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.

Introduction

In the time since Escherichia coli was first engineered to produce ethanol as its major fermentation product [1] and the coinage 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 nutraceuticals [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)

$$E + S \stackrel{k_1}{\longrightarrow} (E-S) \stackrel{k_{cat}}{\longrightarrow} E + P \stackrel{k_{-1}}{\longrightarrow}$$

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

$$V = \frac{v_{max}c_s}{K_m + c_s} \quad \text{Eq} \ 1$$

where

$V$ is the rate of product formation

$v_{max}$ is the maximum rate

$K_m$ is the Michaelis constant

$c_s$ is the substrate concentration

$E$ is the enzyme

$S$ is the substrate

$P$ is the product

$K_{cat}$ is the turnover number

$K_i$ is the inhibitor constant

$k_1$ is the forward rate constant

$k_{-1}$ is the reverse rate constant

$K_m$ is the Michaelis constant

$c_s$ is the substrate concentration

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\[ K_m = \frac{k_1 + k_{cat}}{k_1} \quad \text{Eq 2} \]
\[ CET = c_e + c_{ES} \quad \text{Eq 3} \]
\[ v_{max} = k_{cat} \cdot CET \quad \text{Eq 4} \]

In this manner, \( v \) reflects the overall velocity (rate) of a given reaction as a function of substrate concentration \( c_e \), concentration of active enzyme \( c_{cat} \), Michaelis constant \( K_m \), and turnover number \( k_{cat} \).

Note that \( c_e \) and \( c_{ES} \) 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 \( 10^6 \) to \( 10^7 \) 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 \( 10^8 \) to \( 10^9 \) 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 \( \mu \)M 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, Obta et al. [1] introduced pyruvate decarboxylase (PDC, \( K_{\text{pred}} = 0.4 \) mM) into an existing pyruvate node, where other enzymes (pyruvate formate lyase, \( K_{\text{pred}} = 2.0 \) mM; lactate dehydrogenase, \( K_{\text{pred}} = 7.2 \) mM) were already competing for pyruvate. However, PDC had the lowest \( K_{\text{pred}} \) 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 \( K_{\text{NADPH}} \) of 0.8 \( \mu \)M [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 (\( K_{\text{NADPH}} = 80 \mu \)M) 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 \( K_m \) 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 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 \( K_{\text{F6P}} \) and 3-fold increase in \( k_{\text{cat,F6P}} \), resulting in a 5-fold increase in the \( k_{\text{cat}}/K_m \) 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 \( k_{\text{cat}} \) and thus 40-fold increase in \( k_{\text{cat}}/K_m \) 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 interconvert NADH and NADPH [32,35,46–49]. Another method is to exchange NADPH-dependent enzyme activity for NADH-dependent enzyme activity, either by
selecting an appropriate isozyme or by modifying the NADPH-dependent enzyme. This exchange of NADPH/NADH dependency was recently reviewed [30] and a few key examples are described here.

The reduction of furfural to the less-inhibitory furfuryl alcohol is performed by the NADPH-dependent aldehyde reductase YqhD in wild-type *E. coli* [31]. Deletion or silencing of *yqhD* increases tolerance of approximately 1.0 g/L of furfural by sparing NADPH for biosynthesis [31]. However, this results in a lack of detoxification of furfural to furfuryl alcohol. Wang *et al.* [37] addressed this problem by increasing expression of the NADPH-dependent furfural reductase FucO, enabling a 50% increase in furfural tolerance. Note that the *K_m* of FucO is 0.4 mM, enabling it to outcompete YqhD’s *K_m* of 9 mM [31,37] (Table 1), further highlighting the importance of using enzymes with appropriate *K_m* values.

Watanabe *et al.* [36] used an enzyme modification approach to switch the *P. stipitis* xylose reductase (PsXR) enzyme from a preference for NADPH to a preference for NADH (Table 1). This cofactor switching was motivated by the goal to maintain redox balance with the NADH-dependent xylitol dehydrogenase, the enzyme which is immediately downstream of PsXR in the conversion of xylose to ethanol. The original enzyme had a 10-fold higher *K_m* of NADH relative to *K_m* of NADPH, reflecting a 10-fold lower affinity for NADH, though the *k_cat* was about 30% lower than *k_cat* of NADPH. By contrast, the evolved enzyme had a 10-fold lower *K_m* of NADH relative to *K_m* of NADPH and a 25-fold lower *k_cat* of NADPH relative to *k_cat* of NADH. This combination of changes in *K_m* and *k_cat* means that the evolved enzyme has a 3-fold higher (*k_cat*/*K_m*) of NADH relative to (*k_cat*/*K_m*) of NADPH, relative to the original enzyme’s 20-fold higher (*k_cat*/*K_m*) of NADPH relative to (*k_cat*/*K_m*) of NADH. Simply put, the original enzyme’s preference for NADPH was evolved to a preference for NADH, where this preference is reflected in the *K_m*, *k_cat* and *k_cat*/*K_m* values. Use of this evolved PsXR enzyme in *S. cerevisiae* resulted in increased ethanol production from xylose and decreased formation of the side product xylitol (Table 1) [36].

Ketol-acid reductoisomerase (IIVC) in *E. coli* strain 1993 for anaerobic isobutanol production

Table 1. Improvement in fermentative performance by improving enzyme parameters.

| Enzyme | Substrate | *K_m* (µM) | *k_cat* (s⁻¹) | *k_cat*/*K_m* (M⁻¹ s⁻¹) | Performance | Source |
|--------|-----------|------------|--------------|------------------------|------------|-------|
| native transaldolase (TAL) in *P. stipitis* for ethanol (EtOH) production from xylose (xyl) | | | | | | |
| wild-type | F6P | 560 ± 50 | 9.5 ± 0.7 | 1.7 x 10⁸ | 1.45 ± 0.06 g xyl consumed/L/hr | 0.69 ± 0.05 g EtOH produced/L/hr | [21] |
| Q263R | | 320 ± 10 | 32 ± 1 | 9.8 x 10⁴ | 1.66 ± 0.04 g xyl consumed/L/hr | 0.86 ± 0.05 g EtOH produced/L/hr |
| *L. lactis* alcohol dehydrogenase (AdhA) in *E. coli* + IVC-6E6 for anaerobic isobutanol production | | | | | | |
| wild-type (his6) | | 11,700 | 30 | 2.8x10⁹ | produced 8 g/L isobutanol | [35] |
| RE1: Y50F, I212T, L264V | isobutyraldehyde | 1,700 | 140 | 8.2x10⁶ | produced 13.5 g/L isobutanol |
| *P. stipitis* xylose reductase (PsXR) in *S. cerevisiae* for EtOH production from 15 g/L xyl and 5 g/L glucose | | | | | | |
| wild-type | NADH | 30.5 ± 0.7 | 6.9 ± 0.1 | 2.3 x 10⁹ | produced 8 g/L EtOH and 5 g/L xylitol | [36] |
| R276H | NADPH | 2.5 ± 0.1 | 10.50 ± 0.02 | 4.2 x 10⁹ | | |
| | NADH | 17 ± 2 | 6.8 ± 0.3 | 4.0 x 10⁹ | produced 11 g/L EtOH and 2.5 g/L xylitol | |
| | NADPH | 1.7 ± 0.1 | 0.267±0.003 | 1.6 x 10⁹ | | |
| ketol-acid reductoisomerase (IIVC) in *E. coli* strain 1993 for anaerobic isobutanol production | | | | | | |
| wild-type (his6) | NADH | 1080 | 0.3 | 3.0 x10⁹ | produced 1 g/L isobutanol | [35] |
| R276H | NADPH | 40 | 3.6 | 8.7x10⁶ | produced 3 g/L isobutanol |
| 6E6: A71S, R76D, S78D, Q110V | NADH | 30 | 2.3 | 7.4x10⁶ | | |
| | NADPH | 650 | 0.2 | 4.0x10⁶ | | |
| FucO | furfural | 9,000 | n/a | n/a | deletion increases tolerance to furfural by sparing NADPH for biosynthesis | [31,32] |
| | NADH | n/a | n/a | n/a | | |
| | NADPH | 8 | n/a | n/a | | |
| | furfural | 400,200 | n/a | n/a | overexpression increases tolerance to furfural by reducing furfural to furfuryl alcohol without depleting NADPH | [37,38] |
| | NADH | 2.7 | n/a | n/a | | |
| | NADPH | n.d. | n.d. | n.d. | | |
Table 2. Examples of addressing enzyme inhibition

| Enzyme                                           | Inhibitor                  | $K_i$ (μM) | Performance                              | Source |
|-------------------------------------------------|----------------------------|------------|------------------------------------------|--------|
| homoserine dehydrogenase (ThrA) in E. coli CRS-BuOH 31 | threonine                | n/a        | produced 50 mg/L 1-propanol, 30 mg/L 1-butanol | [51]   |
| wild-type                                      | threonine                  | n/a        | produced >150 mg/L 1-propanol, 100 mg/L 1-butanol |        |
| ThrA<sup>a</sup>B6C                             | n/a                       |            |                                          |        |
| DHAP synthase (AroG) in E. coli K12 ΔtyrR        | L-phenylalanine            | n/a        | produced $6 \pm 1$ mg/L L-tyrosine        | [52,56]|
| wild-type                                      | L-phenylalanine            | n/a        | produced $71 \pm 9$ mg/L L-tyrosine       |        |
| D146N                                          | n/a                       |            |                                          |        |
| chorismate mutase / prephenate dehydrogenase (TyrA) in E. coli K12 ΔtyrR | L-tyrosine               | n/a        | produced $6 \pm 1$ mg/L L-tyrosine        | [52,57]|
| wild-type                                      | L-tyrosine                 | n/a        | produced $86 \pm 9$ mg/L L-tyrosine       |        |
| M531, A354V                                    | n/a                       |            |                                          |        |
| citrate synthase in ethanologenic E. coli KO11  | NADH                      | 2.8        | produced 28 g/L ethanol                   | [53,58]|
| E. coli gltA                                    | NADH                      | 1.00       | produced 7.3 mM ethanol                   | [54,55]|
| B. subtilis citZ                                | NADH                      | 10.0       | produced 125.8 mM ethanol                 |        |
| dihydrolipoamide dehydrogenase (LPD) subunit of PDH in E. coli W3110 | NADH                      |            |                                          |        |
| wild-type                                      | NADH                      |            |                                          |        |
| n/a--- not available                            |                           |            |                                          |        |

Addressing enzyme inhibition ($K_i$)

The Michaelis-Menten parameters described above all relate to an active enzyme, its affinity for the substrate and its speed in forming product. However, many enzymes have at least some degree of post-translational allosteric regulation which serves to fine-tune enzyme activity in response to the abundance of key metabolites. This activity control occurs in the form of both activation and inhibition; here we focus on examples of enzyme inhibition.

As with the Michaelis-Menten model of enzyme activity, there also exist quantitative models for enzyme inhibition. These describe both competitive and non-competitive inhibition. In standard cases of competitive inhibition the inhibitor ($I$) competes with the substrate for binding to the active site, resulting in the additional reaction (rxn 2)

$$E + I \rightleftharpoons (E-I)$$

to the simplified schematic described above. This reversible binding is described with the inhibition parameter $K_i$, which reflects the affinity of the enzyme for the inhibitor according to

$$K_i = \frac{c_i}{c_{i-I}}$$

and the overall velocity $v$ of the reaction is represented by the modified Michaelis-Menten equation

$$\frac{1}{v} = \frac{K_m}{v_{max}c_s} \left(1 + \frac{c_i}{K_i} \right) + \frac{1}{v_{max}}$$

Note that $c_i$ is the concentration of the inhibitor.

By contrast, in standard cases of non-competitive inhibition, the inhibitor binds to a site distinct from the active site and this binding induces a conformational change in the enzyme that decreases enzymatic activity. Thus, in addition to Rxn 2, it is possible for the inhibitor to bind to the E-S complex; this E-S-I complex can revert to E-I by dissociation of the substrate or possibly proceed to product formation, though at a much slower rate than the E-S complex in the absence of bound inhibitor. This non-competitive inhibition is modeled

$$\frac{1}{v} = \frac{K_m}{v_{max}c_s} \left(1 + \frac{c_i}{K_i} \right) + \frac{1}{v_{max}} \left(1 + \frac{c_i}{K_i} \right)$$

Eq 7

Competitive and non-competitive inhibition can be distinguished by the use of Lineweaver-Burk plots, which are not discussed here. The relevance of these equations to the current work is the fact that enzyme sensitivity to inhibition can be quantified by the parameter $K_i$, where a higher value indicates decreased sensitivity to inhibition.

This regulatory control of enzyme activity presumably serves to balance metabolic flux distribution and can be problematic when one desires to produce a single metabolic product at high concentration and yield, as this can conflict with the microbial need to balance production of biomass constituents. Thus, enzyme inhibition is a problem that often needs to be addressed in the fermentative production of biorenewable fuels and chemicals.

As with the other enzyme properties described above, the problem of enzyme inhibition can often be addressed by selecting from existing characterized isozymes. For example, Shen et al [51] observed relatively low metabolic flux through their engineered 1-butanol and 1-propanol pathways that was presumably due to inhibition of homoserine dehydrogenase (ThrA) by threonine, where threonine is an intermediate of the engineered pathway downstream of ThrA.

Replacement of the native E. coli ThrA with a feedback-resistant mutant (ThrA<sup>a</sup>) resulted in a more than 3-fold increase in the final titers of 1-butanol and 1-propanol (Table 2) [51]. Similarly, the use of feedback-resistant mutants of 3-deoxy-D-arabino-heptulosonate-7-phosphate (DHAP) synthase (AroG) and chorismate mutase/prephenate dehydrogenase (TyrA) each increased tyrosine production more than 10-fold when expressed individually (Table 2) and enabled even further increases in production when expressed simultaneously (data not shown) [52]. Note that AroG performs the first dedicated step of the tyrosine biosynthesis pathway and is inhibited by L-phenylalanine. TyrA performs the next-to-last step in tyrosine biosynthesis and is inhibited by tyrosine.
The YgaZH transporter is native to E. coli and has been shown to enable an approximately 50% increase in growth and ethanol production without dependence on NADH formation [62].

Many biorenewable compounds are not naturally produced by the microbial biocatalyst and thus there is an absence of effective export systems. Dunlop et al. [63] generated a library of 43 efflux pumps from 15 different microbes and selected from the mixture based on their ability to increase E. coli's tolerance of limonene, among other biofuels. Introduction of the most useful pump, YP_692684 from Alcanivorax borkumensis, enabled an approximately 50% increase in limonene titer when expressed in an E. coli strain engineered for limonene production.

These three examples highlight the use of native transporters, recombinant transporters and engineered/evolved transporters to increase production of biorenewable fuels and chemicals.

**Summary and Outlook**

Here we have highlighted recent examples of how improvement of enzyme parameters, as reflected in the Michaelis-Menten-type parameters $K_m$, $k_{cat}$ and $V_{max}$, can improve the fermentative performance of a microbial biocatalyst. Each of the examples that we have described represent improved biocatalyst performance in the context of production of biorenewable fuels and chemicals. While the Michaelis-Menten is a simplified model of enzyme kinetics [26, 65-68], these parameters provide a useful quantification of enzyme properties that can be enormously valuable to other researchers when selecting enzymes during pathway design. Databases such as BREnda [69] are a useful repository of this type of information. However, it is critical that researchers continue to quantify and report these parameters for engineered or evolved enzymes so that others can make informed choices and use these enzymes when appropriate.

There are some enzymes that are tantalizing targets for improvement in order to increase production of biorenewable fuels and chemicals, yet these enzymes remain remarkably intractable to such improvement. The most well-known example is photosynthesis pathway enzyme Rubisco, which has a low catalytic efficient and poor substrate specificity [27, 70]. A recent cross-species analysis of the evolutionary landscape for Rubisco has provided interesting insight into why it has proven so difficult to improve its function [27]. Thus, despite the fact that we have described many successful examples of improving strain performance by improving enzyme parameters, it should be noted that enzyme improvement is not always feasible. Note that others have managed to obtain (slightly?) improved Rubisco mutants [70, 71].

### Table 3. Transporter examples

| Transporter | Transporter Substrate | $K_m$ (µM) | Performance | Source |
|-------------|------------------------|------------|-------------|--------|
| wild-type   | valine                 | 0.1        | --          |        |
| +YgaZH      | valine                 | 0.1        | produced 4.34 ± 0.03 g/L valine | [62]   |
| limonene production by engineered derivative of E. coli/DH1 | limonene | 0.1 | produced 35 mg/L limonene | [63]   |
| wild-type   | limonene               | 0.1        | --          |        |
| +YP_692684  | limonene               | 0.1        | produced 55 mg/L limonene | [63]   |
| malic acid production by S. cerevisiaeCEN.PK PYC2 MDH3ASK1 | malate | 1.600 | produced 235 ± 25 mM malate | [60, 64] |
| wild-type   | malate                 | 0.1        | --          |        |
| +S. pombeMAE1 | malate               | 1.600     | produced 235 ± 25 mM malate | [60, 64] |

$n/a$ – not available

**Appropriate transporters for substrate uptake and product export**

Finally, effective pathway flux requires the presence of appropriate uptake systems for the desired substrate and effective means of excreting or sequestering the product compound.

Transporters that are discovered when importing for importers can also be useful as exporters. The *Schizosaccharomyces pombe* malate transporter Mae1 (SpMae1p) was first demonstrated as useful for malate uptake by *S. cerevisiae* [59], but was also able to support a 10-fold increase in the malate titer achieved by a malate-producing *S. cerevisiae* [60].

Product export becomes increasingly important when the target compound is inhibitory to the microbial biocatalyst. Here we discuss two examples of the selection of appropriate exporters in order to improve the microbial production of an inhibitory compound. Despite the fact that it is naturally produced by *E. coli* and is necessary for protein translation, the branched-chain amino acid valine has long been known to be toxic to *E. coli* [61]. Thus, Park et al.'s strain design for valine production included a means to mitigate intracellular valine accumulation via overexpression of the YgaZH transporter [62]. This strategy increased the valine titer by nearly 50% (Table 3). The YgaZH transporter is native to *E. coli* but was not previously recognized as a valine transporter; Park et al. identified it as a potential valine exporter due to its homology with the *brnFE* branched-chain amino acid exporter encoded by *Corynebacterium glutamicum* [62].

Biomass formation by ethanologenic *E. coli* KO11 was limited in defined growth media due to NADH-mediated inhibition of citrate synthase, resulting in limitation of the biomass precursor alpha-ketoglutarate and limitation of overall growth and therefore product formation [53]. Replacement of the native *E. coli* citrate synthase with an NADH-resistant isozyme from *Bacillus subtilis* resulted in a 50% increase in growth and ethanol production in the desired growth condition [53].

An alternative approach to replacing an inhibition-sensitive enzyme with an inhibition-resistant isozyme is to modify the original enzyme so that the inhibition sensitivity is reduced or eliminated. This approach was taken by Kim et al. [54, 55] in regards to pyruvate dehydrogenase (PDH). The PDH complex is normally subject to inhibition by NADH; presumably this serves to balance generation of NADH in glycolysis and the subsequent regeneration of NAD through fermentative pathways. The lack of PDH activity during fermentation, when NADH is abundant, has resulted in reliance on recombinant expression of the *Zymomonas mobilis* PET pathway for ethanol production by *E. coli* [29]. However, mutations within the dihydrolipoamide dehydrogenase (LPD) subunit of PDH reduced this feedback sensitivity approximately 10-fold, resulting in a 10-fold improvement of ethanol production without dependence on the *Z. mobilis* PET pathway (Table 2).

Enzyme optimization for biorenewables production
While this work demonstrates the impact that improved enzyme properties can have on biocatalysts, it is apparent from the literature that additional collaboration between protein engineers and metabolic engineers could result in further advances. For example, Campbell et al. [72] and Machielsen et al. [73] have both demonstrated the ability to switch the cofactor dependence of alcohol dehydrogenase enzymes through rational design. This ability to target specific amino acids could possibly reduce the time needed to acquire useful enzymes relative to enzyme evolution. Additionally, thorough characterization of the resulting mutants adds to our understanding of enzyme design rules and could support further advances in protein engineering. Collaboration between metabolic engineers and protein engineers could ensure that high-impact enzymes are selected for study and that the enzyme modification yields not just a useful enzyme, but also useful information that could further advance our protein engineering capabilities.

It is interesting to note that while improvement of enzyme parameters can improve strain performance, the magnitude of these improvements often differs (Table 1). For example, the Q263R mutation in the P. stipitis transaldolase resulted in a 5-fold increase in its $k_{cat}/K_m$ for F6P, but less than a 30% increase in xylose consumption and ethanol production [21]. Similarly, multiple mutations in the L. lactis alcohol dehydrogenase resulted in a 30-fold increase in its $k_{cat}/K_m$ for isobutryraldehyde, the final isobutanol titer was increased less than 2-fold [35]. This is presumably due to the fact that metabolic flux through a given pathway consists of a series of enzymatic reactions, with each enzyme have its own set of governing parameters. Improvement of the so-called “bottleneck” enzyme will only increase the flux to the limit allowed by the next bottleneck enzyme.

While it makes sense that the fold improvement in enzyme parameters will not result in the same fold improvement in strain performance, the impact that mitigation of enzyme inhibition can have on strain performance is particularly striking (Table 2). Work with three of the examples that we have described, DHAP synthase [52,56], chorismate mutase [52,57] and dihydrolipoamide dehydrogenase [54,55], resulted in a greater than 10-fold increase in product titer. While this work considers only a limited set of enzyme manipulations, it is tempting to conclude that, generally speaking, addressing enzyme inhibition should be a higher priority than improving $k_{cat}$, $K_m$ and $k_{cat}/K_m$.

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References

1. Ohira K, Beall DS, Mejia JP, Shanmugam KT, Ingram LO (1991) Genetic improvement of Escherichia coli for ethanol production - chromosomal integration of Zymomonas mobilis genes encoding pyruvate decarboxylase and alcohol dehydrogenase II. Applied and Environmental Microbiology 57: 893-900.
2. Bailey JE (1991) Toward a science of Metabolic Engineering. Science 252: 1668-1675.
3. Stephanopoulos G, Vallino JJ (1991) Network rigidity and metabolic engineering in metabolite overproduction. Science 252: 1675-1681.
4. Peralta-Yahya P, Zhang F, del Cardaye SB, Keasling JD (2012) Microbial engineering for the production of advanced biofuels. Nature 488: 320-328.
5. Jang YS, Kim B, Shin JH, Choi YJ, Choi S, et al. (2012) Bio-based Production of C2 - C6 Platform Chemicals. Biotechnology and Bioengineering 109: 2437 - 2459.
6. Ye VM, Bhatia SK (2012) Metabolic engineering for the production of clinically important molecules: Omega-3 fatty acids, artemisinin, and taxol. Biotechnology Journal 7: 20-33.
7. Stephanopoulos G, Aristidou A, Nielsen J (1998) Metabolic Engineering: Principles and Methodologies. San Diego, CA: Academic Press.
8. Jarboe LR, Zhang X, Wang X, Moore JC, Shanmugam KT, et al. (2010) Metabolic Engineering for Production of Biorenewable Fuels and Chemicals: Contributions of Synthetic Biology. Journal of Biomedicine and Biotechnology.
9. Alper H, Fischer C, Nevoigt E, Stephanopoulos G (2005) Tuning genetic control through promoter engineering. Proceedings of the National Academy of Sciences of the United States of America 102: 12678-12683.
10. De Mey M, Maertens J, Boogmans S, Soetaert WK, Vandamme EJ, et al. (2010) Promoter knock-in: a novel rational method for the fine tuning of genes. BMC Biotechnology 10.
11. De Mey M, Maertens J, Lequeux GJ, Soetaert WK, Vandamme EJ (2007) Construction and model-based analysis of a promoter library for E.coli: an indispensable tool for metabolic engineering. BMC Biotechnology 7.
12. McClure WR (2009) Application of promoter swapping techniques to control expression of chromosomal genes. Applied Microbiology and Biotechnology 84: 641-648.
13. Huang X, Zhang X-E, Zhou Y-F, Zhang Z-P, Cass AEG (2006) Directed evolution of the 5′-untranslated region of the phaA gene in Escherichia coli simultaneously yields a stronger promoter and a stronger Shine-Dalgarno sequence. Biotechnology Journal 1: 1275-1282.
14. Zhang F, Carothers JM, Keasling JD (2012) Design of a dynamic sensor-regulator system for production of chemicals and fuels derived from fatty acids. Nature Biotechnology 30: 354-U166.
15. Smolke CD, Carrier TA, Keasling JD (2000) Coordinated, differential expression of two genes through directed mRNA cleavage and stabilization by secondary structures. Applied and Environmental Microbiology 66: 5399-5405.
16. Kudla G, Murray AW, Tolleryd D, Plotkin JB (2009) Coding-Sequence Determinants of Gene Expression in Escherichia coli. Science 324: 255-258.
17. Fuglsang A (2003) Codon optimizer: a freeware tool for codon optimization. Protein Expression and Purification 31: 247-249.
18. Te'o VSJ, Ciufersky AE, Bergquist PL, Nevalainen KMH (2000) Codon optimization of xylanase gene xynB from the thermophilic bacterium Dictyoglomus thermophilum for expression in the
Enzyme optimization for biorenewables production

filamentous fungus *Trichoderma reesei*. FEMS Microbiology Letters 190; 13-19.

19. Shiue E, Prather KJ (2012) Synthetic biology devices as tools for metabolic engineering. Biochemical Engineering Journal 65: 82-89.

20. Seo SW, Kim SC, Jung GY (2012) Synthetic regulatory tools for microbial engineering. Biotechnology and Bioprocess Engineering 17: 1-7.

21. Chen S-H, Hwang D-R, Chen G-H, Hsu N-S, Wu Y-T, et al. (2012) Engineering Transaldolase in *Pichia stipitis* to Improve Bioethanol Production. ACS Chemical Biology 7: 481-486.

22. Cluis CP, Ekins A, Narcross L, Jiang H, Gold ND, et al. (2011) Identification of bottlenecks in *Esherichia coli* engineered for the production of CoQ(10). Metabolic Engineering 13: 733-744.

23. Kind S, Jeong WK, Schroeder H, Wittmann C (2010) Systems-wide metabolic pathway engineering in *Corynebacterium glutamicum* for bio-based production of diaminopentane. Metabolic Engineering 12: 341-351.

24. Lutke-Everslooh T, Stephanopoulos G (2008) Combinatorial pathway analysis for improved L-lysine production in *Esherichia coli*: Identification of enzymatic bottlenecks by systematic gene overexpression. Metabolic Engineering 10: 69-77.

25. Redding-Johanson AM, Barth TS, Chan R, Krupa R, Szmidt HL, et al. (2011) Targeted proteomics for metabolic pathway optimization: Application to terpene production. Metabolic Engineering 13: 194-203.

26. Michailis L, Menten ML, Johnson KA, Goody RS (2011) The original Michailis constant: translation of the 1913 Michailis-Menten paper. Biochemistry 50: 82648-8269.

27. Savir Y, Noor E, Milo R, Tzlusty T (2010) Cross-species analysis traces adaptation of Rubisco toward optimality in a low-dimensional landscape. Proceedings of the National Academy of Sciences of the United States of America 107: 3475-3480.

28. Esenthal R, Danson MJ, Hough DW (2007) Catalytic efficiency and k(cat)/K-M: a useful comparator? Trends in Biotechnology 25: 247-249.

29. Jarboe LR, Grabar TB, Yomano LP, Shanmugan KT, Ingram LO (2007) Development of ethanologenic bacteria. In: Olson L, editor. Biofuels. pp. 237-261.

30. Perez JM, Arenas FA, Pradenas GA, Sandoval JM, Vasquez CC (2008) *Esherichia coli* YqbdB exhibits aldehyde reductase activity and protects from the harmful effect of lipid peroxidation-derived aldehydes. Journal of Biological Chemistry 283: 7346-7353.

31. Miller EN, Jarboe LR, Yomano LP, York SW, Shanmugam KT, et al. (2009) Silencing of NADPH-Dependent Oxidoreductase Genes (yqhD and dkgA) in Fusfural-Resistant Ethanologenic *Esherichia coli*. Applied and Environmental Microbiology 75: 4315-4323.

32. Miller EN, Jarboe LR, Turner PC, Pharkya P, Yomano LP, et al. (2009) Fusfural Inhibits Growth by Limiting Sulfur Assimilation in Ethanologenic *Esherichia coli* Strain LY180. Applied and Environmental Microbiology 75: 6132-6141.

33. Layton DS, Ajjarapu A, Choi DW, Jarboe LR (2011) Engineering ethanologenic *Esherichia coli* for levoglucosan utilization. Bioresource Technology 102: 8318-8322.

34. Zhuang XL, Zhang HX (2002) Identification, characterization of levoglucosan kinase, and cloning and expression of levoglucosan kinase cdNA from *Apergillus niger* CBX-209 in *Esherichia coli*. Protein Expression and Purification 26: 71-81.

35. Bastian S, Liu X, Meyerowitz JT, Snow CD, Chen MMY, et al. (2011) Engineered ketol-acid reductoisomerase and alcohol dehydrogenase enable anaerobic 2-methylpropan-1-ol production at theoretical yield in *Esherichia coli*. Metabolic Engineering 13: 345-352.

36. Watanabe S, Abu Saleh A, Pack SP, Annaluru N, Kodaki T, et al. (2007) Ethanol production from xylose by recombinant *Saccharomyces cerevisiae* expressing protein-engineered NADH-prefering xylose reductase from *Pichia stipitis*. Microbiology-SGM 153: 3044-3054.

37. Wang X, Miller EN, Yomano LP, Zhang X, Shanmugam KT, et al. (2011) Increased Furfural Tolerance Due to Overexpression of NADH-Dependent Oxidoreductase FucO in *Esherichia coli* Strains Engineered for the Production of Ethanol and Lactate. Applied and Environmental Microbiology 77: 5132-5140.

38. Obradors N, Cabisco E, Aguilar J, Ros J (1998) Site-directed mutagenesis studies of the metal-binding center of the iron-dependent propanediol oxidoreductase from *Esherichia coli*. European Journal of Biochemistry 258: 207-213.

39. Blagodatski A, Katanaev VI (2011) Technologies of directed protein evolution in vivo. Cellular and Molecular Life Sciences 68: 1207-1214.

40. Carneiro M, Hartl DL (2010) Adaptive landscapes and protein evolution. Proceedings of the National Academy of Sciences of the United States of America 107: 1747-1751.

41. Soskine M, Tawfik DS (2010) Mutational effects and the evolution of new protein functions. Nature Reviews Genetics 11: 572-582.

42. Wang M, Si T, Zhao H (2012) Biocatalyst development by directed evolution. Biosource Technology 115: 117-125.

43. Boeticher D, Borsnscheuer UT (2010) Protein engineering of microbial enzymes. Current Opinion in Microbiology 13: 274-282.

44. Yang JS, Seo SW, Jang S, Jung GY, Kim S (2012) Rational Engineering of Enzyme Allosteric Regulation through Sequence Evolution Analysis. PLoS computational biology 8: e1002612.-e1002612.

45. Atsumi S, Wu T-Y, Eckl E-M, Hawkins SD, Bueltcr T, et al. (2010) Engineering the isobutanol biosynthetic pathway in *Esherichia coli* by comparison of three aldehyde reductase/alcohol dehydrogenase genes. Applied Microbiology and Biotechnology 85: 651-657.

46. Kabus A, Georgi T, Wendisch VF, Bott M (2007) Expression of the *Esherichia coli* pntAB genes encoding a membrane-bound transhydrogenase in *Corynebacterium glutamicum* improves L-lysine formation. Applied Microbiology and Biotechnology 75: 47-53.

47. Rathnasigc R, Raj SM, Lee Y, Catherine C, Ashoka S, et al. (2012) Production of 3-hydroxypropionic acid via malonyl-CoA pathway using recombinant *Esherichia coli* strains. Journal of Biotechnology 157: 633-640.

48. Sanchez AM, Andrews J, Hussein I, Bennett GN, San KY (2006) Effect of overexpression of a soluble pyridine nucleotide transhydrogenase (UdhA) on the production of poly(3-hydroxybutyrate) in *Esherichia coli*. Biotechnology Progress 22: 420-425.

49. Weckbecker A, Hummel W (2004) Improved synthesis of chiral alcohols with *Esherichia coli* cells co-expressing pyridine nucleotide transhydrogenase, NADP(+)-dependent alcohol dehydrogenase and NAD(+)-dependent formate dehydrogenase. Biotechnology Letters 26: 1739-1744.

50. Bastian S, Arnold FH (2012) Reversal of NAD(P)H Cofactor Dependence by Protein Engineering. In: Cheng Q, editor. Microbial Metabolic Engineering: Methods and Protocols. pp. 17-31.

51. Shen CR, Liao JC (2008) Microbial engineering of *Esherichia coli* for 1-butanol and 1-propanol production via the keto-acid pathways. Metabolic Engineering 10: 312-320.

52. Lutke-Everslooh T, Stephanopoulos G (2007) L-tyrosine production by deregulated strains of *Esherichia coli*. Applied Microbiology and Biotechnology 75: 103-110.
53. Underwood SA, Busko ML, Shanmugam KT, Ingram LO (2002) Flux through citrate synthase limits the growth of ethanologenic Enterobacter cloacae K011 during xylose fermentation. Applied and Environmental Microbiology 68: 1071-1081.

54. Kim Y, Ingram LO, Shanmugam KT (2007) Construction of an Enterobacter cloacae K-12 mutant for homoethanologenic fermentation of glucose or xylose without foreign genes. Applied and Environmental Microbiology 73: 1766-1771.

55. Kim Y, Ingram LO, Shanmugam KT (2008) Dihydrolipoamide dehydrogenase mutation alters the NADH sensitivity of pyruvate dehydrogenase complex of Enterobacter cloacae K-12. Journal of Bacteriology 190: 3851-3858.

56. Kikuchi Y, Tsujimoto K, Kurahashi O (1997) Mutational analysis of the feedback sites of phenylalanine-sensitive 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase of Enterobacter cloacae. Applied and Environmental Microbiology 63: 761-762.

57. Litke-Eversloh T, Stephanopoulos G (2005) Feedback inhibition of chorismate mutase/prephenate dehydrogenase (TyrA) of Enterobacter cloacae: Generation and characterization of tyrosine-insensitive mutants. Applied and Environmental Microbiology 71: 7224-7228.

58. Stokell DJ, Donald LJ, Maurus R, Nguyen NT, Sadler G, et al. (2003) Probing the roles of key residues in the unique regulatory NADH binding site of type II citrate synthase of Enterobacter cloacae. Journal of Biological Chemistry 278: 35435-35443.

59. Volschenk H, Viljoen M, Grobler J, Petzold B, Bauer F, et al. (1997) Engineering pathways for malate degradation in Saccharomyces cerevisiae. Nature Biotechnology 15: 253-257.

60. Zelle RM, de Hulster E, van Winden WA, de Waard P, Dijkema C, et al. (2008) Malic acid production by Saccharomyces cerevisiae. Engineering of pyruvate carboxylation, oxaloacetate reduction, and malate export. Applied and Environmental Microbiology 74: 2766-2777.

61. Manten A, Rowley D (1953) Genetic analysis of valine inhibition in the K12 strain of bacterium coli Journal of General Microbiology 9: 226-8.

62. Park JH, Lee KH, Kim TY, Lee SY (2007) Metabolic engineering of Enterobacter cloacae for the production of L-valine based on transcriptome analysis and in silico gene knockout simulation. Proceedings of the National Academy of Sciences of the United States of America 104: 7797-7802.

63. Dunlop MJ, Dossani ZY, Szmidt HL, Chu HC, Lee TS, et al. (2011) Engineering microbial biofuel tolerance and export using efflux pumps. Molecular Systems Biology 7.

64. Camarasa C, Bidard F, Bony M, Barre P, Dequin S (2001) Characterization of Schizosaccharomyces pombe malate permease by expression in Saccharomyces cerevisiae. Applied and Environmental Microbiology 67: 4144-4151.

65. Cao J (2011) Michaelis-Menten Equation and Detailed Balance in Enzymatic Networks. Journal of Physical Chemistry B 115: 5493-5498.

66. Conceicao Bispo JA, Sampaio Bonafe CF, de Souza VB, de Almeida e Silva JB, Mafra de Carvalho GB (2011) Extending the kinetic solution of the classic Michaelis-Menten model of enzyme action. Journal of Mathematical Chemistry 49: 1976-1995.

67. Cornish-Bowden A, Cardenas ML (2010) Specificity of Non-Michaelis-Menten Enzymes: Necessary Information for Analyzing Metabolic Pathways. Journal of Physical Chemistry B 114: 16209-16213.

68. Gunawardena J (2012) Some lessons about models from Michaelis and Menten. Molecular Biology of the Cell 23: 517-519.

69. Scheer M, Grote A, Chang A, Schomburg I, Munaretto C, et al. (2011) BRENDA, the enzyme information system in 2011. Nucleic Acids Research 39: D670-D676.

70. Mueller-Cajar O, Whitney SM (2008) Directing the evolution of Rubisco and Rubisco activase: first impressions of a new tool for photosynthesis research. Photosynthesis Research 98: 667-675.

71. Greene DN, Whitney SM, Matsumura I (2007) Artificially evolved Synechococcus PCC6301 Rubisco variants exhibit improvements in folding and catalytic efficiency. Biochemical Journal 404: 517-524.

72. Campbell E, Weddron IR, Banta S (2010) Broadening the Cofactor Specificity of a Thermostable Alcohol Dehydrogenase Using Rational Protein Design Introduces Novel Kinetic Transient Behavior. Biotechnology and Bioengineering 107: 763-774.

73. Machielsens R, Looger LL, Raeds J, Dijkhuizen S, Hummel W, et al. (2009) Cofactor engineering of Lactobacillus brevis alcohol dehydrogenase by computational design. Engineering in Life Sciences 9: 38-44.

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