Adv Biochem Engin/Biotechnol
DOI: 10.1007/10_2009_63

Impact of Thermodynamic Principles in Systems Biology

J.J. Heijnen

Abstract It is shown that properties of biological systems which are relevant for systems biology motivated mathematical modelling are strongly shaped by general thermodynamic principles such as osmotic limit, Gibbs energy dissipation, near equilibria and thermodynamic driving force. Each of these aspects will be demonstrated both theoretically and experimentally.

Keywords Black box kinetics, Gene regulation, Lin-log kinetics, Metabolic networks, Model reduction, Pseudo-steady state, Stoichiometry, Systems biology, Thermodynamics

Contents

1 Introduction
2 Thermodynamic Principles in Mathematical Models of Biological Systems
3 The Osmotic Limit Dictates Low Concentrations of Intracellular Metabolites
4 Consequences of Low Metabolite Concentrations from a Systems Biology Point of View
5 Thermodynamic Approach to Obtain Network Stoichiometry and Fluxes
  5.1 Thermodynamic Approach to Stoichiometry
  5.2 Thermodynamic Approach for Maximal Growth Rate, μ^{max}
6 Prediction of Gene Regulation of Enzymes Using Energy Optimality
7 Thermodynamics Based Model Reduction in System Biology
  7.1 Black Box Models for Design of Biotechnological Processes: From Complexity to Simplicity Due to Pseudo-Steady State Coupling
  7.2 Metabolic Reaction Network Models to Redesign Organisms: Reducing Complexity

J.J. Heijnen
Department Biotechnology, Bioprocess technology, Delft University of Technology, Julianalaan 67, 2628 BC Delft, The Netherlands
e-mail: j.j.heijnen@tudelft.nl

8 Thermodynamics Inspired Kinetics: Lin-Log Kinetics
8.1 Introduction
8.2 In Silico Studies with Lin-Log Kinetics
8.3 Application of Lin-Log Kinetics to Experimental Data
9 Conclusion
References

1 Introduction

Thermodynamic principles apply to all physical and chemical systems, including biological systems. In this chapter it will be shown how these principles shape their properties, especially from a quantitative model based, systems biology, point of view.

2 Thermodynamic Principles in Mathematical Models of Biological Systems

The key aspect of a living cell is the formation of new cells, called growth.

Growth requires that a cell produce each of the molecules present in the newly formed cells. This occurs in a large and complex metabolic (reaction) network. This network is composed of many reactions, which consume and produce small molecules, called metabolites. Each reaction is catalysed by a specific enzyme, which is under genetic/environmental control. Prediction of growth requires a mathematical model of this network for which the fundamental equations are the mass balances of intracellular metabolites. In vector notation:

$$\frac{\mathrm{d}X}{\mathrm{d}t} = SV\,(e, X, p) - \mu X, \tag{1}$$

where X is the vector containing the individual intracellular metabolites X_j.

V is the vector of the rates of enzyme catalyse reactions, with v_i the rate of reaction catalysed by enzyme present at an amount e_i. The rate v_i depends on the amount of enzyme present, e_i, on the kinetic effect of metabolites X_j involved (e.g. substrate, product and possible allosteric effectors) and the parameters p (e.g. $V^{\max}$, affinities, Hill coefficient etc.). S is the so-called stoichiometric matrix which represents the structure of the reaction network. Its rows represent the metabolites, its columns the reactions [44]. The term μX is the so-called dilution term.

Equation (1) requires information on the dynamic behaviour and values of metabolite concentrations, on the values of stoichiometric coefficients, on enzyme levels resulting from genetic regulation and on the shape/algebraic nature of the

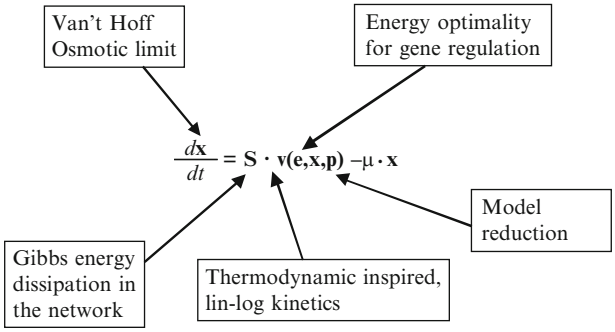

Fig. 1 Impact of thermodynamic principles in systems biology

enzyme kinetic relations, and (1) is the basis of parameter estimation from experimental data and the associated need for model reduction.

In the following sections we will show that thermodynamic principles can be used to shed light on this information (Fig. 1):

- Metabolite concentration levels (X_j) and their control mechanisms
- The stoichiometry of growth (S)
- The genetic regulation of enzyme levels (e_i)
- Principles of model reduction
- The kinetics of enzyme catalysed reactions based on thermodynamic driving force

3 The Osmotic Limit Dictates Low Concentrations of Intracellular Metabolites

Cells have a genome which contains about 5,000 genes. These genes code for about 5,000 proteins, of which about 2,000 are enzymes. Therefore, in a cell, one can expect about 2,000 different metabolites which are small molecules (e.g. metabolites in central metabolism and in pathways for amino acid, nucleotide, lipid and carbohydrate/cell wall synthesis). Many of these metabolites are negatively charged (having phosphate and carboxylate groups) and therefore there are also considerable concentrations of counter cations (K^+, Mg^{2+}). The sum concentration of all these small molecules is limited by a thermodynamic property called osmotic pressure [1]. Because cells contain a cell membrane that is water permeable, the presence of a high intracellular sum concentration of membrane impermeable anionic/cationic small molecules leads to a water activity inside cells that is lower than outside. This creates a flow of water into the cell, leading to increase of intracellular pressure. The water inflow stops when the pressure has reached the

osmotic pressure. According to van 't Hoff the osmotic pressure is linear in the sum concentration C of all intracellular metabolites ($P_{\text{osm}} = CRT$). For example, for C = 1 mol/L it follows that $P_{\text{osm}} = 25$ bar ($C = 1$ mol/L $= 1{,}000$ mol/m^3, $R = 8.314$ J/mol K, $T = 298$ K gives $P_{\text{osm}} = 24.8 \times 10^5 \text{N/m}^2 = 25$bar). From a mechanical point of view, this pressure is counteracted by the mechanical strength of the cell membrane/cell wall, which is obviously bound to physical limits. Therefore there must exist a maximal sum concentration of small intracellular molecules. Assuming a limit of 25 bar gives for this maximal sum $C \approx 1.0$ mol/L, which gives a sum concentration of organic (anionic) metabolites of order 0.5 mol/L. Assuming the presence of about 1,000 different metabolites in cells gives, for the average intracellular metabolite concentration X_j, a value of about 10^{-3} mol/L. Of course, there will be a wide distribution of concentrations, so we can expect an intracellular concentration range of 10^{-2} to 10^{-4} mol/L, which is equivalent to 20–0.20 μmol/g dry biomass. These values are indeed found as shown in Table 1.

4 Consequences of Low Metabolite Concentrations from a Systems Biology Point of View

The general property of low intracellular metabolite concentrations has very important consequences at system level.

A first consequence is the near *absence of spontaneous reactions*. Usually the metabolic network is considered to be totally enzyme catalysed and one assumes implicitly that non-enzymatic reactions (which occur spontaneously) are absent. Given the multitude of reactive molecules inside cells, one would expect much more spontaneous reactions. Such reactions would be disadvantageous because they are not under genetic control and they cause loss of material. The key to suppress such reactions, in favour of enzyme catalysed reactions, is to have a very low metabolite concentration (which kinetically "kills" the rate of a spontaneous reaction) in combination with matching high affinities of enzymes. This is indeed found. So one could state that the osmotic limit enforces *high affinity enzymes*.

A second general consequence is the *need of active export*.

Many biological systems are used in industrial processes (antibiotics, fuels, amino acids, organic acids etc.). From an economic point of view, one aims at high ($\approx$ 1 M) extracellular concentrations of product. This implies that the final step of product metabolism, export, has to deal with an unfavourable concentration gradient of about 10^{-3} M inside and 1 M outside. Clearly, this requires active export [6].

Another general aspect of metabolites is a *fast, order of seconds, turnover time* (t.o.t.) of each metabolite. The t.o.t. of a metabolite X_j is defined as $(\text{t.o.t.})_{X_j} = \frac{X_j}{V_{\text{sum}}}$, with X_j the metabolite concentration and V_{sum} the sum of all production rates of this

Table 1 Intracellular metabolite concentrations and turnover time in glucose limited aerobic cultures of several organisms (*Saccharomyces cerevisiae* from [2], *Penicillium chrysogenum* from [3, 4] *E. coli* from [5])

Metabolites	Intracellular level (μmol/gDW)			Turnover time (s)		
	P. chrysogenum	*S. cerevisiae*	*E. coli*	*P. chrysogenum*	*S. cerevisiae*	*E. coli*
Central metabolites						
G6P	4.64	5.2	1.42	23.3	17	3.6
F6P	0.71	1.4	0.38	5.7	7.3	1.2
T6P	0.55		0.13	47.8		NA
M6P	1.95		0.48			NA
6PG	0.25	0.48	0.10	3.7	4.5	1.1
Mannitol-1P			0.99			NA
G3P		0.13	0.17		57	13.1
FBP	0.9	0.64	0.82	7.2	3.2	2.5
F2,6bP	0.01		0.35			NA
2PG+3PG	0.59	2.8	1.65	2.3	6.6	2.5
PEP	0.24	2.3	1.61	0.9	5.7	2.7
Pyruvate	0.22	1.1	0.75	0.9	1.7	1.5
α-Ketoglutarate	2.05		0.31	22.1		0.6
Succinate	0.23	4.0	2.65	3.3	20	8.9
Fumarate	0.65	0.85	0.22	13.0	4.1	0.7
Malate	3.33	7.3	0.94	19.0	30	2.8
Amino acids						
Alanine	21.7	32	1.34	269	3,268	76.7
Asparagine	1.5	4.7	0.58	459	1,142	81.7
Aspartate	16.3	21	2.57	717	577	35.0
Glutamate	53.0	170	74.69	658	1,112	229.0
Glutamine	28.7	64	6.14	1,243	2,401	80.0
Glycine	2.1	2.9	1.51	244	247	31.0
Histidine	0.72	6.0	0.15	432	3,141	53.8
Isoleucine	0.33	1.6	0.11	111	140	12.9
Leucine	0.73	1.0	0.36	131	125	27.1
Lysine	1.2	4.1	1.21	356	619	119.7
Methionine	0.14	0.20	0.05	58.8	66	10.5
Phenylalenine	0.19	1.6	0.13	61.2	430	23.8
Proline	0.95	3.9	0.66	206	925	101.4
Serine	5.7		0.53	453		8.0
Threonine	5.9	4.0	0.47	758	220	29.3
Tryptophan	0.11	0.51	0.02	130	788	11.9
Tyrosine	0.26	1.6	0.18	145	832	44.3
Valine	2.1	10	0.51	243	490	40.9
Ornithine		4.1	0.49		502	49.1
Adenine nucleotides						
ATP	7.39	7.0	5.95	NA	1.4	2.0
ADP	1.03	1.3	2.31	NA	0.25	0.8
AMP	0.27	0.6	0.91	NA	3.1	9.4

metabolite. Because X_j is low and V_{sum} can be high, one indeed finds t.o.t. of the order of seconds (Table 1).

These *fast t.o.t.* have several important consequences:

- Considering product formation, where a substrate molecule is processed along a multistep pathway to the secreted product, it follows that the time between substrate entrance and product leaving the cell is only of order minutes. Clearly cell factories follow the *just in time* principle.
- Considering the *metabolite mass balances* (1), we can safely neglect:
  - The dilution term μX_i, which is orders of magnitude smaller than the synthesis term SV.
  - The dynamic term $\frac{dX_i}{dt}$ for time scales larger than minutes (which follows from t.o.t. of order seconds). This leads to *pseudo-steady state*.
- This *pseudo-steady state* property, which is a direct consequence of the low metabolite levels due to an osmotic limit, is one of the most important network properties. It allows one to write for the metabolite mass balances:

$$SV = 0 \tag{2}$$

  These balance equations are the basis of the well-known stoichiometric analysis of metabolic networks. We should also realise that, due to the pseudo-steady state property of the metabolic reaction network, these balances also apply to dynamic situations which allow one to formulate so-called black box stoichiometric/kinetic models which are reliable in a wide range of conditions (see also "model reduction").
- A final consequence is *the need for control mechanisms* on the production/consumption of each intracellular metabolite. Cells are, in their natural environment, continuously exposed to perturbations which change the rate of synthesis/consumption of metabolites. Given the low concentration of a metabolite, such a perturbation leads to very quick (second time scale) and drastic (up or down) changes in metabolite concentrations, which propagate through the network leading to potential damaging system responses. Control of metabolite levels is needed to limit these effects, and indeed such control mechanisms are widely found in biological systems and, most interesting, they operate at proper time scales. Most well known are *allosteric feed back inhibition* (e.g. in amino acid synthesis pathways), and *allosteric feed forward activation* (e.g. in glycolysis) mechanisms which operate within seconds. This is exactly the time scale expected from the t.o.t. for metabolite levels. The other mechanisms are slower. The *post translational modification* mechanisms (adenylation, (de)phosphatation, ...) take in the order of minutes, consume ATP and interconvert active/inactive enzyme, but do not change the total enzyme amount. The *genetic mechanisms* (induction, repression) take in the order of tens of minutes and change the amount of enzyme.

5 Thermodynamic Approach to Obtain Network Stoichiometry and Fluxes

To obtain the network fluxes (and therewith the stoichiometry of the network) requires one to solve (2). These balances put linear constraints on the reaction/uptake/secretion rates. The number of rates in a realistic network is typically several hundred; however the number of metabolite mass balances in (2) (= constraints) is also large. A general problem is the ATP-balance which contains uncertain ATP stoichiometric parameters (P/O ratio, unknown growth related ATP $(= K_x)$ and the unknown growth unrelated ATP (m_{ATP}). Van Gulik and Heijnen [7] and Van Gulik et al. [8] have shown how in vivo values for these ATP-parameters can be obtained using extensive experiments. For many organisms this ATP-information is not available. This means that the number of equations in (2) is always at least two lower than the number of rates (underdetermined). This means that solving all rates needs the specification of at least two experimental rates. Without this experimental information one cannot predict stoichiometry. Another approach which has received considerable attention in the past decade, and which aims to predict both rates and stoichiometry of networks, is constraint based modelling [43]. This approach uses an optimality criterion (e.g. maximal biomass yield) to obtain a solution of the underdetermined (2). However, close inspection reveals that this method still requires the above-mentioned experimentally based information:

- Specification of the uncertain ATP stoichiometric coefficients (P/O, growth related and unrelated maintenance values). This information is needed to make stoichiometry predictions!!
- Kinetic information, such as an experimentally determined substrate uptake rate or a maximum O_2-uptake rate. This is needed to calculate fluxes and, e.g. $\mu^{\max}$.

Together with this experimental information the optimality criterion forces ATP requiring processes such as futile cycles to zero and therewith one obtains a unique flux solution and therewith stoichiometry. When the above-mentioned (ATP and kinetic) experimental information is not available, constraint based modelling does not lead to a unique flux solution.

Thermodynamics offers an alternative, more widely applicable, approach to solve the network stoichiometry and fluxes for arbitrary organisms.

5.1 Thermodynamic Approach to Stoichiometry

Thermodynamics allows one, for a given specific growth rate μ (under substrate limited growth in absence of a non-catabolic product, hence only growth), to calculate all uptake/secretion rates. Herewith, all yields are also available (yield is ratio of rates). Heijnen and Van Dijken [9] and Heijnen [10] apply this approach

to any heterotrophic growth system under substrate limited condition. The only information needed is the nature of carbon source, electron donor, electron acceptor, N-source and temperature.

In this method, the ATP-balance in the network equation (2) is replaced by a Gibbs energy balance on all uptake and secretion rates q_i. We can define q_i as a biomass specific rate in mol of *i*/*h* per Cmol biomass. The quantity 1 Cmol of biomass (which has the average composition $C_1H_{1.8}O_{0.5}N_{0.2}$) is the amount (24.6 g organic dry matter) of biomass which contains 12 g of carbon (= 1 mol C-atom).

Compounds taken up have negative q_i values, secreted compounds have positive q_i-values. The Gibbs energy balance follows then as

$$\sum q_i \Delta G_{f_i}^{01} + q_G = 0. \tag{3a}$$

Here $\sum q_i \Delta G_{f_i}^{01}$ is a negative quantity (second law of thermodynamics) and is the total biomass specific rate of Gibbs energy of conversion. q_G is the Gibbs energy produced, which follows from (3a). The second law requires $q_G > 0$.

$\Delta G_{f_i}^{01}$ is the Gibbs energy of formation of compound i at standard condition (1 M, 298 K and at pH = 7.0). In principle, one needs to take actual concentrations into account, but this leads only in special cases to significant changes in q_G [10].

The key to the use of (3a) is to obtain a relation for q_G. Because cells require Gibbs energy for growth and maintenance, we can write a Herbert–Pirt type of relation for q_G, which expresses that (in absence of non-catabolic product) the cell needs Gibbs energy for growth and maintenance:

$$q_G = \frac{1}{Y_{GX}^{\max}} \mu + m_G. \tag{3b}$$

For m_G and $\frac{1}{Y_{GX}^{\max}}$, correlations have been established [9, 11].

5.1.1 Gibbs Energy for Maintenance

All living systems need to generate Gibbs energy for their maintenance (which represents all processes where energy is needed for example to repair degradation and export compounds that entered due to membrane leakage, etc.). Because living cells have similar membranes and composition, it can be assumed that different cells require a similar amount of energy expenditure for maintenance. It has indeed been found that Gibbs energy needed for maintenance is very similar for a large range of microorganisms and only depends on absolute temperature (*T*) [11].

The following *correlation* has been found for m_G $\left(\text{in } \frac{\text{kJ Gibbs energy}/h}{\text{CmolX}}\right)$:

$$m_G = 4.5 \exp\left(\frac{69{,}000}{R}\left(\frac{1}{298} - \frac{1}{T}\right)\right). \tag{4}$$

This correlation shows that at 25°C (298 K) 1 Cmol of cells (≈ 25 g dry matter) spends, and therefore needs to generate, 4.5 kJ of Gibbs energy per hour to cover the energy for maintenance. Also there is a steep temperature effect: for each 8°C temperature increase m_G doubles!!

This correlation applies to aerobic/anaerobic systems and arbitrary electron donors/acceptors. The Gibbs energy is generated from the catabolic reaction, so the maintenance reaction equals the catabolic reaction. For specific cases, one can always set up the catabolic reaction for 1 mol donor and obtain the catabolic Gibbs energy of reaction for 1 mol donor, called $\Delta G^{01}_{\text{cat},D}$, which is negative and is in kJ of Gibbs energy per 1 mol donor consumed in the catabolic reaction. It is then clear that we can write for the substrate (or donor) consumption $m_s\left(\frac{\text{in mol substrate}/h}{\text{CmolX}}\right)$ that must be catabolised for maintenance:

$$m_s = \frac{m_G}{\Delta G^{01}_{\text{cat},D}}. \tag{5}$$

Table 2 shows examples of catabolic reactions consuming 1 mol donor, and the Gibbs energy of catabolism per mol consumed donor $\left(\Delta G^{01}_{\text{cat},D}\right)$. It is obvious that $\Delta G^{01}_{\text{cat},D}$ can be two orders of magnitude different, dependent on the specific catabolism.

For example, consider *Saccharomyces cerevisiae* at 30°C. It follows from (4) that

$$m_G = \frac{7.1\,\text{kJ/h}}{\text{CmolX}}$$

Under *aerobic* conditions using glucose as substrate, catabolism $\Delta G^{01}_{\text{cat},D} = -2{,}843.1$ kJ per mol glucose. This gives

$$m_s = \frac{7.1}{(-2{,}843.1)} = -0.0025\,\frac{\text{mol glucose}/h}{\text{CmolX}}$$

Also $m_{O_2} = 6 \times m_s = -0.015\ \frac{\text{mol O}_2/h}{\text{CmolX}}$. Under *anaerobic* conditions the catabolic ethanol forming reaction shows $\Delta G^{01}_{\text{cat},D} = -225.4$ kJ per mol glucose. This gives $m_s = \frac{7.1}{-225.4} = -0.0315\,\frac{\text{mol glucose}/h}{\text{CmolX}}$ and $m_{\text{ethanol}} = 0.063\,\frac{\text{mol ethanol}/h}{\text{CmolX}}$.

Table 2 Catabolic reactions and their Gibbs energy of reaction, $\Delta G^{01}_{\text{cat,D}}$

Catabolic reactions and their Gibbs energy $\Delta G^{01}_{\text{cat,D}}$(kJ/mol donor)		
Donor	Catabolic reaction	$\Delta G^{01}_{\text{cat,D}}$
Glucose	$C_6H_{12}O_6 + 6O_2 \rightarrow 6HCO_3^- + 6H^+$	−2,843.1
Ethanol	$C_2H_6O + 3O_2 \rightarrow 2HCO_3^- + 2H^+ + 1H_2O$	−1,308.9
Glucose	$C_6H_{12}O_6 + 2H_2O \rightarrow 2C_2H_6O + 2HCO_3^- + 2H^+$	−225.4
Methanol	$CH_4O + 1.20NO_3^- + 0.20H^+ \rightarrow 0.60N_2 + HCO_3^- + 1.60H_2O$	−649.4
Iron (2+)	$Fe^{2+} + \frac{1}{4}O_2 \rightarrow Fe^{3+} + \frac{1}{2}H_2O$ (pH = 1.85)	−33.9
Acetate	$C_2H_3O_2^- + H_2O \rightarrow HCO_3^- + CH_4$	−31.0

So, the same organism has widely different m_s values due to different catabolism, but still has the same Gibbs energy need for maintenance!!

5.1.2 Gibbs Energy for Growth

$\frac{1}{Y_{GX}^{max}}$ is the amount of Gibbs energy needed to synthesise 1 CmolX (in kJ/CmolX). This amount has been found to depend only on two factors [9, 10]: first, the nature of the carbon source for *heterotrophic growth* and, second, the nature of electron donor for *autotrophic growth*.

Regarding the first factor, the nature of the carbon source for *heterotrophic growth*, more Gibbs energy is needed when the carbon source has a smaller number (C) of C-atoms and when its degree of reduction per C-atom (γ) is different from the degree of reduction of biomass (≈ 4.2). The explanation is straightforward that synthesis of biomass monomer molecules (which contain order 6 carbon atoms with $\gamma \approx 4$) requires more C–C-coupling and redox reactions for C-sources with a low number of C-atoms and which need reduction or oxidation because γ of the C-source differs from 4.2. These extra reactions lead to a higher Gibbs energy need. For heterotrophic growth this intuition is quantified in (6a), which is a *correlation*:

$$\frac{1}{Y_{GX}^{max}} = 200 + 18(6 - C)^{1.8} + \exp\left[|3.8 - \gamma|^{0.32} \times (3.6 + 0.4C)\right]. \qquad (6a)$$

This correlation shows that to synthesise 1 Cmol biomass one needs between $\approx$ 236 and 1,087 kJ Gibbs energy (dependent on C-source, e.g. 236 for glucose ($\gamma = 4$, $C = 6$) and 1,087 for CH_4 ($\gamma = 8$, $C = 1$).

Regarding the second factor, the nature of electron donor for *autotrophic growth*, in autotrophic growth CO_2 is the C-source which must be reduced to biomass using electrons from the electron donor. The Gibbs energy needed follows from (6b), which is also a *correlation* [9]:

$$\frac{1}{Y_{GX}^{max}} = 1,000(-\text{RET}) = 3,500(+\text{RET}). \qquad (6b)$$

The nature of the electron donor determines the absence (−RET) or need (+RET) of *R*everse *E*lectron *T*ransport. For several electron donors (e.g. NH_{4^+}, $NO_2{}^-$, Fe^{2+}) the reduction of CO_2 to biomass is not feasible thermodynamically. Therefore cells spend extra Gibbs energy to make the redox potential of electrons obtained from the available donor more negative (e.g. in the production of NADPH from the donor electrons, in a process called RET, NADPH is then used to reduce CO_2 to biomass). This extra Gibbs energy is very considerable (compare in (6b) 3,500 and 1,000). For H_2 as electron donor, (−RET), this problem does not exist (sufficient negative redox potential) and the Gibbs energy need is 1,000 kJ/CmolX (6b).

Summarising, the Gibbs energy needed to make 1 Cmol biomass ranges, dependent on C-source and electron donor, is between 200 and 3,500 kJ and does not depend on the type of electron acceptor. When the carbon source, electron donor and temperature are known, the correlations (4, 6a/6b) give the coefficients $\frac{1}{Y_{GX}^{\max}}$ and m_G in the Gibbs energy Herbert Pirt relation (3b), which completes the Gibbs energy balance (3a). This linear relation can be combined with all metabolite mass balances specified in (2), using the stoichiometric matrix S where the ATP-balance is also absent (due to unknown P/O, K_x, etc.). A constraint of minimal Gibbs energy dissipation will put futile cycles to zero and parallel pathways are also resolved. This set of linear balances gives, for any selected μ, all rates in the network (reaction/uptake/secretion)!! With these known rates all yields are known such as the biomass yield $Y_{SX} = \frac{\mu}{q_s}$ or yield of catabolic products.

This thermodynamic approach has been shown to predict biomass yield with 10–15% error for a wide variety (aerobic/anaerobic/hetero/autotrophic) of microbial systems where Y_{SX} spans a range of near *two orders of magnitude* [9].

5.2 Thermodynamic Approach for Maximal Growth Rate, $\mu^{\max}$

Microorganisms show a very large range (0.001–1 h^{-1}) in $\mu^{\max}$-values and it is relevant to understand why this is so!! A simple approach was proposed [10] which reproduces most of this range. The concept is that cells during evolution ultimately become limited in their *energy production capacity*. Most organisms generate energy by electron transport phosphorylation. This occurs by electron transport proteins embedded in membranes. Because membranes are space limited for protein embedding it is logical to propose that cells have evolved to a maximal electron transport capacity $q_{el}^{\max}\left(\text{in } \frac{\text{mol electrons}/h}{\text{CmolX}}\right)$ which depends mainly on temperature. Also it is known that smaller organisms have higher maximal growth rates, e.g. *E. coli* $\mu^{\max} = 2\ h^{-1}$, *Saccharomyces cerevisiae* $\mu^{\max} = 0.4\ h^{-1}$ and tissue cell cultures $\mu^{\max} = 0.04\ h^{-1}$. This is in line with the smaller surface/volume ratio ($\approx 6/d$, with cell diameter d) which leads to a membrane surface area, hence maximal electron capacity and $\mu^{\max}$ which is inverse to the cell diameter and which is indeed largely observed (e.g. *S. cerevisiae* has a 5× larger cell diameter compared to *E. coli*). This concept has in addition been inspired by the observation that in *E. coli*, for different substrates which lead to different $\mu^{\max}$, the $q_{O_2}^{\max}$-value is nearly constant [12].

The following correlation was proposed:

$$q_{el}^{\max} = 3\ \exp\left[\frac{69{,}000}{R}\left(\frac{1}{298} - \frac{1}{T}\right)\right]. \tag{7a}$$

This electron capacity determines the maximal production rate of Gibbs energy by catabolism ($q_G^{\max}$):

Table 3 Estimated μ^{max} values for different microbial catabolic classes at 25°C based on limiting Gibbs energy production

Catabolic classes	$\Delta G^{01}_{cat,\,D}$ (kJ/mol donor)	γ_D (mol electron/ mol donor)	μ^{max} (h^{-1})
Aerobic/glucose	−2,843.1	24	1.5
Aerobic/acetate	−844.2	8	0.70
Anaerobic/(acetate→CH_4)	−31.0	8	0.015
Aerobic/Fe^{2+} oxidation	−38.6	1	0.030
Aerobic/nitrification	−274.8	6	0.040

$$q_G^{max} = q_{el}^{max} \frac{\left(-\Delta G^{01}_{cat,D}\right)}{\gamma_D}. \tag{7b}$$

Here, γ_D is the number of electrons released in catabolism of 1 mol donor and $\Delta G^{01}_{cat,D}$ is the catabolic Gibbs energy per mol donor. This maximal Gibbs energy sets μ^{max}according to (3b).

Combination of (3b) and (7b), (2) and using the correlations (4), (6a, b) and (7a) for $m_G, Y^{max}_{GX}, q^{max}_{el}$and the available value for $\Delta G^{01}_{cat,D}$ and γ_D immediately allows one to calculate μ^{max}-values for any growth system. These values agree reasonably with known values. Table 3 shows that this simple approach can explain a 100-fold difference in μ^{max}.

Some final remarks:

- This thermodynamic approach uses only three correlations (4, 6a/6b, 7), is simple and general and gives maximal q_i-rates and stoichiometry.
- Effect of temperature is included (in maintenance and μ^{max}).
- The three correlations are based on a wide range of experimental microbial growth systems and reflect that similar biochemical pathways are used (unity of biochemistry). When the predicted μ^{max} or/and stoichiometry are very different from experimental values, this indicates unusual anabolic and/or catabolic routes which might be novel. So this method can act as a filter for unusual behavior of biological systems.

6 Prediction of Gene Regulation of Enzymes Using Energy Optimality

Gene regulation seems at first glance highly complicated. For example, enzyme induction upon exposure to a new catabolic substrate involves many mechanisms between signal transduction, gene expression and production of enzyme for the new catabolic pathway. Model based prediction of enzyme induction therefore seems hopeless. However one could expect that evolution has fine tuned the available

regulation mechanisms such that growth yield is optimal. This optimality principle was tested using mixed substrates with *Saccharomyces cerevisiae*, which was chemostat (aerobic, substrate limited) cultivated at a dilution rate $D = 0.1\ h^{-1}$. Different feed mixtures of glucose and ethanol as substrates were applied between 100% glucose and 100% ethanol) [7, 13, 14].

Growth on glucose differs from that on ethanol. On 100% ethanol the cell has:

- Fully induced:
  - Glyoxylic acid pathway enzymes (isocitrate lyase (ICL) and malate synthase (MS))
  - Gluconeogenic enzymes PEP-carboxykinase (PEPCK) and F16 Bisphosphate (F16BP-ase)
- Fully repressed (or inactivated):
  - Pyruvate kinase/pyruvate carboxylase (PK/PYC)
  - F16 bis P-kinase (PFK)

It is obvious that, under substrate limited condition for mixed substrates, the induction/repression pattern of these enzymes is determined by the residual ethanol and glucose concentration, which would be the basis of a complicated gene regulation mechanism and model to predict the occurrence and concentration of these enzymes as function of the residual ethanol and glucose concentration.

A different approach, based on energy optimality of gene expression, was followed. Using a metabolic stoichiometric model endowed with experimentally obtained stoichiometric values for the ATP-balance (P/O, K_x, m_{ATP}), linear programming was applied with maximal biomass production (or the equivalent minimal energy consumption) as optimality criterion. It was possible to calculate, for each glucose/ethanol supply ratio (which is virtually equal to the ratio of their consumption rates due to the low residual ethanol and glucose concentration) the optimal rates of all reactions in central metabolism. It was observed that, for the above inducible enzymes, clear predictions were made on their need as function of increasing ethanol fraction. This approach predicted:

- The ethanol/glucose feed ratio where a particular enzyme started to be induced
- The enzyme amount then increased linear with increasing ethanol fraction

These predictions were qualitatively, but more surprising also quantitatively, validated using the wild type yeast [13, 14]. Later additional validation was performed with null-mutants in the above enzymes, leading to a predicted maximal ethanol uptake rate of each mutant.

This prediction was again quantitatively confirmed [15]. This example clearly indicates that gene regulation mechanisms might have evolved to provide maximal biomass yield (giving a competitive edge). This maximal biomass yield is the same as Gibbs energy optimality because enzyme induction is such that futile cycles are avoided (e.g. simultaneous activity of FBP-ase/FPK or pyruvate carboxylase/PEP

carboxy kinase). To the authors knowledge this is one of the earliest and most successful examples of experimentally demonstrated thermodynamic optimality of living cells.

7 Thermodynamics Based Model Reduction in System Biology

Mathematical models of biological systems are useful to design processes and/or to redesign organisms using the rec-DNA tool box. Model reduction is an important issue, given the complexity of biological systems. Model reduction aspects will be discussed for two categories of mathematical models.

7.1 Black Box Models for Design of Biotechnological Processes: From Complexity to Simplicity Due to Pseudo-Steady State Coupling

We have observed that the thermodynamically based osmotic limit leads to very low metabolite concentrations in intracellular metabolism. The immediate consequence is that at process time scales larger than about 10 min (as occurs in bath, fed batch processes) the pseudo-steady state condition for all intracellular metabolites holds. The consequence of this condition is that all uptake and secretion rates are directly coupled. This *pseudo-steady state coupling* can be evaluated by linear rearranging the metabolite mass balances (2). Usually, in the absence of by-products, there are only a few (two to three) degrees of freedom, meaning that all uptake/secretion rates can be written as linear combination of only two to three rates (usually growth rate, product formation rate, maintenance). A prime example of such a linear relation is the Herbert–Pirt relation for substrate distribution:

$$q_s = \frac{1}{Y_{SX}^{max}}\mu \; + \; \frac{1}{Y_{SP}^{max}} q_p + m_s. \tag{8a}$$

Furthermore, the substrate uptake relation is usually a hyperbolic relation in the limiting substrate concentration:

$$q_s = q_s^{max}\left(\frac{C_s}{K_s + C_s}\right). \tag{8b}$$

A final relation, which holds generally under *single substrate limited condition* is that there is a unique relation between q_p and μ:

$$q_p = f_p(\mu). \tag{8c}$$

The three equations (8a, b, c) have only *one degree of freedom* (e.g. C_s or μ). All the other uptake/secretion rates $(O_2, CO_2, NH_{4+}, \text{water}, H^+, \text{heat}, \text{etc.})$ can be obtained from q_s, μ and q_p and the conservation relations (elements and charge).

This black box approach for kinetic modeling of biological processes is only possible due to the general biological system property of low intracellular metabolite concentrations, which has its origin in a thermodynamic property (osmotic limit).

Examples of this black box approach can be widely found. A very nice example is the model for penicillin production [8, 16] and the model developed for biological P-removal using mixed cultures in a cyclic process [17]. These black box models show that highly complex biological systems, comprising thousands of reactions, can be effectively modeled with a reduced model containing only about 6–12 parameters. These models are the basis of process design. This simple behavior of complex biological systems (from complexity to simplicity!!) is the direct result of the metabolite pseudo-steady state property which results from an osmotic limit!

7.2 *Metabolic Reaction Network Models to Redesign Organisms: Reducing Complexity*

Genetic intervention in metabolic reaction networks is possible in many ways by, e.g. changing enzyme levels, changing enzyme kinetics (e.g. abolish feed back inhibition), introducing different reactions, etc. The problem is that, following such interventions, the prediction of changes in secretion or uptake rates is very difficult due to the complex nature of the network structure and of the highly non-linear kinetics of the enzymes and gene regulation mechanisms. It is generally recommended that a mathematical model of a metabolic (reaction) network can help to select rationally genetic engineering targets in the redesign of organisms.

Building such a model is essentially straightforward and based on (1). Usually matrix S is known with high confidence. Uncertainties in S (e.g. cycles, parallel reactions, etc.) can be addressed using ^{13}C approaches [18].

A much bigger problem is to obtain kinetic relations for each enzyme, meaning the function $v\,(e, x, p)$ in (1) for each enzyme.

Traditionally, in vitro obtained kinetic functions and parameters have been used. Here two problems arise. First, it was found that in vitro kinetics do not reflect in vivo kinetics [19]. Second, for many enzymes in vitro kinetics are not available. The only solution therefore is to perform experiments with whole cells to obtain in vivo kinetic behavior of all enzymes simultaneously. Here rapid pulse experiments as pioneered by the groups of Reuss [20, 21] and Heijnen [22] offer advantages, but the challenge is the parameter identifiability problem which calls for model reduction as shown by Nikerel et al. [23–25].

Model reduction in biological systems has a thermodynamic basis as will be outlined below.

7.2.1 Pseudo-Equilibrium Reactions

A first approach for model reduction is to replace a kinetic function by an equilibrium relation. This is possible when the enzyme catalysed reaction is so rapid that the reaction remains very close to equilibrium, even when the reaction rates increase.

A strong indication of this situation is that the so-called mass action ratio of a reaction remains nearly constant (independent from flux) and close to the reported equilibrium constant. Many examples have been found which show this behaviour in *Penicillium chrysogenum* [3, 4] and in *Saccharomyces cerevisiae* [26] such as for phospho glucose isomerase, enolase, phosphoglycerate mutase, phosphoglucomutase, fumarase.

Recently a near equilibrium reaction $(F6P + \mathrm{NADH} + H^+ \rightarrow \mathrm{mannitol} - 1 - P + \mathrm{NAD}^+)$was used as a heterologous sensor reaction to obtain the cytosolic NAD/NADH ratio in *Saccharomyces cerevisiae* [2], even under dynamic conditions. It was found that the cytosolic NAD/NADH ratio was about 100. In contrast, a ratio of 4 was obtained by analysing the total amounts of NADH and NAD in whole yeast cells. The large difference is due to the fact that nearly all NADH is present in the mitochondria. Even more important is that, using the cell average NAD/NADH ratio, the ΔG_R of glycolysis between F16BP and (2+3 PG) was > 0, which is impossible. Use of the cytosolic value of 100 leads to ΔG_Rwhich is slightly negative, as expected. It can be expected that metabolism shows much more near equilibrium reactions. However here we need much more accurate data on in vivo equilibrium constants on a genome wide scale!!

7.2.2 Pseudo-Steady State Lumping

We have seen that many metabolites have low concentration levels (due to an osmotic upper limit) which leads to very low turnover times ($<$1 s) of metabolite pools. This allows, even in pulse experiment at a 300-s time scale, lumping of the synthesis and consumption reactions of a fast metabolite pool into 1 "lumped reaction". This model reduction based on pseudo-steady state lumping leads to much less parameters and much less parameter identifiability problems (see [23–25]).

7.2.3 Thermodynamic Inspired Kinetics

Replacing kinetics of individual reactions by equilibrium constants (pseudo-equilibrium) and by lumped kinetics (pseudo-steady state) significantly reduces the number of required kinetic functions and the number of parameters which need to be indentified, with no loss of model performance!! (see [24, 25]).

Nevertheless, there remain a significant number of far from equilibrium reactions into and from metabolite pools with slower turnover times for which we need

to specify a kinetic function. Unfortunately, mechanism based enzyme kinetics provide highly non-linear rate functions which contain many parameters. Examples are bi–bi kinetics, Hill functions, etc.

Although the number of parameters has been significantly reduced (near-equilibrium, pseudo-steady state based reduction) the identification of the remaining parameters still poses enormous problems, due to the non-linear parameter characteristics. Non-linear parameter estimation algorithms need a decent initial set of parameter values, which is not available, and in addition they do not guarantee an optimal result; they often end in a local minimum of the error criterion. Finally, these identification problems scale very unfavourably with increasing network size (e.g. genome scale metabolic models contain in the order of 1,000 reactions!!).

A possible solution is the use of proper approximative kinetic functions [27]. Here lin-log kinetics has been developed recently [28], which has its roots in the concept that the rate of a process is related to the thermodynamic driving force!! [29].

8 Thermodynamics Inspired Kinetics: Lin-Log Kinetics

8.1 Introduction

Lin-log kinetics is an approximative kinetic format which is a generalisation of the driving force concept and has been compared recently to other approaches such as linear, powerlaw, loglin (for review see [27]) and it was concluded that the lin-log format has significant advantages. Therefore we will focus here on use of lin-log kinetics.

Consider (Fig. 2) an enzyme e_1 which is kinetically affected by its substrate x_1 and product x_2, and is also allosterically affected by a metabolite x_8. Moreover, we consider a reference steady state (superscript ^{0}).

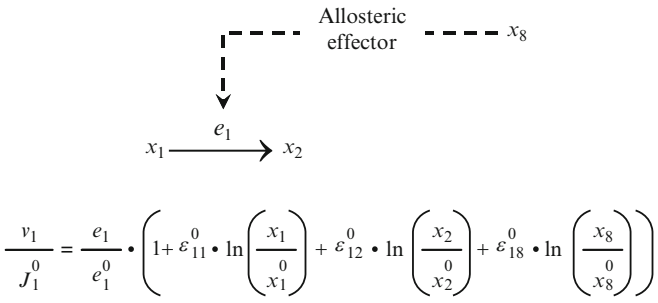

Fig. 2 Lin-log kinetics

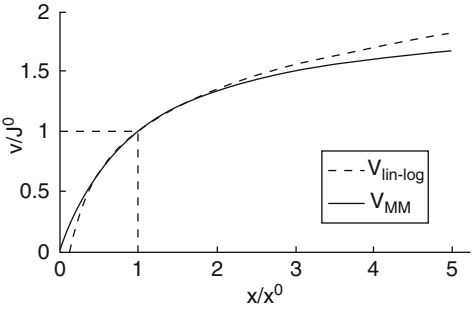

Fig. 3 Comparison between lin-log approximative and hyperbolic mechanistic kinetics

We can then formulate lin-log kinetics:

$$\frac{v_1}{J_1^o} = \frac{e_1}{e_1^o}\left(1 + \varepsilon_{11}^o \ln\frac{x_1}{x_i} + \varepsilon_{12}^o \ln\frac{x_2}{x_2^o} + \varepsilon_{18}^o \ln\frac{x_8}{x_8^o}\right). \tag{9}$$

We can compare approximative lin-log kinetics with traditional mechanistic hyperbolic kinetics and we see (Fig. 3) a very good approximation with respect to large changes (factor 5 up and down) in metabolite concentration.

Other approximative formats, such as power law as S- or GMA-systems and loglin, show similar quality of approximation for such changes in metabolite levels [27].

However metabolic reaction systems not only show *large changes in metabolite concentrations* but especially one faces (in the light of metabolic engineering ambitions) *large changes in enzyme levels*. For example, one can easily achieve enzyme concentration changes of factor 10 up or down due to genetic interventions in gene regulation (promoter libraries) or in gene dosis. Here, lin-log kinetics shows distinct advantages compared to the other approximative kinetic formats, as explained before [27]. A final favourable property of lin-log kinetics is that its parameters (elasticities) are linear in (1) which is significant in the light of parameter identification analysis and parameter estimation algorithms [23–25].

Lin-log kinetics has, after its conception, been successfully applied to kinetic modelling of metabolic reaction networks using in silico studies but has also been applied to experimental systems as will be discussed below.

General important aspects of lin-log kinetics are:

- The format is non-linear in metabolite concentrations.
- The format has a minimum number of kinetic parameters which helps minimise the identification problem.
- The parameters (elasticities) are linear in (1) which has significant advantages with respect to the parameter identifiability and parameter estimation [23–25].
- The elasticity parameters are bounded, e.g. Michaels–Menten kinetics $|\varepsilon| < 1$, for Hill kinetics $|\varepsilon| < n$ (with n subunits in the protein and signs (+ or −) are known. This constrains the ε-estimation problem.

- Lin-log kinetics cannot be applied for datasets where concentrations become zero [28], [30]. An analogous problem also occurs in powerlaw format where an inhibitor concentration cannot become zero.

8.2 In Silico Studies with Lin-Log Kinetics

Although Fig. 3 suggests good performance for lin-log kinetics for an individual reaction, a basic question remains how good does the lin-log approximation work in networks. *This has been studied in several simulation studies*. In a first study, where the lin-log concept was introduced [28], a small branched reaction network, including cofactors, was used as a test case. The strongly non-linear kinetic model was approximated with a lin-log model using the theoretical elasticity parameters. A rapid perturbation experiment, where metabolite concentrations did change several fold, was successfully reproduced. Moreover, use of lin-log kinetics leads to the so-called "design equation", which allows one to specify new fluxes/metabolite levels and to calculate analytically the required large changes in enzyme levels. In a follow up study [31] a non-linear model of glycolysis in *E. coli* was successfully approximated using lin-log kinetics and a connected product pathway was successfully redesigned in silico with respect to the required large changes in enzyme activities. Even more interesting was a recent study of Smallbone et al. [32] in which they showed that a lin-log kinetic model of glycolysis in *Saccharomyces cerevisiae*, in which elasticities were assumed equal to their reaction stoichiometric coefficients, gave surprising agreement with the mechanistic Teusink model!! More recently [30] it was shown that lin-log kinetics could also successfully simulate a genetic network with strongly non-linear kinetics. These simulation results show that lin-log kinetics provide a convenient and satisfying approximation of mechanistic kinetic functions for small and large models (metabolic, genetic), provided that metabolite concentrations do not become zero.

Having ascertained that lin-log kinetics provides a decent approximation to non-linear kinetics of networks, the next important problem is to identify the lin-log parameters (elasticities) from experimental data. The obvious experimental protocol is to perform rapid pulse experiments. The identification of lin-log parameters (elasticities) from such experiments was studied first in silico. Kresnowati et al. [33] showed, for a simple linear pathway, that elasticities can easily be obtained by using the integrated equation (1). The important aspect is that the resulting equations are linear in elasticities, so that linear regression can be used to obtain the elasticities. Subsequently a more complex glycolysis model was used for an in silico study of parameter identification aspects to obtain elasticities from dynamic pulse experiments [23–25]. It was found that, in such rapid pulse experiments, fundamental identification problems occur due to occurrence of pseudo-steady state metabolite pools (with turnover time <1 s) in the rapid pulse experiment. The identification problem could only be resolved by providing additional information (e.g. combined

steady state and dynamic perturbations. Also, a novel non-linear algorithm for elasticity parameter estimation was introduced where, due to properties of the lin-log format, one can obtain a reliable initial estimate of their values using linear regression of the integrated equation (1).

Finally, a completely different approach to the parameter identifiability problem in pulse experiments was shown to be possible due to the unique properties of lin-log kinetics [34]. It was shown that use of lin-log kinetics allows *a priori model* reduction by lumping pseudo-steady state pool coupled reactions. The reduced model reproduced the dynamic pulse experiment (*S. cer*, anaerobic glycolysis) accurately and allowed calculation of, e.g. flux control coefficients [34].

An interesting application of a lin-log kinetic model is to identify the function of so-called silent genes [35, 36] which shows how lin-log kinetic models could be used to resolve gene-annotation problems.

8.3 Application of Lin-Log Kinetics to Experimental Data

Lin-log kinetics allows a general steady state analytical solution of networks which gives fluxes as a non-linear function of enzyme activities with flux control coefficients C^J as parameters [28]. This equation was successfully used to obtain *C^J-values* from experimental data:

- A linear product pathway in Penicillin synthesis using fed batch data on flux and enzymes in the penicillin pathway [37]
- The lower glycolysis in a reconstituted linear three enzyme pathway [38]
- A branch point for lysine or glutamate synthesis [39]

Estimation of *elasticities* from experimental data using lin-log format requires experimental information of fluxes, enzyme levels and metabolite levels. Using an extensive steady state dataset for lower glycolysis [38] showed that elasticity values are easily obtained using linear regression. Also lin-log kinetics was used to set-up a kinetic model for leucine/valine synthesis [40], glycolysis in *Lactococcus lactis* [30] and a batch fermentation [41]. In the last two studies it was shown that lin-log kinetics should not be applied to datasets where metabolite concentrations become zero (which is obvious). Even more impressive was a recent study [42] in which a lin-log model was parameterised on a rapid pulse experiment. The model represents *E. coli* central metabolism and anabolism to all cell compounds, comprising 126 reactions and 130 metabolites (7 conserved moieties). The presence of allosteric mechanisms was taken from the MetaCyc database. In total, 921 elasticities were estimated using evolutionary algorithms and high performance computing. This work is a first, genome like scale, whole cell dynamic model and shows the power of the canonical lin-log format approach.

Parameter identification from experiments, however, remains an important issue. Here recently Nikerel et al. [34] showed the successful application of their

a priori model reduction approach (made possible due to lin-log format) on anaerobic glucose pulse experiments in *Saccharomyces cerevisiae*.

This short overview shows that the recently introduced lin-log kinetics, inspired by thermodynamic principles, shows considerable promise in achieving genome scale kinetic modelling of metabolic networks.

9 Conclusion

Thermodynamic principles do shape the properties of biological systems, with considerable and highly interesting consequences for their mathematical models needed in systems biology. Especially noteworthy are the far reaching consequences of the osmotic limit, such as pseudo-steady state, black box kinetics, just in time, control mechanisms, model reduction etc. Also of future importance is the principle of energy optimality for modelling of genetic mechanisms and of thermodynamic driving force (lin-log kinetics) for kinetic modelling. Lin-log kinetics seems to hold considerable promise to obtain realistic genome scale kinetic models (especially due to the lin-log based possibilities towards model reduction, a priori identifiability analysis and an initial estimate of the elasticity parameters).

Abbreviations and Symbols

X	Intracellular metabolite level	μ mol/g DM
S	Stoichiometry matrix	
e	Enzyme amount per cell mass	
p	Parameter	
μ	Specific growth rate	h^{-1}
q_i	Specific uptake/secretion rate	mol (or kJ/h)/CmolX
P_{osm}	Osmotic pressure	N/m^2
v	Rate of intracellular reaction	mol i/h/CmolX
ΔG^0_{fi}	Standard Gibbs energy of formation	kJ/mol
Y^{max}_{GX}	Maximal yield of biomass on Gibbs energy	CmolX/kJ
Y^{max}_{SX}	Maximal yield of biomass on substrate	CmolX/molS
Y^{max}_{SP}	Maximal yield of product on substrate	molP/molS
m_G	Maintenance Gibbs energy requirement	kJ/h/CmolX
C	Number of C-atoms in carbon source	
γ	Degree of reduction of C-source (per C-atom)	
R	Gas constant 8.314	J/molK
T	Absolute temperature	K
$\Delta G^{01}_{cat,D}$	Gibbs energy of the catabolic reaction per mol donor	kJ/mold
K_s	Affinity for substrate	$molS/m^3$
C_s	Substrate concentration	$molS/m^3$
t.o.t.	Turn over time	s
J	Flux	mol/h/C-molX
ε_{ij}	Elasticity coefficient $\left(\frac{x_j}{v_i}\frac{\partial v_i}{\partial x_j}\right)$ of metabolite j on enzyme i	
m_i	Maintenance coefficient for i (O_2, substrate, ethanol, etc.)	$\frac{\text{mol of } i/h}{\text{CmolX}}$

Subscripts

j	Metabolite j
i	Reaction i
p	Product
S	Substrate
x	Biomass
G	Gibbs energy
o	O_2
el	Electrons
D	Donor
Cat	Catabolic

Superscripts

^{1}	Biochemical standard (pH = 7)
^{0}	Standard condition (1 mol/L, 1 bar, 298 K) or reference condition, also reference steady state

References

1. Atkinson DE (1969) Limitation of metabolite concentrations and the conservation of solvent capacity in the living cell. Curr Top Cell Reg 1:29–43
2. Canelas A, van Gulik WM, Heijnen JJ (2008) Determination of the cytosolic free NAD/NADH ratio in *Saccharomyces cerevisiae* under steady-state and highly dynamic conditions. Biotechnol Bioeng 100(4):734–743
3. Nasution U, van Gulik WM, Kleijn RJ, van Winden WA, Proll A, Heijnen JJ (2006) Measurement of intracellular metabolites of primary metabolism and adenine nucleotides in chemostat cultivated *Penicillium chrysogenum*. Biotechnol Bioeng 94(1):159–166
4. Nasution U, van Gulik WM, Proell A, van Winden WA, Heijnen JJ (2006) Generating short-term kinetic responses of primary metabolism of *Penicillium chrysogenum* through glucose perturbation in the bioscope mini reactor. Metab Eng 5(5):395–405
5. Taymaz-Nikerel H, de Mey M, Ras C, ten Pierick A, Seifar RM, van Dam JC, Heijnen JJ, van Gulik WM (2009) Development and application of a differential method for reliable metabolome analysis in *Escherichia coli*. Anal Biochem 386(11):9–19
6. Maris AJA, Konings WN, Dijken JP, Pronk JT (2004) Microbial export of lactic and 3-hydroxypropanoic acid: implications for industrial fermentation processes. Metab Eng 6 (4):245–255
7. Van Gulik WM, Heijnen JJ (1995) A metabolic network stoichiometry analysis of microbial growth and product formation. Biotechnol Bioeng 48(6):681–98
8. Van Gulik WM, De Laat WTAM, Vinke JL, Heijnen JJ (2000) Application of metabolic flux analysis for the identification of metabolic bottlenecks in the biosynthesis of penicillin-G. Biotechnol Bioeng 68(6):602–618
9. Heijnen JJ, Van Dijken JP (1992) In search of a thermodynamic description of biomass yields for the chemotrophic growth of microorganisms. Biotechnol Bioeng 39(8):833–58
10. Heijnen JJ (1999) Bioenergetics of microbial growth. In: Flickinger MC, Drew SW (eds) Encyclopedia of bioprocess technology: fermentation, biocatalysis and bioseparation. ISBN: 0-471-13822-3. Wiley, New York, pp 267–291

11. Tijhuis L, van Loosdrecht MCM, Heijnen JJ (1993) A thermodynamically based correlation for maintenance Gibbs energy requirements in aerobic and anaerobic chemotrophic growth. Biotechnol Bioeng 42(4):509–19
12. Anderson KB, Meyenburg K von (1980) J Bacteriol 144:114–123
13. De Jong-Gubbels P, Vanrolleghem P, Heijnen S, Van Dijken JP, Pronk JT (1995) Regulation of carbon metabolism in chemostat cultures of Saccharomyces cerevisiae grown on mixtures of glucose and ethanol. Yeast 11(5):407–418
14. Vanrolleghem PA, de Jong-Gubbels P, van Gulik WM, Pronk JT, van Dijken JP, Heijnen S (1996) Validation of a metabolic network for *Saccharomyces cerevisiae* using mixed substrate studies. Biotechnol Progr 12(4):434–448
15. Stuckrath I, Lange HC, Kotter P, Van Gulik WM, Entian KD, Heijnen JJ (2002) Characterization of null mutants of the glyoxylate cycle and gluconeogenic enzymes in *S. cerevisiae* through metabolic network modeling verified by chemostat cultivation. Biotechnol Bioeng 77(1):61–72
16. Van Gulik WM, Antoniewicz MR, DeLaat WTAM, Vinke JL, Heijnen JJ (2001) Energetics of growth and penicillin production in a high-producing strain of *Penicillium chrysogenum*. Biotechnol Bioeng 72(2):185–193
17. Smolders GJF, van der Meij J, van Loosdrecht MCM, Heijnen JJ (1995) A structured metabolic model for anaerobic and aerobic stoichiometry and kinetics of the biological phosphorus removal process. Biotechnol Bioeng 47(3):277–87
18. Wiechert W (2002) Modeling and simulation: tools for metabolic engineering. J Biotechnol 94:37–63
19. Teusink B, Passarge J, Reijenga CA, Esgalhado E, Van der Weijden CC, Schepper M et al. (2000) Can yeast glycolysis be understood in terms of in vitro kinetics of the constituent enzymes? Testing biochemistry. Eur J Biochem 267:5313–5329
20. Rizzi M et al. (1997) In vivo analysis of metabolic dynamics in *Saccharomyces cerevisiae*: II. Mathematical model. Biotechnol Bioeng 55:592–608
21. Theobald U et al. (1997) In vivo analysis of metabolic dynamics in *Saccharomyces cerevisiae*: I. Experimental observations. Biotechnol Bioeng 55:305–316
22. Visser D et al. (2002) Rapid sampling for analysis of in vivo kinetics using the BioScope: a system for continuous-pulse experiments. Biotechnol Bioeng 79:674–681
23. Nikerel IE, Van Winden WA, Van Gulik WM, Heijnen JJ (2006) A method for estimation of in-vivo elasticities in metabolic networks using data from steady-state and rapid sampling experiments with lin-log kinetics. BMC Bioinformatics 7:540
24. Nikerel IE, Van Winden WA, Van Gulik WM, Heijnen JJ (2007) Linear-logarithmic kinetics; a framework for modelling kinetics of metabolic reaction networks. Simulation News Europe 17(1):19–26
25. Nikerel IE, Van Winden WA, Verheijen PJT, Heijnen JJ (2009) Model reduction and a priori kinetic parameter identifiability analysis using metabolome time series for metabolic reaction networks with lin-log kinetics. Metabolic engineering 11:20–30
26. Wu L, Van Dam JC, Schipper D, Kresnowati MTAP, Pröll A, Ras C, Van Winden WA, Van Gulik WM, Heijnen JJ (2006) Short term metabolome dynamics and carbon, electron and ATP balances in chemostat-grown *Saccharomyces cerevisiae* CEN-PK.113-7D following a glucose pulse. Appl Environ Microbiol 72(5):3566–3577
27. Heijnen JJ (2005) Approximative kinetic formats used in metabolic network modelling – review. Biotechnol Bioeng 91(5):534–545
28. Visser D, Heijnen JJ (2003) Dynamic simulation and metabolic re-design of a branched pathway using lin-log kinetics. Metab Eng 5:164–176
29. Westerhoff HV, ven Dam K (1987) Mosaic non-equilibrium thermodynamics and the control of biological free energy transduction. Elsevier, Amsterdam
30. Del Rosario RCH, Mendoza E, Voit EO (2008) Challenges in lin-log modelling of glycolysis in *Lactococcus lactis*. IET Syst Biol 2(3):136–149
31. Visser D, Schmid JW, Mauch K, Reuss M, Heijnen JJ (2004) Optimal redesign of primary metabolism in *Escheria coli* using lin-log kinetics. Metab Eng 6:378–390

32. Smallbone K, Simeonides E, Broomhead D, Kell D (2007) Something from nothing – bridging the gap between constraint – based and kinetic modelling. FEBS J 274:5576–85
33. Kresnowati MTAP, van Winden WA, Heijnen JJ (2005) Determination of elasticities, concentration and flux control coefficients from transient metabolite data using linlog kinetics. Metab Eng 7(2):142–153
34. Nikerel IE, Canelas AB, Jol SJ, Verheijen PJT, Heijnen JJ (2009) Construction of kinetic models for metabolic reaction networks: lessons learned in analysing short-term stimulus response data. Proceedings MATHMOD 09, 805–814. Presented at MATHMOD 09, Vienna, February 11–13, 2009
35. Raamsdonk LM, Teusink B, Broadhurst D, Zhang NS, Hayes A, Walsh MC et al (2001) A functional genomics strategy that uses metabolome data to reveal the phenotype of silent mutations. Nat Biotechnol 19:45–50
36. Wu L, Van Winden WA, Van Gulik WM, Heijnen JJ (2005) Application of metabolome data in functional genomics: a conceptual strategy. Metab Eng 7:302–310
37. Van Gulik WM, van Winden WA, Heijnen JJ (2002) Metabolic flux analysis, modeling and engineering solutions. In Vinci VA, Parekj SR (eds) Handbook of industrial cell culture. Mammalian, microbial, and plant cells. Humana Press, Towota, pp 351–395
38. Wu L, Wang W, Van Winden WA, Van Gulik WM, Heijnen JJ (2004) A new framework for the estimation of control parameters in metabolic pathways using lin-log kinetics. Eur J Biochem 271:3348–3359
39. Heijnen JJ, van Gulik WM, Shimizu H, Stephanopoulus G (2004) Metabolic flux control analysis of branch points: an improved approach to obtain flux control coefficients from large perturbation data. Metab Eng 6:391–400
40. Magnus JB, Hollwedel D, Oldiger M, Takors R (2006) Monitoring and modelling of the reaction dynamics in the valise/leucine synthesis pathway in *Corynebacterium glutamicum*. Biotechnol Progr 22:1071–1083
41. Feng-Sheng Wang, Chih-Lung Ko, Voit EO (2007) Kinetic modelling using S-systems and lin-log approaches. Biochem Eng J 33:238–247
42. Reuss M, Aquilera-Vazquez L, Mauch K (2007) Reconstruction of dynamic network models from metabolite measurements. Top Curr Genet 18:97–127. Nielsen J, Jewett MC (eds) Metabolomics. Springer, Heidelberg
43. Reed JL, Palsson BO (2003) Thirteen years of building constraint-based in-silico models of *Escherichia coli*. J Bacteriol 185(9):2692–2699
44. Heinrich R, Schuster S (1996) The regulation of cellular systems. Science 372 p