condition, i.e., 1 M aqueous solutions. The actually encountered concentrations are considerably lower, especially for the growth-limiting substrate: 5 or 6 orders of magnitudes (1 or 10 μM) lower than the standard state condition is not uncommon [22–25]. This might be expected to contribute significantly to the value of ΔG_a and ΔG_c and therefore it was not neglected (as in Refs. 5, 6 and 9).

Except for the growth-limiting substrate, which was in general below detection level in HPLC analysis (less than 0.1 mM), the physiological concentrations of all compounds are known; biomass concentration is approx. 10 mM, $[HCO_3^-] \approx 1$ mM, $[NH_4^+] \approx 20$ mM, $[O_2] \approx 0.2$ mM and $[H^+] \approx 0.1$ μM. Only growth-limiting concentrations of oxalate (approx. 0.1 mM), formate (approx. 0.2 mM) and glyoxylate (approx. 0.1 mM) at higher growth rates (0.10–0.20 h^{-1}) could be detected.

For sake of simplicity we assumed 10 μM as a good approximation for the concentration of the other growth-limiting substrates [22–25] and together with the above values we calculated the $\Delta G_a/\Delta G_c$ of each substrate limitation. This is also presented in Table I.

The effect of incorporation of actual concentrations of metabolites in the calculations with respect to $\Delta G_a^0/\Delta G_c^0$ in the standard state condition was rather small (except for the formate-limited cultures, see Table I) although not totally negligible (contrast Refs. 5, 6 and 9). Changing the concentration of the limiting substrate by a factor of 10 (1 μM or 100 μM) affects the thermodynamic efficiency by maximally 1%. The concentrations of formate contributes significantly to $\Delta G_a/\Delta G_c$ probably because it is a C-1 compound.

Table I further shows the thermodynamic efficiencies calculated according to Eqn. 2. Because the degree of reduction of biomass is about 4.2, negative thermodynamic efficiencies must be expected when the substrate is more reduced than biomass (downhill process).

In order to evaluate all determined efficiency values of *P. oxalaticus* we made a redraw of the plot of Westerhoff et al. [6] in Fig. 2. For substrates with a reduction level of 3 (citrate) and lower the thermodynamic efficiency of *P. oxalaticus* tends to reach a constant level of 23 ± 3% corresponding closely to the theoretical value of 24% for maximal rate of biomass synthesis at optimal efficiency [10].

The other levels, represented by the dashed lines in Fig. 2, correspond to optimisation for power production (41%), economic biomass synthesis (54%) and economic power production (62%) [6,10].

Negative thermodynamic efficiencies are encountered for growth on highly reduced substrates such as glycerol and ethanol. This is consistent with maximisation towards biosynthesis rate only. Energetic efficiency seems to lose its relevance with reduced substrates and this is in agreement with the idea that in this case lack of carbon rather than free energy becomes the growth-limiting factor [8].

These results with one organism in a well-defined environment fit nicely with the results obtained with many different organisms and conditions as compiled by Westerhoff et al. [5,6]. Thus within one organism maximisation has occurred towards growth rate keeping efficiency as high as possible, as the principle for optimisation.

Westerhoff et al. [5,6] indicated that high thermodynamic efficiencies might be expected when anabolism

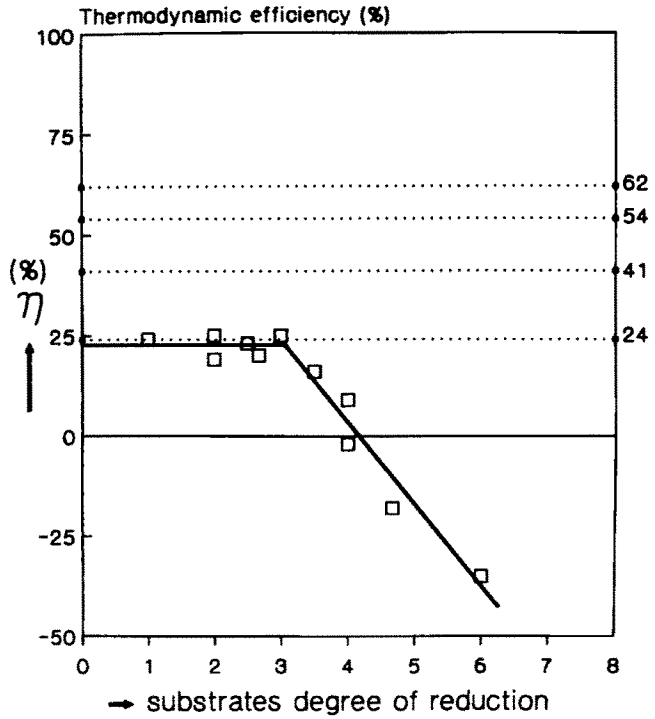

Fig. 2. Thermodynamic efficiencies of growth of *P. oxalaticus* as a function of the degree of reduction of the carbon substrate. Dotted lines represent theoretical optimisation levels (see text and Refs. 6 and 10).

proceeds via the ribulose biphosphate carboxylase pathway after conversion of the carbon source to carbon dioxide. *Paracoccus denitrificans* is unable to use methanol directly for anabolism and assimilates the oxidation product carbon dioxide [26]. Taking the conversion of CO_2 to biomass as the anabolic reaction and after a slight modification of the calculation [5], the thermodynamic efficiency is as high as 33% for methanol-limited cultures of *P. denitrificans*. Almost the same situation appears to exist for formate-limited cultures of *P. oxalaticus*. Also in this case carbon is assimilated via the ribulose bisphosphate carboxylase pathway after oxidation of formate to carbon dioxide [12]. The earlier calculated thermodynamic efficiency of formate-limited *P. oxalaticus* is 19% but it is increased to 33% after redefinition of the anabolic reaction.

This raises a question as to the definition of the anabolic reaction with some substrates, especially when a more oxidised compound is substrate or intermediate in the anabolic metabolism. In these cases, one has to choose between formal production of either CO_2 or O_2 or uptake of H_2 in anabolism. Following Westerhoff et al. [6], we have chosen the second possibility. Possibly autotrophic metabolism in itself behaves different with respect to the optimisation principles as outlined above. Also the few data [27] of carbon monoxide-oxidising bacteria indicate a high thermodynamic efficiency of about 35%.

Another exception to the maximum of 24% may exist in *Bacillus licheniformis*. This organism is able to excrete extracellular proteases in amounts that may consume up to 25% of the total ATP production [28]. When the maintainance and the excreted protein are incorporated in the calculation, we found a thermodynamic efficiency of 28% for this organism during citrate-limited aerobic growth.

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