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Stoichiometric modeling of *Clostridium acetobutylicum* fermentations with non-linear constraints

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Abstract

A stoichiometric model of *Clostridium acetobutylicum* and related strains has been previously derived. The stoichiometric matrix of the model contains a singularity which has prevented the calculation of a unique set of fluxes which describe the primary metabolic activity. To resolve the singularity, we have developed a non-linear constraint relating the acetate and butyrate uptake fluxes. Subsequently, we developed a software package utilizing a model independent heuristic global optimization approach to solve the resultant non-linear problem. We have validated the use of the non-linear constraint by correlating calculated butyrate production pathway flux profiles with measured intracellular pH profiles. Finally, we examined a controled batch fermentation to determine that the acid formation pathways play critical roles throughout solventogenesis. The broader usefulness of reformulating the stoichiometric model as a constrained minimization problem is discussed.

Keywords: Metabolic flux analysis; Non-linear constraints; Solventogenic clostridia

1. Introduction

Strain development of industrial microorganisms has typically been performed through procedures of random mutagenesis and phenotypic selection. Metabolic engineering seeks to replace this 'shotgun' approach with a more rational effort (Bailey, 1991). The metabolic engineering approach is an iterative procedure involving identification of metabolic bottlenecks or limitations followed by manipulation to alter metabolic fluxes (Stephanopoulos and Sinskey, 1993). With the advent of many powerful molecular biology techniques to manipulate the genetics of an organism, the emphasis has been on cloning and sequencing genes for enzymes associated with the desired product. The resultant genetic repertoire is a useful tool to manipulate the genetics of an organism to enhance product yields. However, the underlying assumption that product yields are only affected by the enzymes involved in the final steps of product synthesis is too simple. While levels of enzymes involved in the final steps of

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synthesis are important, significant enhancement of product yields may only be realized by taking a more global approach to cellular metabolism.

A number of techniques have been developed to address the problem of understanding cellular metabolism. The initial efforts resulted in the development of metabolic control theory (MCA), in which information on enzyme kinetics is used to develop a global model of cellular metabolism (Kacser and Burns, 1973). Unfortunately, the lack of sufficient kinetic information renders MCA impractical for most biological systems. An alternative approach, metabolic flux analysis (MFA), using metabolic pathway balances to develop a model of cellular metabolism was reported by Papoutsakis (1984). This model takes the form of a system of linear equations based on species balances and in vivo metabolic pathway fluxes. The system of equations is typically underdetermined and must be further manipulated to be useful. To this end, Papoutsakis utilized biological constraints in the form of pseudo-steady state approximations on metabolic intermediates to reduce the underdetermined nature of the stoichiometric matrix. The reduced matrix was then used to develop a so-called 'fermentation equation' to verify fermentation data consistency, to develop 'gateway' sensors (which estimate unobservable physiological parameters from measurable quantities), and to predict maximum theoretical yields (Papoutsakis 1984). However, the presence of an unresolved singularity has prevented the calculation of some pathway fluxes critical in the metabolism of solventogenic clostridia.

The determination of these unresolved fluxes responsible for acetate, butyrate, and acetone production is required to develop a better understanding of the metabolism of solventogenic clostridia. Batch clostridial fermentations are characterized by two phases, acidogenic and solventogenic. The acidogenic phase involves rapid growth and the production of acetic and butyric acids. The solventogenic phase is characterized by cessation of growth, uptake of acids, and production of acetone, butanol and ethanol. The reutilization of acids occurs primarily through the acetone formation pathways (Hartmanis et al., 1984). Typically, butyrate appears to be reutilized more rapidly than acetate (Ross, 1961; Mermelstein et al., 1993; Walter, 1994). In contrast, in vitro selectivity studies of the enzyme responsible for acetone production, the CoA Transferase (CoAT), indicate a preferential uptake of acetate over butyrate (Wiesenborn et al., 1989a). These contrasting findings may be explained by two different hypotheses. One hypothesis states that in vitro findings do not accurately describe the in vivo activity of the CoAT enzyme. Such contrasting results between in vitro and in vivo activities are possibly due to different in vitro and in vivo conditions (cofactor concentrations, pH, etc.). By this reasoning, the in vivo fluxes of butyrate uptake are larger than the in vivo fluxes of acetate uptake. The other hypothesis states that in vitro findings, while accurately relating in vivo fluxes, cannot be related to observable net production/consumption rates. By this reasoning, acetate is preferentially reutilized in vivo; however, the observed uptake of acetate remains relatively smaller due to continued acetate production during solventogenesis. In essence, the cyclic nature of acetate production and uptake obscures the role of the acetate formation pathways in the metabolism of solventogenic clostridia. Determination of in vivo fluxes can therefore be a useful tool in the understanding of metabolic activity and subsequent metabolic engineering of solventogenic clostridia.

In order to determine in vivo fluxes, various strategies have been developed to resolve singularities. Some researchers have eliminated singularities in the stoichiometric matrix by utilizing in vitro enzyme activity information to remove pathways which were determined to be insignificant (Vallino and Stephanopoulos, 1993). The fluxes involved with the singularity in the clostridial system, however, are known to play important roles in the primary metabolism and cannot be removed. Other researchers have incorporated optimality concepts to develop linear programming methods of resolving singularities (Majewski and Domach, 1990; Varma and Palsson, 1994; Bonarius et al., 1996). However, the optimality principles introduced to date, such as maximal growth or maximal ATP generation, are not capable of resolving the singularity in the clostridial system as discussed later.

In this article, we have returned to the stoichiometric model developed for solventogenic clostridia. In order to resolve the singularity in the stoichiometric matrix, we have developed a non-linear constraint utilizing in vitro information regarding kinetics and selectivity of the CoA Transferase enzyme from *Clostridium acetobutylicum*. Using this additional constraint, the stoichiometric model of solventogenic clostridia was reformulated as a non-linear constrained minimization problem. We also incorporated boundary conditions to prevent reversal of known irreversible reactions. Enforcement of pathway irreversibility results in model calculations which more accurately reflect cellular metabolism. To solve this problem for calculation of pathways fluxes, we have developed a software package implementing a global optimization approach utilizing non-linear programming techniques. Subsequently, we analyzed data from batch cultures of *C. acetobutylicum* ATCC 824 to confirm the validity of the constraint which we have developed. Finally, we speculate on the general utility of reformulating any stoichiometric model as a non-linear constrained minimization problem.

2. Methods

2.1. Software development

The software used in this effort, COMPFlux, was developed in ANSI compliant C. A number of tasks utilized well established subroutines from Numerical Recipes in C (Press et al., 1992). The code was compiled and tested on a Macintosh Quadra 630 using Symantec C + + v6.0 and on a Hewlett Packard 9000 Model 715 33 MHz workstation using the native C compiler.

2.2. Experiments

C. acetobutylicum ATCC 824 was maintained and grown as described by Roos et al. (1985). Uncontroled-pH batch fermentations were performed by Hüsemann in a Bioflo C-30 bioreactor (New Brunswick Scientific, Edison, NJ) with a working volume of approximately 700 ml (Hüsemann, 1989; Hüsemann and Papoutsakis, 1990). A controled-pH batch fermentation was performed by Walter in BioFlo II bioreactor (New Brunswick Scientific, Edison, NJ) with a working volume of approximately 5.5 l (Walter, 1994; Walter et al., 1994).

3. Metabolic flux analysis

3.1. Theory

The basis of metabolic flux analysis has been described in detail previously (Papoutsakis, 1984; Meyer and Papoutsakis, 1988). A brief summary is presented here for background. Given a network of reactions by which a substrate is utilized for the production of biomass and products, a species balance can be formulated. The rate of accumulation of a species is given by the sum of pathways which produce and consume the species:

$$x_i = \sum_j a_{ij} r_j \tag{1}$$

where x_i is the rate of accumulation of species i; a_{ij} is the stoichiometric coefficient of species i in pathway j; and r_j is the flux through pathway j. The set of equations developed from such balances can be represented in matrix notation by:

$$\mathbf{Ar} = \mathbf{x} \tag{2}$$

where $\mathbf{A}$ is a $M \times N$ matrix of stoichiometric coefficients, $\mathbf{r}$ is a N-dimensional flux vector, and $\mathbf{x}$ is a M-dimensional species accumulation vector for a network composed of M species and N reaction pathways. Species can be classified into metabolic intermediates and exchangeable species. Metabolic intermediates are species retained within cells which do not accumulate in significant quantities. Typically, a pseudo-steady state approximation is utilized to set their accumulation terms to 0. Exchangeable species are those which are transported across the cell membrane (nutrients, products, etc.). The accumulation terms for the exchangeable species must be directly measured. If left unmeasured, their corresponding balances must be removed from the stoichiometric matrix when calculating pathway fluxes from ex-

perimental data. The result of the removal of an unmeasured species will be either a decrease in the degree of redundancy or introduction of a singularity in the stoichiometric matrix.

Singularities in a system of linear equations occur if there are more unknowns than linearly independent equations. If the number of unknowns exceeds the number of linearly independent equations by one, then the set of solutions to Eq. (2), $\mathbf{r}'$, can be described by a line,

$$\mathbf{r}' = \mathbf{r}_1 + \alpha \mathbf{r}_0 \quad (3)$$

where $\mathbf{r}_1$ and $\mathbf{r}_0$ can be determined from analysis of the stoichiometric matrix and experimental data but α is an arbitrary scalar constant. In other words, all solutions described by the line in Eq. (3) are mathematically equally valid. In biological systems, however, there must be a specific set of 'true' in vivo fluxes which we can associate with a specific α_{true}. Therefore, resolution of the singularity requires the determination of α_{true} using a physiologically relevant decision process. This terminology can be expanded to situations in which multiple singularities exist, however, we will limit discussions to the case of one singularity for its relevance to the stoichiometric model of solventogenic clostridia.

At this point, it is important to distinguish between features of the system of equations and properties of individual fluxes. 'Redundant' and 'singular' are terms which describe the properties of a system of equations. Václavek developed the classification of individual conversion rates in systems of reactions based on properties of 'balanceability' and 'calculability' (Václavek, 1969; van der Heijden et al., 1994). A 'balanceable flux' is one that is both measured and can be calculated from other measured fluxes. A 'non-calculable' flux is an unmeasured flux which cannot be calculated from measured fluxes. A set of equations may contain some fluxes which are 'balanceable' while other fluxes may be 'non-calculable' (van der Heijden et al., 1994). Consequently, the stoichiometric matrix must be examined for singularities regardless of the dimensions of the matrix. Singularities in the stoichiometric matrix must then be resolved to determine a unique set of metabolic fluxes.

3.2. Stoichiometric model of solventogenic clostridia

The system of balances describing the metabolism of solventogenic clostridia has been described previously (Papoutsakis, 1984). (The metabolic pathways are depicted in Fig. 1.) This model of solventogenic clostridia consists of 16 species and 14 pathway fluxes (Appendix A). The species can be segregated into metabolic intermediates and exchangeable components. The metabolic intermediates are pyruvate, acetyl-CoA, acetoacetyl-CoA, butyryl-CoA, FdRed, and NADH. We have utilized NADH as the only pool of reducing equivalents; however, it is well known that some reactions require NADPH. This simplification has been justified since the conversion of NADH to NADPH is thought to require very little, if any, energy (Papoutsakis, 1984). A pseudo-steady state approximation is used to set the rates of accumulation of the metabolic intermediates to zero. The exchangeable components are biomass, glucose, acetone, ethanol, butanol, acetoin, acetate, butyrate, CO_2, and H_2. The rates of accumulation of these components must be directly measured. In the fermentations described later, CO_2 and H_2 are not measured; therefore, their respective balances have been removed from the stoichiometric matrix for the purpose of calculating pathway fluxes.

The final reduced stoichiometric matrix for solventogenic clostridia that we have developed for the calculation of pathway fluxes is composed of 14 species (eight exchangeables, six intermediates) and 14 metabolic fluxes. Examination of the matrix reveals the presence of a number of 'balanceable' fluxes, namely the biomass, ethanol, and butanol production fluxes. CO_2 and H_2 production fluxes are 'calculable' but not 'balanceable' since their production rates were not measured in the fermentations described later. In addition, singular value decomposition of the matrix has revealed the presence of a singularity which involves the pathways responsible for acetate formation (rPTAAK), butyrate formation (rPTBBK), acetate uptake (rACUP), and butyrate uptake (rBYUP). Such groups of 'non-calculable' pathway fluxes related by a singularity are referred to

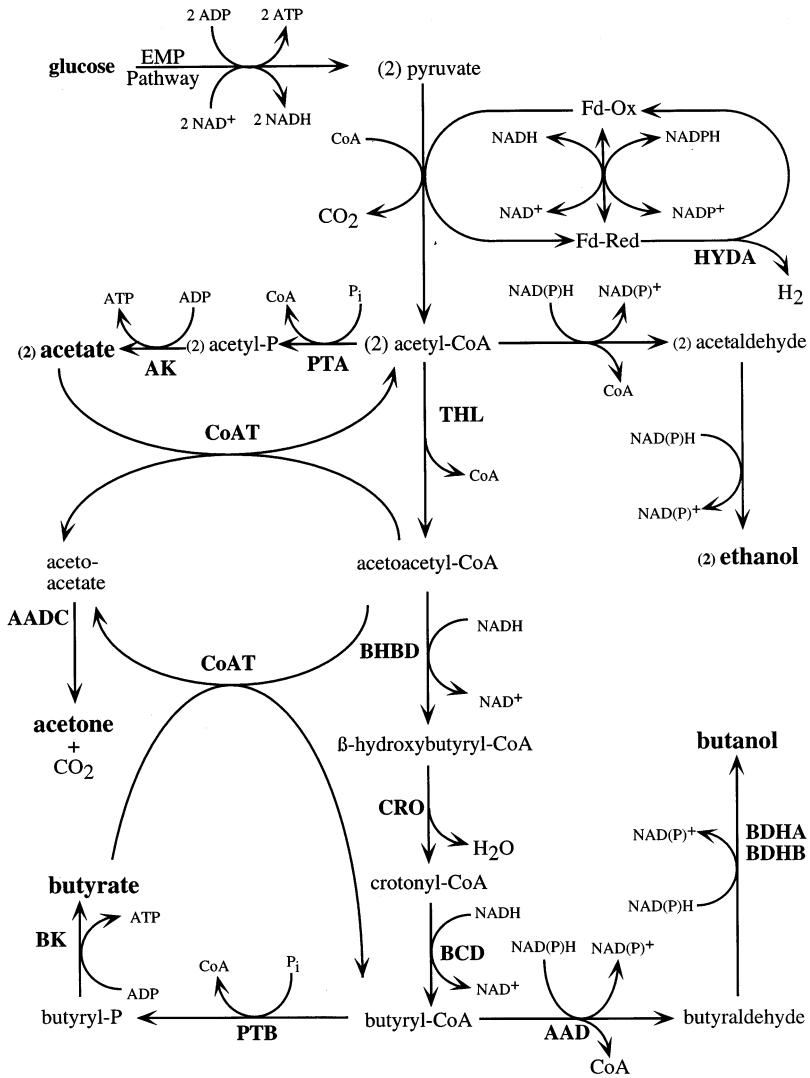

Fig. 1. Biochemical pathways describing the metabolism of solventogenic clostridia. Enzymes for cloned genes are shown in bold type: (HYDA) hydrogenase, (PTA) phosphotransacetylase, (AK) acetate kinase, (THL) thiolase, (CoAT) acetoacetyl-CoA:acetate-butyrate:CoA transferase, (AADC) acetoacetate decarboxylase, (BHBD) β-hydroxybutyryl-CoA dehydrogenase, (CRO) crotonase, (BCD) butyryl-CoA dehydrogenase, (PTB) phosphotransbutyrylase, (BK) butyrate kinase, (AAD) aldehyde/alcohol dehydrogenase, (BDHA&B) butanol dehydrogenase A & B.

as singular groups (Vallino and Stephanopoulos, 1993).

The fluxes in the singular group of the clostridial system (rPTAAK, rACUP, rBYUP and rPTBBK) are related by their involvement in acetone production. Acetone is produced via two pathways which result in the simultaneous uptake of either acetate or butyrate. The relative significance of these acetone formation pathways could ideally be determined from the relative rates of acetate and butyrate uptake. However, the presence of the respective acid formation pathways results in the same net reaction for both acetone formation pathways, as described in Eqs. (4)–(9).

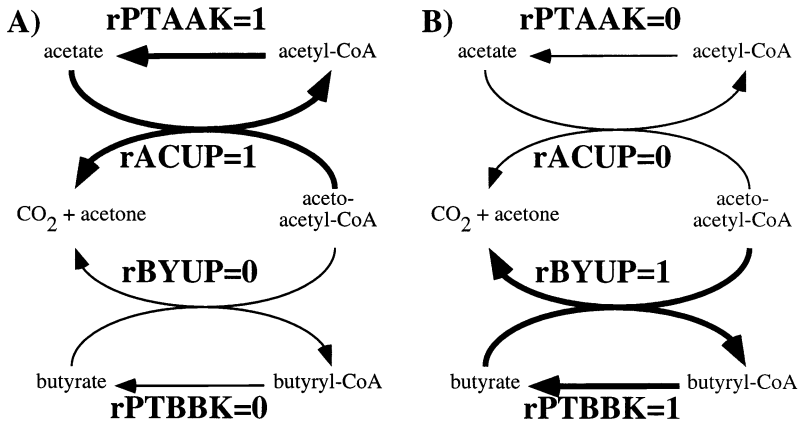

Fig. 2. Possible scenarios for the production of 1 mol of acetone from 1 mol of acetoacetyl-CoA with zero net production of acids: (A) extreme case of only acetate uptake; and (B) extreme case of only butyrate uptake.

rPTAAK: acetyl-CoA $\leftrightarrow$ acetate + ATP (4)

rACUP: acetoacetyl-CoA + acetate $\rightarrow$ acetone + CO_2 + acetyl-CoA (5)

NET: acetoacetyl-CoA $\rightarrow$ acetone + CO_2 + ATP (6)

rPTBBK: butyryl-CoA $\leftrightarrow$ butyrate + ATP (7)

rBYUP: acetoacetyl-CoA + butyrate $\rightarrow$ acetone + CO_2 + butyryl-CoA (8)

NET: acetoacetyl-CoA $\rightarrow$ acetone + CO_2 + ATP (9)

The calculation of the in vivo fluxes becomes a non-trivial issue due the interactions of the pathways. For example, the production of 1 mol of acetone from 1 mol of acetoacetyl-CoA with zero net production of acetate and butyrate can be explained by many sets of flux values. The two extreme cases of exclusive acetate uptake and exclusive butyrate uptake are depicted in Fig. 2. Essentially, the four in vivo pathway fluxes, rPTAAK, rACUP, rBYUP, and rPTBBK cannot be determined from a species balance.

To eliminate the singularity, the acetone pathways can be grouped together to form a new set of net pathways.

acetyl-CoA $\leftrightarrow$ acetate + ATP (10)

butyryl-CoA $\leftrightarrow$ butyrate + ATP (11)

acetoacetyl-CoA $\rightarrow$ acetone + CO_2 + ATP (12)

The system of balances formed from these pathways no longer contains a singularity; however, the in vivo fluxes have been replaced with net production rates of acetate, butyrate, and acetone. Papoutsakis utilized this grouping technique in the development of a stoichiometric equation which describes the interrelations among the products and biomass in fermentations of solventogenic clostridia (Papoutsakis, 1984). Unfortunately, this technique results in a loss of information concerning the physiologically relevant in vivo fluxes.

Another method by which singularities may be removed is by measuring one of the fluxes in the singular group. By fixing one such flux using additional measurements, the remaining fluxes in the singular group can be determined from the stoichiometric matrix. Using the analogy of representing the singularity as a line (Fig. 3(A)), the choice of α in Eq. (3) is made by measuring one in vivo flux, which fixes the entire solution (Fig. 3(B)). Typically, in vitro information is used to set a pathway flux to zero. For example, for the growth of *Corynebacterium glutamicum* on glucose containing media, the flux of the glyoxylate shunt was set to zero due to the low measured in vitro activity of the isocitrase lyase (Vallino and Stephanopoulos, 1993). Unfortunately, direct

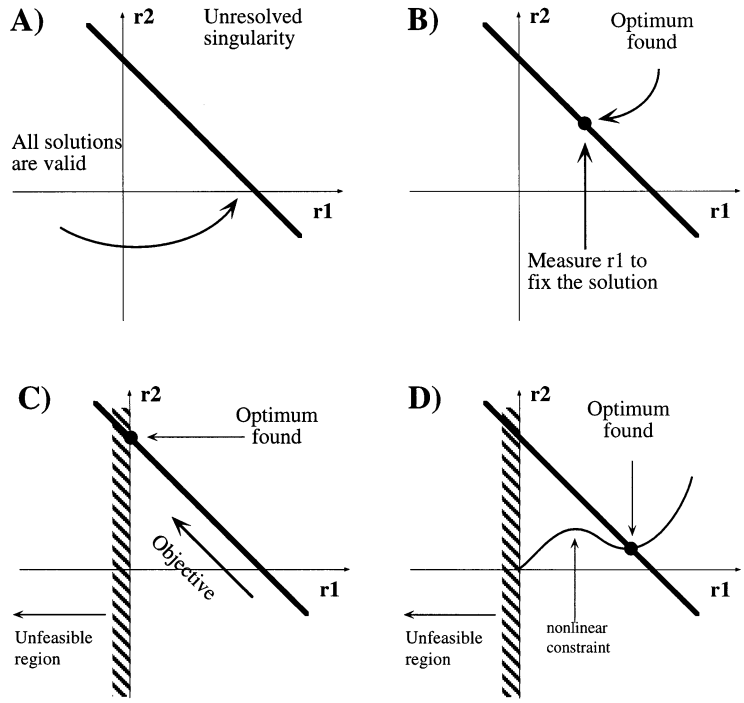

Fig. 3. Graphical representation of the methods of resolving singularities. (A) The unresolved singularity results in a set of solutions defined by a line. (B) Measurement of an in vivo flux involved with the singularity fixes the solution at a unique point. (C) The use of linear optimization techniques to determine the set of fluxes which best fit the objective of the reaction network. Hatched area indicates beginning of unfeasible region. Objective function increases along the line from bottom right to top left. (D) The use of a non-linear equation to relate two fluxes involved with the singularity fixes the solution at a unique point. Hatched area indicates beginning of unfeasible region.

measurement of an unresolveable flux is not possible in fermentations using *C. acetobutylicum*. In addition, since all of the unresolveable fluxes (rPTAAK, rPTBBK, rACUP and rBYUP) play important roles in a *C. acetobutylicum* fermentation, the singularity cannot be removed by setting a flux to zero.

An alternative method of resolving singularities is the introduction of optimality principles. The hypothesis behind the introduction of optimality principles is that metabolic networks have evolved for a specific purpose such as generation of energy or biosynthetic precursors required for growth. A number of researchers have successfully used linear programming techniques to resolve singularities by introducing objectives such as minimizing the norm of the flux vector (Bonarius et al., 1996), maximizing growth rate (Varma and Palsson, 1994), and maximizing ATP generation (Majewski and Domach, 1990). Linear constraints such as experimentally determined maximal glucose or oxygen utilization (Varma and Palsson, 1994) and maximal flux through the TCA cycle (Majewski and Domach, 1990) have been used to establish feasible regions for the optimization. Subsequently, linear optimization algorithms determine the set of fluxes which match the desired objective. Graphically, the singularity is resolved by using linear programming methods to indirectly choose the scalar α in Eq. (3) (Fig. 3(C)). First, the addition of linear constraints such as minimum and maximum allowable fluxes constrain the possible solution to a feasible region. Next, a linear objective function is used to determine the one solution which results in the optimal (minimum or maximum) value of the objective function.

Since acetone is produced by the same net pathways (Eqs. (6) and (9)), the objectives of maximal growth or maximal ATP generation are incapable of differentiating between acetate uptake and butyrate uptake. Finally, the objective of minimizing the norm of the flux vector does not seem to have any physiological significance for the differentiation of acetate uptake from butyrate uptake.

An alternative approach to resolve the singularity is to utilize the fact that both acid uptake reactions are catalyzed by the same enzyme, the CoAT. We can utilize available in vitro information to develop an equation relating acetate and butyrate uptake fluxes. Since the clostridial system is deficient by one equation, an equation relating fluxes of acetate and butyrate uptake can resolve the singularity (Fig. 3(D)). The CoAT has been purified and well characterized kinetically. The apparent K_m values of 1200 mM for acetate and 660 mM for butyrate (Wiesenborn et al., 1989a) are above the reported intracellular concentrations of 60–311 (Hüsemann and Papoutsakis, 1988) and 161–311 mM (Gottwald and Gottschalk, 1985; Terracciano and Kashket, 1986) for acetate and butyrate, respectively. Hence, physiologically, the fluxes should be proportional to the respective acid concentration. In addition, in vitro selectivity studies (Wiesenborn et al., 1989a) have indicated ratios of butyrate uptake to acetate uptake of 0.27 with both acids at 100 mM and 0.32 with both acids at 200 mM. Furthermore, this selectivity ratio increased to 0.71 with 100 mM acetate and 200 mM butyrate. This in vitro assay selectivity information seems to be in direct contrast to qualitative experimental evidence which indicates preferential uptake of butyrate (Jones and Woods, 1986). However, the determination of the true in vivo fluxes may resolve this apparent contradiction between the behavior of the enzyme responsible for acid uptake and the observed acid uptake patterns.

To that end, we utilized the available kinetic and selectivity information to develop the following correlation relating the ratio of uptake fluxes:

$$\frac{\text{rBYUP}}{\text{rACUP}} = 0.315\frac{[\text{butyrate}]_i}{[\text{acetate}]_i} \tag{13}$$

where rBYUP and rACUP are the uptake rates of butyrate and acetate, respectively, and the subscript i denotes the intracellular concentration of the respective acid. The selectivity parameter in Eq. (13), 0.315, has been calculated as the average of the three reported selectivity ratios normalized to a [butyrate]/[acetate] ratio of 1.0 (0.27, 0.32, 0.355). The sensitivity of calculated in vivo fluxes to this selectivity parameter is discussed later.

The constraint described by Eq. (13) is not immediately useful since intracellular levels of acids are not commonly measured. However, intracellular acid concentrations have been related to extracellular concentrations by the following relationship (Rottenberg 1979):

$$[\text{acid}]_i = \left(\frac{10^{\text{pH}_i} + 10^{\text{p}K_\text{A}}}{10^{\text{pH}_e} + 10^{\text{p}K_\text{A}}}\right)[\text{acid}]_e \tag{14}$$

where [acid] refers to the total (dissociated and undissociated) acid concentration and subscripts i and e refer to intracellular and extracellular concentrations. This relationship has also been verified experimentally for acetate and butyrate levels in *C. acetobutylicum* (Terracciano and Kashket, 1986; Hüsemann and Papoutsakis, 1988). Next, since the pK_As of acetic acid and butyric acid are very similar, 4.82 and 4.76 respectively, we can make the following approximation using Eq. (14):

$$\frac{[\text{butyrate}]_i}{[\text{acetate}]_i} = \frac{[\text{butyrate}]_e}{[\text{acetate}]_e} \tag{15}$$

Combining, Eqs. (13) and (15), we have an equation which relates the in vivo uptake through the acetone formation pathway of butyrate to that of acetate (the CoAT constraint), namely:

$$\frac{\text{rBYUP}}{\text{rACUP}} = 0.315\frac{[\text{butyrate}]_e}{[\text{acetate}]_e} \tag{16}$$

Since the acetate and butyrate concentrations are functions of rPTAAK, rACUP, rBYUP and rPTBBK, Eq. (16) is a non-linear constraint. The stoichiometric balances can now be used with the CoAT constraint to determine a unique set of in vivo metabolic pathway fluxes capable of describing the metabolism of solventogenic clostridia.

3.3. Calculation of in vivo fluxes

Including Eq. (16), we have a system of equations which can be solved for a unique set of fluxes. However, as discussed previously, the clostridial system contains a number of balanceable fluxes. Redundant systems can only be solved in a least-squares sense. The least-squares solution to the problem is the set of fluxes that minimizes the sum of (weighted) squared residuals between observed values and calculated values.

An additional feature that can be introduced at this point is the enforcement of pathway irreversibility. While all enzymatic reactions can proceed in either of their two directions, most operate in vivo in one direction in most situations. For our specific case, ethanol, acetone, or butanol uptake have never been experimentally observed and thus it is unlikely that ethanol will be converted back to acetyl-CoA, that acetone will be converted back to acetoacetyl-CoA, or that butanol will be converted back to butyryl-CoA. Therefore, a model incorporating the irreversibility of these pathways will result in a more accurate representation of cellular metabolism. Pathway irreversibility can be directly incorporated into the problem formulation as boundary constraints on the flux vector.

In summary, we have reformulated the stoichiometric model of solventogenic clostridia as a constrained minimization problem of the form:

Minimize the objective

$$\|\mathbf{W}^{-1}\mathbf{A}\hat{\mathbf{f}} - \mathbf{W}^{-1}\mathbf{x}\|^2 + (\mathrm{rBYUP[acetate]}_e - 0.315\mathrm{rACUP[butyrate]}_e)^2$$

with respect to $\hat{\mathbf{f}}$ subject to

$$\mathbf{l} \le \hat{\mathbf{f}} \le \mathbf{u} \tag{17}$$

where the first term of the objective is a sum of weighted squared residuals and the second term is a reformulation of Eq. (16). In this problem, **A** is the stoichiometric matrix, **x** is the species accumulation vector, $\hat{\mathbf{f}}$ is the desired pathway flux vector, **bf** is the vector defining the lower limit of $\hat{\mathbf{f}}$, **u** is the vector defining the upper limit of $\hat{\mathbf{f}}$, and **W** is the weighting matrix. **W** is a diagonal matrix which contains weighting factors associated with each element of **x**. These weighting factors serve two purposes: (1) determine the relative importance of the measured accumulation terms; and (2) enforce the pseudo-steady state approximation on metabolic intermediates. For measured components (glucose, biomass, acetate, etc.), the weighting factor is simply the measurement standard deviation. For metabolic intermediates, an applicable weighting factor is the largest magnitude pathway flux in which the intermediate appears. However, in practice the weighting factor for each metabolic intermediate is further scaled by a factor ranging from 0.01 to 0.10 since the accumulation of a metabolic intermediate should be smaller than the rate of production or consumption according to the pseudo-steady state approximation (Papoutsakis, 1984). These scaling factors affect the level of accumulation of a metabolic intermediate which is considered significant. For example, NADH (see discussion above) is a strictly conserved metabolic cofactor, as has been previously documented (Papoutsakis, 1984; Papoutsakis and Meyer, 1985a,b). Therefore, a scaling factor of 0.01 was used to keep NADH accumulation below 1 μM, a level considered significant in batch cultures of *C. acetobutylicum* (Meyer and Papoutsakis, 1989). In comparison, the levels of acetyl-CoA and butyryl-CoA are known to vary by as much as 500 μM in *C. acetobutylicum* (Boynton et al. 1994), therefore, a scaling factor of 0.10 was sufficient to enforce the pseudo-steady state approximation for these species. In essence, the scaling factor affects the level of enforcement of the pseudo-steady state approximation.

To solve the non-linear constrained minimization problem formulated above, we have implemented a global optimization algorithm using non-linear programming techniques to determine the set of fluxes which best fit the stoichiometric model and the CoAT constraint. A global optimization algorithm attempts to determine the global optima of a real valued objective function. However, most non-linear programming methods have been developed to determine local optima. In general, for non-linear objective functions, several local optima may exist and the difficulty

becomes the determination of the global optima (Rinnooy Kan and Timmer, 1987a). We have implemented a global optimization algorithm based on the multi-level single linkage algorithm (MLSL; Rinnooy Kan and Timmer, 1987b). Our implementation utilizes both the direction set method (Acton, 1970) and the downhill simplex method (Nelder and Mead, 1965) to perform local minimizations.

4. Results and discussion

4.1. Validation of the non-linear constraint

Mathematically, the addition of any linearly independent equation relating the members of a singular group can resolve the singularity. However, we are interested in a constraint that is physiologically valid and that can accurately determine the set of fluxes occurring in vivo. Therefore, the validity of Eq. (16) must be confirmed. Since the constraint affects the resolution of all of the fluxes in the singular group, experimental evidence regarding any of the four fluxes can be used to validate Eq. (16). Conclusive proof of the validity of our constraint requires measurement of in vivo fluxes through one of the pathways responsible for acetate, acetone, and butyrate formation. However, tracer experiments, typically used for such measurements, are not trivial and are not capable of resolving the fluxes in our system. Therefore, we examined the available information on the pathways responsible for acetate, acetone, and butyrate formation for an alternative method of corroboration.

The enzymes catalyzing the reactions of the butyrate formation pathway have been found sensitive to pH changes. Therefore, we may indirectly validate the CoAT constraint if the calculated flux through the butyrate formation pathway correlates with measured pH, a parameter not incorporated into the stoichiometric model. The butyrate formation pathway converts butyryl-CoA to butyrate as follows:

$$\text{butyryl}-\text{CoA} \overset{\text{PTB}}{\leftrightarrow} \text{butyryl}-\text{P} \overset{\text{BK}}{\leftrightarrow} \text{butyrate} \qquad (18)$$

Phosphotransbutyrylase (PTB) and butyrate kinase (BK) are sensitive to pH changes. The relative activity (% of activity at optimum pH) of both enzymes decreases as pH decreases from 7.5 to 5.5. Within the physiological range of pH 7–5.5, the relative PTB activity in the butyrate forming direction (forward) rapidly decreases to nearly zero ($<5\%$ of maximum) below pH 6 (Wiesenborn et al., 1989b). Relative PTB activity in the reverse direction is less sensitive to pH and remains significant ($\sim 25\%$ of maximum) at pH 5.5. The second enzyme in the pathway, BK, is less sensitive to pH changes than PTB and relative activity remains significant at pH 5.5 ($\sim$ 10% of maximum) (Hartmanis, 1987). Since levels of both enzymes are relatively constant we expect the magnitude of in vivo fluxes at low pH to be much smaller than at high pH. However, the in vivo flux can reverse direction depending upon the fermentation conditions (Hüsemann and Papoutsakis, 1989). Therefore, if the constraint relating acid uptake fluxes is physiologically valid, then the calculated flux rPTBBK should decrease with the measured intracellular pH.

With these expectations, we analyzed data from cultures of *C. acetobutylicum* ATCC 824 for which intracellular pH measurements were available: BC # 1, batch culture (control); BC # 2, batch culture with 12 mM exogenous butyrate addition; and BC # 3, batch culture with 30 mM exogenous acetate addition (Hüsemann, 1989; Hüsemann and Papoutsakis, 1990). Subsequently, we compared the calculated in vivo flux rPTBBK to the measured intracellular pH (Fig. 4). In all three experiments, as the intracellular pH decreased to 5.5, the calculated flux rPTBBK also decreased to zero or below zero. In cultures BC # 2 and BC # 3, the reversal of flux (in the butyryl-CoA forming direction) becomes significant, approaching 10% of the maximum flux achieved in the forward direction. In fact, the calculated flux rPTBBK behaves exactly as expected based on the sensitivity of the PTB and BK enzymes to pH in all three experiments. This indirect validation gives us confidence in the developed constraint.

4.2. Sensitivity analysis

In the development of a physiologically relevant constraint to resolve the singularity in the stoichiometric model of solventogenic clostridia, we have utilized information from in vitro kinetic and selectivity studies. We have developed a constraint based on 'soft' information as opposed to 'hard' information such as stoichiometry. Specifically, we utilized experimentally calculated selectivity ratios to determine the selectivity parameter (0.315) in Eq. (13). However, these selectivity ratios are subject to measurement errors. Furthermore, the value of the selectivity parameter which we have incorporated is an average value from three experiments. The actual value of this selectivity parameter ranged from 0.27 to 0.355 (see above). Therefore, we analyzed culture data using values of 0.27 and 0.355 as the selectivity parameter to assess the sensitivity of the calculated in vivo fluxes. These values reflect 14 and 12% change in the selectivity parameter. The fluxes rPTAAK and rPTBBK were found to vary by approximately 3 and 2%, respectively. The fluxes rACUP and rBYUP were found to vary by approximately 5 and 10%, respectively, when their respective pathways supported significant fluxes. We conclude that for the selectivity parameter in the expected range, the variation in the calculated fluxes is not more than the variation in the calculated fluxes due to standard measurement errors of the measured components (biomass, glucose, etc.).

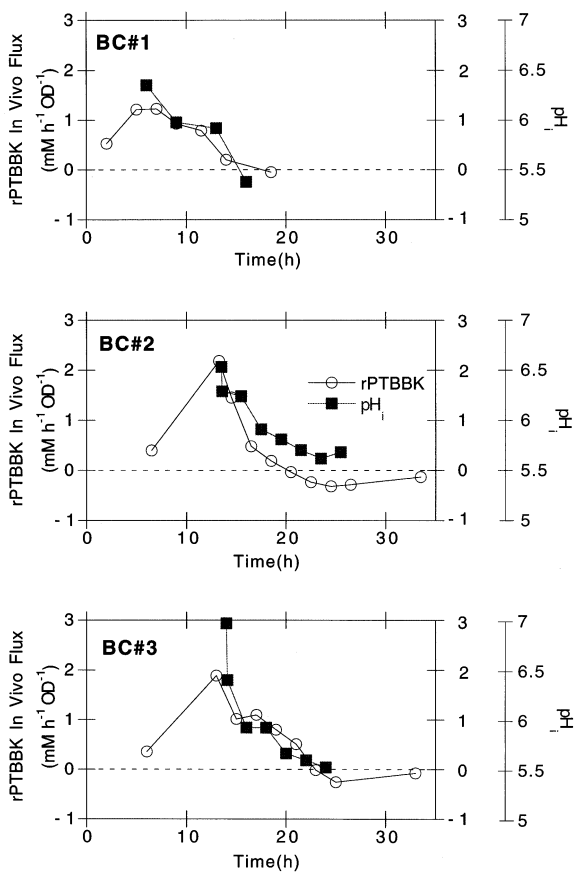

Fig. 4. Correlation of calculated flux profiles to measured intracellular pH profiles: BC # 1-batch culture (control); BC # 2-batch culture with 12 mM exogenous butyrate addition; and BC # 3-batch culture with 30 mM exogenous acetate addition. Dashed line indicates zero flux.

4.3. Metabolism of solventogenic clostridia

The ability to resolve the fluxes involved with acetone production can improve our understanding of the metabolism of solventogenic clostridia. The current paradigm of metabolic activity suggests that acid formation pathways play important roles during acidogenic phase of the fermentation. At the shift to solventogenic phase, the significance of acid formation pathways diminish in favor of the solvent formation pathways. To verify this model of metabolic activity, we examined a controled pH batch culture of *C. acetobutylicum* ATCC 824 in which significant levels of solvents are produced (Fig. 5; Walter, 1994; Walter et al., 1994). During the acidogenic phase, acid formation pathways support significant fluxes while solvent formation pathways are inactive. As the culture shifts to the solventogenic phase, we observe the net consumption of butyrate and the net production of acetate (Fig. 6(A)). From the net rates of change of acetate, acetone, and butyrate, a model of metabolic activity may be inferred (Fig. 6(B)). The butyrate uptake rates cannot entirely account for acetone production, therefore, some cycling of either acetate or butyrate must account for the remaining acetone production. This means that both of the

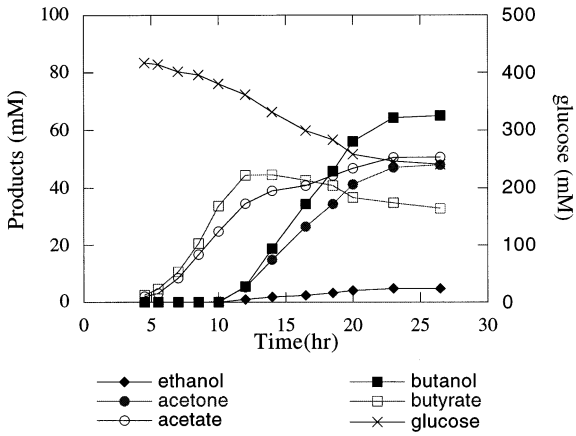

Fig. 5. Substrate and product concentration profiles of a batch fermentation of *C. acetobutylicum* ATCC 824 controled at pH 4.5 (Walter, 1994; Walter et al., 1994).

acid formation pathways may continue to produce acids during the solventogenic phase. This model of metabolic activity also suggests that the butyrate uptake pathway is at least as significant as the acetate uptake pathway. However, examination of the calculated in vivo fluxes suggests a different scenario. The calculated in vivo fluxes suggest that acetone formation occurs primarily through acetate uptake while significant butyrate uptake occurs through both the butyrate formation pathway and the acetone formation pathway (Fig. 7(A)). Furthermore, the acetate formation pathway supports a significant flux throughout solventogenesis. The calculated in vivo fluxes suggest a different model of metabolic activity than that suggested by examining net rates of change (Fig. 7(B)). In this model the butyrate formation pathway serves to uptake butyrate as opposed to producing butyrate. These results suggest that enhanced levels of PTB and BK during solventogenesis may result in increased butyrate uptake without acetone formation. Finally, we may also speculate that the large degree of cycling of acetate during solventogenesis allows the cells to continue to generate additional ATP in the presence of toxic or inhibitory levels of acetate. This description of metabolic activity would seem to suggest that the acid formation pathways play a significant role during both acidogenesis and solventogenesis.

5. Conclusions

We have developed a tool for the metabolic engineering of solventogenic clostridia. In the development of this tool, we reformulated the stoichiometric model as a non-linear constrained minimization problem. Consequently, we were able to resolve the singularity in the stoichiometric model of solventogenic clostridia by developing a physiologically based non-linear constraint. Using intracellular pH measurements, we were able to indirectly validate the constraint that we developed. Finally, we examined the metabolic activity of solventogenic clostridia in a controled batch fermentation to find that the acid formation pathways may play significant roles during the solventogenic phase of the culture.

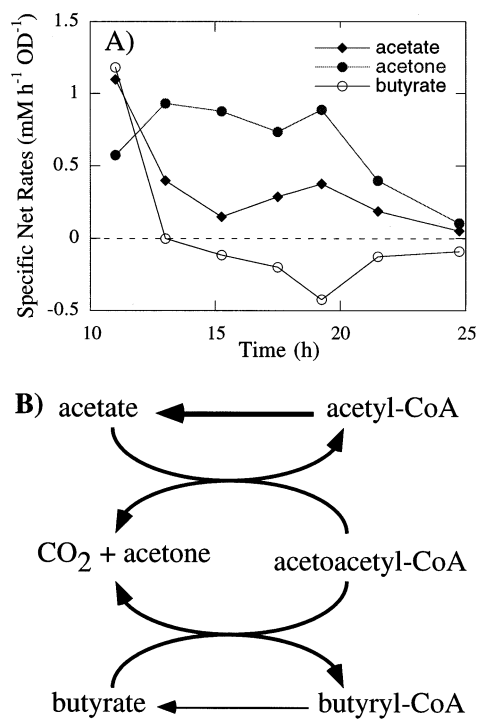

Fig. 6. Net production of acetate, acetone, and butyrate during the solventogenic phase of the fermentation depicted in Fig. 5. (A) Calculated net rates of acetate, acetone, and butyrate production. Dashed line indicates zero net production. (B) Model of metabolic activity based upon observable net rates.

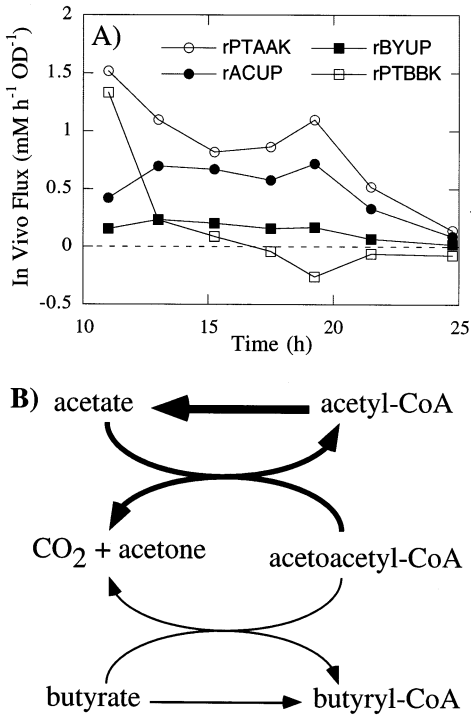

Fig. 7. Model calculations of in vivo fluxes during the solventogenic phase of the fermentation depicted in Fig. 5. (A) Calculated in vivo fluxes of the pathways involved with acetone formation. Dashed line indicates zero flux. (B) Model of metabolic activity based upon calculated in vivo fluxes.

The reformulation of the stoichiometric model as a constrained minimization problem offers many advantages over traditional methods of linear algebra. Obviously, the greatest advantage is the ability to incorporate non-linear equations into a stoichiometric model. We have used this to our advantage in the development of a non-linear constraint capable of resolving the singularity in the clostridial stoichiometric matrix. However, this method of dealing with singularities is also applicable to other systems. For example, in the stoichiometric model of *C. glutamicum*, the glyoxylate pathway was removed from the system to eliminate a singularity. The validity of this procedure in the analysis of cultures containing high glucose concentrations has been demonstrated (Vallino and Stephanopoulos, 1993). However, at lower glucose levels the glyoxylate shunt may become significant. The dynamic emergence of the glyoxylate shunt may be easily dealt with by developing a constraint relating activities of isocitrate dehydrogenase (TCA cycle) to isocitrate lyase (glyoxylate shunt) in the presence of varying levels of glucose. Qualitatively, we may expect the ratio of activities to behave as a logistic curve where isocitrate lyase is predominant at low glucose levels and isocitrate dehydrogenase is predominant at high glucose levels. By incorporating such a constraint into the stoichiometric model, a more physiologically relevant assessment of in vivo fluxes could possibly be realized.

The ability to incorporate non-linear equations also allows for the incorporation of optimality principles. The introduction of optimality principles may be required to analyze grossly underdetermined systems (Savinell and Palsson, 1992; Varma and Palsson, 1993). The constrained non-linear minimization problem we have formulated is essentially a more generalized form of the problem solved by linear programming. However, with the implementation of non-linear programming techniques, non-linear optimality principles may be introduced to stoichiometric modeling.

In addition to the incorporation of non-linear constraints, reformulation of the stoichiometric model has also allowed the enforcement of pathway irreversibility using boundary constraints. An accurate model of in vivo fluxes must observe irreversibility of pathways. For example, in the validation of a metabolic network for *Saccharomyces cerevisiae* (Vanrolleghem et al., 1996), enforcement of pathway irreversibility required the construction four different metabolic networks in order to describe the metabolism for a range of glucose/ethanol mixtures. By reformulating the stoichiometric model as a constrained minimization problem, a single metabolic network is capable of describing the metabolism of a range of substrate mixtures without a priori determination of the fluxes which are insignificant at a given substrate mixture.

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Appendix A

Pathway stoichiometry describing metabolism of solventogenic clostridia, as described previously (Papoutsakis, 1984). Reversible reactions are indicated with '$\pm$' while irreversible reactions are indicated with '+'.

($\pm$ rBIO)[a]; glucose + 0.873 NADH + x ATP

= 6 biomass

(+ rGLY1)[b]; glucose

= 2 pyruvate + 2 NADH + 2 ATP

(+ rGLY2); pyruvate

= acetyl-CoA + CO_2 + FdRed

(+ rACTN)[b]; 2 pyruvate = acetoin + 2 CO_2

(+ rETOH)[b]; acetyl-CoA + 2 NADH = ethanol

($\pm$ rPTAAK)[b]; acetyl-CoA = acetate + ATP

($\pm$ rTHL); 2 acetyl-CoA = acetoacetyl-CoA

(+ rACUP)[b]; acetoacetyl-CoA + acetate

= acetone + CO_2 + acetyl-CoA

(+ rBYUP)[b]; acetoacetyl-CoA + butyrate

= acetone + CO_2 + butyryl-CoA

($\pm$ rBYCA)[b]; acetoacetyl-CoA + 2 NADH

= butyryl-CoA

($\pm$ rPTBBK)[b]; butyryl-CoA = butyrate + ATP

(+ rBUOH)[b]; butyryl-CoA + 2 NADH

= butanol

(+ rHYD); FdRed = H_2

($\pm$ rFDNH); FdRed = NADH

ATP has not been considered in this modeling effort. Essentially, all ATP produced is assumed to be consumed through growth and non-growth maintenance requirements. In addition, NADH has been used as the single pool of reducing equivalents.

[a] The flux rBIO was used as a measure of both growth and lysis by dynamically shifting the stoichiometric coefficients of glucose and NADH depending on the direction of rBIO. For positive values of rBIO, the indicated coefficients were used. For negative values of rBIO, the coefficients of glucose and NADH were changed to 0.

[b] Biochemical pathway has been reduced to a net pathway.

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