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Development of a metabolic network design and optimization framework incorporating implementation constraints: A succinate production case study

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Abstract

We have developed a pathway design and optimization scheme that accommodates genetically and/or environmentally derived operational constraints. We express the full set of theoretically optimal pathways in terms of the underlying elementary flux modes and then examine the sensitivity of the optimal yield to a wide class of physiological perturbations. Though the scheme is general it is best appreciated in a concrete context: we here take succinate production as our model system. The scheme produces novel pathway designs and leads to the construction of optimal succinate production pathway networks. The model predictions compare very favorably with experimental observations.
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1. Introduction

The processes of concern to the metabolic engineer typically involve relatively few metabolites engaged in relatively many reactions. The balance of fluxes therefore leads to significantly underdetermined systems. These extra degrees of freedom then provide organisms and engineers the opportunity to engineer optimal pathways (see Bonarius et al., 1997 for an early review of the application of the methodology of linear programming to such design questions). It was soon recognized that the optimization problem itself may have many solutions (Lee et al., 2000) and that this additional freedom would permit one to investigate notions of robustness (Edwards and Palsson, 2000) as well as genetic constraints (Burgard et al., 2003).

However, most studies often concentrate on optimization aspects and frequently neglect the implementation constraints imposed by the network. In particular, very few studies have addressed the issue of operating conditions. For example, a process designed for aerobic or anaerobic conditions will cause many of the underlying genes to undergo aerobic or anaerobic activation and repression, which will subsequently affect the associated enzyme activities. As such, operating conditions must be taken into account in addition to a traditional pathway optimization approach.

The current study is designed to bridge the gap between modeling/theoretical studies and that of experimental implementation. A metabolic network design and optimization framework that will take experimental results and implementation constraints into consideration is developed. The approach will be illustrated with a moderate

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sized system, that of succinate production. This target network is ideal since it is small enough such that it can be implemented and evaluated experimentally and yet large enough to contain the salient features of a large network.

2. Background

The valuable specialty chemical succinate and its derivatives have extensive industrial applications. They can be used as additives and flavoring agents in foods, pharmaceutical supplements, surfactants, detergent extenders, foaming agents, and ion chelators (Zeikus et al., 1999). Examples of succinate derivatives that are commodity chemicals include 1,4-butanediol, tetrahydrofuran, and γ-butyrolactone. These chemicals are applied in areas such as polymers, solvents, and additives (Zeikus et al., 1999). Currently, succinate is produced through petrochemical processes that can be expensive and can lead to pollution problems. Much effort has shifted toward making biocatalysts a viable and improved alternative for the production of succinate. The success of microbial fermentation coupled with the use of renewable carbohydrates would significantly improve the economics of the succinate market (Schilling, 1995).

3. Synthesis of succinic acid (native system)

Succinate is a metabolite that is formed under both anaerobic and aerobic conditions. During anaerobic conditions, *Escharichia coli* undergoes mixed-acid fermentation that yields acetate, ethanol, formate, and lactate as its major fermentation products with only a small amount of succinate formed. Succinate is formed under anaerobic conditions through a carboxylation reaction and a series of reductive reactions (Fig. 1A). In the first enzymatic step, the precursor phosphoenolpyruvate (PEP) is converted to oxaloacetate (OAA) through fixation of CO_2. This reaction is catalyzed by phosphoenolypyruvate carboxylase (PEPC). OAA is then reduced to malate, and eventually converted to succinate. During this process, 2 mol of NADH are oxidized to 2 mol of NAD^+. Therefore, for every mole of succinate formed from PEP, 1 mol of CO_2 and 2 mol of NADH are required. Since only 2 mol of NADH will be formed per mole of glucose converted to PEP or pyruvate, the maximum yield for succinate is one (mole of succinate formed/mole of glucose consumed) under purely anaerobic conditions.

Under the aerobic condition, succinate is only an intermediate of the tricarboxylic acid (TCA) cycle or the glyoxalate shunt metabolism (Van der Werf et al., 1997) (Fig. 1B). It is eventually oxidized to supply electrons for oxidative phosphorylation (ATP generation) and to regenerate OAA. Succinate is, therefore, normally not produced as a byproduct under aerobic conditions in *E. coli*. PEPC is also active under aerobic condition to help replenish OAA. However, succinate, under aerobic conditions, is usually formed at a minimal level from glucose with a yield of 0.1 mol succinate per mole glucose in wild type cell fermentations (Bock and Sawers, 1996).

4. Synthesis of succinic acid (genetically engineered systems)

Metabolic engineering studies in *E. coli* have increased succinate levels through introduction of a malic enzyme to convert pyruvate to the succinate precursor, malate (Stols and Donnelly, 1997; Stols et al., 1997). This reversal of the normal direction of action of the malic enzyme in combination with a host, which could not metabolize pyruvate by the pyruvate:formate lyase (PFL) or lactate dehydrogenase (LDH) pathways allowed succinate to be produced in moderate yield; however, the low final level achieved limited economic appeal (Stols and Donnelly, 1997; Stols et al., 1997). The *pfl* and *ldh* mutations required in the host strain for this route typically lead to slow growth and low cell density.

Several groups have reported *E. coli* genetically engineered to increase succinate production; optimization of culture parameters for the strain has also been examined (Vermuri et al., 2002a, b), and theoretical analysis of yields has been performed. General models of metabolism have included succinate (Papin et al., 2002). Recent work from the Argonne group has used a *pfl ldhA ptsG* mutant to increase succinate yields, therefore, producing a strain that yielded 1 mol of succinate, 0.5 mol of acetate, and 0.5 mol of ethanol per mole of glucose (Chatterjee et al., 2001). Studies have employed cloned PEPC (Millard et al., 1996), malic enzyme (Stols et al., 1997; Stols and Donnelly, 1997), or pyruvate carboxylase (Gokarn et al., 1998, 2000, 2001; Vemuri et al., 2002b) to improve yields. Some attention to other routes has appeared. Conversion of fumarate to succinate was improved by overexpressing native fumarate reductase (*frd*) in *E. coli* (Goldberg et al., 1983; Wang et al., 1998). The function of the glyoxylate pathway in succinate formation under various redox conditions was considered (Vemuri et al., 2002a). Mutant hosts where conversion of pyruvate to lactate or formate has been reduced showed improved yields (Hong and Lee, 2001) and metabolic flux analysis was extended to try to account for intracellular concentrations of pyruvate and succinate.

5. Implementation constraints and concerns

As mentioned before, most modeling studies often concentrate on the optimization aspects and frequently neglect the implementation constraints imposed by the network. In particular, very few studies have addressed the issue of operating conditions. In particular, we will focus on two potential implementation constraints shown below as examples:

(1) *Operating conditions*: The operating condition may lead to gene activation or repression, which will undoubtedly alter the metabolic network significantly. For example, some of the genes shown in Fig. 1 will

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