ACCELERATED PUBLICATION

Metabolic Pathway Analysis of a Recombinant Yeast for Rational Strain Development

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Abstract: Elementary mode analysis has been used to study a metabolic pathway model of a recombinant *Saccharomyces cerevisiae* system that was genetically engineered to produce the bacterial storage compound poly-β-hydroxybutyrate (PHB). The model includes biochemical reactions from the intermediary metabolism and takes into account cellular compartmentalization as well as the reversibility/irreversibility of the reactions. The reaction network connects the production and/or consumption of eight external metabolites including glucose, acetate, glycerol, ethanol, PHB, CO_2, succinate, and adenosine triphosphate (ATP). Elementary mode analysis of the wild-type *S. cerevisiae* system reveals 241 unique reaction combinations that balance the eight external metabolites. When the recombinant PHB pathway is included, and when the reaction model is altered to simulate the experimental conditions when PHB accumulates, the analysis reveals 20 unique elementary modes. Of these 20 modes, 7 produce PHB with the optimal mode having a theoretical PHB carbon yield of 0.67. Elementary mode analysis was also used to analyze the possible effects of biochemical network modifications and altered culturing conditions. When the natively absent ATP citrate-lyase activity is added to the recombinant reaction network, the number of unique modes increases from 20 to 496, with 314 of these modes producing PHB. With this topological modification, the maximum theoretical PHB carbon yield increases from 0.67 to 0.83. Adding a transhydrogenase reaction to the model also improves the theoretical conversion of substrate into PHB. The recombinant system with the transhydrogenase reaction but without the ATP citrate-lyase reaction has an increase in PHB carbon yield from 0.67 to 0.71. When the model includes both the ATP citrate-lyase reaction and the transhydrogenase reaction, the maximum theoretical carbon yield increases to 0.84. The reaction model was also used to explore the possibility of producing PHB under anaerobic conditions. In the absence of oxygen, the recombinant reaction network possesses two elementary modes capable of producing PHB. Interestingly, both modes also produce ethanol. Elementary mode analysis provides a means of deconstructing complex metabolic networks into their basic functional units. This information can be used for analyzing existing pathways and for the rational design of further modifications that could improve the system's conversion of substrate into product. *Biotechnol Bioeng* **79**: 121–134, 2002.
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INTRODUCTION

Knowing, a priori, the possible effects of genetic modifications could greatly simplify strain development. However, predicting the effects of topological modifications, such as the expression of a recombinant gene or the knocking-out of an existing gene, is often difficult. Incomplete databases of critical, organism-specific parameters, including gene regulation, gene expression levels, mRNA stability, and enzyme kinetics, limit the ability of kinetic models to accurately predict the effects of topological changes. Other tools such as linear programming give some insight but are often limited by the scope of the results. Instead of giving all possible flux states, usually only a single flux state is found for a given optimization criterion. This can result in missing potentially interesting and useful suboptimal flux states. A few tools, based on a biochemical network's structural invariants, have been developed that are capable of partially circumventing some of these problems. The

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methods focus on system invariants such as conservation relationships between metabolites and cofactors, balanced subnetworks (compartmentalization), and thermodynamic constraints such as the reversibility/irreversibility of reactions to deconstruct the metabolic networks (Fell and Small, 1986; Mavrovouniotis et al., 1990; Schilling and Palsson, 1998, 2000; Schuster et al., 1994, 1996). A review of these methods can be found in Schilling et al. (1999). One of the recent approaches is elementary mode analysis. This generalized method uses concepts from convex analysis to identify all possible, unique, nondecomposable biochemical pathways for a steady-state system (Schuster et al., 1994, 1996). In Schuster et al. (1994), elementary modes are described as "those flux patterns that remain after inhibition of a number of enzymes, such that inhibition of a further still active enzyme leads to cessation of any non-zero flux." A formal mathematical definition can be found in Schuster et al. (1994, 1996) and Pfeiffer et al. (1999). Elementary mode analysis has been used for a number of applications, including the analysis of glycolysis, the pentose phosphate pathway, and the citric acid cycle, in models representing a number of organisms, including human red blood cells, *Escherichia coli*, and *Treponema pallidum* (Dandekar et al., 1999; Schuster et al., 1996, 1999, 2000).

We have used elementary mode analysis to examine a recombinant *Saccharomyces cerevisiae* strain that expresses the three-gene poly-β-hydroxybutyrate (PHB) pathway from *Ralstonia eutropha* (β-ketothiolase [phbA], reductase [phbB], and synthase [phbC]) (see Fig. 2 and reactions phbA, phbB, and phbC in Appendix). This natural, biodegradable thermoplastic has been studied widely for its material properties and as a possible alternative to some petroleum-based thermoplastics (for a recent review see Lee [1996]). The existence of relatively simple gas-chromatography analytical techniques also makes this acetyl-CoA-derived biopolymer an attractive tool for probing in vivo intracellular metabolite pools (Riis and Mai, 1988). Elementary mode analysis was used to identify and to study all optimal and suboptimal material flux patterns available for producing PHB. The analysis was also used to gain information on network modifications and environmental conditions that could be utilized to rationally improve the recombinant PHB production system.

EXPERIMENTAL SYSTEM

The described theoretical analysis was based on experimental data from a culture of *S. cerevisiae* strain D603 (MATa/MATα *ura3-52 lys2-801 met his3 ade2-101 reg1-501* [cir+]) (Srienc et al., 1986) that was engineered to produce PHB. The detailed construction and characterization of this strain will be given elsewhere, but briefly *S. cerevisiae* strain D603 was transformed with two plasmids containing the three *R. eutropha* PHB genes (β-ketothiolase [phbA], reductase [phbB], and synthase [phbC]). All three genes were regulated by the bidirectional GAL1-10 promoter. Descriptions of some aspects of PHB synthesis kinetics have been included because they influenced how the present study was conducted. Strain D603 possesses the *reg1-501* mutation, which partially releases catabolite repression of the GAL1-10 promoter (Matsumoto et al., 1983). In the presence of glucose and galactose, the promoter is active. The specific PHB production rate remains essentially constant for approximately 20 h after the exhaustion of the two sugars and the cessation of promoter induction (see Fig. 1). Such observations suggest the PHB enzymes are relatively stable and that the PHB is not produced directly from glucose or galactose but rather some metabolic byprod-

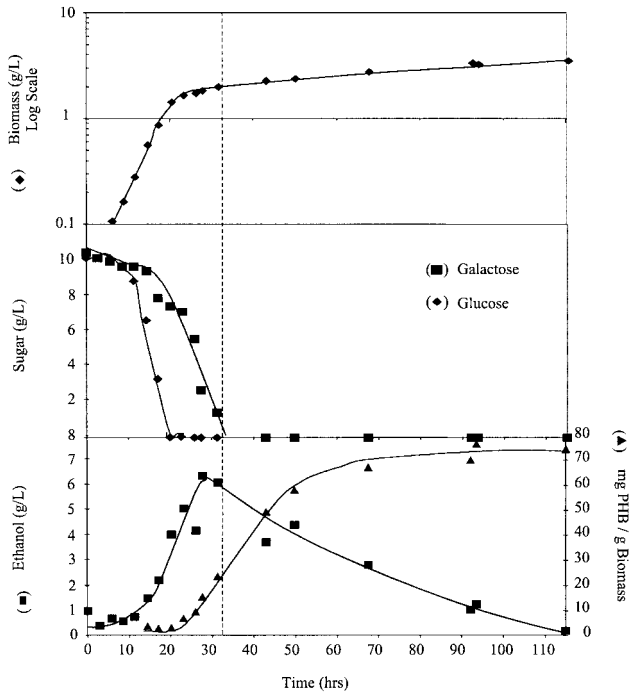

Figure 1. Bioreactor time profiles from a recombinant *S. cerevisiae* strain expressing the *Ralstonia eutropha* PHB pathway. The construction and characterization of this strain will be given elsewhere, but briefly *S. cerevisiae* strain D603 (MATa/MATα *ura3-52 lys2-801 met his3 ade2-101 reg1-501* [cir+]) (Srienc et al., 1986) was transformed with two high-copynumber 2-μm-based plasmids containing the three *R. eutropha* PHB genes (β-ketothiolase [phbA], reductase [phbB], and synthase [phbC]). All three genes were regulated by the bidirectional GAL1-10 promoter. The plasmids utilized either a uracil or a histidine selection marker. Strain D603 possesses the *reg1-501* mutation, which partially releases catabolite repression of the GAL1-10 promoter (Matsumoto et al., 1983). In the presence of glucose and galactose, the promoter is active. The strain was cultured in a 5-L bioreactor at 30°C in defined media (glucose 10 g/L, galactose 10 g/L, yeast nitrogen base with ammonium sulfate 6.7 g/L, adenine 100 mg/L, methionine 100 mg/L, and lysine 150 mg/L). The pH was maintained at 4.5 with acid (2% H_3PO_4) and base (1 *M* NaOH) additions, the reactor was sparged with 1 vessel volume of air per minute (1 vvm) and agitated at 300 rpm. Biopolymer accumulation begins after the cells enter a slow growth phase and continues linearly for approximately 20 h after both glucose and galactose are exhausted. The dashed line indicates the beginning of the culturing period represented by the elementary mode model.

ucts. This period of PHB accumulation occurs after the exponential growth phase during a slow growth phase.

The elementary mode analysis technique, as with most pathway analysis techniques, is based on a steady-state system (Schuster et al., 1996). Whereas a batch-cultivated system does not produce steady states, a culturing period was identified in which a pseudo-steady state could be applied for theoretical purposes. The culturing period between approximately hours 33 and 50 served as the basis for the elementary mode model (see Fig. 1). During this period, the sugars are exhausted yet PHB production continues to accumulate linearly for close to 20 h with very little cell growth.

CONSTRUCTION OF THE METABOLIC REACTION NETWORK

A *S. cerevisiae* biochemical reaction model was constructed that includes reactions of the central metabolism and of PHB synthesis (see Fig. 2). The model is based on current literature and incorporates what is presently known about yeast intermediary metabolism. Sixty-four different reactions are included in the model (see Appendix for specific reactions and references). Of these reactions, 26 are classified as reversible, whereas 38 reactions are classified as irreversible. For simplicity, the reactions are not necessarily the product of a single enzyme. In some cases, the reaction is the overall stoichiometry of a linear series of enzymes. The reactions are written with 67 metabolites. These metabolites are classified as either "external" or "internal" (Schuster et al., 1996). The model includes 59 "internal" metabolites that are defined as metabolites constrained by the steady-state assumption of no accumulation ($dc/dt = 0$). The model also uses eight "external" metabolites: glucose, acetate, ethanol, glycerol, PHB, ATP, CO_2, and succinate. By the elementary mode definition of an external metabolite, these metabolites are not constrained by the steady-state assumption and serve as sources or sinks (Schuster et al., 1994, 1996, 1999, 2000). The physical location of "external" metabolites is not necessarily "external" to the cell. For instance, PHB is accumulated as insoluble

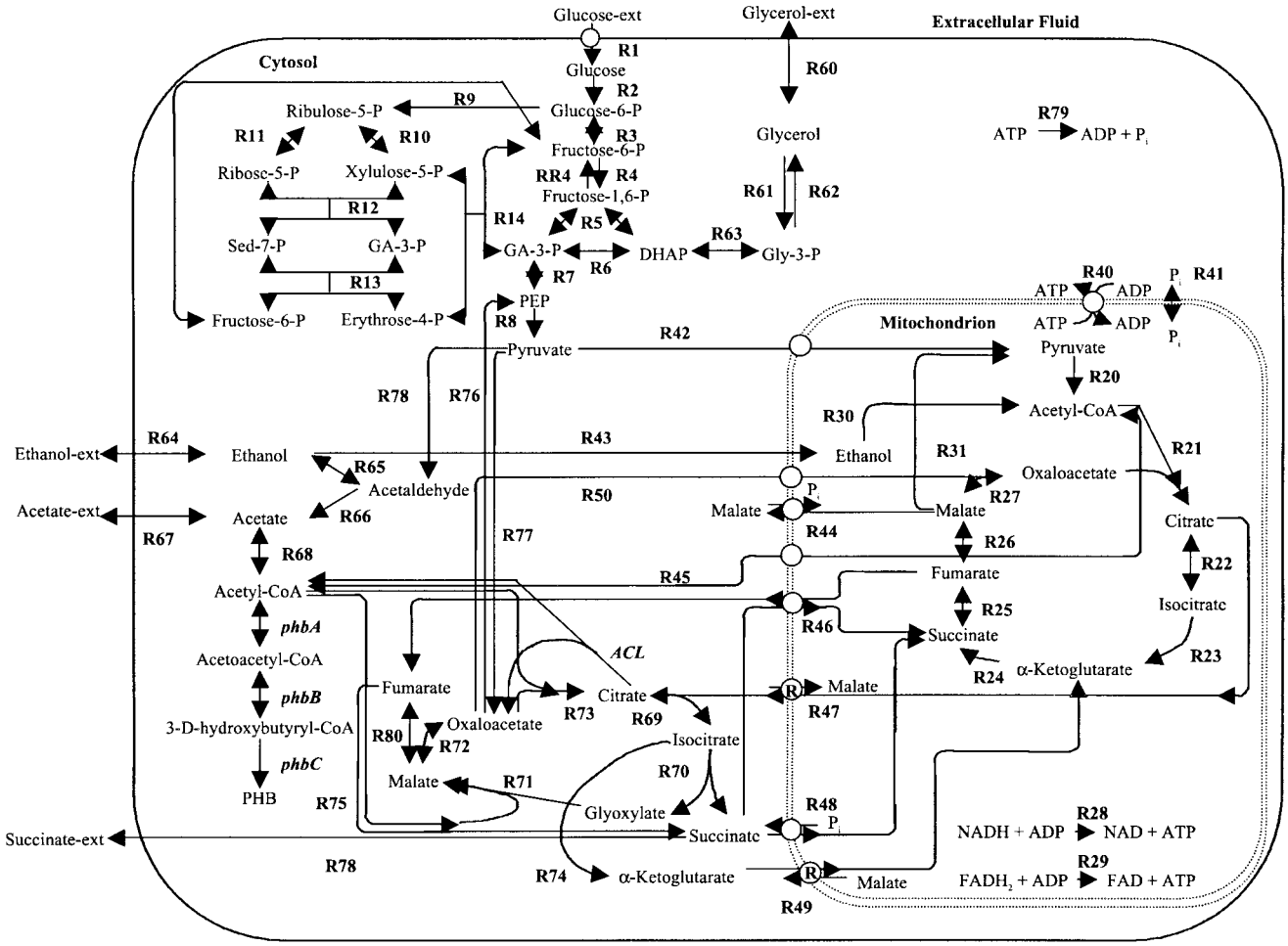

Figure 2. Biochemical network model for a recombinant *S. cerevisiae* strain expressing the three-gene PHB pathway (β-ketothiolase [phbA], reductase [phbB], and synthase [phbC]). The model contains 64 reactions, with 28 being reversible, and 67 metabolites, with 8 considered external. The external metabolites include glucose, acetate, ethanol, glycerol, PHB, ATP, CO_2, and succinate. Reactions and metabolites are partitioned between extracellular, cytosolic, and mitochondrial compartments. Numbers next to arrows refer to designated reaction number. See Appendix for details.

granules within the cytosol (Leaf et al., 1996). Based on experimental data suggesting that PHB accumulation occurs during a slow growth phase (see Fig. 1), the model assumes no growth and does not contain a biomass term. The special adenosine triphosphate (ATP) term is included as a "dummy" variable that permits the dissipation of excess energy (reaction R79, see Appendix). Although this variable permits excess ATP to be consumed by implicit maintenance processes, all elementary modes must produce at least enough ATP to meet the individual mode's requirements. O_2 consumption is implicitly included in the oxidative phosphorylation reactions wherein NADH and $FADH_2$ are used to generate ATP (reactions R28 and R29, see Appendix). The ATP yield per mole of $NADH/FADH_2$ is set to 1.0 based on the analysis of Vanrolleghem et al. (1996). Water and proton balances are not enforced due to the aqueous nature of biological systems, and because, under standard bioreactor operating conditions, the pH is controlled by acid and base additions. Because the metabolism of galactose proceeds through glucose-6-phosphate, glucose and galactose are considered interchangeable for purposes of the model (Johnston, 1987).

Due to physical constraints imposed by the selective permeability of cell membranes, the metabolites and the reactions are partitioned between the extracellular, intracellular, and mitochondrial compartments. The partitioning between the various cellular compartments and the incorporation of various transporters are based on current literature. Due to the lack of data, the energy requirements associated with the transporters are neglected. The reaction model and relevant references are listed in the Appendix.

All elementary mode analysis was done with the publicly available METATOOL v3.1 program found at http://mudshark.brookes.ac.uk/sware.html or ftp://ftp.bioinf.mdc-berlin.de/Pub/metabolic/metatool/ (Pfeiffer et al., 1999; Schuster et al., 1994).

The METATOOL output file includes a concise listing of all elementary modes in a matrix format. An MS EXCEL template has been developed to facilitate analysis of this matrix. Carbon yields are determined for each product based on the utilized substrates. Logical statements determine whether a metabolite is used as a reactant or a product. For instance, glycerol, acetate, and ethanol can be either substrates or products and the calculated yields need to account for the direction of the flux to calculate the proper yield. In addition to calculating yields, the EXCEL template permits easy sorting and plotting of results based on such properties as products formed, substrates utilized, reactions (enzymes) utilized, or economic characteristics such as mode profit.

RESULTS

Elementary mode analysis of the *S. cerevisiae* model was used to examine existing network characteristics and then to identify catalytic activities and culturing conditions that could increase product yield, change mode byproducts, or change the number of possible pathways leading to a desired product.

"Metabolic Richness" of Wild-Type System

The "metabolic richness" of the wild-type biochemical network was analyzed by determining the total number of elementary modes possible (Schuster et al., 1999). The wild-type system utilizing seven external metabolites, glucose, acetate, ethanol, glycerol, ATP, CO_2, and succinate, has 241 unique, balanced elementary modes. The large number of modes illustrates the complexity of the branched system and the difficulty in determining all possible reaction combinations manually. When the modes were sorted for the involvement of ethanol, 111 modes were found to produce ethanol, whereas 43 consumed ethanol. Of the 111 modes forming ethanol, 107 were found to coproduce glycerol. Ninety-three of the 111 ethanol-forming modes utilized both acetate and glucose as substrates whereas the remaining 18 modes used only glucose. The ethanol carbon yield, which is defined as the ratio of the number of carbon atoms found in the produced ethanol to the total number of carbon atoms found in all utilized substrates ($C_{EtOH}/C_{SUBSTRATE(S)}$), was found to range from 0.04 to 0.67 for the ethanol-producing modes. The highest yielding ethanol mode converted 1 mol of glucose into 2 mol of ethanol and 2 mol of CO_2 with a net production of 2 ATP.

Pathway Analysis: PHB Production

To study the production of PHB in the recombinant *S. cerevisiae* system, the wild-type model was modified to include the PHB pathway, and was further modified to simulate the experimental conditions when PHB accumulates. Based on experimental data, *S. cerevisiae* appears to produce PHB from a metabolic byproduct and not directly from glucose or galactose (see Fig. 1). For this reason, the reaction for the uptake of extracellular glucose (reaction R1) was removed from the model. It should be noted that ethanol, acetate, glycerol, and succinate are all common byproducts of *S. cerevisiae* cultures grown on high concentrations of glucose and galactose and would be found in the extracellular environment (Gancedo and Serrano, 1989; Oura, 1977).

The experimental system has a total of 20 modes, 7 of which produce PHB. The large reduction in the total number of modes from the wild-type system is due in part to the elimination of glucose as an external metabolite. The PHB-producing modes are shown in Table I. Table I shows only four reaction stoichiometries, because three modes, although unique, share the same overall stoichiometry as the other modes. The seven

Table I. PHB-producing elementary modes from experimental reaction network.[a]

Mode stoichiometry	PHB carbon yield	Mitochondrial oxidation of:		Source of NADPH
		Acetate	Ethanol	
2 acetate + EtOH = PHB + 2 CO_2	0.67	No	Yes	ICD
65 acetate + 31 EtOH = 30 PHB + 72 CO_2	0.63	No	Yes	PPP
10 acetate + 16 EtOH = 5 PHB + 17 CO_2 + 5 glycerol	0.38	No	Yes	ALDH
15 acetate + 16 EtOH = 5 PHB + 27 CO_2 + 5 glycerol	0.32	Yes	Yes	ALDH

[a]Model included acetate, ethanol, glycerol, PHB, ATP, CO_2, and succinate as external metabolites. Seven elementary modes produced PHB, however, while each mode is a unique combination of reactions some modes shared the same overall stoichiometry. Each overall stoichiometry is only listed once. ICD, cytosolic isocitrate dehydrogenase; PPP, pentose phosphate pathway; ALDH, cytosolic aldehyde dehydrogenase.

PHB-producing modes were sorted according to PHB carbon yield, which is defined as the ratio of the number of carbon atoms in produced PHB to the total number of carbon atoms found in all utilized substrates (C_{PHB}/$C_{SUBSTRATE(S)}$). The modes had carbon yields ranging from 0.32 to 0.67 (see Table I). The highest yielding mode used mitochondrial oxidation of ethanol (reaction R30) to supply the ATP required to convert externally supplied acetate into acetyl-CoA and used cytosolic isocitrate dehydrogenase (reaction R74) to supply the NADPH required by the PHB reductase enzyme (reaction phbB). The top-yielding elementary mode is shown in Figure 3. The second highest yielding mode, which had a PHB carbon yield of 0.63, also used ethanol and acetate as substrates, but used the pentose phosphate pathway to produce cytosolic NADPH (see Table I).

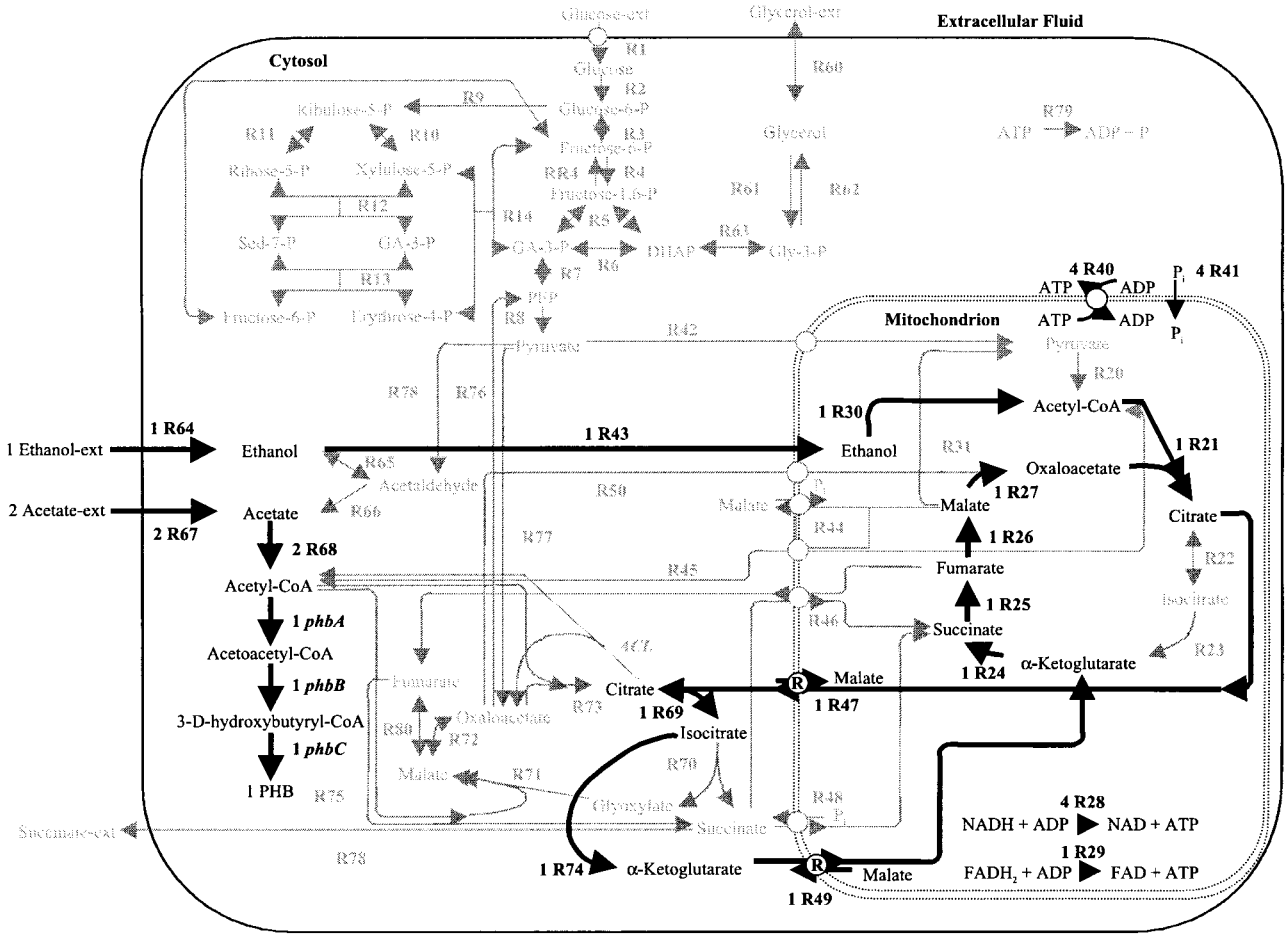

Figure 3. Heavy lines indicate the elementary mode with highest PHB carbon yield for the basic experimental network model. The number preceding the reaction number indicates relative flux through that particular reaction. The analysis used acetate, ethanol, glycerol, PHB, ATP, CO_2, and succinate as external metabolites. This model is based on experimental results and does not include glucose as an external metabolite. The highest-yielding mode has an overall stoichiometry of 2 acetate + ethanol = 2 CO_2 + PHB, and has a PHB carbon yield of 0.667.

Table II. Summary of elementary mode analysis results.[a]

	Total number of elementary modes	Number of PHB yielding elementary modes	Most efficient carbon yield for PHB
Experimental model	20	7	0.67
Experimental model + ACL	496	314	0.83
Experimental model + transhydrogenase	88	38	0.71
Experimental model + transhydrogenase +ACL	1010	447	0.84
Experimental model, anaerobic	8	2	0.24

[a]The basic experimental model included acetate, ethanol, glycerol, PHB, ATP, CO_2, and succinate as external metabolites. "ACL" refers to the addition of the ATP citrate-lyase reaction to the model and "transhydrogenase" refers to the addition of a transhydrogenase reaction to the model.

Network Modification: ATP Citrate-Lyase (ACL)

Elementary mode analysis was also used to investigate other topological modifications, such as the expression of additional recombinant genes. This study's focus was on genes that could supply metabolites used in PHB production, namely acetyl-CoA and NADPH.

Yeasts that accumulate high levels of fatty acids produce the precursor, cytosolic acetyl-CoA, primarily by the ATP citrate-lyase (ACL) enzyme, which cleaves cytosolic citrate into acetyl-CoA and oxaloacetate (Boulton and Ratledge, 1981). *S. cerevisiae* does not natively possess this gene (Evans et al., 1983; sequence analysis of *S. cerevisiae* genome). Because cytosolic acetyl-CoA is a precursor of PHB, expression of the ACL reaction is investigated as a theoretical means of improving PHB production.

Addition of the ACL-catalyzed reaction to the *S. cerevisiae* reaction network significantly increases both the total number of possible modes and PHB's maximum theoretical carbon yield. The number of modes increases from 20 to 496 with 314 modes producing PHB (see Table II). Introduction of the ACL reaction also improves the maximum theoretical yield of PHB from 0.67 to 0.83. The two top-yielding stoichiometries with ACL are shown in Table III. The top-yielding elementary mode with the ACL reaction is shown in Figure 4. The mode uses mitochondrial oxidation of ethanol to supply ATP (reaction R30), cytosolic aldehyde dehydrogenase (reaction R66) to produce NADPH, and both the pyruvate dehyrogenase bypass (reactions R78, R66, and R68) and the ACL-mediated cleavage of citrate to produce acetyl-CoA. The mode with the second highest PHB carbon yield uses ethanol and glycerol as substrates and utilizes the pentose phosphate pathway for NADPH production (not shown).

Network Modification: Transhydrogenase Reaction

NADH is typically formed during catabolic reactions, whereas NADPH is consumed in anabolic reactions. Although many bacterial and animal cells possess a transhydrogenase activity that permits the conversion of NADH and $NADP^+$ to NAD^+ and NADPH and vice versa, *S. cerevisiae* does not (Bruinenberg et al., 1985; Hoek and Rydström, 1988). *S. cerevisiae* must carefully regulate a balance between the production and consumption of NADPH and NADH to maintain a favorable redox balance. The lack of a transhydrogenase system has implications on the expression of foreign proteins and on the yields of products such as ethanol or PHB. For instance, glycerol is produced during anaerobic growth to reoxidize the NADH formed during biomass production (van Dijken and Scheffers, 1986). Glycerol formation can significantly affect the economics of processes such as ethanol production by lowering yields (Anderlund et al., 1999; Oura, 1977). A theoreti-

Table III. Overall stoichiometry from the two top-yielding elementary modes.[a]

A. 2 acetate + EtOH = 2 CO_2 + PHB	0.67
65 acetate + 31 EtOH = 30 PHB + 72 CO_2	0.63
B. 12 EtOH = 5 PHB + 4 CO_2	0.83
77 EtOH + 31 glycerol = 48 PHB + 4 acetate + 47 CO_2	0.78
C. 13 EtOH + 15 acetate = 10 PHB + 16 CO_2	0.71
EtOH + 20 acetate + 5 glycerol = 10 PHB + 17 CO_2	0.70
D. 5 EtOH + 3 glycerol = 4 PHB +3 CO_2	0.84
EtOH + glycerol = PHB +CO_2	0.80
E. 25 glucose + 24 acetate = 12 PHB + 48 EtOH + 54 CO_2	0.24
3 glucose + acetate = PHB + 4 EtOH + glycerol + 5 CO_2	0.20

[a](A) Basic experimental model that included acetate, ethanol, glycerol, PHB, ATP, CO_2 and succinate as external metabolites; (B) basic experimental model with the addition of the ATP citrate-lyase reaction; (C) basic experimental model with transhydrogenase activity; (D) basic experimental model with the addition of the ATP citrate-lyase reaction and transhydrogenase activity; and (E) system under anaerobic conditions.

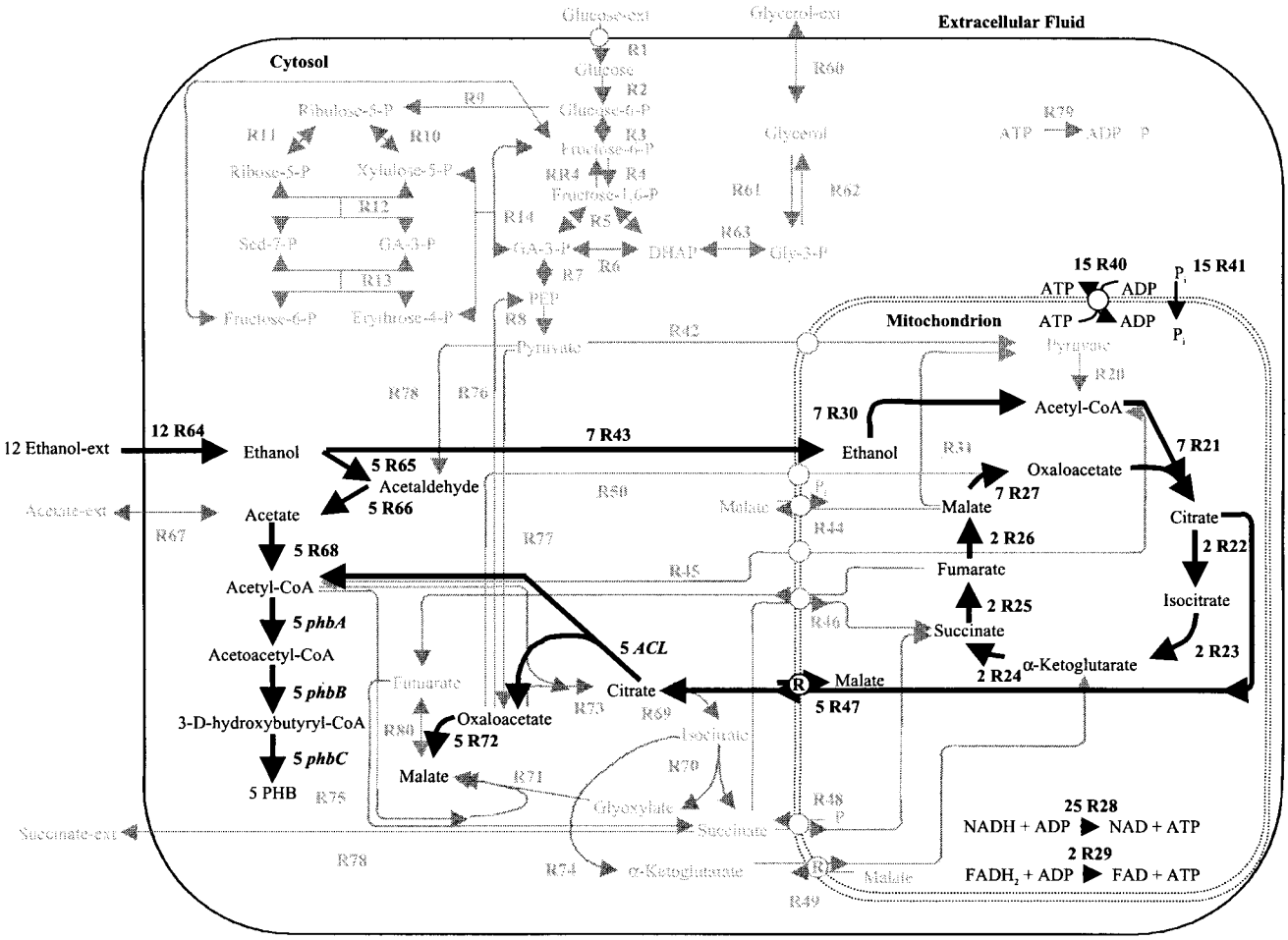

Figure 4. Heavy lines indicate the elementary mode with highest PHB carbon yield for the basic experimental network model with ATP citrate-lyase (ACL) activity. The number preceding the reaction number indicates relative flux through that particular reaction. The analysis includes acetate, ethanol, glycerol, PHB, ATP, CO_2, and succinate as external metabolites. The highest-yielding mode has an overall stoichiometry of 12 ethanol $=$ 4 CO_2 $+$ 5 PHB, and has a PHB carbon yield of 0.83.

cal analysis of the stoichiometric implications of a transhydrogenase system was investigated using the *S. cerevisiae* model. The transhydrogenase reaction was implemented by assuming that NADH and NADPH were equivalent and mutually exchangeable in the reactions involving these coenzymes.

Introducing the transhydrogenase activity to the experimental reaction model increases the total number of modes from 20 to 88, with 38 of these modes producing PHB. The maximum theoretical carbon yield for PHB increases from 0.67 to 0.71 (see Table II). The highest yielding mode utilizes mitochondrial oxidation of ethanol (reaction R30) for ATP production, whereas alcohol dehydrogenase (reaction R65) and aldehyde dehydrogenase (reaction R66) supply the reducing equivalents required for biopolymer formation. Acetate and ethanol both serve as substrates for acetyl-CoA production (see Fig. 5). The transhydrogenase reaction removes the need for either isocitrate dehydrogenase (reaction R74) or the pentose phosphate pathway (reactions R9 through R14). By avoiding these decarboxylating reactions, the loss of carbon through CO_2 is minimized, which improves the overall carbon yield.

When both the transhydrogenase reaction and the ACL reaction are added to the experimental model, the number of modes increases to 1010 with 447 producing PHB (see Table II). The maximum theoretical yield also increases slightly to 0.84. In the highest yielding mode, both ethanol and glycerol serve as substrates for PHB production. The reducing equivalents are formed during the oxidation of glycerol to pyruvate and all the cytosolic acetyl-CoA is derived from the ACL-mediated cleavage of citrate (see Fig. 6).

Altered Culturing Conditions: Anaerobic PHB Production

Culturing conditions can affect which substrates and which reactions are available to balance the internal workings of a cell. By limiting various sources and sinks, the overall character of the network can be changed. The stoichiometric constraints associated with aerobic

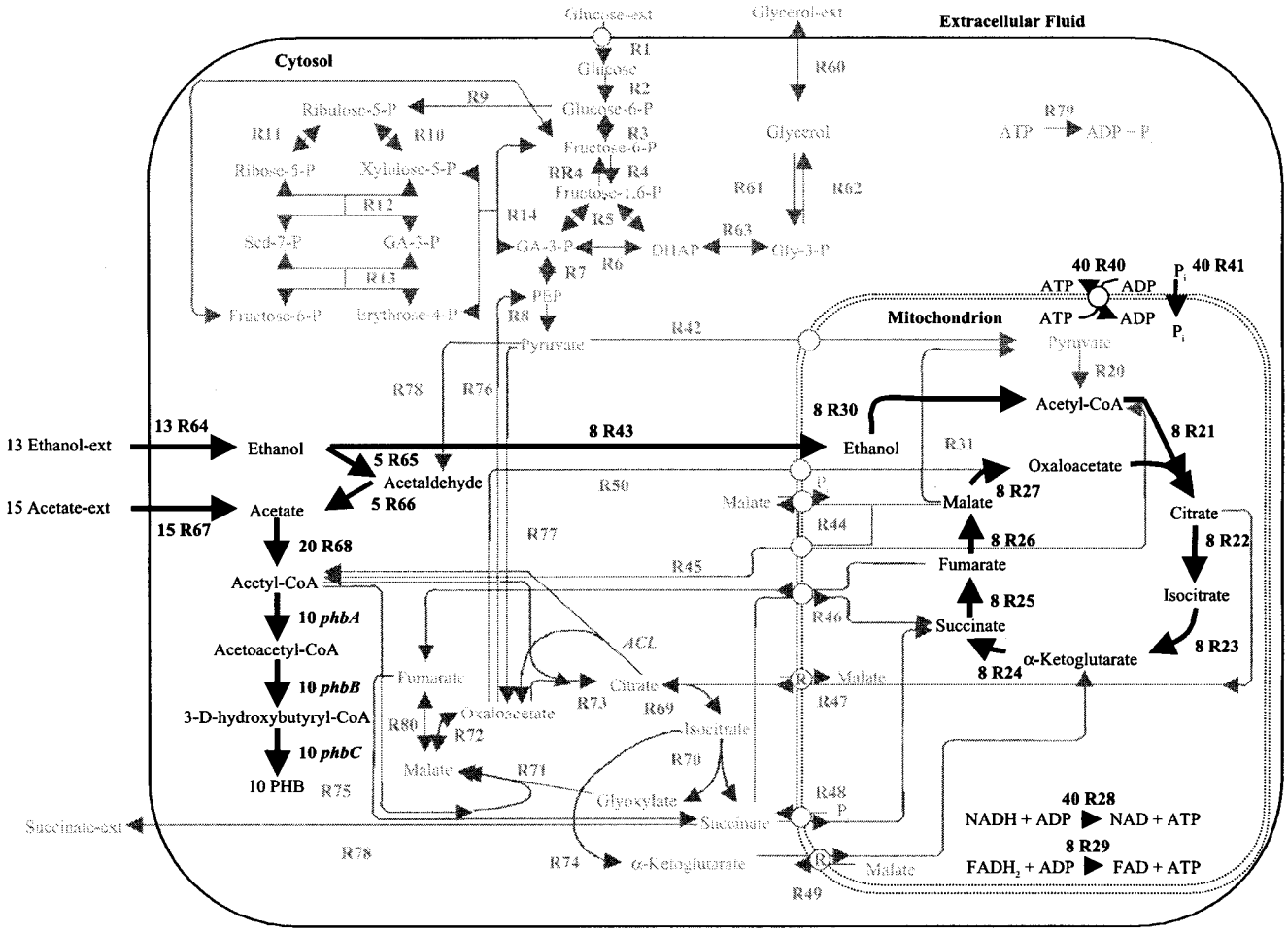

Figure 5. Heavy lines indicate the elementary mode with highest PHB carbon yield for the basic experimental network model with transhydrogenase activity. The number preceding the reaction number indicates relative flux through that particular reaction. The analysis uses acetate, ethanol, glycerol, PHB, ATP, CO_2, and succinate as external metabolites. The highest yielding mode has an overall stoichiometry of 15 acetate + 13 ethanol = 16 CO_2 + 10 PHB, and has a PHB carbon yield of 0.71.

and anaerobic conditions were investigated to determine the feasibility of alternative culturing conditions. For economic reasons, particularly with regard to equipment design and operational costs, it is of interest to produce PHB anaerobically. Anaerobic conditions were simulated by removing the oxidative phosphorylation reactions (reactions R28 and R29) from the model. Under anaerobic conditions, a fermentable carbon source is necessary so the glucose uptake reaction (reaction R1) was included.

Elementary mode analysis reveals eight anaerobic elementary modes with two modes capable of producing PHB. Interestingly, both of these anaerobic modes coproduce PHB and ethanol (see Table III). The highest yielding mode catabolizes glucose into ATP and NADPH using glycolysis and the pentose phosphate cycle and produces ethanol and CO_2 as byproducts. The ATP and NADPH derived from glucose permits 100% conversion of acetate carbon into PHB synthesis (see Fig. 7). The overall carbon yield for PHB is 0.24 and the carbon yield for ethanol is 0.48 for a combined carbon yield of 0.72. The analysis did not include the transhydrogenase reaction or the ACL reaction. Including these reactions did not have a major effect on PHB production (data not shown).

Yield Trends

Elementary mode analysis was also used to examine trends in the yields of various compounds. Stoichiometric constraints related to the redox and carbon balances can influence which combinations of reactions are available for the synthesis of a desired product. The available combinations of reactions can then have an influence on what metabolites are produced as byproducts.

The carbon yield of PHB versus the carbon yield of ethanol and the carbon yield of PHB versus the carbon yield of glycerol are shown in Figure 8. The results are from a model using glucose, acetate, ethanol, glycerol, PHB, ATP, CO_2, and succinate as external metabolites. The system did not include the ACL reaction or the transhydrogenase reaction.

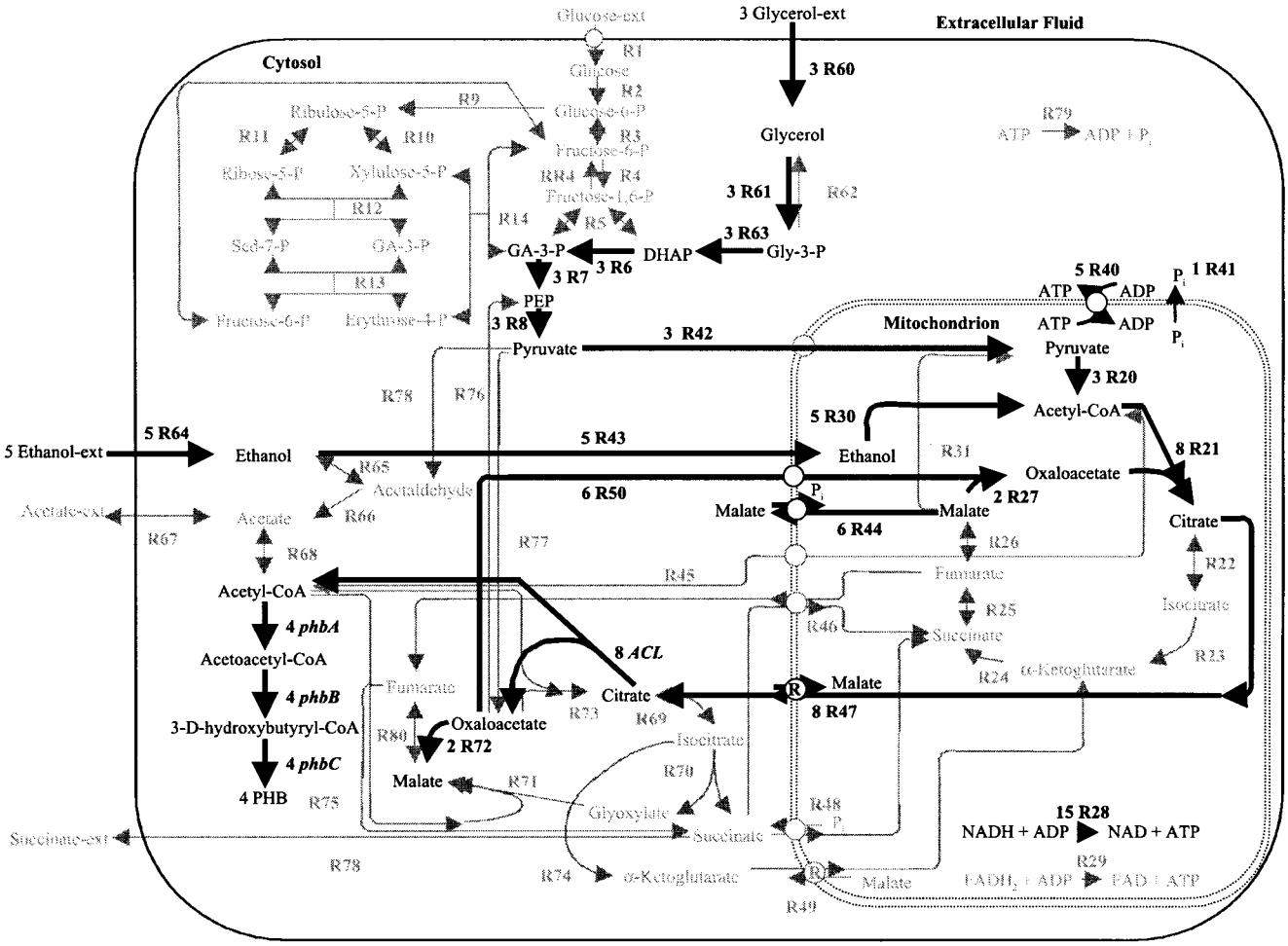

Figure 6. Heavy lines indicate the elementary mode with highest PHB carbon yield for the basic experimental network model with ATP citrate-lyase (ACL) activity and transhydrogenase activity. The number preceding the reaction number indicates relative flux through that particular reaction. The analysis includes acetate, ethanol, glycerol, PHB, ATP, CO_2, and succinate as external metabolites. The highest yielding mode has an overall mode stoichiometry of 3 glycerol + 5 ethanol = 3 CO_2 + 4 PHB, and has a PHB carbon yield of 0.84.

Figure 8A and B shows an overall mass conservation trend. As the yield of one metabolite increases, the other metabolite's yield decreases. Figure 8B also shows an interesting relationship between PHB production and glycerol production. A large number of modes producing PHB also produce glycerol, which is illustrated by the mode population above the *x*-axis. This trend is not seen in the Figure 8A, wherein the majority of the modes producing PHB are on the *x*-axis, indicating they did not coproduce ethanol. This trend is in part due to redox constraints. While PHB consumes electrons in the form of NADPH, additional cytosolic reducing equivalents are often formed during substrate catabolism. A number of the modes then use glycerol as an electron sink to dispose of the excess reducing equivalents.

DISCUSSION

Understanding the basic structure of the biochemical network and the system's working boundaries are important first steps in characterizing an organism. Pathway analysis tools such as elementary mode analysis are useful for deconstructing complex networks into their basic functioning units. These units can then serve as simplified focal points for further study and possible modification. We have used elementary mode analysis to study a recombinant *S. cerevisiae* system engineered to produce the biodegradable plastic PHB. The analysis reveals what possibilities and what limitations the system's reaction stoichiometry, compartmentalization, and thermodynamics place on the system. For instance, the results suggest that, under aerobic conditions, the system requires both ethanol and acetate to produce PHB (Table I). Knowing what substrates are required for PHB synthesis and knowing what combination of reactions gives the best yields, it is possible to develop strategies that encourage metabolite flux toward certain desired modes. In addition to feeding appropriate substrates, the existing network could be manipulated to partition the metabolites at critical branch points toward a desired pathway. Techniques such as gene

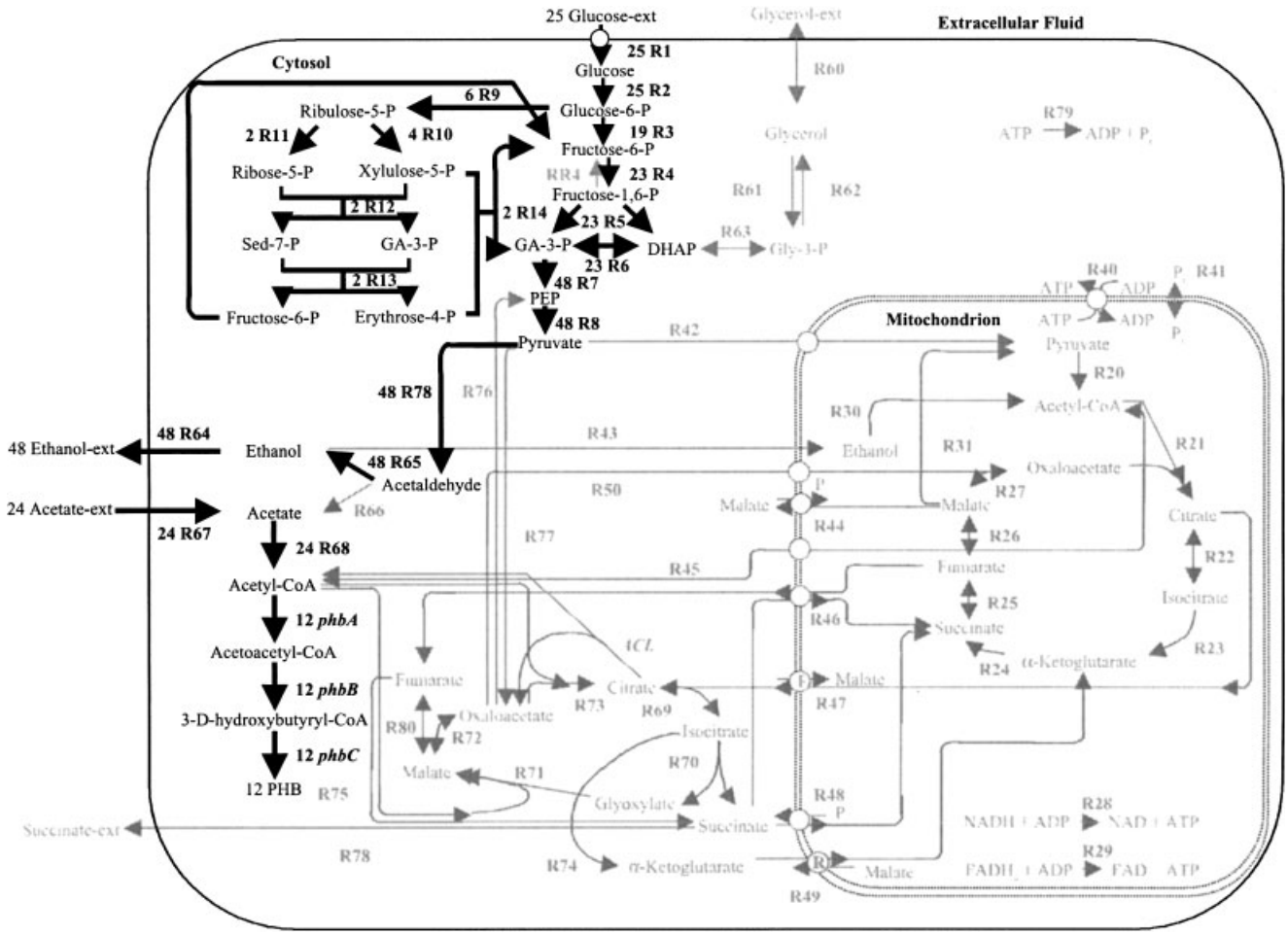

Figure 7. Heavy lines indicate the elementary mode with highest PHB carbon yield for the original experimental network model under anaerobic conditions. The number preceding the reaction number indicates relative flux through that particular reaction. The analysis includes glucose, acetate, ethanol, glycerol, PHB, ATP, CO_2, and succinate as external metabolites. The highest yielding mode has an overall mode stoichiometry of 25 glucose + 24 acetate = 12 PHB + 48 ethanol + 54 CO_2 and has a PHB carbon yield of 0.24 and an ethanol yield of 0.48.

overexpression, gene knock-outs, or metabolite channeling through the creation of fusion proteins could all be used to influence carbon flux to favor certain modes (Liao et al., 1996; Sumegi et al., 1993). For instance, one difference between the highest yielding mode and the second highest yielding mode for the aerobic experimental system is the alternative sources of NADPH (see Table I). If both modes were operating, it would be possible to knock-out the lower yielding mode by inhibiting the pentose phosphate pathway through the deletion of the gene D-ribulose-5-phosphate 3 epimerase (Miosga and Zimmerman, 1996). Without this enzymatic activity, the lower yielding mode could not operate. Culturing conditions could also be used to influence which modes are available to balance a cell's internal workings (de Jong-Gubbels et al., 1995). The presence or absence of oxygen has a strong effect on which modes are available. Switching culturing conditions could have an effect on which substrates are used and which products are formed. For instance, by switching from aerobic to anaerobic conditions, the analysis suggests that ethanol could switch from a substrate used to form PHB to a product coproduced with PHB (see Table III).

Elementary mode analysis is also well suited for exploring the possible effects of topological changes such as the addition of new catalytic activities. Adding ATP citrate-lyase to the model significantly increased the number of possible pathways leading to PHB. Not only did this reaction permit the more efficient conversion of substrate into product but it also changed the distribution of non–PHB-forming modes to PHB-forming modes. With the addition of ACL, 63% of all possible modes produced PHB, whereas only 35% of the original experimental systems modes led to PHB production. The addition of the ACL also changes the requirement of ethanol and acetate for the production of PHB. The highest yielding mode with ACL used only a single substrate, ethanol, to produce PHB, whereas the second highest yielding mode used a combination ethanol and glycerol.

Elementary mode analysis of the recombinant *S. cerevisiae* system has provided insight into the basic

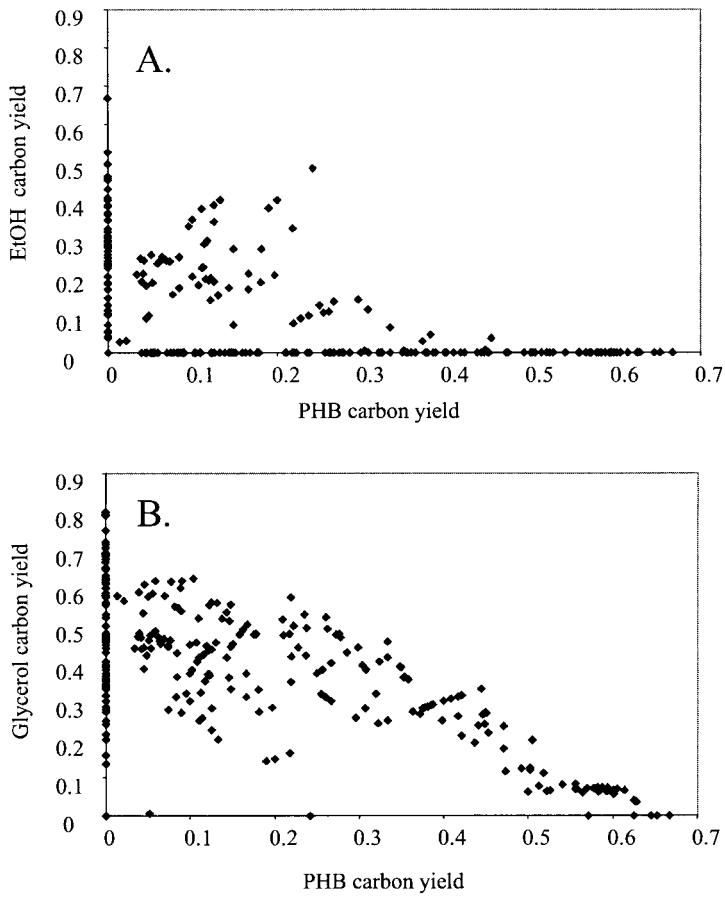

Figure 8. Yield trends for elementary modes found during analysis of the recombinant *S. cerevisiae* biochemical reaction model. The external metabolites include glucose, acetate, ethanol, glycerol, PHB, ATP, CO_2, and succinate. For each mode, the carbon yield for PHB is plotted against the carbon yield for ethanol (A) or the carbon yield for PHB is plotted against the carbon yield for glycerol (B).

biochemical structure of this fungus and into the production of PHB in this host. The predictions about the effect of additional modifications will serve as the basis for continued research and future strain development with this system. In addition, this type of analysis could be invaluable for studying and sorting the large volumes of data being generated by advances in the areas of genomics research. Due to the shear magnitude of the accumulated data and the complexity of the branched networks, the value of this information is best appreciated when it can be simplified into understandable units.

The predicted feasibility of anaerobic PHB synthesis in *S. cerevisiae* has recently been confirmed experimentally (manuscript in preparation). The results support the validity of the metabolic network model.

APPENDIX

Listed in what follows is the METATOOL elementary mode analysis program input file. The file includes the reversible reactions and the irreversible reactions. These reactions are followed by the internal metabolites, the external metabolites, and the actual biochemical reactions. References relevant to the model are listed after the METATOOL input file.

-ENZREV
R3r R5r R6r R7r R10r R11r R12r R13r R14r R22r R25r R26r R27r R41r R43r R45r R60r R63r R64r R67r R68r R69r R72r R80r phbA phbB

-ENZIRREV
R1 R2 R4 RR4 R8 R9 R20 R21 R23 R24 R28 R29 R30 R31 R40 R42 R44 R46 R47 R48 R49 R50 R61 R62 R65 R66 R70 R71 R73 R74 R75 R76 R77 R78 R79 R81 phbC ACL

-METINT
GLU_cyt ATP_cyt ADP_cyt P_cyt GLU_6_P FRU_6_P FRU_BIS_P DHAP GA_3P NAD_cyt NADH_cyt NADPH NADP RIBULOSE_5_P XYL_5_P RIBOSE_5_P SED_7_P ERYTH_4_P PYR_cyt MALATE_cyt
CITRATE_cyt OXALO_cyt CoASH_cyt ACETYL_CoA_cyt GLYCEROL_P PEP AKG_cyt ISOCIT_cyt

PYR_mit CITRATE_mit OXALO_mit MALATE_mit CoASH_mit ACETYL_CoA_mit ATP_mit ADP_mit P_mit
NADH_mit NAD_mit FADH_mit FAD_mit AKG_mit ISOCIT_mit bhbcoa acaccoa ETOH_cyt
ACEADH_cyt ACETATE_cyt GLYCEROL_cyt SUCC_mit GLYOX_cyt ETOH_mit
FUMARATE_mit SUCC_cyt FUMARATE_cyt NADP_mit NADPH_mit FAD_cyt FADH_cyt

-METEXT
ATP_base phb ACETATE_ext CO2 SUCC_ext ETOH_ext GLYCEROL_ext GLU_ext

-CAT

glycolysis:
R1: GLU_ext = GLU_cyt.
R2: GLU_cyt + ATP_cyt = GLU_6_P + ADP_cyt.
R3r: GLU_6_P = FRU_6_P.
R4: FRU_6_P + ATP_cyt = FRU_BIS_P + ADP_cyt.
RR4: FRU_BIS_P = FRU_6_P + P_cyt.
R5r: FRU_BIS_P = DHAP + GA_3P.
R6r: GA_3P = DHAP.
R7r: GA_3P + ADP_cyt + P_cyt + NAD_cyt = PEP + ATP_cyt + NADH_cyt.
R8: PEP + ADP_cyt = PYR_cyt + ATP_cyt.

pentose phosphate pathway:
R9: GLU_6_P + 2 NADP = RIBULOSE_5_P + 2 NADPH + CO2.
R10r: RIBULOSE_5_P = XYL_5_P.
R11r: RIBULOSE_5_P = RIBOSE_5_P.
R12r: RIBOSE_5_P + XYL_5_P = SED_7_P + GA_3P.
R13r: GA_3P + SED_7_P = ERYTH_4_P + FRU_6_P.
R14r: ERYTH_4_P + XYL_5_P = GA_3P + FRU_6_P.

mitochondrial reactions:
R20: PYR_mit + CoASH_mit + NAD_mit = ACETYL_CoA_mit + NADH_mit + CO2.
R21: OXALO_mit + ACETYL_CoA_mit = CITRATE_mit + CoASH_mit.
R22r: CITRATE_mit = ISOCIT_mit.
R23: ISOCIT_mit + NAD_mit = AKG_mit + NADH_mit + CO2.
R24: AKG_mit + NAD_mit + ADP_mit + P_mit = NADH_mit + ATP_mit + SUCC_mit + CO2.
R25r: SUCC_mit + FAD_mit = FUMARATE_mit + FADH_mit.
R26r: FUMARATE_mit = MALATE_mit.
R27r: MALATE_mit + NAD_mit = OXALO_mit + NADH_mit.
R28: NADH_mit + ADP_mit + P_mit = NAD_mit + ATP_mit.
R29: FADH_mit + ADP_mit + P_mit = FAD_mit + ATP_mit.
R30: ETOH_mit + CoASH_mit + 2 ATP_mit + 2 NAD_mit = ACETYL_CoA_mit + 2 ADP_mit + 2 P_mit + 2 NADH_mit.
R31: MALATE_mit + NADP_mit = PYR_mit + NADPH_mit + CO2.

mitochondrial transporters:
R40: ADP_cyt + ATP_mit = ADP_mit + ATP_cyt.
R41r: P_cyt = P_mit.
R42: PYR_cyt = PYR_mit.
R43r: ETOH_cyt = ETOH_mit.
R44: MALATE_mit + P_cyt = MALATE_cyt + P_mit.
R45r: ACETYL_CoA_mit = ACETYL_CoA_cyt .
R46: FUMARATE_mit + SUCC_cyt = FUMARATE_cyt + SUCC_mit.
R47: CITRATE_mit + MALATE_cyt = CITRATE_cyt + MALATE_mit.
R48: SUCC_cyt + P_mit = SUCC_mit + P_cyt.
R49: AKG_cyt + MALATE_mit = AKG_mit + MALATE_cyt.
R50: OXALO_cyt = OXALO_mit.

cytosolic reactions:
R60r: GLYCEROL_cyt = GLYCEROL_ext.
R61: GLYCEROL_cyt + ATP_cyt = GLYCEROL_P + ADP_cyt.
R62: GLYCEROL_P = GLYCEROL_cyt + P_cyt.
R63r: DHAP + NADH_cyt = GLYCEROL_P + NAD_cyt.
R64r: ETOH_cyt = ETOH_ext.
R65: ACEADH_cyt + NADH_cyt = ETOH_cyt + NAD_cyt.
R66: ACEADH_cyt + NADP = ACETATE_cyt + NADPH.
R67r: ACETATE_cyt = ACETATE_ext.
R68r: ACETATE_cyt + CoASH_cyt + 2 ATP_cyt = ACETYL_CoA_cyt + 2 ADP_cyt + 2 P_cyt.
R69r: CITRATE_cyt = ISOCIT_cyt.
R70: ISOCIT_cyt = GLYOX_cyt + SUCC_cyt.
R71: GLYOX_cyt + ACETYL_CoA_cyt = MALATE_cyt + CoASH_cyt.
R72r: OXALO_cyt + NADH_cyt = MALATE_cyt + NAD_cyt.
R73: OXALO_cyt + ACETYL_CoA_cyt = CITRATE_cyt + CoASH_cyt.
R74: ISOCIT_cyt + NADP = AKG_cyt + NADPH + CO2.
R75: FUMARATE_cyt + FADH_cyt = SUCC_cyt + FAD_cyt.
R76: OXALO_cyt + ATP_cyt = PEP + ADP_cyt + CO2.
R77: PYR_cyt + ATP_cyt + CO2 = ADP_cyt + P_cyt + OXALO_cyt.
R78: PYR_cyt = ACEADH_cyt + CO2.

R79: ATP_cyt + ATP_base = ADP_cyt + P_cyt.
R80r: MALATE_cyt = FUMARATE_cyt.
R81: SUCC_cyt = SUCC_ext.

PHB pathway:
phbA: 2 ACETYL_CoA_cyt = acaccoa + CoASH_cyt.
phbB: acaccoa + NADPH = bhbcoa + NADP.
phbC: bhbcoa = phb + CoASH_cyt.

ATP citrate lyase:
ACL: CITRATE_cyt + ATP_cyt + CoASH_cyt = ACETYL_CoA_cyt + OXALO_cyt + ADP_cyt + P_cyt.

References for *S. cerevisiae* elementary mode model are as follows:

General pathway information: Gancedo and Serrano (1989)

Acetyl-CoA synthetase: Fernandez et al. (1992); van den Berg et al. (1996)

ADP/ATP carrier: Klingenberg (1993)

ATP citrate-lyase: Boulton and Ratledge (1981); Evans et al. (1983)

Carnitine acetyltransferase: Kohlaw and Tan-Wilson (1977)

Cytosolic and mitochondrial aldehyde dehydrogenases: Wang et al. (1998); Nissen et al. (1997); von Jagow and Klingenberg (1970); Tamaki and Hama (1982); Jacobson and Bernofsky (1974)

Succinate dehydrogenase/fumarase: Muratsubaki and Enomoto (1998); Muratsubaki (1987)

General dicarboxylate carriers: Lancar-Benba et al. (1996); Perkins et al. (1973); Pederson (1993)

Isocitrate lyase/malate synthase: Lopez-Boado et al. (1988); Fernandez et al. (1992); Taylor et al. (1996); Palmieri et al. (1997); Duntze et al. (1969)

Malic enzyme: Boles et al. (1998)

Oxaloactetate transporter: Palmieri et al. (1999a)

Stoichiometry of oxidative phosphorylation: Vanrolleghem et al. (1996)

Succinate-fumarate carrier: Palmieri et al. (1999b, 1997)

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