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Optimization of enzyme parameters for fermentative production of biorenewable fuels and chemicals

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Abstract: Microbial biocatalysts such as *Escherichia coli* and *Saccharomyces cerevisiae* have been extensively subjected to Metabolic Engineering for the fermentative production of biorenewable fuels and chemicals. This often entails the introduction of new enzymes, deletion of unwanted enzymes and efforts to fine-tune enzyme abundance in order to attain the desired strain performance. Enzyme performance can be quantitatively described in terms of the Michaelis-Menten type parameters K_m, turnover number k_{cat} and K_i, which roughly describe the affinity of an enzyme for its substrate, the speed of a reaction and the enzyme sensitivity to inhibition by regulatory molecules. Here we describe examples of where knowledge of these parameters have been used to select, evolve or engineer enzymes for the desired performance and enabled increased production of biorenewable fuels and chemicals. Examples include production of ethanol, isobutanol, 1-butanol and tyrosine and furfural tolerance. The Michaelis-Menten parameters can also be used to judge the cofactor dependence of enzymes and quantify their preference for NADH or NADPH. Similarly, enzymes can be selected, evolved or engineered for the preferred cofactor preference. Examples of exporter engineering and selection are also discussed in the context of production of malate, valine and limonene.

Mini Review Article

Introduction

In the time since *Escherichia coli* was first engineered to produce ethanol as its major fermentation product [1] and the coining of the term "metabolic engineering" in that same year [2,3], a variety of microbes have been engineered for the production of a wide range of products. These products include, but are not limited to, fuels [4], chemicals [5] and neutraceuticals [6]. Here we focus on the use of microbial biocatalysts to produce biorenewable fuels and chemicals.

Metabolic Engineering is defined as "the directed improvement of production, formation or cellular properties through the modification of specific biochemical reactions or the introduction of new ones with the use of recombinant DNA technology" [7]. Straightforward expression of a new pathway is often sufficient for production of the desired compound. However, an economically viable process requires that the target compound be formed at high titer (concentration), yield and rate, where the target values for these parameters can obviously vary according to the value of the product. Deletion of competing pathways and increasing expression of the target pathway are standard tools for increasing titer, yield and rate [8]. A variety of tools exist for increasing gene and enzyme abundance including the use of inducible promoters [9-12], engineering or evolution of the promoter and ribosome binding region [13], mutation of transcriptional regulators [14], transcript stabilization [15], optimization of translation initiation [16], codon optimization [17,18] and others [8,19,20].

However, pathway function is determined by more than just the expression level of the constituent enzymes. The affinity of an enzyme for substrate(s) and/or cofactor(s), catalytic efficiency, cofactor requirements and allosteric regulation, as well as substrate uptake and product export, are all important drivers of flux through the desired pathway. Here we describe key examples where knowledge and manipulation of these parameters have enabled increased process performance in terms of the production of biorenewable fuels and chemicals. Note that it is often difficult to determine *a priori* which enzyme is limiting biocatalyst performance. There are several recent examples of methods for identifying problematic, or "bottleneck" enzymes [21-25]; this topic is not addressed in this review.

Overview of Michaelis-Menten Parameters

The enzymatic conversion of substrate S to product P by enzyme E can be represented by the following simplified two-step reaction schematic (rxn 1)

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} (E\text{-}S) \xrightarrow{k_{cat}} E + P$$

In this model, formation of the enzyme-substrate complex (E-S) is reversible, but formation of product P is irreversible. This schematic is represented mathematically by the Michaelis-Menten equation

$$v = \frac{v_{max} c_s}{K_m + c_s} \qquad \text{Eq 1}$$

where

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$$K_m = \frac{k_{-1}+k_{cat}}{k_1} \quad \text{Eq 2}$$

$$c_{ET} = c_E + c_{E\text{-}S} \quad \text{Eq 3}$$

$$v_{max} = k_{cat}c_{ET} \quad \text{Eq 4}$$

In this manner, v reflects the overall velocity (rate) of a given reaction as a function of substrate concentration c_s, concentration of active enzyme c_{ET}, Michaelis constant K_m, and turnover number k_{cat}. Note that c_E and $c_{E\text{-}S}$ represent the concentration of enzyme in the unbound and substrate-bound states, respectively. This formulation was first described in 1913 and has recently been translated into English and revisited with some interesting insights [26].

v_{max} and K_m are the two most-commonly quantified values for a particular enzyme-substrate pair, as they can be determined by measuring reaction rate v over a range of substrate concentrations. When the substrate concentration becomes saturating, the reaction velocity will approach v_{max}. K_m is the substrate concentration at which the reaction velocity is one half of v_{max}. Thus, K_m reflects the affinity of an enzyme for its substrate, with a lower value indicating a stronger affinity. k_{cat}, also known as the turnover number, represents the speed at which a particular enzyme can convert substrate to product; higher values represent a faster-acting enzyme. The theoretical upper limit of k_{cat} is generally considered to be in the range of $10^6 - 10^7$ s^{-1} [27]. The ratio of k_{cat}/K_m is often referred to as the 'specificity constant' and used to compare the activity of a particular enzyme with multiple substrates; the theoretical upper limit of k_{cat}/K_m is estimated as 10^8-10^9 $M^{-1}s^{-1}$ [27]. This ratio is also said to reflect an enzyme's catalytic efficiency, though there are concerns about the validity of this term [28]. A recent compilation and analysis of data for more than 1,800 enzymes reported that median values for k_{cat}, K_m and k_{cat}/K_m are 13.7 s^{-1}, 130 μM and 125,000 $M^{-1}s^{-1}$, respectively [27].

Impact of Michaelis-Menten parameters on biocatalyst performance

K_m values are especially important at metabolic nodes, where multiple enzymes compete for one substrate. When engineering *E. coli* for homoethanol production, Ohta *et al* [1] introduced pyruvate decarboxylase (PDC, $K_m^{pyruvate}$ = 0.4 mM) into an existing pyruvate node, where other enzymes (pyruvate formate lyase, $K_m^{pyruvate}$ = 2.0 mM; lactate dehydrogenase, $K_m^{pyruvate}$ = 7.2 mM) were already competing for pyruvate. However, PDC had the lowest $K_m^{pyruvate}$ and was able to effectively out-compete the other enzymes, enabling production of ethanol at 95% of the theoretical yield without deletion of the competing enzymes [1,29].

Metabolic cofactors, such as ATP and NAD(P)H can be considered among the most highly-connected metabolic nodes. In these cases, enzymes with a high affinity (low K_m) for these valuable metabolites can be problematic for a well-performing strain if these enzymes are not involved in product formation. For example, *E. coli*'s YqhD is an NADPH-dependent promiscuous aldehyde reductase that normally functions to reduce the toxic aldehydes that are produced by lipid peroxidation [30]. It has a K_m^{NADPH} of 0.8 μM [29,31]. However, in the presence of exogenous aldehydes, such as the furfural that can be relatively abundant in hydrolyzed biomass, YqhD-mediated furfural reduction results in depletion of the NADPH pool [31,32]. This depletion is so extreme that there is insufficient NADPH for sulfite reductase (K_m^{NADPH} = 80μM) to produce the hydrogen sulfide required for production of cysteine [31,32]. This depletion of cysteine results in a lack of growth and therefore a lack of product formation. Elimination of this NADPH depletion via silencing or removal of *yqhD* results in increased furfural tolerance, both in terms of biocatalyst growth and product formation [31,32].

A high K_m value can be problematic when it results in incomplete substrate utilization. A demonstration of this problem is the levoglucosan kinase (LGK) enzyme. Levoglucosan is an anhydrosugar produced during biomass pyrolysis that can be utilized with the same ATP and redox demand as glucose [33]. However, LGK has a relatively high $K_m^{levoglucosan}$ of 75 mM [34]. The problem incurred by this K_m value is reflected by the fact that a substantial amount of levoglucosan is left unutilized, resulting in a loss in product formation [33]. This problem could potentially be alleviated by modifying the enzyme to have a lower K_m; examples of this type of modification are described below.

Improving K_m, k_{cat} and k_{cat}/K_m to improve strain performance

As highlighted above, the use of enzymes with appropriate Michaelis-Menten parameters can enhance the performance of a microbial biocatalyst. The question becomes how to obtain enzymes with the appropriate parameters. In some cases, there exist characterized isozymes for a given enzymatic reaction. However, in many cases it becomes necessary to generate variants of an enzyme in order to obtain the desired function. These variants can either be generated by evolution [39-42] or through rational design [43,44].

Chen *et al* [21] recently provided an excellent example of the how improving the Michaelis-Menten parameters of one enzyme can improve process performance. Having identified transaldolase (TAL), a component of the non-oxidative branch of the pentose phosphate pathway, as the enzyme limiting the utilization of pentose sugars by ethanol-producing *Pichia stipitis*, Chen *et al* set out to generate improved variants of this enzyme through directed evolution and screening. The most promising variant (Gln263Arg) had a two-fold decrease in K_m^{F6P} and 3-fold increase in k_{cat}^{F6P}, resulting in a 5-fold increase in the k_{cat}/K_m ratio (Table 1). When the fermentative performance of the strain expressing this improved enzyme was compared to the strain with the original TAL enzyme, an increase in both the xylose consumption rate and ethanol production rate were observed (Table 1).

As part of an engineered pathway for isobutanol production, the *Lactococcus lactis* alcohol dehydrogenase (AdhA) was demonstrated as effective for converting isobutyraldehyde to isobutanol, though the K_m value was higher than other existing enzyme alternatives [45]. Screening of nearly 4,000 random variants identified amino acid changes that were useful in lowering the K_m. Three of these changes were engineered into a final mutant termed REI [35]. REI showed a 10-fold decrease in K_m, 4-fold increase in k_{cat} and thus 40-fold increase in k_{cat}/K_m and enabled a nearly 2-fold increase in isobutanol titer (Table 1).

Cofactor requirements

The above example of YqhD-mediated drainage of NADPH highlights the importance of this valuable cofactor. Relative to the glycolysis-associated NADH, NADPH can be relatively scarce. Therefore pathway designs in which NADPH is required for production of the target compound can suffer from a lack of NADPH availability. One method for dealing with this problem is to use transhydrogenase enzymes to intercovert NADH and NADPH [32,35,46-49]. Another method is to exchange NADPH-dependent enzyme activity for NADH-dependent enzyme activity, either by