Metabolic Flux Balancing: Basic Concepts, Scientific and Practical Use

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Recently, there has been an increasing interest in stoichiometric analysis of metabolic flux distributions. Flux balance methods only require information about metabolic reaction stoichiometry, metabolic requirements for growth, and the measurement of a few strain-specific parameters. This information determines the domain of stoichiometrically allowable flux distributions that may be taken to define a strain's "metabolic genotype". Within this domain a single flux distribution is sought based on assumed behavior, such as maximal growth rates. The optimal flux distributions are calculated using linear optimization and may be taken to represent the strain's "metabolic phenotype" under the particular conditions. This flux balance methodology allows the quantitative interpretation of metabolic physiology, gives an interpretation of experimental data, provides a guide to metabolic engineering, enables optimal medium formulation, and provides a method for bioprocess optimization. This spectrum of applications, and its ease of use, makes the metabolic flux balance model a potentially valuable approach for the design and optimization of bioprocesses.

Given the need to quantitatively understand metabolic physiology in order to design and optimize bioprocesses, considerable attention has been devoted towards the mathematical description of metabolic function. Several attempts have been made to systematically model metabolic dynamics[1-6] and reviews are available[7,8]. A complete dynamic description of metabolism requires the knowledge of intricate regulatory features. However, the enzyme kinetic and regulatory information required to formulate such detailed dynamic models has proved difficult to obtain. Thus, the synthesis of detailed dynamic descriptions of metabolism that account for the kinetics and regulation of individual enzymes has proven difficult.

Recently, several studies have appeared that attempt to overcome this limitation. These studies are based on a steady-state analysis of metabolic pathway stoichiometry along with known metabolic requirements for growth to describe metabolic flux distributions and cell growth. This approach has yielded accurate and valuable information about how microbial cells utilize their metabolic fluxes and optimize their growth rates. Here we discuss how these models are formulated, their basic scientific utility, and their practical application.

Formulation of Flux Balance Models

Metabolic flux models are based on the assumption that metabolic transients are more rapid than both cellular growth rates and the dynamic changes in the organism's environment. Metabolism typically has transients that are shorter than a few minutes and thus metabolic fluxes are in a quasi-steady state relative to growth and typical process transients.

The mathematical formulation of the flux balance model requires two items of metabolic information. First, metabolic stoichiometry is required to write down all the chemical reactions that take place in the metabolic network of interest. In most cases this includes all of intermediary metabolism. For *Escherichia coli* this information is readily available[9], and catabolic[10] as well as biosynthetic stoichiometric models[11] have been formulated. Eukaryotic metabolic information can be obtained from standard literature sources and stoichiometric models have been formulated for these metabolic networks[12,13].

The second item that is needed is information about the demands that are placed on the metabolic system. These demands include biomass synthesis, maintenance requirements and, in selected cases, the secretion of an important product. For prokaryotic systems the major metabolic requirements are the provision of material for biomass synthesis. These requirements can be determined from a chemical composition analysis of the particular cell of interest. The metabolic requirements for growth have been estimated for *E. coli*[14-16] and also for murine hybridoma cells[13]. Maintenance requirements can be obtained from strain-specific experiments[17].

Obtaining the metabolic information required to formulate stoichiometric models is relatively straightforward. Once obtained, this information is put into the appropriate mathematical framework and the metabolic flux distributions are obtained by assuming that the cell is striving to meet a particular objective.

Mathematical structure. As shown in Figure 1, the catabolic pathways serve to degrade the carbon source into precursors from which monomers are synthesized which are then polymerized and assembled into cellular components[14]. Material balances can be written around a system comprised of a network of metabolic reactions. Inputs to the system include the carbon source provided in the culture medium while the outputs include by-products, additional biomass generated, as well as any maintenance requirements. The dynamic material balance determines metabolite concentrations, provided that the kinetics of the enzymatic reactions are known. The flux balance model eliminates this requirement by treating the metabolic reaction fluxes as the unknown quantities that need to be determined.

A metabolic quasi-steady state is assumed. This assumption is based on the fact that metabolic transients are typically rapid compared to cellular growth rates and environmental changes. The consequence of this assumption is that all metabolic fluxes leading to the formation and degradation of any metabolite must balance, leading to the flux balance equation[12,13]:

$$S \cdot v = b \qquad (1)$$

where S is a matrix containing the stoichiometry of the catabolic reactions, v is a vector of the 'n' metabolic reaction rates, and b is a vector containing the net metabolite uptake by the cell. Equation (1) is typically underdetermined since the number of fluxes normally exceeds the number of metabolites.

Therefore, a plurality of solutions exists and the cell is faced with an infinite number of choices on how it can distribute its

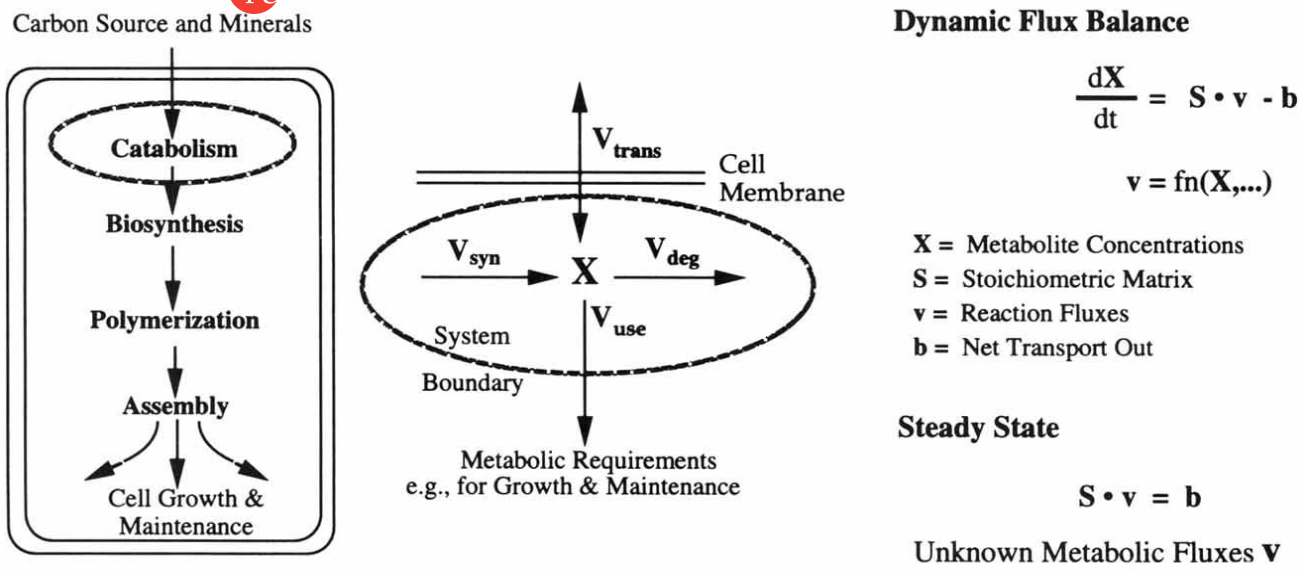

FIGURE 1. Flux balance models use material balances around each metabolite in a metabolic network. Inputs to the metabolic network include the carbon source provided in the culture medium while the outputs include by-products, additional biomass generated as well as the maintenance requirements.

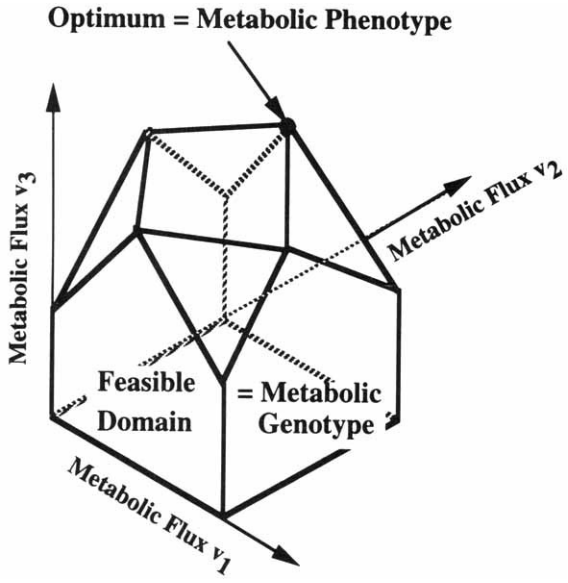

FIGURE 2. Stoichiometric constraints defined by Eqn. (1) define a feasible domain in an 'n' dimensional space where 'n' is the number of metabolic pathways. The feasible domain is thus conceptualized as the "metabolic genotype" since it is determined by the 'n' enzyme catalyzed metabolic pathways. A particular choice of flux distribution is represented by a point within the feasible domain and is conceptualized as the "metabolic phenotype" since the particular flux distribution is a characteristic of the organism's response to the culture environment.

metabolic fluxes. The choices are constrained by the stoichiometric matrix and these constraints form a domain of stoichiometrically allowable behavior as illustrated in Figure 2. This domain may be thought of as defining the "metabolic genotype" of the strain since it describes the metabolic flux distributions that can be achieved with the metabolic enzymes that the strain possesses. The enzyme "portfolio" of a strain thus determines its "metabolic genotype". It can be changed through genetic engineering.

The mechanisms by which metabolic flux distributions are chosen is a complex interplay of enzymatic and genetic regulatory events. Not all of these events are known in detail. However, the evolutionary selection process forces micro-organisms to make these choices such that the survivability of the cell is enhanced and the network acts in concert to attain this "optimal metabolism". For a given environment a given cell chooses to express and regulate a specific set of metabolic enzymes which act in concert to produce a particular metabolic flux distribution, which may be thought of as the "metabolic phenotype" of that strain under those conditions (Fig. 2). It is expected that the metabolic phenotype of wild-type strains is defined by a tendency to optimize their growth rates, at least in nutritionally rich environments such as those found in the majority of bioprocesses.

Why optimize? A particular solution for the distribution of metabolic fluxes may be found using linear optimization by stating an objective and seeking its maximal value within the stoichiometrically defined domain (Fig. 2). The reasons for choosing optimal metabolic behavior fall into three basic categories. First, optimization of growth usually confers a growth advantage that is selected for by natural processes. Thus, the determined flux distribution can be used to describe experimental results and to predict how cells will respond to changes in their environment. Second, one can determine the maximum allowable production capability of a particular strain and appropriate metabolic engineering strategies can be developed for strain design. Third, a bioprocess engineer can seek to optimize process design and control, for instance, through optimal medium formulation.

To illustrate, a maximal growth objective is implemented as follows[18]:

$$\text{Minimize} \quad Z = -V_{gro} \tag{2}$$

$$\sum_{\text{all } M} d_M \cdot M \xrightarrow{V_{gro}} biomass \tag{3}$$

where d_M are the requirements in mmol/g biomass of the M biosynthetic precursors and cofactors required for biomass production. V_{gro} is the growth flux (g biomass produced) which, with an appropriate basis (1 g DW-hr), reduces to the growth rate (g biomass produced/g DW-hr).

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FIGURE 3. Optimal aerobic growth and secretion of by-products predicted by the flux balance model for *E. coli* during glucose limited growth. Reproduced from ref. 20.

The linear optimization problem described above has an interesting mathematical duality[19]. The solution to the dual problem, the dual variables (γ_i), have a useful interpretation as the shadow prices. The shadow price defined in Eqn. (4) provides a useful intrinsic measure of the value of a metabolic intermediate towards optimizing the objective.

$$\gamma_i = \frac{\partial Z}{\partial b_i} \tag{4}$$

The shadow price (γ_i) measures the marginal increase in objective (Z) possible due to the addition of the i^{th} metabolite. With growth as the objective, the shadow price indicates the marginal usefulness of a metabolite towards accelerating the growth rate. The shadow price of metabolites have been used to explain the phenomenon of by-product secretion in *E. coli*[20] and also to determine the trade-off between growth and biochemical production[11]. The usefulness of a product, such as acetate, represented by the product's shadow price essentially determines the secretability of the product. Shadow prices may also be used to evaluate the ability of various medium components to enhance the stated objective.

Applications of Stoichiometric Flux Balance Models

Stoichiometric models have been used to address a variety of issues including basic metabolic physiology, simulation and interpretation of experimental data, metabolic engineering, optimal medium formulation, and process design. They have also been used for educational purposes[21,22].

Quantitative metabolic physiology. Flux balance models have been used to examine the physiology of ATP production during fat synthesis by adipocytes[12] and also the phenomenon of metabolic by-product secretion by microorganisms. Ethanol secretion by yeast[23] and acetate secretion by *Escherichia coli*[24,25,20,17] have been shown to arise from a balance between oxidative and reductive pathways.

The application of flux balance models to the interpretation of metabolic physiology can be illustrated by the computation of by-product secretion. Figure 3, reproduced from ref. 20, shows the optimal by-product secretion computed using the flux balance model formulated for *E. coli*. The figure shows the computed relationship between nutrient uptake rates and by-product secretion rates as a function of the growth rate. As growth rate increases the nutrient consumption *per* cell is found to increase. However, at high growth rates the oxygen utilization capacity is reached and the cell faces a condition of surplus reductive potential. For higher growth rates the metabolic fluxes redistribute[20] leading to a secretion of by-products as a means to eliminate surplus redox. Acetate secretion has been experimentally observed corresponding to the flux balance model predictions for *E. coli*[17,25,26]. Thus, flux balance models provide a prediction and interpretation of the metabolic physiology of microorganisms.

Interpretation of experimental data. The ability to extract intracellular information on pathway utilization using the flux balance model suggests that the model is suitable to serve as a process model for process control applications. A detailed flux balance model specified for the 'wild type' *E. coli* W3110 strain has been shown to accurately predict growth and by-product secretion of the bacterium. Figure 4, reproduced from ref. 17, illustrates the ability of the model to predict growth, glucose consumption, acetate secretion and reconsumption from the culture medium. Thus, knowing the response of cells to a particular culture environment, optimal feed strategies can be formulated to optimize a particular bioprocess.

As an alternative to linear optimization one can sometimes reduce the dimensionality of Eqn. (1) by experimentally measuring some of the metabolic fluxes. Thus, flux balance techniques may be coupled to extracellular measures of culture parameters in order to uniquely determine the intracellular metabolic flux distribution[27-29]. Stoichiometric flux balance techniques have used this approach to analyze microbial fermentation data in order to determine the intracellular reaction fluxes[30-34]. These methods show promise in determining yields and selectivities as well as the fermentation biochemistry.

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FIGURE 4. Flux balance model predictions compared to experimental data for aerobic fed-batch culture with continuous glucose injection at 0.2g Glc/l-hr, reproduced from ref. 17. The time profile of cell density, glucose, and acetate concentrations are shown with model predictions given as the solid line.

Metabolic engineering. Flux balance models provide a quantitative method enabling metabolic pathway analysis and design for bioprocesses optimization. Metabolic pathway engineering as a field of study has been recently reviewed[35-37]. Its primary goal is to design engineered strains to achieve higher efficiencies in metabolite overproduction through alterations in the metabolic flux distribution. Flexibility of the metabolic flux through key pathways has been determined for *Corynebacterium glutamicum*[38] in order to find 'principal' and 'rigid' nodes and reviews are available[36,37].

To illustrate the use of flux balance analysis for metabolic engineering, consider the production of the amino acid tryptophan. Figure 5a, shows a metabolic flux distribution that is optimized for tryptophan production which may be contrasted to the pathway utilization for optimal growth[11]. Using the flux balance techniques, one is able to determine the changes needed to shift the metabolic pathway utilization from producing biomass towards producing the desired biochemical species. Thus, it is possible to state production targets and desirable strain manipulations using flux balance models.

Optimal medium formulation. Stoichiometric techniques have been applied to a rational development of cell culture medium[39,40]. Instead of considering the entire metabolic pathways these studies have determined the stoichiometric rates of nutrient consumption. By optimizing the nutrient feed, accumulation of waste products has been reduced several fold for a hybridoma culture[39].

Quantification of metabolic fluxes has been carried out for bacterial cultures[41], and insect cell cultures[42]. In these studies it has been possible to determine the pathways of nutrient consumption and evaluate nutrient consumption. Flux balance techniques have also been applied to murine hybridoma cell metabolism and consumption of medium components[13,43].

Generally, a flux balance model may be formulated using known metabolic stoichiometry and metabolic demands. The optimal uptakes rates of various nutrients may be determined by linear optimization and this information can be used to state the desired medium composition. Several criteria can be used for optimization such as maximizing growth rate, minimizing waste products, maximizing product formation, etc. In addition, different optimal culture criteria can be specified for different phases of a culture.

Process design and optimization. A flux balance model of *E. coli* has been used to determine process conditions that may enhance strain stability. Figure 5b illustrates the conditions for increased stability during tryptophan production. Stability of a production strain is enhanced at the minimum growth advantage, *S*. The growth advantage is defined as a ratio of the growth rate of a non-producing network to the growth rate of a product producing metabolic network. The results computed using the flux balance model show that an optimal glucose to oxygen supply ratio can enhance the stability of a production strain. Thus, optimal feed strategies can be designed that allow for higher productivities with the aid of a flux balance model.

Conclusion

The examples outlined in this review illustrate the potential usefulness of flux balance based metabolic models. Their applications span the interpretation of experimental data, quantitative insights into metabolic physiology, design methods for metabolic engineering, optimal medium formulation, and bioprocess design and optimization. The formulation of flux balance models is relatively simple and requires only the statement of metabolic reaction stoichiometry, enumeration of the demands that a metabolic network must meet, and the experimental measurement of strain specific parameters[17]. Their ease of formulation, versatility in use and broad spectrum of applications, make metabolic flux balance models a potentially significant new method for the analysis of metabolic physiology and the design of optimal bioprocesses.

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FIGURE 5. (a) Optimal pathway utilization computed using the flux balance model for aerobic tryptophan production reproduced from ref. 11. (b) Population stability (S) during tryptophan production is computed from a flux balance model as a function of the oxygen supply. Population stability *S* is defined as the growth rate ratio between a non-producing network (μ_n) over a tryptophan producing metabolic network (μ_p). Reproduced from ref. 44.

References

1. Garfinkel, D., Garfinkel, L., Pring, M., Green, S. B. and Chance, B. 1970. Computer applications to biochemical kinetics. Ann. Rev. Biochem. **39:**473–498.
2. Heinrich, R., Rapoport, S. M. and Rapoport, T. A. 1977. Metabolic regulation and mathematical models. Prog. Biophys. Mol. Biol. **32:**1–82.
3. Joshi, A. and Palsson, B. O. 1989. Metabolic dynamics in the human red cell. Part I. A comprehensive model. J. Theor. Biol. **141:**515–528.
4. Kacser, H. and Burns, J. A. 1973. The control of flux. Symp. Soc. Exp. Biol. **27:**65–104.
5. Reich, J. G. and Sel'kov, E. E. 1981. Energy Metabolism of the Cell. Academic Press, New York.
6. Savageau, M. A. 1969. Biochemical systems analysis. II. The steady state solutions for an n-pool system using a power-law approximation. J. Theor. Biol. **25:**370–379.
7. Athel Cornish-Bowden and Maria Luz Cardenas (Eds.). 1990. Control of Metabolic Processes. NATO ASI Series A: Lifesciences Vol. 190. Plenum Press, New York.
8. Srere, P. A., Jones, M. E. and Matthews, C. K. (Eds.). 1990. Structural and Organizational Aspects of Metabolic Regulation. UCLA Symposia on Molecular and Cellular Biology, New Series, Vol. 133. Wiley-Liss, New York.
9. In Neidhardt, F. C. (Ed.). 1987. *Escherichia coli* and *Salmonella typhimurium*.

Cellular and molecular biology. American Society for Microbiology, Wash., DC.
10. Varma, A. and Palsson, B. O. 1993. Metabolic capabilities of *Escherichia coli*: I. Synthesis of biosynthetic precursors and cofactors. J. Theor. Biol. **165:**477–502.
11. Varma, A., Boesch, B. W. and Palsson, B. O. 1993. Biochemical production capabilities of *Escherichia coli*. Biotech. Bioeng. **42:**59–73.
12. Fell, D. A. and Small, J. A. 1986. Fat synthesis in adipose tissue. An examination of stoichiometric constraints. Biochem. J. **238:**781–786.
13. Savinell, J. M. and Palsson, B. O. 1992. Network analysis of intermediary metabolism using linear optimization: I. Development of mathematical formalism. J. Theor. Biol. **154:**421–454.
14. Ingraham, J. L., Maaloe, O. and Neidhardt, F. C. 1983. Growth of the Bacterial Cell. Sinauer Associates Inc., Sunderland, Massachusetts.
15. Neidhardt, F. C. 1987. Chemical composition of *Escherichia coli*, p. 3–6. *In: Escherichia coli* and *Salmonella typhimurium*. Cellular and Molecular Biology. Neidhardt, F. C. (Ed.). American Society for Microbiology, Wash., D.C.
16. Neidhardt, F. C., Ingraham, J. L. and Schaechter, M. 1990. Physiology of the Bacterial Cell. A Molecular Approach. Sinauer Associates, Sunderland, Massachusetts.
17. Varma, A., and Palsson, B. O. 1994. Stoichiometric flux balance models quantitatively predict growth and metabolic by-product secretion in wild type *Escherichia coli* W3110. Appl. and Environ. Microbiol. *In press*.
18. Varma, A. and Palsson, B. O. 1993. Metabolic capabilities of *Escherichia coli*: II. Optimal growth patterns. J. Theor. Biol. **165:**503–522.
19. Murty, K. G. 1983. Linear Programming. John Wiley & Sons, New York.
20. Varma, A., Boesch, B. W. and Palsson, B. O. 1993. Stoichiometric interpretation of *Escherichia coli* glucose catabolism under various oxygenation rates. Appl. and Environ. Microbiol. **59:**2465–2473.
21. Watson, M. R. 1984. Metabolic maps for the Apple II. Biochem. Soc. Trans. **12:**1093–1094.
22. Watson, M. R. 1986. A discrete model of bacterial metabolism. Computer Applications in the Biosciences. **2:**23–27.
23. Sonnleitner, B. and Kappeli, O. 1986. Growth of *Saccharomyces cerevisae* is controlled by its limited respiratory capacity: Formulation and verification of a hypothesis. Biotech. Bioeng. **28:**927–937.
24. Ko, Y., Bentley, W. E. and Weigand, W. A. 1993. An integrated metabolic modeling approach to desccribe the energy efficiency of *Escherichia coli* fermentations under oxygen-limited conditions: Cellular energetics, carbon flux, and acetate production. Biotech. Bioeng. **42:**843–853.
25. Majewski, R. A. and Domach, M. M. 1990. Simple constrained-optimization view of acetate overflow in *E. coli*. Biotech. and Bioeng. **35:**732–738.
26. Bajpai, R. 1987. Control of bacterial fermentations. Ann. N.Y. Acad. Sci. **506:**446–458.
27. Cooney, C. L., Wang, H. Y. and Wang, D. I. C. 1977. Computer-aided material balancing for prediction of fermentation parameters. Biotech. Bioeng. **19:**55–67.
28. Humphrey, A. E. 1974. Current developments in fermentation. Chemical Engineering **81:**98–112.
29. Vallino, J. J. and Stephanopoulos, G. N. 1987. Intelligent sensors in biotechnology. applications for the monitoring of fermentations and cellular metabolism. Ann. N. Y. Acad. Sci. **506:**415–430.
30. Papoutsakis, E. T. 1984. Equations and calculations for fermentations of butyric acid bacteria. Biotech. Bioeng. **26:**174–187.
31. Papoutsakis, E. T. and Meyer, C. L. 1985. Equations and calculationsof product yields and preferred pathways for butanediol and mixed-acid fermentations. Biotech. and Bioeng. **27:**50–66.
32. Papoutsakis, E. T. and Meyer, C. L. 1985. Fermentation equations for propionic-acid bacteria and production of assorted oxychemicals from various sugars. Biotech. and Bioeng. **27:**67–80.
33. Tsai, S. P. and Lee, Y. H. Application of metabolic pathway stoichiometry to statistical analysis of bioreactor measurement data. Biotech. Bioeng. **32:**713–715.
34. Vallino, J. J. and Stephanopoulos, G. 1990. Flux determination in cellular bioreaction networks: Applications to lysine fermentations, p. 205–219. *In:* Frontiers in Bioprocessing. Bier, M. and Todd, P. (Eds.). CRC Press, Boca Raton, Florida.
35. Bailey, J. E. 1991. Toward a science of metabolic engineering. Science **252:**1668–1675.
36. Stephanopoulos, G. and Sinskey, A. J. 1993. Metabolic engineering—methodologies and future prospects. Trends in Biotechnol. **11:**392–396.
37. Stephanopoulos, G. and Vallino, J. J. 1991. Network rigidity and metabolic engineering in metabolite overproduction. Science **252:**1675–1681.
38. Vallino, J. J. and Stephanopoulos, G. 1993. Metabolic flux distributions in *Corynebacterium glutamicum* during growth and lysine overproduction. Biotech. Bioeng. **41:**633–646.
39. Xie, L. and Wang, D. I. C. 1994. Fed-batch cultivation of animal cells using different medium design concepts and feeding strategies. Biotech. Bioeng. **43:**1175–1189.
40. Xie, L. and Wang, D. I. C. 1994. Stoichiometric analysis of animal cell growth and its application in medium design. Biotech. Bioeng. **43:**1164–1174.
41. Goel, A., Ferrance, J., Jeong, J. and Ataai, M. M. 1993. Analysis of metabolic fluxes in batch and continuous cultures of *Bacillus subtilis*. Biotech. Bioeng. **42:**686–696.
42. Ferrance, J. P., Goel, A. and Ataai, M. M. 1993. Utilization of glucose and amino acids in insect cell cultures: Quantifying the metabolic flows within the primary pathways and medium development. Biotech. Bioeng. **42:**697–707.
43. Savinell, J. M. and Palsson, B. O. 1992. Network analysis of intermediary metabolism using linear optimization: II. Interpretation of hybridoma cell metabolism. J. Theor. Biol. **154:**455–473.
44. Varma, A. and Palsson, B. O. 1994. Predictions for oxygen supply control to enhance population stability of engineered production strains. Biotech. Bioeng. **43:**275–285.