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Review Article

Biothermodynamics: Bridging Thermodynamics with Biochemical Engineering

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Abstract

As opposed to chemical process technology, bioprocess engineering has hardly benefitted from the potential of thermodynamics at all. This leads still today to the need of extensive and expensive experimental work when developing bio-products and bioprocesses. It is expected that this need could be limited if it becomes possible to apply thermodynamics to living systems more successfully. This represents quite a challenge in view of the daunting complexity of living systems. This contribution briefly reviews the progress already made and the hurdles still remaining in bridging thermodynamics with biochemical engineering.

Keywords: Biothermodynamics; Thermodynamics; Biochemical

Introduction

Since the 19th century, Thermodynamics has had an enormous impact on a very wide variety of fields, including chemistry, biology, physics, geology, and especially also on process engineering disciplines such as chemical engineering. In the area of basic chemical process technology, dealing with gas and oil processing, it was possible already 20 years ago to design whole new plants and even to develop new processes with only a bare minimum of experimental work, if any [1]. This was true because chemical thermodynamics enabled scientist and engineers to predict the behavior of organic molecules in mixtures based on advanced molecular models and on the availability of excellent experimental databases.

In the area of Bioprocess Engineering the situation is radically different. Still today, new processes cannot be developed without extensive experimental trials, which are often done on high throughput platforms allowing massively parallel experimentation. These may be impressive to see, but they also demonstrate our vast lack of knowledge that forces us to try everything out empirically.

About 20 years ago a group of scientists created an advanced course on thermodynamics in biochemical engineering in the hope of making the available knowledge in biothermodynamics better known amongst the biochemical engineers and to stimulate some more research in order to curtail the need for the extensive and costly experimentation in bioprocess development. This course was given 7 times at various locations around Europe, and finally the material that had accumulated was made available to a larger audience in the form of the book [2]. This book will serve partially as a base for the following short overview.

It turns out that the literature in this area can be divided into 3 large subdomains according to the scale at which the biological systems are formalized and thermodynamically analyzed (cf. Figure 1) [3].

Figure 1: Subdividing biothermodynamics into 3 areas according to the scale at which the biological system is described. Adapted from von Stockar [3].

The most fundamental description would be right at the molecular level and appears at the bottom of Figure 1. This so-called bio molecular thermodynamics is of course of fundamental importance for developing downstream processes, for understanding structural and functional stability of large biomolecules and the like and is by far the best developed of the three areas shown in Figure 1. When dealing with live cells things become so complex on the molecular level that simplifications must be introduced. Thermodynamics is then applied to whole metabolic pathways, thus giving rise to thermodynamics of metabolism. This subdomain appears on the intermediate level in Figure 1. In applying thermodynamics to whole cellular cultures further simplifications seem necessary, such as treating live cells simply as black boxes. This area appears at the top in Figure 1 and could be termed whole cell thermodynamics.

Biomolecules

Applying biomolecular thermodynamics to downstream process (DSP) development is expected to make major contributions as a tool for predicting phase equilibrium behavior of biomolecules in L/L extraction, in solid-liquid partition for salting in or out or for chromatographic applications, in aqueous two phase partitioning (ATPS) and many similar applications. A central topic is the prediction of activity coefficients γ_i that are indispensable for linking the chemical potentials μi to the actual concentrations or mole fractions x_i:

The most popular way to predict these in organic chemistry are probably so-called excess Gibbs energy models such as UNIQUAC, NRTL, and COSMO-RS. These estimate the excess partial Gibbs energy g_i^E. Unfortunately, they do not seem to work very well for aqueous solutions of biomolecules as demonstrated in Figure 2.

Partition coefficients must be known for the design of the extraction process of penicillin G, that is produced by microbial cultures in aqueous phase at a very large scale, into suitable organic extractants such as alkyl acetate esters. Despite the fact that Pen G is still very far away from really large biological molecules, the widely used thermodynamic model UNIFAC overestimates the experimentally determined partition coefficients by about two orders of magnitude, and, what is worse, predicts tendencies for homologous series of solvents incorrectly.

Figure 2: Comparison of experimental partition coefficients for PenG (K PenG) between water and alkyl acetate esters with those predicted by UNIFAC [1,4].

One reason for these difficulties is clearly the fact that biological molecules often bear pH dependent electrostatic charges. It has therefore been proposed to estimate the activity coefficients by means of the extended Debye-Hückel theory, but as will be demonstrated later, this is probably not very reliable either.

An alternative way to the excess Gibbs energy models is to predict activity coefficients based on Equations of State (EOS). These yield fugacity coefficients from which the activity coefficient can be calculated as

where φ_i stands for the fugacity coefficient of the i-th compound in the mixture and φ_i^0 for its fugacity coefficient in the chosen standard state. It was recently demonstrated by Held and Sadowski [5] that one such theory known as electrolyte Perturbated-Chain Statistical Associating Fluid Theory (ePC-SAFT) is clearly superior to excess energy models in predicting partition coefficients. Research continues along these lines and is expected to improve the situation decisively.

Other important applications of thermodynamics to biomolecules include

- Research into structural and functional stability of large biomolecules. Owing to thermodynamics and calorimetry the forces that hold proteins and DNA together as well as those responsible for denaturation can be studied. Research shows the different types of denaturation and how these can be avoided [6-8].
- Prediction and manipulation of reaction equilibria in biotransformation processes. The effects of temperature, pressure, pH and co-solutes on the stability and activity of enzymes and of whole-cell biocatalysts, and especially the use of non-aqueous solvents have been studied intensively since the eighties of last century. As a result there exists today a quite solid basis for bioreaction equilibrium engineering in order to shift the reaction equilibrium towards the desired product [9].

Thermodynamics of metabolism

The application of thermodynamics to metabolic networks has been introduced less by bio-process engineers than by scientists active in the field of systems biology. One of the main goals of this field is the prediction of all enzymatic reaction rates occurring inside live cells as well as of all the substance consumption and excretion rates between the cell and its environment. This would yield a complete overview of the product distribution and provide the necessary insight into the metabolism needed to genetically modify it as a basis for designing and tailor-making novel biocatalysts for bio-refineries.

One of the core tools of systems biology is metabolic flux analysis. This consists of a construction of a model of the whole cellular network based on the knowledge of the important enzymes present in the cell from genomics and of the important metabolites from metabolomics. For each metabolite, a molar balance is formulated as shown in Figure 3. Each line represents the balance of a particular metabolite, whereby v_j stands for the rate of the j-th enzymatic reaction and $s_{i,j}$ for the stoichiometric coefficient of the i-th metabolite in the j-th reaction. As shown in Figure 3 the entirety of these equations can be written as a matrix equation with S representing the stoichiometry matrix with a number of lines equal to the number of metabolites and the number of columns equal to the number of enzymatic reactions. v represents the vector of enzymatic rates. The idea consists of assuming the cell to be at steady state, rendering the vector of the concentration derivatives to zero, and of solving the equation for the vector v.

Figure 3: Molar balances in metabolic flux analysis.

Unfortunately, the number of metabolites in networks is always much smaller than the number of enzymatic reactions. This can already be concluded from the very simple hypothetical network shown in Figure 4, where only 4 balances can be written for 5 different enzymatic rates, even if we assume that the feed rate of A and the excretion rate of D have been measured.

Figure 4: Mass balance equations for networks are usually underdetermined.

A typical example for a genome-wide metabolic flux analysis was published by Feist, et al. where 1668 metabolites were

opposed to 2381 reactions [10]. This obviously corresponds to a vastly undertermined system of equations, which will not yield a single solution, but only a solution space.

Many different methods have been proposed to reduce this solution space. One proposition was to use constraints from thermodynamics for this purpose. Indeed, according to the 2nd Law of Thermodynamics each and every enzyme reaction must obey the following constraint:

$$\Delta_{rj} \bullet v_j < 0. \quad (3)$$

This equation can be used for determining the direction in which the reaction is occurring. If the direction of the reaction is known beforehand, the equation reduces to $\Delta_{rj}G < 0$ and can be used to place limits on the concentration distribution such that the equation is satisfied. The problem of the constraints based on thermodynamics is the fact that they introduce a host of new unknowns, so that the system tends to remain undetermined.

The equation $\Delta_{rj}G < 0$ is the basis of so-called Thermodynamic Feasibility Analyses. In 1993 Mavrovouniotis presented an algorithm permitting to determine whether concentration distributions exist in linear pathways that satisfy this equation for all the constituent enzymatic reactions [11]. If such a concentration distribution can be found, the pathway is deemed to be thermodynamically feasible, otherwise it is assumed to be impossible. Alternative algorithms have also been developed permitting to integrate the thermodynamic constraints into genome-wide mathematical reconstructions of cellular metabolism [12-14]. Thermodynamic Feasibility Analysis would be of great use to assess the thermodynamic feasibility of envisaged molecular genetic modifications of the metabolism before undertaking costly experimental developments.

In order to investigate what additional data is required with what accuracy in thermodynamic analysis of metabolic pathways the thermodynamic feasibility analysis was subjected to a test by applying it to the well known central pathway of glycolysis [15,16]. Not only much of the data needed is better known for glycolysis than for other pathways, but the result of such an analysis must clearly demonstrate the thermodynamic feasibility of this pathway as it is operative in most of the living cells on this planet.

When, however, published concentration ranges of the participating metabolites found in the literature were used as maximum and minimum possible concentrations in the analysis, glycolysis turned out to be essentially forbidden. It is possible that one of the reasons for this clearly erroneous result was the fact that the activity coefficients were calculated using the ex-tended Debye-Hückel theory. According to what was said in section on bio-molecular thermodynamics, this analysis ought to be repeated based on the ePC-SAFT. But the study also showed that correctly assessing the thermodynamic feasibility requires experimental values for, among other things, the apparent standard Gibbs energies of reaction in some clearly de-fined reference state, the activity coefficients of all the participating species, the equilibrium constants of all the complexes the involved species can undergo with hydrogen and magnesium ions, as well as realistic ranges of the pH, pMg, ionic strength and of the concentrations of the involved compounds in the cytosol of the live cells. Therefore, quite some research is required before the thermodynamic analysis of metabolic networks can be used to their promising full potential.

Live cellular cultures

Thermodynamics may also be applied to whole cultures of live microbial or animal cells, but due to the daunting complexities of such processes it is appropriate to simplify the description of the system even further. Figure 5 depicts such a culture growing in a bio-reactor schematically.

Figure 5: System considered for establishing energy balance for heat measurements. i, i-th chemical compound; e, e-th mass exchange port.

The highly complex biochemical transformations may sometimes be described by one or a few so-called macro-chemical equations with fixed stoichiometries, of which the following is an example:

Where S stands for a C-mole of a carbon and energy substrate, such as glucose, X for a C-mole of dry biomass, P for a product, and Yi/X for the stoichiometric coefficient, or yield, of substance i per C-mole of dry biomass grown. Neglecting all exo- or endothermic side processes other than growth an energy balance may be written around this bioreactor based on the First Law of Thermodynamics [3]:

$$\rho V \cdot \bar{c}_p \cdot \frac{dT}{dt} = \dot{Q} + \dot{W} + \sum_e \dot{V}_e \rho_e \bar{c}_{pe} (T_e - T) - \Delta_r H_X \cdot r_X V$$

where p, V, c_p and T stand for, respectively, the density, the volume, the mean specific heat capacity and the temperature of the culture broth, where as p_e, V_e, c_p and T_e denote the density, the volume flow rate, the mean specific heat capacity and the temperature of the mixture entering into the system through the e-th mass exchange port. Q and W stand for the total heat flux transferred into the bioreactor and for the mechanical and electrical power done to the culture (kW), respectively. $\Delta_r H_X$ and r_X are the heat and the rate of the macro-chemical growth reaction per C-mole of dry biomass grown. The heat transfer flux Q may often be measured on-line in bioreactors by adjusting the cooling rate such that the temperature T remains constant, either by observing the temperature difference T – Te needed to do that, or by establishing a heat balance around the cooling circuit. In small bioreactors, uncontrolled heat losses by-passing the cooling device must be suppressed and residual temperature variations dT/dt as well as the second and the third right hand term of Eq. 5 must be corrected for. Monitoring the heat generation rate becomes particularly suitable in large-scale bioreactors because they essentially operate adiabatically, such that the heat flux Q is dominated by the cooling coils or jacket.

Based on Equation 5 it is possible to either

Predict the heat evolution rate Q of the culture if all the other elements of the balance are known, which is of prime importance when designing the necessary cooling capacity of the bioreactor, or

Observe the biological activity on-line in terms of Q and measure the growth rate r_X on-line if $\Delta_r H_X$ is known, or

Observe the biological activity on-line in terms of Q and measure the heat of growth $\Delta_r H_X$ online if r_x is known. The heat of growth contains information on the stoichiometry of the growth reaction.

The most productive way to use the on-line information of Q is to combine it with other on-line rate information such as the oxygen uptake rate, the carbon dioxide evolution rate, and the base consumption rate needed to keep the pH constant. Using these signals in a combination with elemental-, energy and charge balances often allows to obtain on-line information on the rates of substrate consumption, product secretion and growth [17,18].

Applying the Second Law of Thermodynamics to growing cellular cultures has been shown to be useful in predicting important culture performance parameters such as growth yields, product yields, growth rates and maintenance requirements. Estimation of growth yields is of central importance because this parameter measures how much biomass can be grown from a given amount of carbon and energy substrate and determines how much, how fast and at what costs a desired product may be obtained from a cellular culture.

The ease at which research can be done on a given strain also depends on this yield. On the other hand, biomass yields for given microbial strains vary from values as low as 0.015 to 0.8 C-mole/C-mol. Many methods have been proposed and explored in order to come up with biomass yield predictions, but this discussion will be limited to those based on the 2nd Law. A more complete overview can be found in the literature [3]. Also, the following discussion is restricted to microbial cultures.

At first sight, the 2nd Law appears counterintuitive to cellular growth because the latter occurs spontaneously and obviously is a highly irreversible phenomenon. The 2nd Law has it that such processes must be coupled with a large production of entropy, and yet, microbial (and animal) cell growth transform simple nutritional molecules into new cells, representing a highly organized form of matter. One intuitively gets the impression that cellular growth reduces the entropy rather than producing it.

This contradiction may be resolved on the basis of an open-system entropy balance around a cell [19]. It is easier, however, to contemplate a Gibbs energy balance. If one considers the anabolic, ie the biosynthetic reactions alone, the living cell indeed seems to catalyze the transition of matter from a certain Gibbs energy

level to a higher one because of the polymerization and the high organizational state of the fresh biomass produced (upper green curved arrow on Figure 6). This reaction tends to have a somewhat positive Gibbs energy of reaction

Figure 6: Gibbs energy transduction from a catabolic, ie energy- yielding to an anabolic, ie biosynthetic reaction in a growing cell. This transduction is symbolized by a cog-wheel mechanism (two circles).

Reaction (blue arrow showing upwards). The Gibbs energy of reaction may be understood as its driving force: in the same way a vehicle on a slope has a tendency to roll down the slope the faster the steeper the slope is, ie the more potential energy is lost per meter of advancement, chemical phenomena have a tendency to occur the faster the more Gibbs energy is dissipated per mole of reaction advancement, ie the more negative the Gibbs energy of reaction $\Delta_r G$ is. As the Gibbs energy change of the biosynthetic reaction shown on Figure 6 is positive, it is subject to a driving force in the wrong direction, disintegrating the fresh biomass into simpler molecules.

In order to prevent that and to force the anabolic reaction "up-hill" against its own driving force, it is coupled through the biochemical machinery (ATP, NADH etc) to the energy yielding, "catabolic" reaction (curved downhill pointing red arrow in Figure 6). This reaction often starts from the same type of molecule (called carbon and energy substrate) as the biosynthetic reaction, but degrades it into much simpler fragments such as carbon dioxide and water, which are depleted of energy. Due to the large generation of entropy the catabolic reaction is associated with a strongly negative Gibbs energy process change $\Delta_r G$ and thus carries a strong driving force, which can be transferred to the biosynthetic reactions.

The coupling of an endergonic reaction with a driving force in the wrong direction to a strongly exergonic driving reaction with a highly negative $\Delta_r G$ can be compared to a mountain cable car used to bring tourists to the top of a mountain (Figure 7). Traditionally such cable cars were operated by filling a tank of the car at the top of the mountain with water, making it heavier than the car at the bottom with the tourists. The car with the water tank therefore has a larger driving force for rolling down the slope than the car with the tourists and can thus force this car with its payload to roll up-hill against its own driving force.

Figure 7: Traditional mountain cable car being driven up-hill against its own driving force by another car made heavier by a tank full of water attached to it.

The driving force for the combined system is obviously proportional to the weight difference between the two cars and decreases with the payload, ie the number of tourists transported up-hill. The situation is analogous in the metabolism of growing cells, but the payload in this case is the biomass yield $Y_{X/S}$ that indicates the amount of dry biomass X that the cell can synthesize per amount of substrate S consumed. In both the cellular metabolism and the cable car the energy efficiency may be increased by increasing the payload (less energy or water spent per payload) but as the combined driving force decreases, the whole process is slowed down. This may be seen quantitatively

by calculating the driving force for the combined growth reaction from the following equation:

where $Y_{X/S}$ is the biomass yield in C-mole/C-mole, ΔG_{cat} represents the strongly negative Gibbs energy change of the energy yielding reaction, ΔGan stands for the positive Gibbs energy change of the biosynthetic reaction, and $\Delta_r GS$ denotes the Gibbs energy change of the combined process per C-mole of substrate totally consumed.

Equation 6 can be used to estimate the thermodynamically maximum possible biomass yield, which is reached when $\Delta_r G_S$ goes to zero. Without driving force, the growth would then proceed infinitely slowly because the system would be locked in a thermodynamic equilibrium. In the cable car analogue this would correspond to a transport of so many tourists that the two cars have the same weight.

Figure 8 shows a plot of the Gibbs energy of the whole growth process calculated by a similar equation as Eq. 6 above. The difference is that ΔG^0 is not evaluated per C-mole of consumed substrate, but per C-mole of freshly grown dry biomass. As larger values of the latter are assumed, the ΔG^0 value (black downward pointing arrow) decreases, ie becomes less negative, and reaches 0 at the maximal possible biomass yield of about 1.04 C-mole/C-mole. As can be seen, the experimentally observed biomass yields are considerably lower than this theoretical maximum. The reason is obviously the need for a sufficiently large driving force, which forces the cultures to dissipate between -250 and -500 kJ of Gibbs energy per C-mole of freshly grown dry biomass.

Figure 8: Gibbs energy of growth (black curve) and enthalpy of growth (red curve) as a function of assumed biomass yields for aerobic growth on glucose. Black symbols: measured biomass yields; red symbols: enthalpies of growth determined by reaction calorimetry as a function of measured biomass yields. : E. coli; : C. pseudotropicalis; : C. utilis: x: K. fragilis; +: S. cerevisiae. Adapted from [20].

The red curve represents a similar calculation for the enthalpy of growth ΔH^0, which for aerobic growth practically coincides with $\Delta_r G_x 0$. The values can therefore be compared with experimental calorimetric measurements as shown by the red symbols. Although they scatter quite a bit they still confirm the general trend of the red line.

The relationship shown as the black curve in Figure 8 can be used to predict the probable growth yield for a microbial strain as long as ΔG_{cat}, ΔG_{an} and $\Delta_r G_X$ are known. This could either be done on the basis of a plot such as Figure 8 or by using an equation similar to Eq 6 but yielding $\Delta_r G_X$ rather than $\Delta_r G_S$ and solving it for the biomass yield. The Gibbs energy of catabolism ΔG_{cat} can easily be evaluated based on a knowledge of the catabolic reaction employed by the strain. Values for ΔG_{an} can be found in the literature [20].

The Gibbs energy dissipation per C-mole of newly synthesized dry biomass $\Delta_r G_X$ would also be required, but is more difficult to estimate. It must be sufficiently negative to provide a driving force supporting a reasonable growth rate, but still small enough for supporting a large biomass yield. The Gibbs energy of the growth reaction $\Delta_r G_X$ has been calculated from measured and published biomass yields and correlated to known characteristics in various ways. Heijnen and coworkers developed a correlation with the degree of reduction and the number of carbon atoms of the carbon substrate [21-23]. The standard error of correlation (SEC) was about 10 absolute percent points. More recently, Liu, et al. proposed a considerably simpler correlation based on the degree of reduction of the energy substrate and obtained a SEC of ± 9% [24].

More recently, it was proposed that an optimal Gibbs energy dissipation could be predicted by incorporating the Gibbs energy dissipation due to maintenance requirements into a model [25]. The microbial cell was therefore assumed to perform 3 parallel reactions (Figure 9): i) the catabolic reaction for growth, which is coupled through ATP to the ii) the anabolic growth reaction, and iii) the catabolic reaction generating additional energy for maintenance tasks.

In a living cell, membranes constantly degrade and get leaky, protein molecules denature thermally with time, and DNA accumulates errors. These problems are continuously being fixed by dedicated maintenance reactions, but these are also endergonic and need to be driven up-hill by an additional extent of catabolism. This maintenance requirement of substrate has been measured for many microbial strains in chemostats. Tijhuis, *et al*. (1993) have analyzed a large body of such data and concluded that the rate of Gibbs energy dissipation for maintenance per C-mole of dry biomass in the culture could be correlated by a single Arrhenius-type equation depending only on the absolute temperature [26]. If

during evolution the Gibbs energy for growth (processes i and ii) of a microbial strain is reduced in order to increase the bio-mass yield (Figure 8), growth will become slower and more maintenance Gibbs energy will be required to maintain the slowly growing cell. The total Gibbs energy dissipation must thus have a minimum between the two extremes of a rapid metabolism with large driving forces but small biomass yields, and a slow metabolism with large Gibbs energy expenditures for maintenance. A metabolism with that minimum dissipation will have the highest possible biomass yield. By mathematically searching for this biomass yield maximum, a new way to predict yields can be developed [27]. It could eventually also be used to predict the optimal growth yield.

Figure 9: Representing cell metabolism by 3 simple reactions: i) catabolism for driving growth, ii) anabolism yielding new biomass, and iii) additional catabolism for maintenance requirements.

Different thermodynamic considerations have led to the proposal of methods for estimating microbial culture performance parameters other than optimal biomass yields. Tijhuis, et al. have shown how to estimate growth yields in situations where the growth rate is maintained at an artificially low level for instance in chemostats operating at low dilution rates [6]. Assuming that the liberation of Gibbs energy in the electron transport chain is the growth limiting phenomenon, Heijnen developed an expression for the maximum growth rate μ_{max} [3]. He also presented thermodynamic arguments for estimating the minimum substrate concentration at which growth ceases and the threshold concentration at which no substrate can be taken up any more. The methods presented above for estimating growth yields may also be used to compute the yields of products from catabolism, which is important in bio-refinery design [3].

Conclusion

Considerable progress has been made in bio-molecular thermodynamics. It is expected that future research into the models for predicting activity coefficients will further improve the situation and will contribute to a considerable reduction of the need for experiments in bio-process development. Biochemical engineers and industrial biotechnologists ought to be encouraged to make use of the available expertise. The thermodynamics of metabolism has a considerable potential in metabolic engineering and synthetic biology and is expected to be highly useful for developing novel biocatalysts for bio-refineries. A considerable amount of research is still needed before this potential can be brought to full fruition. This research will probably benefit from progress made in bio-molecular thermodynamics. Whole-cell thermodynamic is useful as a basis for on-line monitoring of pilot-plant and industrial-scale cultures based on their heat dissipation rates. Thermodynamic analysis of their Gibbs energy dissipation has been shown to be a basis for estimating important culture performance parameters before starting costly experimental trials. However, such methods are limited for the moment to microbial culture yielding catabolic products only. As soon as more complex metabolic stoichiometries are involved as for instance encountered in cultures synthesizing secondary metabolites or in animal cell cultures, it will be necessary to open up the black box of the cell and predict their behavior based on the progress expected in the thermodynamic analysis of systems biology.

Bibliography

1. Von Stockar U and van der Wielen LAM. “Back to Basics Thermodynamics in Bio-chemical Engineering”. *Advances in Biochemical Engineering/Biotechnology* 80 (2003): 1-17.

2. Von Stockar. “Biothermodynamics: The role of thermodynamics in biochemical engineering”. *EPFL Press, Lausanne* (2013).

3. Von Stockar U. “Biothermodynamics of live cells: a tool for biotechnology and biochemical engineering”. *Journal of Non-Equilibrium Thermodynamics* 35.4 (2010): 415-475.

4. Reschke M and Schügerl K. “Reactive extraction of pennicillin II: distribution coefficents and degrees of extraction”. *Chemical Engineering Journal* 28 (1984): B11-B20.

5. Held C and Sadowski G. "Thermodynamics of Bioreactions". *Annual Review of Chemical and Biomolecular Engineering* 7 (2016): 395-414.

6. Norde W. "Proteins. In: von Stockar (ed), Biothermodynamics, EPFL Press, Lausanne, Chap 12 (2013).

7. Chi EY., et al. "Thermodynamics if the Physical Stability of Protein solutions". In: von Stockar (ed), Biothermodynamics, EPFL Press, Lausanne Chap 14 (2013).

8. Hughesman CB., et al. "Measuring, Interpreting and Modeling the Stabilities and Melting Temperatures of B-Form DNA's that Exhibit a Two-State Helix- to-Coil Transition". In: von Stockar (ed), Biothermodynamics, EPFL Press, Lausanne, Chap 15 (2013).

9. Straathof AJJ. "Thermodynamics in Multiphase Biocatalysis". In: von Stockar (ed), Biothermodynamics, EPFL Press, Lausanne, Chap 13 (2013).

10. Feist AM., *et al*. "A genome scale metabolic reconstruction for Escherichia coli M.G.1655 that accounts for 1260 ORF's and thermodynamic information". *Molecular Systems Biology* 3 (2007): 1-18.

11. Mavrovouniotis ML. "Identification of qualitatively feasible metabolic pathways. In: L. Hunter (ed), Artificial intelligence and molecular biology", MIT Press Classic Series and AAAI Press, Boston (1993): 325-364.

12. Kümmel A., *et al.* "Putative regulatory sites unraveled by network-embedded thermodynamic analysis of metabolic data". *Molecular Systems Biology* 2 (2006): 0034.

13. Henry CS., *et al.* "Genome-Scale thermdynamic analysis of Escherichia coli metabolism". *Biophysical Journal* 90.4 (2006): 1453-1461.

14. Henry CS., *et al*. "Thermodynamics-based metabolic flux analysis". *Biophysical Journal* 92.5 (2007): 1792-1805.

15. Th Maskow and Von Stockar U. "How reliable are thermodynamic feasibility state-ments of biochemical pathways?". *Biotechnology and Bioengineering* 92.2 (2005): 223-230.

16. Vojinović V and Von Stockar U. "Influence of Uncertainties in pH, activity coefficients, metabolic concentrations, and other factors on the analysis of the thermodynamic feasibility of metabolic pathways". *Biotechnology and Bioengineering* 103.4 (2009): 780-795.

17. Jungo C., *et al*. "Mixed feeds of glycerol and methanol can improve the performance of Pichia pastoris cultures: A quantitative study based on concentration gradients in transient continuous cultures". *Journal of Biotechnology* 128.4 (2007a): 824-837.

18. Jungo C., *et al.* "A quantitative analysis of the benefits of mixed feeds of sorbitol and methanol for the production of recombinant avidin with Pichia pastoris". *Journal of Biotechnology* 131.1 (2007): 57-66.

19. Von Stockar U. "Biothermodynamics in Live Cells. In: Biothermodynamics, EPFL Press, Lausanne, Chap 19 (2013).

20. Von Stockar U., *et al*. "Thermodynamics of microbial growth and metabolism: An Analysis of the Current Situation". *Journal of Biotechnology* 121.4 (2006): 517-533.

21. Heijnen JJ and Van Dijken JA. "In Search of Thermodynamic Description of Bio-mass Yields for the Chemotrophic Growth of Micro-organisms". *Biotechnology Bioengineering* 39.8 (1992): 833-858.

22. Heijnen JJ., *et al*. "A black box mathematical model to calculate auto- and heterotrophic biomass yields based on Gibbs energy dissipation". *Biotechnology Bioengineering* 40.10 (1992b): 1139-1154.

23. Heijnen JJ. "Bioenergetics of microbial growth". In: M. C. Flickiger, S. W. Drew (eds) Bioprocess Technology: Fermentation, Biocatalysis and Bioseparation, J Wiley & Sons, Inc (1999): 267-291.

24. Liu JS., *et al*. "A comparison of various Gibbs energy dissipation correlations for predicting microbial growth yields". *Thermochimica Acta* 458.1-2 (2007): 38-46.

25. Von Stockar U. "Optimal Energy Dissipation in Growing Microorganisms and Rectification Columns". *Journal of Non-Equilibrium Thermodynamics* 39.1 (2014): 3-11.

26. Tijhuis L., *et al.* "A Thermodynamically Based Correlation for maintenance Gibbs Energy Requirements in Aerobic and Anaerobic Chemotrophic Growth". *Biotechnology Bioengineering* 42.4 (1993): 509-519.

27. Von Stockar U. The growth yield of microorganisms represent an optimal me-tabolism between fast growth with large Gibbs energy dissipation needed as driving force and slow metabolism with a large Gibbs energy dissipation for maintenance, in preparation (2019).

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