JOURNAL REVIEW

Dynamic Models of Metabolism: Review of the Cybernetic Approach

Doraiswami Ramkrishna and Hyun-Seob Song
School of Chemical Engineering, Purdue University, West Lafayette, IN 47907

DOI 10.1002/aic.13734
Published online February 2, 2012 in Wiley Online Library (wileyonlinelibrary.com).

The cybernetic approach to metabolic modeling tracing its progress from its early beginnings to its current state with regard to its relationship to other modeling approaches, applications to bioprocess modeling, metabolic engineering, and future prospects are described. The framework is shown to handle large metabolic networks in making dynamic predictions from limited data with looming prospects of extending to genome scale networks. © 2012 American Institute of Chemical Engineers *AIChE J,* 58: 986–997, 2012

Keywords: dynamic metabolic models, the cybernetic approach, metabolic engineering, elementary modes, cellular regulation

Introduction

Cellular metabolism involves the uptake of nutrients followed by transformation through a complex network of reactions catalyzed by highly specific enzymes, producing numerous intracellular components known as metabolites and other metabolic products that are excreted into the abiotic phase. A distinct aspect of metabolism is the presence of regulatory processes which endow the organisms to route chemical changes by the control of the levels and activities of various enzymes that catalyze metabolic transformations. Consequently, the metabolic state of an organism could be a function of its environment as much as of its genetic background.

Modeling of metabolism is of engineering interest in that its products cover a large variety of diverse applications. Quantitative understanding of metabolic processes is essential not only for the optimization and control of biological processes but also toward engineering genetic changes for improved metabolic performance. It is the objective of this article to examine the status of modeling in this area and to expound the interrelationship of various approaches in this regard, ratiocinating ultimately in support of the framework of dynamic cybernetic models. The cybernetic framework, since its inception,[1] has evolved progressively transcending various limitations to its current stage of development,[2–4] that holds unparalleled promise for a rational, quantitative description of metabolism. These developments will be reviewed in this article, providing a discussion of their relationship to "steady state" methods, known as the flux balance approach that has hitherto been more popular (than dynamic models) with analysis of metabolic data.

Correspondence concerning this article should be addressed to D. Ramkrishna at ramkrish@ecn.purdue.edu.

In the sequel, we first present the metabolic system as a set of chemical reactions including transport of the external nutrients into the cell, followed by a complex network of internal cellular reactions resulting in the production of various metabolites, and other products of metabolism. Internal organization in the cell could be accommodated by partitioning the state vector comprising the concentration levels of various chemical species into separate vectorial entities representing those in different spatially distributed compartments. A theoretical framework representing continuous spatio-temporal distribution of the state vector within the cell is conceivable but difficult from a computational perspective. Thus, the compartmental model must be viewed as a "macroscopic" version obtained by averaging the continuous perspective over each of the compartments within the cell. However, our focus, for the present, will be limited to a single state vector, say $\boldsymbol{\psi} \equiv \left[\psi_1, \psi_2, \ldots, \psi_{n_\psi}\right]$ of n_ψ components, whose application is most suited to prokaryotic organisms, although higher organisms have often been treated by modelers as an approximation. We deem the vector $\boldsymbol{\psi}$ to comprise the concentrations of all external species including nutrients and excreted metabolic products, intracellular variables referred to the biomass that include, the various enzymes that catalyze the different reactions. The inclusion of enzyme levels in the state vector is most fundamental to the cybernetic models that have made them distinctive. The reactions constituting metabolism may be represented by

$$\sum_{j=1}^{n_\psi} a_{ij}\Psi_j = 0,\ i = 1, 2, \ldots, n_r \tag{1}$$

where Ψ_j denotes the species whose concentration is ψ_j. Eq. 1 represents n_r reactions of metabolism which subsume both transport and chemical reaction with a_{ij} the stoichiometric coefficient which, as per the usual convention, is positive,

negative, or zero, in respective accordance with Ψ_j being a product, reactant, or nonparticipant in the ith reaction. The rate associated with the ith reaction is denoted by r_i. As each reaction is catalyzed by a specific enzyme, the level of this enzyme and its activity would determine the rate of the reaction besides the concentrations (as dictated by the kinetics) of species participating in the reaction.

The Nature of Regulatory Processes

Metabolism in microorganisms is subject to regulation by which the levels of various enzymes and their activities are subject to control. The well-known diauxic growth of bacteria in mixtures of sugars, discovered by Monod,[5] provides a classic example of regulation in which enzymes are preferentially synthesized for the utilization of one of the sugars. Similarly, the activities of enzymes are also subject to preferential control. Although detailed mechanisms of specific regulatory processes are known, mechanistic information about the phenomenon on the whole is largely unknown. From a modeling viewpoint, however, the dynamic description of metabolic processes must comprehensively address regulatory phenomena. This requirement, which is enforced on dynamic models, becomes irrelevant for flux balance approaches which relate intracellular fluxes through stoichiometric coupling to external fluxes that are experimentally measured. Consequently, such approaches cannot be wholly predictive; neither are they suitable to describe the state of the organism as determined by the concentration levels of all intracellular metabolites.

The Cybernetic Approach

In the face of inadequate information about mechanistic details of regulatory processes, the need for their incorporation into dynamic modeling calls for an approach that is radically different from that used for modeling physical systems. The cybernetic modeling strategy developed by Ramkrishna and coworkers must be regarded in this light. It builds on the idea that biological systems derive their robustness to diverse environmental changes from being able to respond by manipulating their metabolism in ways perhaps with at least as much diversity. It is generally understood that this capacity of living systems is an acquisition from adaptation to eons of exposure to extreme evolutionary pressures. The implication of the foregoing observation is that regulation of metabolism must be attached to a survival goal of the organism. The term "cybernetic," used to connote this goal-seeking aspect of system behavior, was first introduced by Wiener.[6] This idea of goal seeking behavior, although a long-standing feature of teleological reasoning in biology, however, has come under criticism as being antithetical to the usual scientific explanation of phenomena as a natural sequence of cause and effect. Furthermore, natural selection through evolution is itself not considered to be goal-oriented. In this connection, Mayr[7] has observed that such goal seeking behavior could be understood in light of how a genetic program can develop through progressive learning that formulates and enforces an optimal response. From this point of view, Mayr has proposed the use of the word "teleonomy" in place of the infamous "teleology" for the investigation of goal oriented systems within the framework of rational science.

The speculative nature of the actual survival goal has often led many researchers to be critical about the cybernetic methodology. This criticism ignores the value of inductive reasoning in science that leads to understanding of systems through successive formulation of hypotheses and revision by comparison with observation. It would appear that the study of biological systems should include cybernetic mechanisms that can provide bases for mathematical models and suitable experiments for their verification. Indeed such practice already has come into being in quantitative investigation of biological phenomena. For example, the flux balance analysis (FBA) has relied on the use of linear programming based on the postulate that the organism maximizes the biomass yield.[8,9] Although this approach effectively resolves the mismatch between the large number of variables (fluxes) and the considerably less number of (steady state mass balance) equations, there has been no articulation by the practitioners of FBA on the significance of the assumption of maximizing the biomass yield.[10] Further, although the use of an optimization principle such as maximizing the yield of biomass would seem to imply a cybernetic basis, its qualification for the same is rendered suspect as the strategy contains no element of timeliness, an attribute that would appear to be essential for survival in a dynamically varying environment. Consequently, the objective functions in our cybernetic models would stress maximization of the rates of one aspect of metabolic performance or another. We deem this point of departure of cybernetic models from FBA to be significant not only from the point of view of applications in various ways but also in the elegance with which model predictions relate to those that emerge from FBA.

The means for manipulating metabolism lies in control of the syntheses and activities of the enzymes that catalyze the reactions in metabolism. The reactions are thus competitors for a limited amount of resources essential for the synthesis of enzymes. We define two vectors $\mathbf{u} \equiv [u_1, u_2, \ldots, u_{n_r}]$ and $\mathbf{v} \equiv [v_1, v_2, \ldots, v_{n_r}]$ that we refer to as cybernetic variables. The vector $\mathbf{u}$ is associated with the fractional allocations of resource for enzyme synthesis for the different reactions, so that $\sum_{i=1}^{n_r} u_i = 1$. The vector $\mathbf{v}$ represents the activities of the different enzymes. Thus, we must have $0 \leq u_i, v_i \leq 1$, $i = 1,2,\ldots,n_r$. The v-variables are not required to sum to 1 as they are not viewed as based on allocation of a common resource. Although the possible candidates for this resource have been discussed in various papers,[11,12] it has not become necessary to identify them precisely. The cybernetic variables in $\mathbf{u}$ would be required for enzyme balances. In other words, if the maximum enzyme synthesis rate is r_{E_i} for enzyme E_i, the regulated rate is $u_i r_{E_i}$. The cybernetic variables in $\mathbf{v}$ are required to calculate the regulated reaction rates. Thus, if r_i is the rate of the ith reaction in (1) when the enzyme E_i is fully active, then the regulated rate of the ith reaction is $v_i r_i$. As the reaction rates are determined by the specification of $\boldsymbol{\psi}$, $\mathbf{u}$, $\mathbf{v}$, we have, for the description of system dynamics, the differential equation

$$\frac{d\boldsymbol{\psi}}{dt} = \mathbf{f}(\boldsymbol{\psi}, \mathbf{u}, \mathbf{v}) \tag{2}$$

We now posit that the goal of the organism is to maximize (or minimize) at any instant t some functional related to its survival (or threat to existence) over the time interval $(t, t + \tau)$ that can be defined in terms of the state vector $\boldsymbol{\psi}$. Denoting this functional by $\Delta J \equiv J(t+\tau) - J(t)$, the cybernetic goal of the organism is given by

$$\underset{\mathbf{u}(t),\mathbf{v}(t)}{\text{Max}}\ \Delta J \quad \text{such that} \sum_{i=1}^{n_r} u_i = 1,\ \ 0 \le u_i, v_i \le 1 \tag{3}$$

Notice in particular that the cybernetic variables of interest are only at time t although the "planning horizon" is the finite interval $(t, t + \tau)$. When the solution to Eq. 3 has been obtained to identify the cybernetic variables **u** and **v**, Eq. 2 is a fully defined model except for the knowledge of kinetic parameters contained in the various reaction rates. The evolution of cybernetic models in the last three decades has occurred along the following directions.

a) Formulation of objective functions.

b) Solution of the optimal control problem.

c) Metabolic scale, referring to the level of pathway details.

We discuss each of these directions in the following sections.

Cybernetic Variables and Their Evaluation

The formulation and solution of the optimal control problem was first considered by Dhurjati et al.[13] Their concern was the growth of bacteria in a mixture of substitutable substrates such as glucose and xylose. Substitutable substrates satisfy the same nutritional needs of an organism.[14] No structure was attributed to biomass other than the key enzymes required to metabolize the two substitutable substrates, denoted by S_1 and S_2. Growth on each of the substrates could then be represented by

$$S_i + B \xrightarrow{E_i} (1 + Y_i)B + \cdots,\ \ i = 1, 2 \tag{4}$$

Equation 4 is of course a gross oversimplification of the metabolism of the organism on either substrate. Thus, the state vector $\boldsymbol{\psi}$ in the work of Dhurjati et al.[13] was given by $\boldsymbol{\psi} \equiv [s_1, s_2, c; e_1, e_2]$. The semicolon in brackets is used to separate the intracellular variables (which include only the enzymes for the present case) from the extracellular nutrients and the biomass concentration. The intracellular variables are described as mass fractions of biomass. The objective function was the maximization of the biomass produced in the time interval $(t, t + \tau)$. Thus, $J(t) = c(t)$ and the maximization was sought of $\Delta J = \Delta c$.

In view of the linear dependence of the reaction rate on the enzyme levels, the function $\mathbf{f}(\boldsymbol{\psi}, \mathbf{u}, \mathbf{v})$ has a linear dependence on the cybernetic variables making the optimality problem a singular control problem.[13] The solution of the singular control problem was accomplished by these authors to predict diauxic growth in a batch culture of bacteria with utilization of the faster growth supporting substrate, S_1 (glucose) first and the slower growth supporting substrate next. The computational overhead for solution of the singular control problem, however, appeared unsuitable for more general applications. There were various other conceptual issues[1] in the foregoing that found favor with a different approach as recounted below.

The matching and proportional laws

If the planning horizon is reduced to only the instant t, a policy that Young[2] referred to as "greedy," then the goal here becomes $\lim_{\tau \to 0} \frac{\Delta c}{\tau} \equiv$ biomass production rate at t. Thus, the maximization in this case became one of allocating resources for enzyme syntheses for S_1 and S_2 such that the combined growth rate on the two substrates is maximized. The maximization is performed under the assumption that progressive investment on any substrate leads to diminishing returns as measured by the growth rate. This problem has been analyzed by Kompala et al.[11] to arrive at the matching law result

$$u_i = \frac{r_i}{\sum_{j=1}^{2} r_j}, \quad i = 1, 2 \tag{5}$$

Expression (5) is a remarkably simple heuristic to describe the regulatory process of catabolite repression. The control of enzyme activity was determined by Kompala et al.[11] to be given by the proportional law

$$v_i = \frac{r_i}{\max(r_1, r_2)}, \quad i = 1, 2 \tag{6}$$

which was derived based on providing the maximum activity for the enzyme supporting the fastest reaction while activating the other enzymes proportionally to the reaction rates from them. As the reaction rates depend on the levels of enzymes that catalyze reactions, dynamic enzyme balances are called for. The enzyme balance is written in terms of the mass fraction of enzymes in cell mass as follows.

$$\frac{de_i}{dt} = \alpha_i + u_i r_{E_i} - (\beta_i + r_G)e_i \tag{7}$$

The foregoing balance accounts for a constitutive rate α_i, an inductive rate r_{E_i} which is regulated, a degeneration rate β_i, and a dilution term due to growth rate r_G.[15] The differential equations for biomass and substrates are readily written as in Kompala et al.[11]

Diauxic growth

Kompala et al.[11] have presented the successful application of the cybernetic laws for diauxic growth in a batch reactor on several substrate pairs based on parameters obtained from experiments on each of the substrates singly. The differential equations for batch growth are readily identified and may be found in the above publication. Some fine tuning of the single substrate parameters improves the model simulations for mixed substrate growth while not noticeably affecting single substrate behavior. Their simulations alongside experimental data for glucose and xylose are shown in Figure 1.

Growth in low carbon substrate environments

The growth of microorganisms in a medium of very low (carbon) substrate concentration, however, presents a different regulatory scenario even with single substrates than that predicted by the model of Kompala et al.[11] Turner et al.[16,17] discuss the issue of maintenance processes and their modeling. They postulated the appearance of nongrowth associated maintenance processes for growth rates below their maximum value. They further proposed that the primary objective was the maximization of the growth rate while the secondary objective was maximizing the total substrate uptake for growth and maintenance. The resulting model is shown to describe the behavior of slowly growing batch cultures of *Klebsiella oxytoca* perturbed by the addition of pulses of

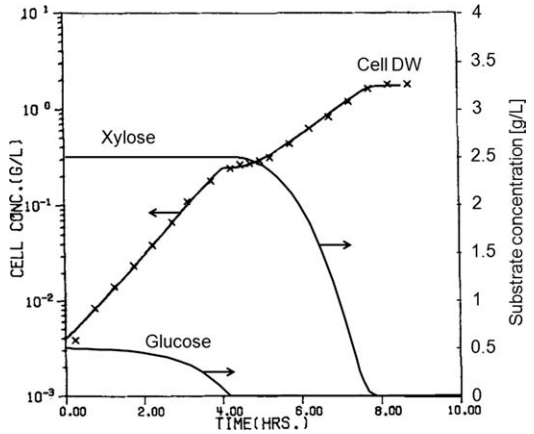

Figure 1. Data of Kompala et al.[11] on diauxic growth and model simulations: transient concentrations of biomass and substrates.

Cell growth profile and data reprinted with permission from John Wiley and Sons.

glucose. The behavior in mixed cultures was investigated by Turner et al.[16] Retaining the same enzymes for growth and maintenance with each substrate, and the same objective functions, they were able to accurately describe growth on glucose perturbed by pulse additions of arabinose, fructose, and xylose. However, Baloo and Ramkrishna[18,19] noted that transient behavior in the form of overshoots and undershoots of biomass in continuous cultures following step-ups and step-downs in dilution rate were not described adequately by the models of Turner et al.[16] At severely lower levels of substrate concentrations encountered in continuous cultures, Baloo and Ramkrishna envisaged growth and maintenance processes to be in competition for cellular resources. In other words, the survival strategy in very lean environments was viewed to favor maintenance of viability in preference to engage in active growth. The models were able to describe complex transient behavior found in experiments with step-ups and step-downs in dilution rates. An example is shown in Figure 2.

Simultaneous uptake patterns

Narang et al.[20] performed experiments with *Escherichia coli* on substrate mixtures of glucose and organic acids such as fumarate and pyruvate and found that uptake of substrates could also take place simultaneously. With glucose pyruvate mixtures, the uptake pattern depended on preculturing; sequential utilization (with glucose first) when precultured on glucose and simultaneous utilization when precultured on pyruvate. The cybernetic model of Ramakrishna et al.,[21] which was able to describe these uptake patterns successfully, was built by expanding the gross pathway (4) to include an anabolic component involving synthesis of biomass from two different kinds of growth precursors produced from the breakdown of the two substrates. Thus

$$\begin{aligned} &S_i + B \xrightarrow{E_i} M_i, \quad i = 1,2 \\ &M_1 \underset{E_4}{\overset{E_3}{\rightleftharpoons}} M_2 \\ &M_1 + M_2 \rightarrow B \end{aligned} \tag{8}$$

The above model derives its success from predicting simultaneous consumption when the precursor pools are filled more expeditiously by consuming the two substrates than when consuming alone. Conversely, diauxic growth on a specific substrate occurs when that substrate is more effective on filling the precursor pools. The dependence on the uptake pattern on preculturing is represented by initial enzyme levels that can tip the balance in favor of one or the other. In regard to the foregoing model, it is well to recall a slight correction in the growth rate by Namjoshi and Ramkrishna,[22] which helps to ensure mass conservation.

The enhanced capacity of the model by Ramakrishna et al.[21] is a consequence of additional details on the growth process.

Growth on complementary substrates

Alexander and Ramkrishna[23] developed a cybernetic model for the production of siderophores (iron-chelating agents) associated with iron-limiting conditions. Their data showed that, while final biomass levels were governed by exhaustion of the carbon source, iron limitations also contributed to determination of the biomass yield. They developed a relatively detailed structured model including an iron-limited energy resource production, which was able to quantitatively account for this apparent dual-substrate limitation over a wide range of batch and continuous operating conditions. The experimental data featured large variations in iron uptake over the wide range of operating conditions and iron levels investigated. The model, which included a low and high (siderophore-mediated) affinity iron transport, and siderophore production, however, closely simulated results that were in good quantitative agreement with the siderophore, medium and cell iron levels, in both batch and steady-state continuous cultures for a wide range of operating conditions. Alexander's models[24] involved more pathway details than those of his predecessors. However, a more systematic attempt to look at metabolic pathways in terms of basic units was due to Straight.[25]

Pathway Considerations

Straight,[25] in his doctoral dissertation, introduced interesting ideas in the extension of cybernetic models to more detailed consideration of pathway structure. This extension[26,27] recognized that metabolic networks contain basic units such as linear segments, converging and diverging units, and cyclic pathways. The matching and proportional laws of Kompala et al.[11] were extended to local objectives

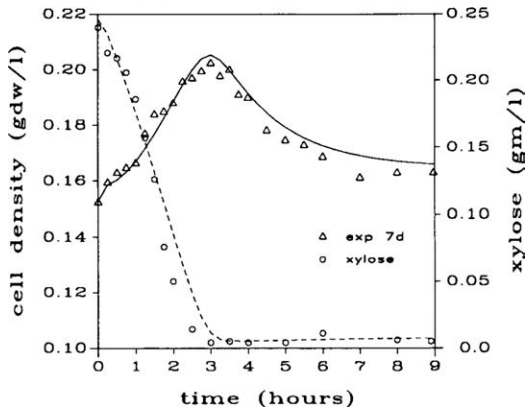

Figure 2. Simulations of Baloo and Ramkrishna[19] for continuous culture with mixed feed of glucose and xylose, showing overshoot of biomass transients during shift-down of dilution rate.

Reprinted with permission from John Wiley and Sons.

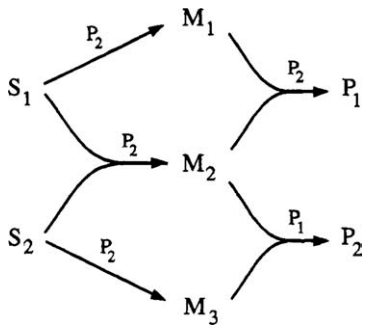

Figure 3. Simplified metabolic network of Straight and Ramkrishna[26] accommodating both interactive and noninteractive behavior of complementary substrates.

Reprinted with permission from John Wiley and Sons.

for each of the units. For example, a diverging unit leading to multiple products could use a local objective of maximizing either the products' sum or product, the latter yielding more aggressive control than the former. Similar strategies were used for cyclic pathways. The common underlying regulatory feature was the optimal distribution of resources with a fixed allotment for each unit toward accomplishing the local goal. Straight and Ramkrishna[26] applied these ideas successfully to bacterial growth on carbon and nitrogen substrates under dual limiting conditions. To describe growth on complementary substrates under interactive and noninteractive situations, they introduced the concept of multiple biosynthetic intermediates all of which were necessary for biomass synthesis but some of which could arise from either of the complementary substrates. Their figure is reproduced alongside simply to show the type of network structure used to produce interactive behavior (Figure 3). The diverse predictive potential of such a minimal model can be seen from Straight and Ramkrishna.[26] They also investigated the growth of *E. coli* in continuous cultures fed with carbon and nitrogen substrates under various limiting conditions such as (i) carbon-limited, (ii) nitrogen-limited, and (iii) dual carbon and nitrogen-limited.[27] The simplified network considered by them is presented in Figure 4. The network can be seen to feature the basic metabolic units considered by these authors for cybernetic formulations by assigning local objectives as mentioned earlier. Figure 5 presents their steady state profiles under carbon limitation on the left and under nitrogen limitation on the right. The biomass trends are remarkably different at lower dilution rates in the two cases because of overflow metabolism and accumulation of storage compounds. Similarly, data are compared of steady state yields of biomass as a function of dilution rate under different limiting conditions. In addition, the studies also included transient effects following shift-ups and shift-downs of dilution rate under different limiting conditions. Although, for a full discussion of the quality of performance of the models, the reader is referred to Straight and Ramkrishna,[27] their model has had considerable success in describing data notably diverse in nature and difficult to describe with kinetic models devoid of regulatory features. Indeed we are even unaware of kinetic models that have addressed such diverse transient patterns.

The concepts developed in Straight's work[25] were remarkable in extending the success of cybernetic ideas to describing complex interactive behavior in environments of complementary substrates with relatively simple networks. However, the recognition of basic metabolic units toward the mission of modeling larger metabolic networks lacked an actual manner of synthesis. The underlying conundrum was to conjure a collection of smaller subnetworks with an allocation strategy for resources toward synthesis of enzymes that would support a global objective of the organism. Further, the attractiveness of the idea of distributing a resource among many metabolic subunits was confounded by the combinatorial complexity of their choice, and the hazard of throttling fluxes through indiscriminate application of the local objectives for subunits.

Varner and Ramkrishna[28,29] addressed detailed metabolic networks in which transcriptional details were included for the synthesis of enzymes. They introduced cybernetic variables to regulate the synthesis of m-RNA's for different proteins. Subsequently, Varner[30] presented a model of central carbon metabolism of *E coli*, describing batch aerobic growth on glucose, in which transcription, translation, and activity of the gene products of 45 genes were included. The model featured 122 species including metabolites, enzymes, mRNA pools, and biomass with 46 reactions. An attractive feature of Varner's model was in its capacity to evaluate the effect of various gene knockouts. For example, his model accurately captured the metabolic reprogramming results from the deletion of pyruvate kinase, a fact that went unnoticed in the subsequent work of Segre et al.[31] Varner's expansion of the cybernetic framework with the potential to incorporate proteomic and microarray data could be important in the future.

Dynamic Models with Local and Global Cybernetic Variables

Elementary Modes

We will now turn our attention to the new optimality considerations that Young[2] (see also Young and Ramkrishna[4]) introduced. Young revisited the optimality problem (Eqs. 2 and 3) with the proposition that the planning horizon for the organization at any instant is a time interval τ is small but

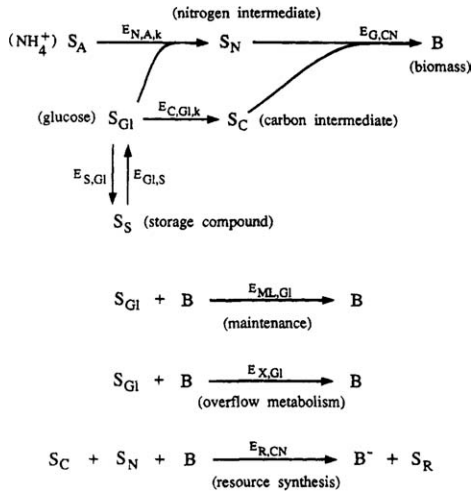

Figure 4. Simplified pathway of Straight and Ramkrishna[27] growth of *E. coli* under carbon and nitrogen limitations.

Reprinted with permission from John Wiley and Sons.

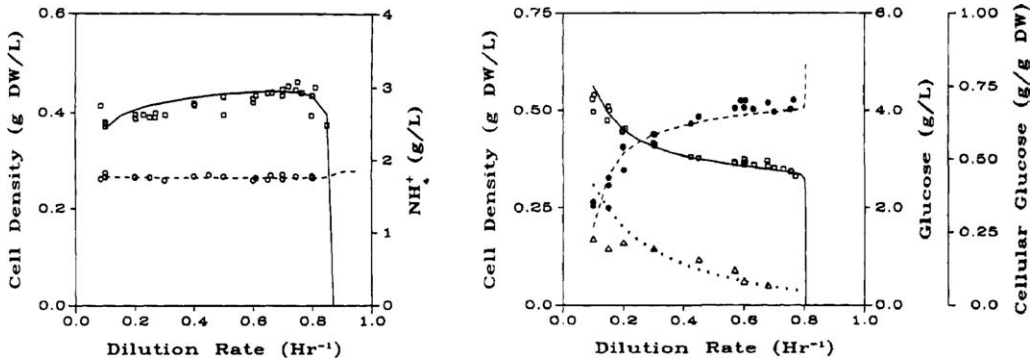

Figure 5. Model simulations (continuous, dashed and dotted lines) of Straight and Ramkrishna[27] alongside data obtained for *E. coli* in continuous cultures with glucose and ammonia substrates.

The figure to the left is under carbon-limiting conditions while that to the right is under nitrogen-limiting conditions. Reprinted with permission from John Wiley and Sons.

distinct from zero. The smallness of τ permits linearization of the function $\mathbf{f}(\psi, \mathbf{u},\mathbf{v})$ with respect to ψ, while introducing a quadratic penalty function on the control (cybernetic) variables to account for diminishing returns from each investment. This perspective was all that was required for optimal control theory to yield the most elegant solution to represent this framework. The cybernetic variables could be determined analytically for arbitrary objective functions. A distinctive quality of Young's development[2] is that the optimality perspective at any instant over a finite (albeit short) time interval lent an anticipatory aspect to the cell's planning process without it being unreasonably futuristic. Young appropriately termed this policy as temperate as against the greed implied in the matching law development of Kompala et al.[11] that was based on a zero planning horizon. As a result, the framework based on Young's work will display a sense of robustness that has for long been held to be a property of biological systems without a concomitant effort to incorporate it in models.

With the optimality framework firmly secured through the application of mathematical control theory, Young's doctoral dissertation[2] also addressed the problem of developing a view of the metabolic network for identification of appropriate objective functions. In accomplishing this, the decomposition of a metabolic network into so-called elementary modes, which represent metabolic pathway options for the organism, became a key aspect of the development. This decomposition has its roots in the steady state view of metabolism in which all intracellular metabolites (with some exceptions such as those involved in overflow metabolism that accumulate within the cell that have slower dynamics) are at steady state with respect to the uptake of nutrients by the cell. We will indulge in some detail on introducing elementary modes in this review because it provides us with an effective way of comparing our dynamic cybernetic models to the flux balance approach. The steady state mass balance for the metabolites, neglecting the dilution term due to growth, leads to the homogeneous equation

$$\mathbf{S_m r} = \mathbf{0}, \mathbf{r} \geq \mathbf{0} \tag{9}$$

where $\mathbf{S_m}$ is the stoichiometric matrix associated with intracellular participants in all metabolic processes and $\mathbf{r}$ is the metabolic flux vector. Eq. 9 reveals the metabolic flux vector to be in the null space of $\mathbf{S_m}$; however, $\mathbf{r}$, in view of its components being intrinsic reaction rates that are non-negative, must lie within and on the boundary of a "flux cone," a convex set in the non-negative orthant of $\Re^{n_r}$. This flux cone clearly has its vertex at the origin. Each solution vector espouses reactions that form a sub-pathway of the overall network. The cone edges are special solutions that are in fact elementary modes mentioned earlier (it is customary in the metabolic literature to distinguish between elementary modes, extreme pathways, and generating modes, the distinction among which comes about when there are reversible reactions. This set of elementary modes defines a convex basis set of solution vectors in the sense that every solution of Eq. 9 is a convex combination of these vectors. For a full discussion of elementary modes, the reader is referred to Schuster et al.,[32,33] Klamt and Stelling,[34] and Wagner and Urbanczik.[35] See also Trinh et al.[36] for the review of recent development and applications of elementary modes). It is also convenient to represent the potential metabolic states in a yield vector space in which the yields of all extracellular products are represented.[37] The flux cone transforms to a convex hull in this space. The edges of the flux cone collapse into the vertices of the foregoing convex hull. The steady state metabolic performance will clearly be a point in this hull obtained by a convex combination of its vertices. Each elementary mode is viewed as a metabolic option of the organism for substrate uptake. All fluxes in the mode are calculable from a specification of this uptake rate. Young's cybernetic perspective[4] proposed that the organism invests its total resources available for enzyme synthesis among elementary modes chosen to realize a global objective of the organism such as maximizing biomass or uptake of carbon during the time interval t to $t + \tau$ (in other words the average rate of metabolism during this interval). Thus, elementary modes are viewed to compete for resources to drive metabolism effectively to meet the foregoing global objective. Individual reactions in each mode, however, compete for resources allocated to that mode through the realization of a local objective, which is to maximize the harmonic mean of all the fluxes in that mode. No reaction in the mode has therefore the potential to thwart the flux through that mode because of deficiency in the amount or activity of the enzyme catalyzing the reaction in question, a feature that also further contributes to the property of robustness. The joint realization of the global and local objectives of the organism will therefore ensure the survival effort of the organism. The incorporation

of elementary modes by Young[2] resolved the basic difficulty alluded to earlier associated with finding subunits and an appropriate resource allocation strategy that would allow a coordinated flow of fluxes.

The application of Young's approach to *E. coli* is presented by Young et al.[3] They show that parameters can be identified for multiple strains of the organism with identical values for overlapping parameters so that their metabolic performance can be evaluated which includes the rates of growth and substrate uptake. This predictive element of the cybernetic model is one that distinguishes it from other approaches to modeling metabolism. As no quasi-steady state assumption is involved for intracellular variables in the model of Young et al.,[3] metabolic transients will appear as a curve in yield space terminating at a point when steady state is attained for all intracellular variables. We note that this steady state point could be an interior point of the convex hull while, FBA, in view of its linear programming strategy to maximize the biomass yield, would require it to be at some vertex.[38] This would be the case even with the dynamic version of FBA for describing metabolic transients. We will again return to this issue in our discussion of the so-called hybrid cybernetic models (HCM).

Young's models obviously retain the property of their precursors to represent regulatory phenomena without the aid of parameters in addition to those that must of course be used for reaction kinetics. However, the parameter identification issue for the foregoing models is a formidable one especially for large metabolic networks as it would require the full blown power of metabolomic measurements with accuracy. That identification can, however, be accomplished for relatively smaller networks with dynamic measurements of extracellular variables as shown by Young et al.[3] and reproduced in Figure 6 here.

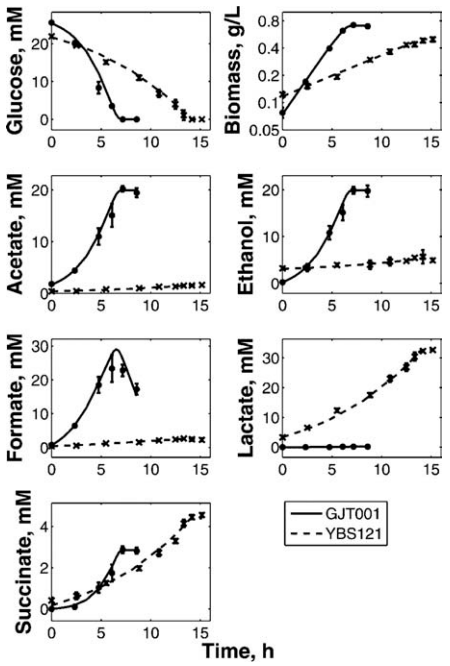

Figure 6. Comparison of Young's model[3] simulations with data on two different strains of *E. coli*.

Reprinted with permission from John Wiley and Sons.

HCM

The viewpoint of elementary modes being metabolic options for an organism leads naturally to an attractive model framework, which, in its application to larger metabolic networks, could retain the cybernetic features introduced earlier by Kompala et al.[11] This framework which we shall refer to as the HCM accomplishes an interesting synthesis of the early cybernetic approach with the quasi-steady state assumption for intracellular variables used in FBA. An exposition of this approach can be found in Kim et al.[39] The quasi-steady state assumption relegates consideration of regulatory processes associated with the reactions in each elementary mode to that of substrate uptake through that mode. In other words, a single enzyme is assigned to each mode the control of whose synthesis and activity will represent the regulation of all reactions in the elementary mode. Thus, regulation is viewed to distribute resources among different modes so that a global objective of the organism is satisfied. Note in particular that, in realizing the above objective, which involves the maximization of a metabolic rate, a convex combination of many modes is involved. It is well to reflect at this stage the point of departure of HCM from that of FBA, as the latter, in focusing on metabolic yield, seeks out a single (except when the linear programming has multiple solutions) mode (vertex in the convex hull) as the operating metabolic state in yield space. The differences between the two approaches are, however, deeper. FBA relegates regulatory effects to the measurement of uptake rates so that the consequence of regulation falls outside the scope of prediction. Attempts to correct for this through the development of kinetic expressions for uptake rates would in fact eliminate the input of regulation through experiment.

The intracellular fluxes in each mode are determined once the uptake rate through the mode is known. The only dynamic variables of the HCM are the concentrations of extracellular variables such as substrates, fermentation products, and cell mass. The model parameters are the kinetic constants associated with uptake of substrate through each mode, which must be determined by comparing the dynamic predictions of extracellular variables with suitably spaced measurements. As large metabolic networks lead to a large number of elementary modes, the consequent imbalance between the number of parameters and the number of available measurements could greatly complicate the identification of such models. To contain such an imbalance, it becomes necessary to explore a process of mode reduction and/or expand the measurements of extracellular variables to include as many of the intracellular fluxes as possible through, for example, C13 labeling.[40–42]

Song and Ramkrishna[37] have developed strategies for mode reduction (see also Provost et al.[43] for other approaches) based on what they refer to as metabolic yield analysis, which involves exploring metabolic data on the yield vector space. As observed earlier, the modes appear as vertices of a convex hull in yield vector space and the point, that represents the metabolic function in terms of the yields of all extracellular products, is a convex combination of all the vertices (Figure 7). It is now possible to select a minimal set of modes whose convex combination can yield the given point. If, it turns out that the point lies outside the convex hull either due to experimental measurement errors or perhaps due to missing modes, a minimal set of the existing ones is selected based on their proximity to the point in question. In fact Song and Ramkrishna[37] have a well laid

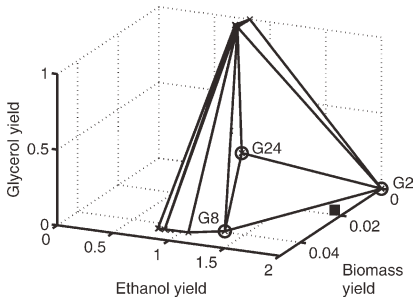

Figure 7. Convex hull in yield vector space from Song and Ramkrishna[37] showing elementary modes as vertices.

Reprinted with permission from John Wiley and Sons.

out strategy for substantial mode reduction that has been applied in various cases.[44,45]

The mode reduction strategy is inspired by the belief that an organism's explosive repertoire of elementary modes is a consequence of evolutionary experience not all of which may be essential to respond to a controlled laboratory or industrial reactor environment. Thus, highly reduced lower order models could be envisaged for control applications to bioprocesses. Conversely, an exuberant effort to eliminate modes runs the risk of limiting metabolic engineering directives that could accrue from models. Thus, some balance is called for in condensing the number of modes. The reader is referred to Song and Ramkrishna[37] for a more detailed account of the mode reduction process.

The hybrid model equations are given by differential equations for biomass, substrates, fermentation products, and enzyme levels for uptake of substrates together with steady state balances for intracellular variables. The intracellular fluxes relate to the uptake rates through all the elementary modes. The growth rate is expressed as a known linear combination of intracellular fluxes. Kim et al.[39] produced two reduced order models of *E coli*, one with nine elementary modes and the other containing 20. Their model simulations are shown along with those of FBA and Young's model for comparison with experimental data obtained by Kim et al.[39] (Figure 8). Of particular significance is the fact that the formation of succinate in experiments is upheld by both HCM and Young's model while linear programming, which maximizes the biomass yield in FBA, selects out a mode that produces no succinate.

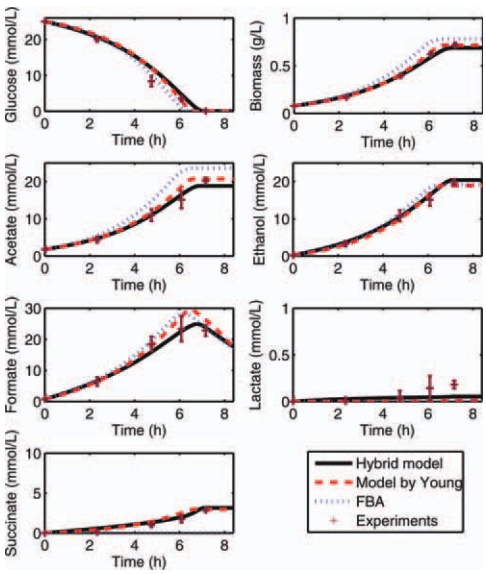

Figure 8. Comparison of simulation of *E. coli* metabolism by Kim et al.[39] with data.

Reprinted with permission from John Wiley and Sons. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Song et al.[44] have successfully applied HCM to the co-utilization of glucose and xylose by a recombinant strain of *Saccharomyces*. Figure 9 shows the HCM simulations (continuous lines) alongside data obtained by Krishnan. Dashed lines are simulations of what has been termed as macroscopic bioreaction model (MBM) due to Provost et al. The MBM serves as a useful comparison of regulatory effects considered by HCM in the optimal distribution of substrate uptake among the different elementary modes of metabolism as against a fixed distribution in MBM. Harsher scenarios can be conceived for comparing the two models, however. In particular, it will be of interest to examine how HCM can predict events in a chemostat, an issue to be addressed in a subsequent section (see Nonlinear Behavior of Cybernetic Models).

As pointed out before, the focus of FBA on maximizing biomass yield results in a daring selection of a single elementary mode to represent metabolism under some stipulated growth or uptake rate. Of course variability occurs in the mode selected with change in the metabolic rate but with the proviso of having to specify the latter from experimental observation. This is a constraint that strips the methodology of the essential need of a modeling framework to contribute to experiment. In other words, a quest for the conditions under which specific metabolic states can manifest is without direction. We shall have more to say about this issue in a subsequent section of this article.

Lumped HCM

The size of the network which HCM can effectively handle would depend on the tradeoff between the number of available extracellular measurements and intracellular fluxes (as by using isotopic carbon labeling) and the number of elementary modes that can be accommodated. The attractiveness of FBA lies in its ability to address the estimation of

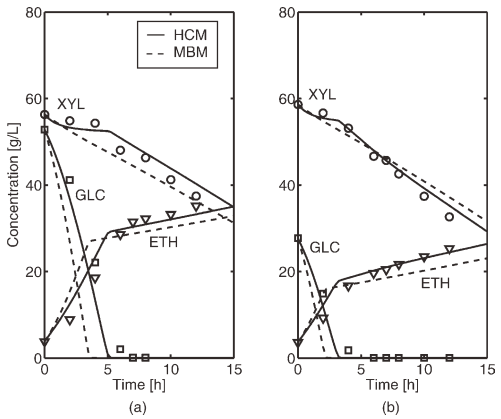

Figure 9. HCM simulations of Song et al.[44] for recombinant Saccharomyces producing bioethanol versus experimental data of Krishnan et al.[46] in glucose-xylose environments.

Reprinted with permission from John Wiley and Sons.

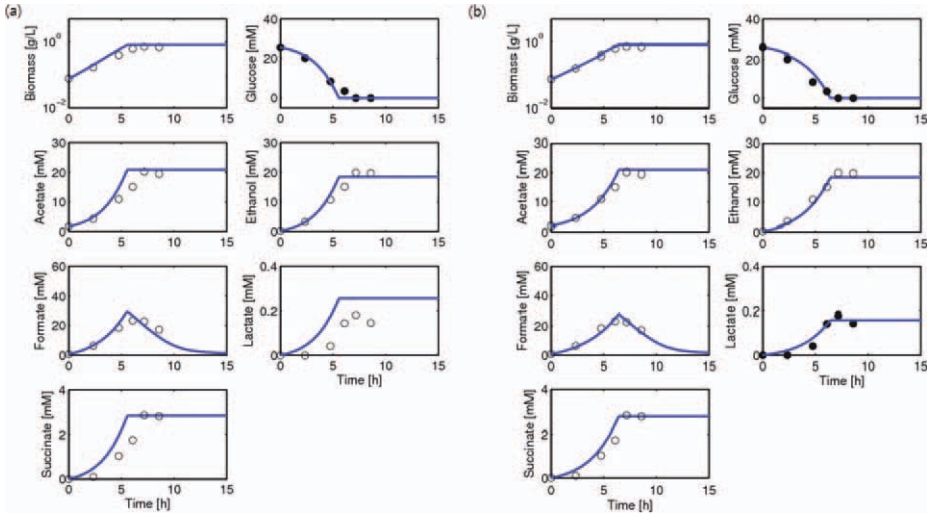

Figure 10. L-HCM predictions (continuous lines) of Song and Ramkrishna[48] for dynamic fermentation data shown in open circles, based on measurements shown in solid circles.

Predictions (a) based on measurements of glucose alone leads to good predictions of biomass, formate, ethanol but not of lactate and succinate. When measurements of glucose and lactate are both used for model identification (b), all predictions (shown in continuous lines) are considerably improved. Reprinted with permission from John Wiley and Sons. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

metabolic fluxes with the minimum of data as, for example, the substrate uptake rate. It is of interest to see to what extent cybernetic models, with their dynamic features intact, can be exploited to obtain information on metabolic performance of large metabolic networks with minimal data. In this connection, we discuss next what we have termed Lumped HCM (L-HCM), which provide dynamic models for very large networks that can be identified with limited data. Further, L-HCM can be used to interpret the cybernetic models of the older generation. Song and Ramkrishna[47,48] have shown how it may be possible to recognize "families" of elementary modes each containing several elementary modes with one or more unifying characteristics with respect to metabolic function. Thus, key enzymes are envisaged for each of the EM families, their syntheses receiving resources which are then shared among the various modes within each family. This sharing is of course related to their contribution to satisfying the organism's goal that was chosen to be the maximization of carbon uptake rate. A notable additional feature of L-HCM is a simplification, enabled by analysis of the enzyme synthesis equations, which relates the cybernetic variables for jth EM in a given family to a quantity termed "structural return on investment" denoted as η_j. This quantity, which has a purely stoichiometric origin, represents the "quality" of the EM in its contribution to the organism's goal. Furthermore, "tuning parameters" were introduced into the formulation that would use dynamic experimental data to distill out the significant EMs in a family that were obscured by lumping. For details, the reader is referred to the publications of Song and Ramkrishna.[47,48] Here, we take particular note of a change in lumping rule between the two cited publications with the later one showing improvement in predictive quality. Their results are included in Figure 10 below which shows how L-HCM, with parameters identified by some measured variables (represented in solid circles in Figure 10), is able to predict the dynamics of other variables shown in open circles. It follows from Figure 10 that L-HCM can make dynamic predictions with considerably less data because of the reduction in parameters. However, the quality of prediction depends upon its sensitivity to the data chosen for model identification. Song and Ramkrishna[48] have presented several more successful dynamic predictions.

It is useful to reflect on the old cybernetic models such as those of Kompala et al.[11] and Baloo and Ramkrishna[18,19] in light of current developments. As they were designed only to deal with biomass and extracellular variables (aside from key enzymes), and were free of EMs, it will be convenient to refer to them as lumped cybernetic models (LCM). Figure 11 shows a conceptual comparison of LCM shown in (a) and HCM in (b). Part (c) shows L-HCM with two families, one consuming S_1 and the other consuming S_2, while (d) shows the LCM of Baloo and Ramkrishna.[18] It should become clear that HCM and L-HCM provide avenues for linking extracellular variables to intracellular metabolism.

Nonlinear Behavior of Cybernetic Models

A discussion on nonlinear behavior is spurred by fundamental as well as practical issues. At the fundamental level, it behooves one to inquire into how the inherent nonlinearity of the cybernetic model leads to dynamic variations in the manner in which pathway options are negotiated by the organism toward satisfying its dynamic goal (e.g., maximize carbon uptake rate). At the practical level, it is of interest to determine the transient and steady state behavior of continuous bioreactors.

Although nonlinearity arises in the kinetics of reactions in metabolism, the primary source of nonlinearity in the cybernetic model may be attributed to the cybernetic variables. As the cybernetic variables are functions of the reaction rates, they also inherit the nonlinearity of the kinetic expressions. The underlying feature of nonlinear behavior of cybernetic models is the dynamic manipulation of pathway options by the organism to survive in a dynamic environment. Thus, metabolic processes may change in the extent to which specific reactions are commissioned by the organism as survival measures. Steady state multiplicity could therefore arise

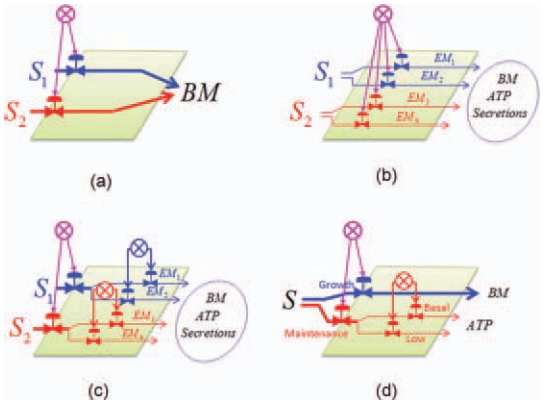

Figure 11. Comparative illustration of cybernetic modeling concepts (from Song and Ramkrishna[47]): (a) LCM (e.g., Kompala et al.[11]), (b) HCM, (c) L-HCM, and (d) LCM (e.g., Baloo and Ramkrishna[18]).

Reprinted with permission from John Wiley and Sons. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

because of the availability of multiple metabolic choices that ensure survival criteria. Oscillatory dynamic behavior may also come into play because the interaction of the organism with its environment may call for dynamically periodic switches in metabolism.

Namjoshi and Ramkrishna[22] show how the cybernetic models of Kompala et al.[11] and Ramakrishna et al.[21] for bacteria in a chemostat (continuous reactor) fed with a mixture of carbon sugars such as glucose and xylose may lead to multiple steady states for a range of dilution rates and feed compositions. This multiplicity arises from the model being able to meet a specific growth rate by metabolizing the mixture of glucose and xylose with differing preferences, one in which more glucose is used and the other in which more xylose is used. Their results are shown in Figure 12. The mechanism for multiplicity can be clearly seen from the figure as changing uptake patterns with different preferences for the carbon sources as represented by the cybernetic variable u_1. For example, at low concentrations of glucose in the feed and high dilution rates the "upper" steady state shows preference for glucose while the "lower" one shows a preference for xylose because of very low residual concentration of glucose. A similar situation has been observed by Kim[49] for *E. coli* both experimentally and using HCM. Kim's model displays as many as five steady states (three stable and two unstable) with different uptake patterns for certain ranges of dilution rate and fraction glucose in a glucose–pyruvate mixture as feed (unpublished work). Such operating ranges, which can only be diagnosed by the use of dynamic models, are difficult to discover by any form of experimental design. It was this issue to which we referred earlier in regard to the importance of dynamic models.

Multiple steady states have been observed by Hu and coworkers[50] and independently by Stephanopoulos and coworkers[51] in mammalian cell cultures. A cybernetic model formulated by Namjoshi et al.[52] provides a close interpretation of the observed multiplicity as shown in Figure 13. Jones and Kompala[53] showed that oscillations observed in cultures of *Saccharamomyces cerevisiae* could be explained by a cybernetic model. Although oscillatory phenomena have been known in yeast cultures, instances of such oscillations in bacterial cultures are evident. Kim's analysis[49] shows that oscillations could be expected in continuous cultures of *E. coli* at very low dilution rates with glucose feed. Experimental confirmation is yet to be made of such phenomena. Straight and Ramkrishna[54] found fairly bizarre dynamic behavior in the simulated growth of *K. oxytoca* on lactose because of metabolic switches. It must be said, however, that the extent to which nonlinear phenomena of metabolic systems have been investigated hardly probes the surface of what could well be a treasure-trove of pathological behaviors. In this connection, one is tempted to speculate that detailed determination of gene expression profiles could serve as fine diagnostic tools for such nonlinear behavior.

Process Applications

We are concerned in this section about modeling the use of microbes for bioprocesses in which the focus is on increasing productivity of the bioproduct by (i) varying process conditions and (ii) metabolic engineering of strains through genetic manipulation. In addressing (i), the dynamic nature of cybernetic models is a crucial asset that can be used not only to optimize bioprocesses for maximum productivity but also institute continuous control.[55] Control based on cybernetic models has the advantage of accounting for regulatory responses of the organism to variation of its environment. Song et al.[56] have recently shown how bioethanol productivity can be notably improved by varying process configurations using HCM on fermentation of a mixture of glucose and xylose.

Metabolic engineering

Metabolic engineering, initiated by Bailey,[57] is an exciting area of application of metabolic models. The special claims of cybernetic models to being an effective tool in metabolic engineering are derived from its dynamic nature which enables focus on productivity of the metabolite of interest. Steady state approaches are constrained to address the improvement of yield often beset by a drop in productivity due to a drop in growth rate caused by "metabolic burden." Cybernetic models, which have a mechanism to address metabolic burden, therefore represent an attractive tool in this

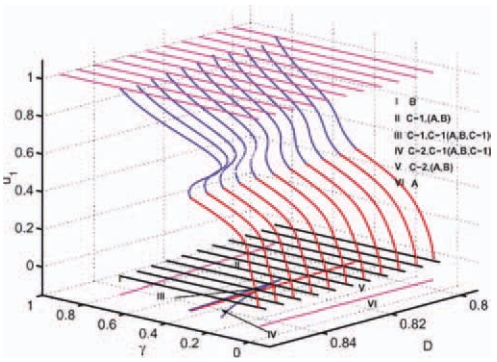

Figure 12. Steady state multiplicity in bioreactor fed with glucose and xylose shown as a function of dilution rate D and fraction glucose in the feed γ (from Namjoshi and Ramkrishna[22]).

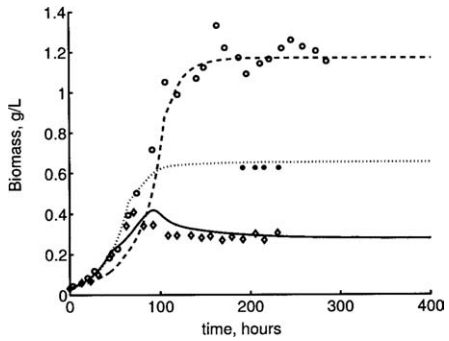

Figure 13. Cybernetic model simulations of Namjoshi et al.[52] alongside with experimental data of Europa et al.,[50] showing multiple steady states in continuous cultures of hybridoma cells.

Reprinted with permission from John Wiley and Sons.

regard. Young's models are specially suited for metabolic engineering as they address intracellular reactions without the assumption of steady state. However, their applicability is constrained by the current limitation in the size of the metabolic network that can be used. A similar limitation in network size applies to HCM. Although HCM assumes steady state for intracellular metabolites, the uptake rate associated with an EM could be varied to examine its effect on the productivity of any metabolite. Thus, the metabolic engineering quest would first focus on determining the EMs that would most contribute to increase the productivity of the metabolite, followed by a search for intracellular reactions that would most effectively accomplish the required change in uptake rates of the EMs.[58] The L-HCM, because of its ability to handle large metabolic networks represents a perhaps more promising direction for metabolic engineering. Recent estimates by Song and Ramkrishna (*Metabol Eng*., submitted) shows impressive dynamic predictions of several KO strains of *E. coli* based on limited data on the wild-type strain. Considerable further work remains in this area, however.

Future Prospects

The future of the cybernetic approach is enhanced not only by the progress to date in handling increasingly larger sized metabolic networks but also in the upcoming prospects of being able to handle genome scale networks. FBA derives its capability for genome scale networks because of the facility for solving large scale linear programming problems but, in view of its focus on maximizing biomass yield, a great collection of pathway options is discarded. The L-HCM approach of Song and Ramkrishna[47,48] recruits many pathway options that could be superior to those that maximize biomass yield because of higher uptake rates. Thus, a dynamic genome scale L-HCM represents a distinct possibility in the metabolic modeling horizon.

Literature Cited

1. Ramkrishna D. A cybernetic perspective of microbial-growth. *Acs Symp Ser*. 1983;207:161–178.
2. Young JD. *A System-Level Mathematical Description of Metabolic Regulation Combining Aspects of Elementary Mode Analysis with Cybernetic Control Laws*. West Lafayette: Purdue University, 2005.
3. Young JD, Henne KL, Morgan JA, Konopka AE, Ramkrishna D. Integrating cybernetic modeling with pathway analysis provides a dynamic, systems-level description of metabolic control. *Biotechnol Bioeng*. 2008;100:542–559.
4. Young JD, Ramkrishna D. On the matching and proportional laws of cybernetic models. *Biotechnol Prog*. 2007;23:83–99.
5. Monod J, editor. *Recherches sur la croissance des cultures bacteriennes*. Paris, France: Hermann et Cie, 1942.
6. Wiener N. *Cybernetics or Control and Communication in the Animal and the Machine*. New York: Wiley, 1948.
7. Mayr E. *Toward a New Philosophy of Biology: Observations of an Evolutionist*. Cambridge, Massachusetts: Harvard University Press, 1988.
8. Orth JD, Thiele I, Palsson BO. What is flux balance analysis? *Nat Biotechnol*. 2010;28:245–248.
9. Reed JL, Palsson BO. Thirteen years of building constraint-based in silico models of Escherichia coli. *J Bacteriol*. 2003;185:2692–2699.
10. Schuster S, Pfeiffer T, Fell DA. Is maximization of molar yield in metabolic networks favoured by evolution? *J Theor Biol*. 2008;252: 497–504.
11. Kompala DS, Ramkrishna D, Jansen NB, Tsao GT. Investigation of bacterial-growth on mixed substrates - experimental evaluation of cybernetic models. *Biotechnol Bioeng*. 1986;28:1044–1055.
12. Kompala DS, Ramkrishna D, Tsao GT. Cybernetic modeling of microbial-growth on multiple substrates. *Biotechnol Bioeng*. 1984;26: 1272–1281.
13. Dhurjati P, Ramkrishna D, Flickinger MC, Tsao GT. A cybernetic view of microbial-growth - modeling of cells as optimal strategists. *Biotechnol Bioeng*. 1985;27:1–9.
14. Baltzis BC, Fredrickson AG. Limitation of growth-rate by 2 complementary nutrients - some elementary but neglected considerations. *Biotechnol Bioeng*. 1988;31:75–86.
15. Turner BG, Ramkrishna D. Revised enzyme-synthesis rate expression in cybernetic models of bacterial-growth. *Biotechnol Bioeng*. 1988;31:41–43.
16. Turner BG, Ramkrishna D, Jansen NB. Cybernetic modeling of bacterial cultures at low growth-rates - mixed-substrate systems. *Biotechnol Bioeng*. 1988;32:46–54.
17. Turner BG, Ramkrishna D, Jansen NB. Cybernetic modeling of bacterial cultures at low growth-rates - single-substrate systems. *Biotechnol Bioeng*. 1989;34:252–261.
18. Baloo S, Ramkrishna D. Metabolic-regulation in bacterial continuous cultures. 1. *Biotechnol Bioeng*. 1991;38:1337–1352.
19. Baloo S, Ramkrishna D. Metabolic-regulation in bacterial continuous cultures. 2. *Biotechnol Bioeng*. 1991;38:1353–1363.
20. Narang A, Konopka A, Ramkrishna D. New patterns of mixed-substrate utilization during batch growth of Escherichia coli K12. *Biotechnol Bioeng*. 1997;55:747–757.
21. Ramakrishna R, Ramkrishna D, Konopka AE. Cybernetic modeling of growth in mixed, substitutable substrate environments: Preferential and simultaneous utilization. *Biotechnol Bioeng*. 1996;52:141–151.
22. Namjoshi AA, Ramkrishna D. Multiplicity and stability of steady states in continuous bioreactors: dissection of cybernetic models. *Chem Eng Sci*. 2001;56:5593–5607.
23. Alexander ML, Ramkrishna D. Cybernetic modeling of iron-limited growth and siderophore production. *Biotechnol Bioeng*. 1991;38: 637–652.
24. Alexander ML. *Cybernetic Modeling of Bacterial Metabolite Production*. West Lafayette: Purdue University, 1990.
25. Straight JV.Microbial Growth in Continuous Cultures Subject to Single and Multiple Limitations Involving Carbon and/or Nitrogen. Ph.D. Thesis, West Lafayette: Purdue University, 1991.
26. Straight JV, Ramkrishna D. Cybernetic modeling and regulation of metabolic pathways - growth on complementary nutrients. *Biotechnol Prog*. 1994;10:574–587.
27. Straight JV, Ramkrishna D. Modeling of bacterial-growth under multiply-limiting conditions - experiments under carbon-limiting or/ and nitrogen-limiting conditions. *Biotechnol Prog*. 1994;10:588–605.
28. Varner J, Ramkrishna D. Metabolic engineering from a cybernetic perspective. 1. Theoretical preliminaries. *Biotechnol Prog*. 1999;15:407–425.
29. Varner J, Ramkrishna D. Metabolic engineering from a cybernetic perspective. 2. Qualitative investigation of nodal architectures and their response to genetic perturbation. *Biotechnol Prog*. 1999;15: 426–438.
30. Varner JD. Large-scale prediction of phenotype: concept. *Biotechnol Bioeng*. 2000;69:664–678.
31. Segre D, Vitkup D, Church GM. Analysis of optimality in natural and perturbed metabolic networks. *Proc Natl Acad Sci USA*. 2002;99:15112–15117.

32. Schuster S, Fell DA, Dandekar T. A general definition of metabolic pathways useful for systematic organization and analysis of complex metabolic networks. *Nat Biotechnol*. 2000;18:326–332.
33. Schuster S, Hilgetag C, Woods JH, Fell DA. Reaction routes in biochemical reaction systems: algebraic properties, validated calculation procedure and example from nucleotide metabolism. *J Math Biol*. 2002;45:153–181.
34. Klamt S, Stelling J. Two approaches for metabolic pathway analysis? *Trends Biotechnol*. 2003;21:64–69.
35. Wagner C, Urbanczik R. The geometry of the flux cone of a metabolic network. *Biophys J*. 2005;89:3837–3845.
36. Trinh CT, Wlaschin A, Srienc F. Elementary mode analysis: a useful metabolic pathway analysis tool for characterizing cellular metabolism. *Appl Microbiol Biotechnol*. 2009;81:813–826.
37. Song HS, Ramkrishna D. Reduction of a set of elementary modes using yield analysis. *Biotechnol Bioeng*. 2009;102:554–568.
38. Song HS, Ramkrishna D. When is the quasi-steady-state approximation admissible in metabolic modeling? When admissible, what models are desirable? *Ind Eng Chem Res*. 2009;48:7976–7985.
39. Kim JI, Varner JD, Ramkrishna D. A hybrid model of anaerobic E. coli GJT001: combination of elementary flux modes and cybernetic variables. *Biotechnol Prog*. 2008;24:993–1006.
40. Young JD, Walther JL, Antoniewicz MR, Yon H, Stephanopoulos G. An elementary metabolite unit (EMU) based method of isotopically nonstationary flux analysis. *Biotechnol Bioeng*. 2008;99:686–699.
41. Young JD, Shastri AA, Stephanopoulos G, Morgan JA, Mapping photoautotrophic metabolism with isotopically nonstationary 13C flux analysis, *Metab Eng*. 2011;13:656–665.
42. Wiechert W. C-13 metabolic flux analysis. *Metab Eng*. 2001;3:195–206.
43. Provost A, Bastin G, Agathos SN, Schneider YJ. Metabolic design of macroscopic bioreaction models: application to Chinese hamster ovary cells. *Bioprocess Biosyst Eng*. 2006;29:349–366.
44. Song HS, Morgan JA, Ramkrishna D. Systematic development of hybrid cybernetic models: application to recombinant yeast coconsuming glucose and xylose. *Biotechnol Bioeng*. 2009;103:984–1002.
45. Franz A, Song HS, Ramkrishna D, Kienle A. Experimental and theoretical analysis of poly(beta-hydroxybutyrate) formation and consumption in Ralstonia eutropha. *Biochem Eng J*. 2011;55:49–58.
46. Krishnan MS, Xia Y, Ho NWY, Tsao GT. Fuel ethanol production from lignocellulosic sugars - studies using a genetically engineered Saccharomyces yeast. *Fuels Chem Biomass*. 1997;666:74–92.
47. Song HS, Ramkrishna D. Prediction of metabolic function from limited data: lumped hybrid cybernetic modeling (L-HCM). *Biotechnol Bioeng*. 2010;106:271–284.
48. Song HS, Ramkrishna D. Cybernetic models based on lumped elementary modes accurately predict strain-specific metabolic function. *Biotechnol Bioeng*. 2011;108:127–140.
49. Kim JI.A Hybrid Cybernetic Modeling for the Growth of Escherichia coli in Glucose-Pyruvate Mixtures. Ph.D. Thesis, West Lafayette: Purdue University, 2008.
50. Europa AF, Gambhir A, Fu PC, Hu WS. Multiple steady states with distinct cellular metabolism in continuous culture of mammalian cells. *Biotechnol Bioeng*. 2000;67:25–34.
51. Follstad BD, Balcarcel RR, Stephanopoulos G, Wang DIC. Metabolic flux analysis of hybridoma continuous culture steady state multiplicity. *Biotechnol Bioeng*. 1999;63:675–683.
52. Namjoshi AA, Hu WS, Ramkrishna D. Unveiling steady-state multiplicity in hybridoma cultures: the cybernetic approach. *Biotechnol Bioeng*. 2003;81:80–91.
53. Jones KD, Kompala DS. Cybernetic model of the growth dynamics of Saccharomyces cerevisiae in batch and continuous cultures. *J Biotechnol*. 1999;71:105–131.
54. Straight JV, Ramkrishna D. Complex growth dynamics in batch cultures - experiments and cybernetic models. *Biotechnol Bioeng*. 1991;37:895–909.
55. Ramkrishna D. On modeling of bioreactors for control. *J Process Control*. 2003;13:581–589.
56. Song HS, Kim SJ, Ramkrishna D. Synergistic optimal integration of continuous and fed-batch reactors for enhanced productivity of lignocellulosic bioethanol. *Ind Eng Chem Res*. 2011; doi:10.1021/ie200879s.
57. Bailey JE. Toward a science of metabolic engineering. *Science*. 1991;252:1668–1675.
58. Song HS, Morgan JA, Ramkrishna D. *Towards increasing the productivity of lignocellulosic bioethanol: rational strategies fueled by modeling*. In: Lima MAP, editor. Bioethanol, InTech: Rijeka, Croatia, in press.

Manuscript received July 19, 2011, revision received Nov. 19, 2011, and final revision received Jan. 10, 2012.