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Addition of Autotrophic Carbon Fixation Pathways to Increase the Theoretical Heterotrophic Yield of Acetate

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Abstract The production of valuable biological products from sugars via fermentation is of importance in the chemical industry. One key parameter by which processes are evaluated is the product carbon yield on substrate. In the normal growth of heterotrophic organisms on fermentable sugars, some carbon is lost as CO_2. In the production of acetate from hexose or pentose sugars, homoacetogens using the reductive acetyl CoA pathway have a theoretical yield of acetate on glucose of 3. We hypothesized that the addition of enzymes necessary for fixation of the lost CO_2 would enhance the theoretical carbon yield of acetate in the dark in organisms without the reductive acetyl CoA pathway. Using computational flux balances of central metabolism of *Escherichia coli*, we show that the addition of the enzymes for the reductive pentose phosphate (Calvin Benson Bassham) cycle increases the theoretical yield of acetate from glucose or xylose. An even more significant increase in the theoretical carbon yield of acetate was obtained by adding the reactions for the reductive tricarboxylic acid cycle (rTCA), matching that of homoacetogens. However, addition of these pathways did not result in an increase in the theoretical yield of other excreted metabolites or biomass.

Keywords flux balance analysis, reductive citric acid cycle, optimization, acetic acid, Calvin cycle

1 Introduction

Industrial biotechnology is a growing area with an increasing number of chemical companies interested in producing valuable products from renewable resources [12]. The improved production of biochemicals from organisms through rational genetic engineering is an active area of research, commonly referred to as metabolic engineering [24]. From this knowledge, targets for metabolic engineering are identified and tested. The problem of selecting an optimal enzymatic route between substrates and products has been the interest of several research groups [20,23,26]. One of the key criteria used to select pathways is the theoretical metabolic yield, defined as the number of moles of product formed per mole of substrate utilized.

Numerous metabolic pathways exist for the oxidative catabolism of substrates, e.g. the oxidative pentose phosphate pathway and oxidative citric acid cycle. These pathways each contain reactions that lead to the loss of CO_2. Conversely, a set of

pathways exist in the reductive direction in autotrophs [11], namely, the reductive pentose phosphate cycle (rPP and also designated as the Calvin-Benson-Bassham cycle) and the reductive tricarboxylic acid cycle (rTCA) [1]. In this work, we hypothesize that by adding carbon fixation pathways to a heterotrophic organism some of the carbon lost as CO_2 in oxidative pathways could be recovered, leading to increased theoretical yields of fermentation products.

We selected the production of acetate as a test case that has the theoretical yield limited by efficiency of carbon utilization. Acetate is an important industrial bulk chemical that is currently obtained from petroleum [30]. Currently, several routes for the biological production of acetate exist. A two stage process in which yeast ferments sugars to ethanol that is subsequently oxidized by *Acetobacter* strain is used for vinegar production (3). Single stage aerobic production of acetate by *Dekkera* or *Brettanomyces* yeasts [10] has been reported. Recently, Ingram et al. successfully demonstrated the metabolic engineering of *E. coli* strains for the sole production of acetate from glucose (5). In these cases, the theoretical yield of acetate on hexose is 2.0. However, homoacetogens such as *Clostridia* strains and even in cyanobacteria have a theoretical yield of 3 as they possess the reductive Acetyl CoA pathway to refix the lost CO_2 [6,16].

For the study of theoretical yields of products on specific substrates, manual computations are sufficient for short and relatively linear pathways. However, computer models are useful for cases that involve complex pathways such as biomass formation [13]. Generally, the models for generation of routes between substrates and products do not involve regulatory information but are based upon stoichiometric balancing [4, 9]. We selected this type of modeling to test the hypothesis that the addition of enzymes such as those found in photoautotrophs would enable the (re)fixation of carbon that is normally lost as CO_2. Even though the focus of the study is on carbon yield, the flux balance analysis incorporates all of the reactions of primary metabolism leading to biomass formation and therefore cellular growth and maintenance can be considered while explicitly balancing cofactors.

2 Materials and Methods

2.1 Construction of the metabolic pathways for the in silico organism

We constructed a metabolic network to analyze the production of a specific compound given a substrate based only on the stoichiometry of enzyme reactions. The enzymes in this constructed network can be input from different organisms. In this study, we selected *E. coli* as the host organism and thus used the stoichiometric central metabolic reaction network [27,28]. The stoichiometric model was also supplemented with anapleurotic reactions and a reaction to represent fumarate reductase which catalyzes the synthesis of succinate from fumarate [8].

Additions to *E. coli* central metabolism model

Anaplerotic reactions

$PEP + CO_2 + ADP <-> OA + ATP$ phosphoenolpyruvate

carboxykinase

PEP + CO_2 --> OA	phosphoenolpyruvate carboxylase
NADP + MAL <-> NADPH + CO_2 + PYR	malic enzyme
NAD + MAL --> NADH + CO_2 + PYR	malic enzyme
ICT --> GOX + SUC	isocitrate lyase
CoA + MAL <-> AcCoA + GOX	malate synthase

TCA reaction

Fumarate + QH2 → succinate + Q	fumarate reductase

In addition, we included enzymes necessary to complete the reductive citric acid cycle and the reductive pentose phosphate cycle. Specifically, the following enzymes were added to the model: phospho-ribulokinase and ribulose-1,5-biphosphate carboxylase for the Calvin cycle, and α-ketoglutarate synthase and ATP citrate lyase for the rTCA, respectively.

Based on the enzyme information in the database, stoichiometric relationships among the substrate, intermediates, and products were constructed resulting in a set of linear equations when applying the steady state assumption. The flux balance analysis (FBA) technique [29] uses this set of linear equations to predict the theoretical yield of a product. Besides the stoichiometric relationships from the enzyme database, information about the requirements for growth and the measurement of a few strain-specific parameters are input as constraints [17]. If an objective such as maximizing the growth yield or maximizing the yield of a metabolite is specified, the problem is solved as a linear program.

2.2 Prediction of theoretical yields using FBA

In typical flux balance analysis, the objective is usually maximizing the flux of biomass production [29]. This is perceived to be an evolutionary objective for an organism. However, there can be other rational choices for the objective, for example, maximizing the flux to a certain metabolite or ATP. Since the quantity of interest in this work is the theoretical yield of some product *P*, the objective function is set to be the maximization of the flux v_p, constrained by the flux v_s of a certain substrate *S*. The theoretical yield is thus represented by $|v_p/v_s|$. For convenience in formulating the problem, we assume the substrate flux v_s = -1 mole (the negative sign indicates the consumption of the metabolite). The complete formulation is as follows:

$$
\begin{aligned}
&\max v_p \\
&\text{subject to} \\
&\sum_j s_{ij} v_j = 0 \quad \forall i \in M_i \\
&\sum_j s_{ij} v_j \le 0 \quad \forall i \in M_r \\
&\sum_j s_{ij} v_j \ge 0 \quad \forall i \in M_p \\
&\sum_j s_{s,j} v_s = -1 \\
&v_j \ge 0
\end{aligned}
\tag{1}
$$

where s_{ij} is the stoichiometric coefficient of the i^{th} metabolite in the j^{th} reaction, v_j is the flux of the j^{th} reaction, M_i is the set of internal metabolites, M_r is the set of reactants other than the substrate, and M_p is the set of products. The matrix $\mathbf{S} = \{s_{ij}\}$ represents the reaction network structure. Eq. (1) is a linear program, which was solved by the CPLEX software, which is an optimization product of ILOG (http://www.ilog.com).

The framework described in the previous section predicts the theoretical yield. To estimate the effect of cellular maintenance and growth on theoretical yields, two constraints are added into the original formulation.

$$v_{ATP} \ge v_{ATP,\min} \tag{2}$$

$$v_{biom} \ge v_{biom,\min} \tag{3}$$

The maintenance cost, $v_{ATP,\min}$, was formulated in terms of a required ATP flux of 4 mole ATP/mole glucose for *E. coli* [29]. The $v_{biom,\min}$, which is prespecified arbitrarily, denotes the minimum yield of the biomass, which indicates the minimum requirement of the growth.

Because of the underdetermined nature of the FBA problem, more than one network topology could exist for a given maximum yield. We used CellNetAnalyzer software [16] to generate all the elementary modes containing substrate and acetate. From the elementary modes, we selected those that gave the maximal biomass yield.

3 Results

As a case study, we selected acetate to test the hypothesis that the addition of carbon fixation pathways could enhance the theoretical yield of metabolic products. The theoretical yield of acetate on glucose in *E. coli* is 2.0 (12). In the production of

acetate from 1 mol of glucose, two moles of pyruvate are made from glycolysis and decarboxylated to acetyl CoA, which is subsequently converted to acetate. This pathway is shown as the upper half of Figure 1a. The net reaction is

$$1 \text{ glucose} \rightarrow 2 \text{ acetate} + 2\ CO_2 \quad (4)$$

We used the metabolic model containing carbon fixing pathways to determine if the theoretical yield of acetate could be increased by adding the enzymes for the rTCA cycle. Indeed, the yield could be increased to 3.0 moles acetate/ mol glucose (Figure 1a). This indicated that there was enough excess reducing power to re-fix the CO_2 released in the decarboxylation of pyruvate. In another case, the presence of the only 2 additional enzymes to complete the reductive pentose phosphate (rPP) pathway, the yield of acetate was 2.91 mol/mol glucose (Figure 1b).

While the results are impressive, this theoretical yield is unobtainable because realistic conditions of growth and maintenance were not included in the analysis. Therefore, we repeated the problem of maximizing acetate yield, under the constraint of a maintenance flux of 4 mol ATP/ mol glucose. As expected, the theoretical yield of acetate decreased with the additional constraint. The theoretical yield was 2.56 for the CBB pathway and 2.94 mol acetate/ mol glucose for the reductive TCA (rTCA) cycle. Further, we analyzed the yield of acetate as a function of the growth rate of *E. coli*, by specifying realistic growth rates in the flux balance model, and then optimizing for acetate production. The theoretical yield of acetate with the reductive TCA cycle always remained greater than the original theoretical yield of 2.0 which was computed without any growth or maintenance constraints.

The optimum solution from the FBA analysis is not-unique. Therefore, we enumerated all the solutions that produced the same theoretical yield using elementary modes analysis. There were a total of 8 pathways that resulted in the optimal yield of acetate. For all the enumerated pathways, the common feature was the presence of the 2 enzymes needed to complete the reductive TCA cycle. The other optimal solutions included variations in the anapleurotic pathways.

We also examined whether the addition of the rTCA or rPP cycle would enhance the synthesis of biomass or other excreted compounds. No increase in the theoretical biomass yield was calculated under conditions of no maintenance. Similarly for other fermentation products, e.g. succinate, ethanol, lactate there was no increase in theoretical yields above wild-type *E. coli*. Furthermore, no increase in theoretical yields of biomass or succinate was calculated with a maintenance flux included in the model. To synthesize these products requires additional reducing equivalents (ferredoxin or NAD(P)H) for the reduction of acetyl CoA and CO_2 to pyruvate. From the FBA model, we determined that after the refixation of lost CO_2 in the rTCA cycle, no additional reducing equivalents are available, which explains why the increase in theoretical yield is limited to acetate.

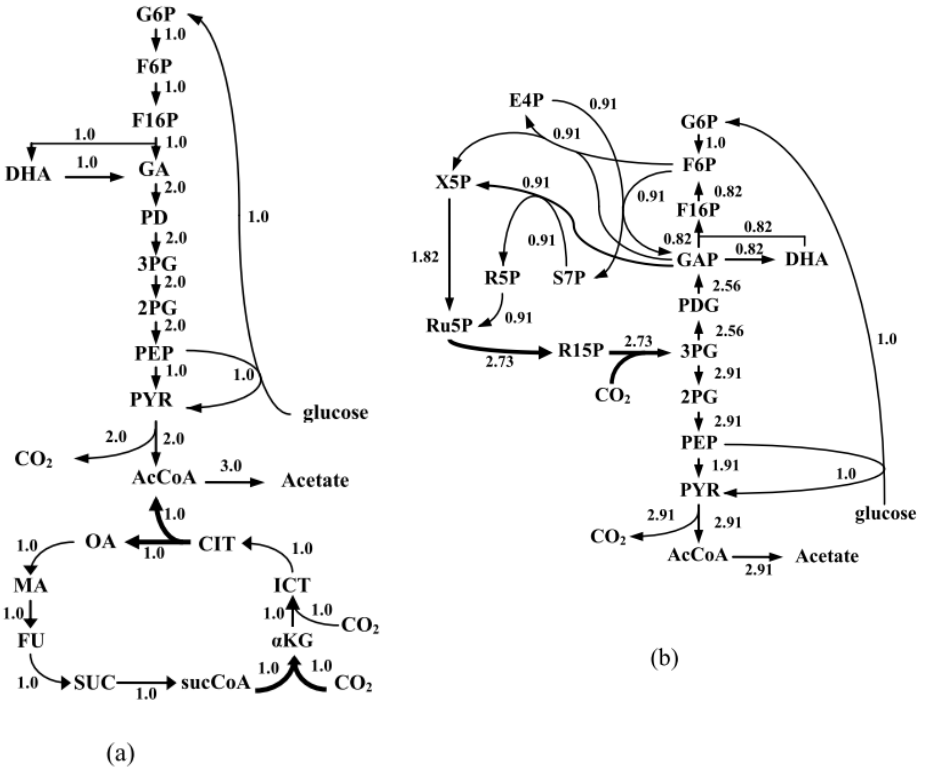

Figure 1. Central metabolic pathways to synthesize acetate: (a) wild type *E. coli*; *E. coli* modified with reductive TCA enzymes; (b) *E. coli* with additional reductive pentose phosphate pathway enzymes. The additional enzymes required to complete the carbon fixation cycles are depicted in bold.

The yield of acetate from xylose was investigated because of its presence in nutrient broths derived from renewable resources such as cellulose. Without maintenance and growth the theoretical yield of acetate from wild type *E. coli* is 1.67. In the computationally modified strains, the theoretical yield of acetate was 2.38 and 2.50 mol/mol xylose from the rPP cycle and rTCA cycle, respectively. Similar to the yield of acetate on glucose, the theoretical yield of acetate on xylose is 50% greater with the addition of the rTCA cycle.

4 Discussion

The hypothesis that adding genes encoding carbon dioxide fixing pathways from an autotrophic organism to heterotrophic organisms increases the theoretical carbon yield of acetate was confirmed. The testing of the *in silico* model, indicated that the pathways are feasible as the entire central metabolic pathway necessary for growth was included in the analysis. Either the addition of the enzymes necessary to

complete the rPP pathway or for the reductive TCA cycle enhanced yield of acetate. No synergy was found when both pathways were present simultaneously. In an optimization with both pathways present, the reductive TCA cycle was selected as the more efficient pathway, which agrees with calculations of the per mole cost of CO_2 fixation in autotrophic organisms [11].

In this work, *E. coli* was selected as a host since it has been successfully used for several other metabolic products and secretes significant amounts of acetate. Additionally, *E. coli* also has the advantages of significant growth on minimal medium and utilization of both hexose and pentose sugars, which are important in producing a value added product. However, the strategy of adding carbon fixing pathways to heterotrophic organism to increase theoretical yields of metabolic products should be applicable to a broad range of hosts.

Obviously, the implementation of such a metabolic engineering strategy may not work for several reasons. As indicated earlier, the stoichiometric modeling approach does not take into account enzyme kinetics or regulation of enzyme concentrations. For example, the kinetics of enzymes responsible for carbon fixation such as Rubisco are relatively slow and have been implicated in limiting the rate of carbon fixation [22]. Furthermore, the enzymes necessary to achieve the proper fluxes may not be expressed at the correct ratios. Other factors such as thermodynamic and allosteric regulation could prevent the reductive cycles from operating in the necessary direction or at significant enough rates to sustain growth or acetate production. The loss of CO_2 through diffusion would have to be restricted. A gas stream enriched in CO_2 such as combustion flue gas could be sparged reducing the driving force for CO_2 loss. An additional criterion for success is the host organism must be tolerant of the product. For example, Ingram and coworkers reported engineering an *E. coli* strain for homoacetate production and indicated that at 85% of theoretical limit of 2 mol acetate/ mol glucose the tolerance level was likely being approached [5]. However, others have demonstrated the ability to evolve an organism to become more tolerant of an organic acid by genome shuffling [19]. Alternatively, a process design could be implemented to extract the produced acetate [7,15,18]. The fact that the process is anaerobic is economically beneficial because the lack of required oxygen significantly reduces the energy requirements for compression of air or oxygen.

Interestingly, we discovered that the addition of CO_2 fixation pathways never increased the biomass yield. Apparently, the carbon dioxide could be reduced to the acetyl CoA level, but there are no more reducing equivalents available for the conversion of this central precursor into biomass or reduced metabolites. The work is being extended to computationally examine the effect of adding other autotrophic pathways. For example, several additional carbon dioxide fixing pathways are reported to exist in Nature; the 3-hydroxypropionate malyl-CoA cycle [26], the 3-hydroxypropionate/4-hydroxybutyrate cycle [2], and the dicarboxylate/4 -hydroxybutyrate cycle [14]. Alternatively to this strategy outlined in this manuscript, one could engineer the pathway for acetate production into an autotrophic organism.

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References

[1] Atomi H: Microbial enzymes involved in carbon dioxide fixation. *J Biosci Bioeng* 2002, **94**(6):497-505.

[2] Berg IA, Kockelkorn D, Buckel W, Fuchs G,A 3-hydroxypropionate/4-hydroxybutyrate autotrophic carbon dioxide assimilation pathway in archaea. *Science* 2007, **318**(5857):1782-1786.

[3] Berraud C Production of highly concentrated vinegar in fed-batch culture. *Biotech Lett* 2000, **22**(6):451-454.

[4] Burgard AP, Maranas CD Optimization-based Framework for Inferring and Testing Hypothesized Metabolic Objective Functions. *Biotech Bioeng* 2003, **82**:670-677.

[5] Causey TB, Zhou S, Shanmugam KT, Ingram LO: Engineering the metabolism of *Escherichia coli* W3110 for the conversion of sugar to redox-neutral and oxidized products: Homoacetate production. *PNAS* 2003, **100**(3):825-832.

[6] Diekert, G. and Wohlfarth, G. (1994) Metabolism of homoacetogens **66**: 209-221.

[7] Dionysiou DD, Tsianou M, Botsaris GD: Investigation of the conditions for the production of calcium magnesium acetate (CMA) road deicer in an extractive crystallization process. *Crystal Res Technol* 2000, **35**(9):1035-1049.

[8] Edwards JS, Palsson BO: Robustness Analysis of the Escherichia coli Metabolic Network. *Biotechnol Prog* 2000, **16**:927-939.

[9] Edwards JS, Covert M, Palsson BO: Metabolic modelling of microbes: the flux-balance approach. *Environ Microbiol* 2002, **4**(3):133-140.

[10] Freer SN, Dien B, Matsuda S: Production of acetic acid by *Dekkera/Brettanomyces* yeasts under conditions of constant pH. *World J Microbiol Biotechnol* 2003, **19**(1):101-105.

[11] Fuchs G, Stupperich E: Evolution of autotrophic CO_2 fixation. In: *Evolution of prokaryotes.* Edited by Schleifer K, Stackebrandt E. London: Academic Press; 1985: 235-251.

[12] Herrera S: Industrial biotechnology-a chance at redemption. *Nat Biotechnol* 2004, **22**(6):671-675.

[13] Hong S, Moon S, Lee S: Prediction of maximum yields of metabolites and optimal pathways for their production by metabolic flux analysis. *J Microbiol Biotechnol* 2003, **13**(4):571-577.

[14] Huber H, Gallenberger M, Jahn U, Eylert E, Berg IA, Kockelkorn D, Eisenreich W, Fuchs G: A dicarboxylate/4-hydroxybutyrate autotrophic carbon assimilation cycle in the hyperthermophilic Archaeum *Ignicoccus hospitalis*. *PNAS USA* 2008, **105**(22):7851-7856.

[15] Katikaneni SPR, Cheryan M: Purification of fermentation-derived acetic acid by liquid-liquid extraction and esterification. *Ind Eng Chem Res* 2002, **41**(11):2745-2752.

[16] Klamt S, Saez-Rodriguez J, Gilles ED: Structural and functional analysis of cellular networks with CellNetAnalyzer. *BMC Syst Biol* 2007, **1**:2.

[17] Oberhardt MA, Palsson BO, Papin JA: Applications of genome-scale metabolic reconstructions. *Mol Syst Biol* 2009, **5**:320.

[18] Ozadali F, Glatz BA, Glatz CE: Fed batch fermentation with and without on-line extraction for propionic and acetic acid production by *Propionibacterium acidipropionici*. *Applied Microbiology and Biotechnology* 1996, **44**(6):710-716

[19] Patnaik R, Louie S, Gavrilovic V, Perry K, Stemmer WPC, Ryan CM, del Cardayre S: Genome shuffling of *Lactobacillus* for improved acid tolerance. *Nature Biotechnology* 2002, **20**(7):707-712.

[20] Pharkya P, Burgard AP, Maranas CD: OptStrain: A computational framework for redesign of microbial production systems. *Genome Res* 2004, **14**(11):2367-2376.

[21] Ragsdale SW: Enzymology of the Acetyl-CoA Pathway of CO_2 Fixation. *Crit Rev Biochem Mol* 1991, **26**(3-4):261-300.
[22] Raines CA: The Calvin cycle revisited. *Photosynth Res* 2003, **75**(1):1-10
[23] Schilling CH, Edwards JS, Letscher D, Palsson BO: Combining Pathway Analysis with Flux Balance Analysis for the Comprehensive Study of Metabolic Systems. *Biotech Bioeng* 2001, **71**(4):286-306.
[24] Stephanopoulos G: Metabolic fluxes and metabolic engineering. *Metabol Eng* 1999, **1**:1-11.
[25] Thauer RK: Microbiology - A fifth pathway of carbon fixation. *Science* 2007, **318**(5857):1732-1733.
[26] Trinh CT, Carlson R, Wlaschin A, Srienc F: Design, construction and performance of the most efficient biomass producing *E. coli* bacterium. *Metabol Eng* 2006, **8**(6):628-638.
[27] Varma A, Palsson BO: Metabolic Capabilities of *Escherichia coli*: I. Synthesis of Biosynthetic Precursors and Cofactors. *J Theor Biol* 1993, **165**:477-502.
[28] Varma A, Palsson BO: Metabolic Capabilities of *Escherichia coli*: II. Optimal Growth Patterns. *J Theor Biol* 1993, **165**:503-522
[29] Varma A, Palsson BO: Stoichiometric Flux Balance Models Quantitatively Predict Growth and Metabolic By-Product Secretion in Wild-Type Escherichia coli W3110. Appl Environl Microbiol 1994, 60(10):3724-3731.
[30] Wilke D: Chemicals from biotechnology: molecular plant genetics will challenge the chemical and the fermentation industry. Appl Microbiol Biotechnol 1999, 52(2):135-145.