

Supplementary material 1

Boltzmann statistics are underlying microbial growth

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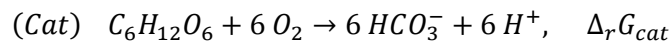
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17 **Exergy balance for an elementary microbial division**

18 Gibbs energy balance of growth can be estimated using different methods summed up in [1]. The
19 notations of this paper were slightly modified in order to use the meaningful concept of exergy,
20 which represents the maximum work available for a microorganism during a chemical
21 transformation. For more clarity, let us consider *E. coli* growing aerobically on glucose as a case
22 study. We use the Gibbs energy dissipation method initially proposed by Heijnen to determine the
23 growth stoichiometry.

24 The catabolism for aerobic growth on glucose is associated with a Gibbs energy variation, denoted
25 $\Delta_r G_{cat}$:

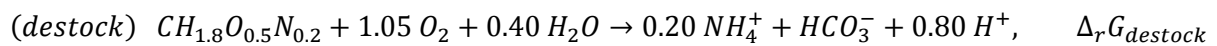


26 Thus, catabolic exergy (chemical energy available for the microbe) is:

$$27 \quad E_{cat} = -\Delta_r G_{cat} \text{ if } \Delta_r G_{cat} < 0$$

$$28 \quad E_{cat} = 0 \text{ otherwise}$$

29 Let us then define microbial exergy E_M as the chemical energy available in a C-mol of microbe (or in a
30 microbe), in other words the energy a microbe could virtually obtain by consuming its own biomass.
31 It should be noted that E_M depends on the type of microbial metabolism: for a given cell
32 composition, the exergy of cell constituents degraded through aerobic respiration is greater than the
33 exergy of the same constituents degraded into methane and carbon dioxide. For an aerobic microbe,
34 biomass oxidation can be written as follows:



35 Thus, microbial exergy is:

$$36 \quad E_M = -\Delta_r G_{destock} \text{ if } \Delta_r G_{destock} < 0$$

$$37 \quad E_M = 0 \text{ otherwise}$$

38 $CH_{1.8}O_{0.5}N_{0.2}$ represents a generic elemental composition of one C-mole of biomass. The standard
39 Gibbs energy of formation of all constituents is known, including the Gibbs energy of formation of
40 one C-mol of biomass [1]. E_{cat} and E_M can thus be calculated from tables. The standard Gibbs
41 energies for (cat) and (destock) corrected for pH=7, denoted $\Delta_r G^{01}$, are:

$$\Delta_r G_{cat}^{01} = -2852 \text{ kJ/mol}$$

$$\Delta_r G_{destock}^{01} = -474 \text{ kJ/C - mol}$$

42 The metabolic equation of growth of *E. coli* on glucose is a linear combination of equations
43 (destock) and (cat).

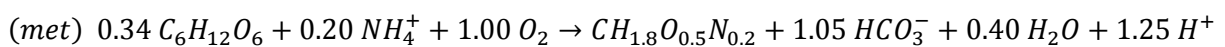
$$(met) = v(cat) - (destock), \quad \Delta_r G_{met}$$

44 The metabolic exergy dissipated during growth ($E_{dis} = -\Delta_r G_{met}$) is equal to the exergy of the ν
 45 substrates molecules consumed during the synthesis of one C-mol of biomass (or of one cell) minus
 46 exergy stored in the biomass.

47 Exergy balance thus enable to calculate the factor ν [1]. Using the Gibbs energy dissipation method,
 48 $\Delta_r G_{met}$ can be calculated for one C-mol of biomass knowing the number of carbon atoms in a
 49 substrate molecule and its oxidation degree [1]. For simplicity, it is possible to consider $E_{dis} =$
 50 $500 \text{ kJ/C} - \text{mol}$ as a good approximation of dissipated exergy [2]. The factor ν can then be obtained:

$$\nu = \frac{E_M + E_{dis}}{E_{cat}}$$

51 Thus for *E. coli* growing on glucose in standard conditions corrected for pH=7, $\nu = 0.34$, leading to
 52 the following stoichiometric metabolic growth equation:



53

54 Boltzmann distribution of activated microbes

55 Let us first consider the system constituted by a 3D physical space in which a clonal population of N
 56 microbes is consuming a total pool of k molecules of substrate S and dividing. Each individual
 57 microbe has access to a volume (V_{harv}) in which it can harvest the substrate. The n elementary
 58 volumes ($n = V_{tot}/V_{harv}$) constituting the 3D space define the statistical units of our model. Then,
 59 let us also consider a microstate consisting of a specific distribution of substrate molecules in the
 60 different statistical units. Such a microstate can be viewed as a part of a microcanonical ensemble as
 61 defined in the framework of statistical physics. Let us assume that the total volume accessible to the
 62 microbes ($N \cdot V_{harv}$) is small compared to the rest of the system. Thus, three systems can be defined:

- 63 - Σ the system constituted by the 3D physical space, with $\Omega(n, k)$ its number of microstates.
- 64 - Σ' the subsystem constituted by the N statistical units containing microbes (representing the
 65 volume accessible to the microbes).
- 66 - R the reservoir, $R = \Sigma - \Sigma'$.

67 Therefore, the microstates consisting of distributions of substrate molecules in the statistical units
 68 occupied by microbes (Σ') constitute a canonical ensemble. Consequently, the probability for a
 69 microbe to have i_0 molecules in its harvesting volume follows a Boltzmann distribution:

$$p(i_0) = \frac{1}{Z} \exp(-\beta \cdot i_0)$$

70 with $Z = \sum_{i=0}^k \exp(-\beta \cdot i)$ the partition function and $\beta = \frac{\partial \ln(\Omega(n,k))}{\partial k}$ by definition.

71 β can be inferred from the expression of $\Omega(n, k)$, which corresponds to the number of ways of
 72 distributing k indistinguishable objects in n boxes:

$$\Omega(n, k) = C_{n+k-1}^k = \frac{(n+k-1)!}{k!(n-1)!}$$

73 Using Stirling's formula, $\ln(\Omega(n, k))$ can be expressed as a function of n and k :

$$\ln(\Omega(n, k)) \approx n \cdot \ln\left(\frac{n+k}{n}\right) + k \cdot \ln\left(\frac{n+k}{k}\right)$$

74 then

$$\beta = \frac{\partial}{\partial k} \left(n \cdot \ln\left(\frac{n+k}{n}\right) + k \cdot \ln\left(\frac{n+k}{k}\right) \right) = \ln\left(1 + \frac{n}{k}\right)$$

75 In the case for which $k \gg n$ (the number of molecules is large compared to the number of statistical
76 units, cf. discussion below) we obtain:

$$\beta \approx \frac{n}{k}$$

77 Knowing that $n = \frac{V_{tot}}{V_{harv}}$ and $k = V_{tot} \cdot [S]$, it leads:

$$\beta = \frac{1}{V_{harv} \cdot [S]}$$

78 The proportion of activated microbes (N^\ddagger/N) is then given by the probability of finding occupied
79 elementary volumes with more than ν substrates molecules:

$$\frac{N^\ddagger}{N} = \sum_{i=\nu}^k p(i) = \exp(-\beta \cdot \nu) \cdot \frac{1}{Z} \sum_{i=0}^{k-\nu} \exp(-\beta \cdot i)$$

80 Finally, since $\nu \ll k$ (the number of molecules required for one division is small compared to the
81 total number of molecules in the system):

$$\frac{N^\ddagger}{N} = \exp(-\beta \cdot \nu)$$

82 In the case of energy limited growth, we previously established that:

$$\nu = \frac{E_M + E_{dis}}{E_{cat}}$$

83 Thus :

$$\frac{N^\ddagger}{N} = \exp\left(-\frac{E_M + E_{dis}}{V_{harv} \cdot [S] \cdot E_{cat}}\right)$$

84

85 **Generalization of our law to multiple substrates and to the case of elemental growth limitation**

86 We can now generalize this equation to multiple substrates $\{S_i\}$. If the different substrates
87 distributions are independent, the joint probability is then the product of Boltzmann distributions for
88 the different substrates and the proportion of activated microbes is given by:

$$\frac{N^\ddagger}{N} = \prod_i \exp(-\beta_i \cdot \nu_i)$$

89 with

$$90 \quad \beta_i = \frac{1}{V_{harv} [S_i]}$$

91 In the case of elemental limitation of growth, the coefficient ν represents the stoichiometric
 92 coefficient of the molecule supplying the limiting element. ν can thus be directly obtained from the
 93 elemental composition of biomass ($CH_{1.8}O_{0.5}N_{0.2}$). Indeed if nitrogen is limiting and ammonium ions
 94 are the nitrogen source: $\nu = 0.2 \frac{mol NH_4^+}{C-mol biomass}$.

95 A more detailed chemical composition of biomass [3] can be used in order to model microbial growth
 96 under various types of elemental limitation (P, S, K, Mg, Ca,...) applying the same principle to obtain
 97 ν .

98

99 **Range of values commonly encountered in practical growth situations and validity of our**
 100 **approximations**

101 The demonstration of Boltzmann distribution of activated microbes requires two main
 102 approximations:

- 103 • First, in order to use the canonical ensemble formalism and to derive the statistical
 104 distribution of molecules in elementary units, the volume not accessible to the microbes
 105 should constitute a reservoir of molecules. In practice, this situation is fulfilled when the
 106 volume of Σ' is a small fraction of the volume of Σ ($N \cdot V_{harv} \ll V_{tot}$). In environmental
 107 conditions such as seawater, bacterial densities commonly encountered range around
 108 10^6 cells/ml [4]. In these conditions, one cell is, on the average, surrounded by $10^6 \mu m^3$ of
 109 medium. In these cases, a harvesting volume (V_{harv}) up to $10^5 \mu m^3$ assures a ratio less than
 110 1/10 between occupied and non-occupied volumes. This harvesting volume is consistent with
 111 the volume estimated from Figure 1b (fitted $V_{harv} \approx 1 \cdot 10^5 \mu m^3$ see sup. mat. 2). Our first
 112 hypothesis is therefore justified in many environmental conditions. However, in high density
 113 cultures, such as those obtained in laboratory conditions, *E. coli* can grow up to a density of
 114 10^9 cells/ml. In these cases, the free volume around each cell is only $10^3 \mu m^3$ on the average
 115 and the harvesting volume should be less than $100 \mu m^3$ to assure a ratio 1/10 between
 116 occupied and non-occupied volumes. Our model might therefore reach the limits of its
 117 validity domain under such high density conditions. However, as growth is exponential, it
 118 would still be applicable for most of the bacterial generations before reaching the saturation
 119 plateau, and thus for the major part of the growth curve. The development of an equation
 120 applicable to such high population densities would require further theoretical developments
 121 by considering a fully microcanonical ensemble without the canonical approximation, which
 122 is beyond the scope of the current paper.

123 • The second approximation stipulates that the total number of statistical units n is negligible
124 with respect to the number k of molecules in the media ($k \gg n$). The fraction n/k can be
125 expressed as a function of substrate concentration and harvesting volume (cf. above):

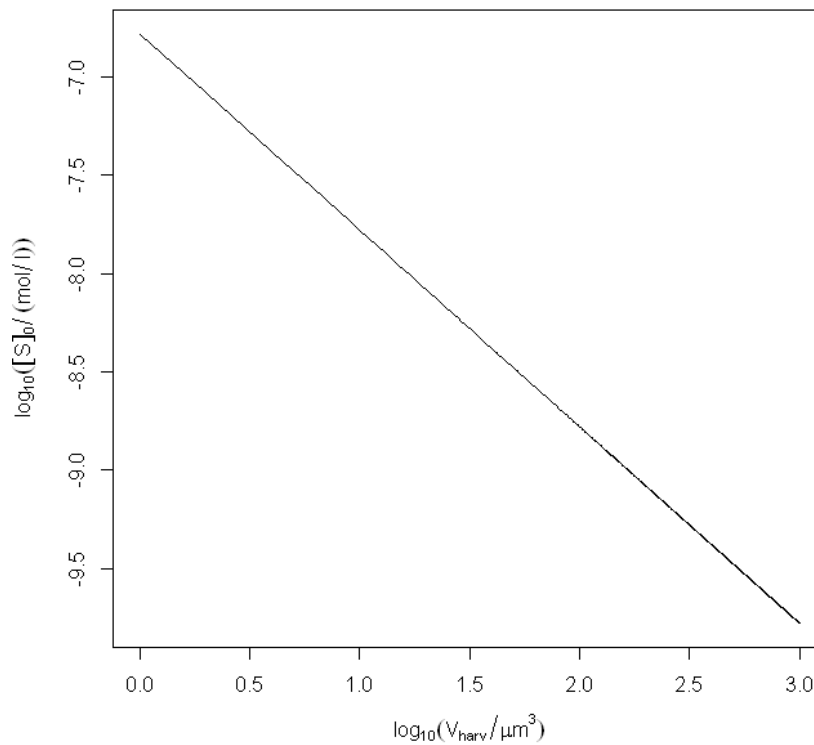
$$\frac{n}{k} = \frac{1}{V_{harv} \cdot [S] \cdot N_a}$$

126 In this case, to allow practical evaluations, $[S]$ is expressed in mol/l and N_a (Avogadro
127 number) is therefore introduced in the expression.

128 If we take $\frac{k}{n} = 100$ as a threshold ratio, we can compute a corresponding limit concentration:

$$[S]_0 = \frac{100}{V_{harv} \cdot N_a}$$

129 The hypothesis ($k > 100 \cdot n$) is then fulfilled if $[S] > [S]_0$. In order to estimate the
130 robustness of this hypothesis, let us plot $\log([S]_0)$ as a function of $\log(V_{harv})$:



131

132 In the least favourable cases, corresponding to small harvesting volumes ($1 \mu\text{m}^3$ to $10^3 \mu\text{m}^3$),
133 threshold concentrations are still very low ($[S]_0 < 1 \mu\text{mol}/l$). The second hypothesis is
134 therefore not limiting the applicability of the model.

135

136

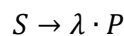
137 μ_{max}

138 μ_{max} is introduced as a parameter of Equation 3 that represents the probability for an activated
139 microbe to divide by time unit. However, it is also fundamentally related to the distribution of
140 catabolic exergy around the microbes. Its literal expression requires another important theoretical
141 development that is underway.

142

143 **The microbial isotopic fractionation phenomenon as viewed from our theory**

144 Let us consider the biologic fraction due to the following reaction catalyzed by microbes:



145 with S the substrate, P the product and λ a stoichiometric coefficient. Substrate and product with a
146 heavy isotope will be denoted S^h and P^h , substrate and product with a light isotope will be denoted
147 S^l and P^l .

148 The kinetic fractionation factor $\alpha_{S/P}$ of substrate towards product (sometimes $\alpha_{P/S} = 1/\alpha_{S/P}$ is used
149 instead) [5] of the reaction is:

$$\alpha_{S/P} = \frac{\frac{[S^h]}{[S^l]}}{\frac{\partial[P^h]}{\partial[P^l]}}$$

150 With $\delta[P^l] = -\lambda \cdot \delta[S^l]$ and $\delta[P^h] = -\lambda \cdot \delta[S^h]$, let us rewrite $\alpha_{S/P}$:

$$\alpha_{S/P} = \frac{\frac{\partial[S^l]}{[S^l]}}{\frac{\partial[S^h]}{[S^h]}}$$

151 With our theory, kinetic isotopic fractionation can be explained by the difference between exergy of
152 molecules containing light or heavy isotopes. Indeed the harvesting rates r^h and r^l for a microbe
153 consuming only heavy or only light substrate are given by:

$$r^h = -v \cdot [M] \cdot \mu^h = -v \cdot [M] \cdot \mu_{max}^h \cdot \exp\left(-\frac{E_M + E_{dis}}{V_{harv} \cdot [S] \cdot E_{cat}^h}\right)$$
$$r^l = -v \cdot [M] \cdot \mu^l = -v \cdot [M] \cdot \mu_{max}^l \cdot \exp\left(-\frac{E_M + E_{dis}}{V_{harv} \cdot [S] \cdot E_{cat}^l}\right)$$

154 μ_{max}^h and μ_{max}^l are the maximal growth rates for heavy and light substrate respectively.

155 E_{cat}^h and E_{cat}^l represents the catabolic exergy for the catabolic reaction with heavy or light substrates
156 respectively (see thermodynamic properties of isotopomers below).

157 In practice, the microbe is always consuming a mixture of light and heavy molecules. Consumption
 158 rates of heavy and light substrates can be expressed in respect to the relative proportion of light
 159 ($[S^l]/[S]$) and heavy ($[S^h]/[S]$) molecules:

$$\frac{\partial[S^h]}{\partial t} = \frac{[S^h]}{[S]} \cdot r^h$$

$$\frac{\partial[S^l]}{\partial t} = \frac{[S^l]}{[S]} \cdot r^l$$

160 Let us then calculate the kinetic fractionation factor:

$$\alpha_{S/P} = \frac{\frac{\partial[S^l]}{[S^l]}}{\frac{\partial[S^h]}{[S^h]}} = \frac{r^l}{r^h} = \frac{\mu_{max}^l}{\mu_{max}^h} \cdot \exp\left(-\frac{E_M + E_{dis}}{V_{harv} \cdot [S]} \left(\frac{1}{E_{cat}^l} - \frac{1}{E_{cat}^h}\right)\right)$$

$$\Rightarrow \alpha_{S/P} = \frac{\mu_{max}^l}{\mu_{max}^h} \cdot \exp\left(-\frac{E_M + E_{dis}}{V_{harv} \cdot [S] \cdot (E_{cat}^h \cdot E_{cat}^l)} (E_{cat}^h - E_{cat}^l)\right)$$

161 Considering that $E_{cat}^h \approx E_{cat}^l$ we have $E_{cat}^h \cdot E_{cat}^l \approx E_{cat}^2$

162 Let us denote $\Delta E_{cat}^{h-l} = E_{cat}^h - E_{cat}^l$

$$\Delta E_{cat}^{h-l} = (\Delta G^{l0} + RT \cdot \log(\Pi)) - (\Delta G^{h0} + RT \cdot \log(\Pi)) = \Delta G^{l0} - \Delta G^{h0}$$

163 This difference is independent from chemical concentrations. It only depends on intrinsic
 164 characteristics of the molecules considered, i.e. the stabilization of the electronic bonds by the
 165 isotopes within the molecules as predicted from quantum theory [6]. Then comes the expression:

$$\alpha_{S/P} = \alpha_0 \cdot \exp\left(-\frac{E_M + E_{dis}}{V_{harv} \cdot [S_{lim}] \cdot E_{cat}^2} \Delta E_{cat}^{h-l}\right)$$

166 With S_{lim} the limiting substrate and $\alpha_0 = \frac{\mu_{max}^h}{\mu_{max}^l}$.

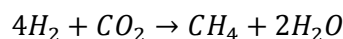
167 Contrary to the classical derivation of a constant kinetic fractionation factor [5] this expression
 168 predicts the dependency of the fractionation to exergy and substrate concentration as recently
 169 evidenced in various experimental reports [7-10].

170

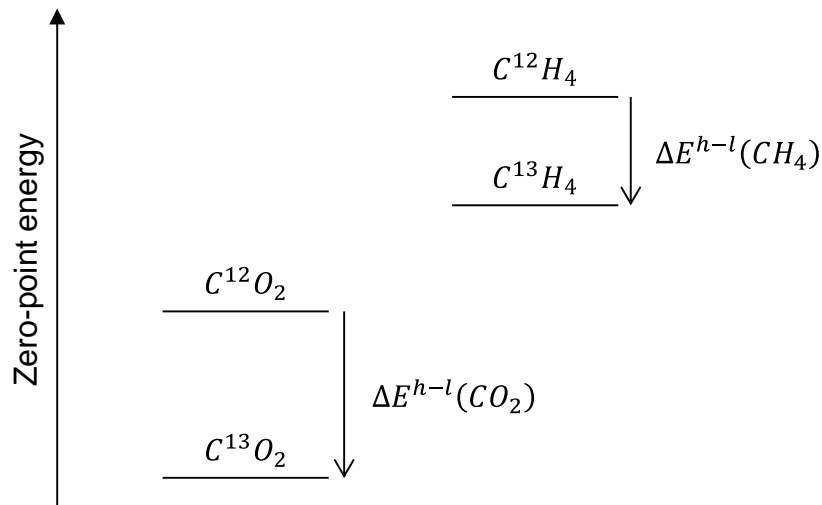
171 Thermodynamic properties of isotopomers

172 Different isotopomers have different energy levels [6]. Indeed a molecule containing a heavy isotope
 173 has stronger bonds than a molecule with a light isotope.

174 Let us consider hydrogenotrophic methanogenesis:



175 Putative zero point energies for light and heavy carbonate and methane are shown on the graph
 176 below:



177

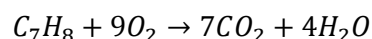
178 Covalent bonds are stabilized by heavy atoms thus exergies of heavy molecules are lower than
 179 exergies of light molecules. In addition C-O bonds are relatively more stabilized by C¹³ than C-H bonds
 180 (Zeebe and Wolf-Gladrow 2001), thus heavy carbonate is relatively more stabilized than heavy
 181 methane:

$$\Delta E_{cat}^{h-l} = \Delta E^{h-l}(CH_4) - \Delta E^{h-l}(CO_2) < 0$$

182 This calculation is consistent with known carbon equilibrium fractionation for the system CO₂-CH₄
 183 over a wide range of temperature [11]. Together with Equation 4, this simple argument thus predicts
 184 overfractionation for hydrogenotrophic methanogenesis as observed in the experiments of [9] and
 185 [10] (cf. figure 2b).

186

187 If we now consider aerobic degradation of toluene:



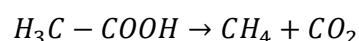
188 Bigeleisen reports that equilibrium fractionation factor of protium-deuterium between toluene and
 189 water is smaller than one [12]. Thus water is relatively more stabilized by deuterium than toluene. It
 190 is consistent with a simple rule established by Bigeleisen: "The heavy isotope goes preferentially to
 191 the chemical compound in which the element is bound most strongly." [12]. Thus:

$$\Delta E_{cat}^{h-l} = \Delta E^{h-l}(H_2O) - \Delta E^{h-l}(C_7H_8) > 0$$

192 Again, the sign of this inequality together with Equation 4 implies microbial underfractionation which
 193 is indeed supported by previous experimental evidences [8] (cf. figure 2c).

194

195 For acetoclastic methanogenesis:



196 Evaluating the isotope stabilization effect on a polyatomic molecule such as acetate is a complicated
197 task because of the existence of multiple vibrational and rotational modes. Moreover, experimental
198 data on equilibrium fractionation of ^{13}C between acetate and methane/carbon dioxide had never
199 been reported to our knowledge, precluding any theoretical prediction about the nature of the
200 aceticlastic microbial fractionation phenomenon. However, if our theory is correct, the microbial
201 underfractionation phenomenon deduced from [7] (cf. figure 2d) conversely implies that ΔE_{cat}^{h-l} of
202 the catabolic microbial reaction is positive. It thus suggests that ^{13}C stabilizes more methane than
203 acetate.

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