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Review Article

Metabolic Engineering for Production of Biorenewable Fuels and Chemicals: Contributions of Synthetic Biology

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Production of fuels and chemicals through microbial fermentation of plant material is a desirable alternative to petrochemical-based production. Fermentative production of biorenewable fuels and chemicals requires the engineering of biocatalysts that can quickly and efficiently convert sugars to target products at a cost that is competitive with existing petrochemical-based processes. It is also important that biocatalysts be robust to extreme fermentation conditions, biomass-derived inhibitors, and their target products. Traditional metabolic engineering has made great advances in this area, but synthetic biology has contributed and will continue to contribute to this field, particularly with next-generation biofuels. This work reviews the use of metabolic engineering and synthetic biology in biocatalyst engineering for biorenewable fuels and chemicals production, such as ethanol, butanol, acetate, lactate, succinate, alanine, and xylitol. We also examine the existing challenges in this area and discuss strategies for improving biocatalyst tolerance to chemical inhibitors.

1. Introduction

Human society has always depended on biomass-derived carbon and energy for nutrition and survival. In recent history, we have also become dependent on petroleum-derived carbon and energy for commodity chemicals and fuels. However, the nonrenewable nature of petroleum stands in stark contrast to the renewable carbon and energy present in biomass, where biomass is essentially a temporary storage unit for atmospheric carbon and sunlight-derived energy. Thus there is increasing demand to develop and implement strategies for production of commodity chemicals and fuels from biomass instead of petroleum. Specifically, in this work we are interested in the microbial fermentation of biomass-derived sugars to commodity fuels and chemicals.

In order for a fermentation process to compete with existing petroleum-based processes, the target chemical must be produced at a high yield, titer and productivity. Sometimes there are additional constraints on the fermentation process, such as the presence of potent inhibitors in biomass hydrolysate or the need to operate at an extreme pH or temperature [1]. These goals can be difficult to attain with naturally-occurring microbes. Therefore, microorganisms with these desired traits often must be developed, either by modification of existing microbes or by the *de novo* design of new microbes. While significant progress has been made towards *de novo* design [2, 3], this work focuses on the modification of existing microbes.

Humanity has long relied on microbial biocatalysts for production of fermented food and beverages and eukaryotic biocatalysts for food and textiles. We have slowly modified these biocatalysts by selecting for desirable traits without understanding the underlying biological mechanisms. But upon elucidation of the biological code and the development of recombinant DNA technology, we now have the tools to do more than just select for observable traits—we are now able to rationally modify and design metabolic pathways, proteins, and even whole organisms.

Much of this rational modification has been in the form of Metabolic Engineering. Metabolic Engineering was

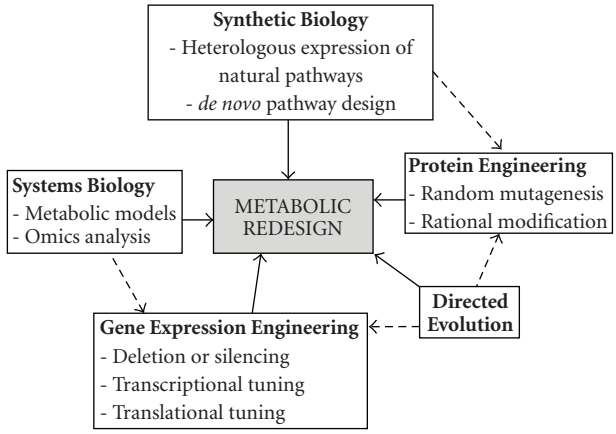


Figure 1: Overview of tools for metabolic redesign.

defined in 1991 [4, 5] and here we use the definition of "*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* " [6]. While Metabolic Engineering has enabled extraordinary advances in the production of commodity chemicals and fuels from biomass, some of which are discussed in this work, we have now reached the point where biological functions that do not exist in nature are desired. Synthetic biology aims to develop and provide these nonnatural biological functions.

For many years, the term Synthetic Biology was used to describe concepts that would be classified today as Metabolic Engineering [7]. However in the last 10 years, terms such as "unnatural organic molecules" [7], "unnatural chemical systems [8], "novel behaviors" [9], "artificial, biology-inspired systems" [10], and "functions that do not exist in nature" [11] have been used to describe Synthetic Biology. For the purpose of this review, we will apply the Synthetic Biology definition of "*the design and construction of new biological components, such as enzymes, genetic circuits, and cells, or the redesign of existing biological systems*" [12].

Synthetic biology has application to many fields, including cell-free synthesis [13], tissue and plant engineering [14] and drug discovery [15], but here we are interested in the modification of microbes for the biorenewable production of commodity chemicals and fuels. Other recent reviews have also dealt with this topic [16–18].

Synthetic biology for the production of a target compound can be expressed as a sequence of the following events, each of which will be discussed in more detail and demonstrated below. (1) Design the metabolic pathways and phenotypic properties of the desired system. What are the desired substrates and products? What are the expected environmental stressors? (2) Choose an appropriate host organism (chassis) based on the following criteria. Which organisms display at least some of the desired properties? How well characterized and annotated are these organisms? Are there molecular biology tools for modification of this chassis? (3) Formulate an implementation approach. What modifications are necessary to achieve the pathways and properties identified in step (1)? Do metabolic pathways need to be added, removed, or tuned? Does the desired pathway or phenotype exist in nature, or does it need to be designed *de novo*? (4) Optimize the redesigned system and assess the system properties relative to the ideal. Can the chassis be improved further?

Even a simple biocatalyst, such as the laboratory workhorse *Escherichia coli*, is a complex system of an estimated 4603 genes, 2077 reactions, and 1039 unique metabolites [19, 20], and while the steps outlined above are relatively straightforward, it is still difficult to quickly and reliably engineer a biocatalyst to perform desired behaviors [21]. Systems biology, the standardization of biological systems, and metabolic evolution are all vital to the compensation for this disconnect between the expected and actual biocatalyst behaviors. Through a combination of these powerful techniques, biocatalysts have been redesigned for the production of an astounding array of commodity fuels and chemicals, both natural and unnatural (Figure 1 and Table 1). Here we discuss successful examples involving the production of commodity fuels and chemicals, with a focus on D- and L-lactate, L-alanine, succinate, ethanol, and butanol.

2. Methods and Tools for Biocatalyst Redesign

2.1. Chassis. A robust and stable chassis enables efficient and economical production of fuels and chemicals at an industrial level. Since we are specifically interested in biocatalysts that can utilize biomass, a desirable chassis has the following characteristics: (1) growth in mineral salts medium with inexpensive carbon sources, (2) utilization of hexose and pentose sugars, so that all the sugar components in lignocellulosic biomass can be converted to the desired product, (3) high metabolic rate, essential for high rate of productivity, (4) simple fermentation process to reduce the manipulation cost and minimize failure risks in large-scale production, (5) robust organism (high temperature and low pH where possible) to reduce the requirement for external cellulase during cellulose degradation, as well as to reduce the required amount of base addition, (6) ease of genetic manipulation and genetic stability, (7) resistance to inhibitors produced during the biomass pretreatment process, and (8) tolerance to high substrate and product concentrations in order to obtain high titers of target compound.

Enteric bacteria, especially *E. coli*, have many of the above mentioned physiological characteristics and are, thus, an excellent chassis for synthetic biology. Most of the examples discussed here use *E. coli*, but other important microbial model systems have been redesigned, including *Clostridium acetobutylicum* [28], *Corynebacterium glutamicum* [29], *Saccharomyces cerevisiae* [30], and *Aspergillus niger* [31]. *E. coli* has been used as a model organism since the beginning of genetic engineering [32]. While K-12 strain MG1655 (ATCC# 47076) is one of the most commonly used *E. coli* strains [33], there are other lineages, such as B (ATCC# 11303), C (ATCC# 8739), and W (ATCC# 9637), that are also generally regarded as safe since they are unable to

Table 1: Summary of engineered *E. coli* biocatalysts for production of renewable fuels and chemicals in our laboratory.

Product	Fermentation condition[(1)]	Titer (g/L)	Yield(g/g)	Productivity (g/L/h)	Reference
Redesign through modification of existing pathways					
D-lactate	Anaerobic, batch	118	0.98	2.88	[22]
Acetate	Aerobic, fed-batch	53	0.50	1.38	[23]
Succinate	Anaerobic, batch	83	0.98	0.90	[24]
Redesign through introduction of foreign pathways					
Ethanol	Anaerobic, batch	43	0.48	2.00	[25]
L-lactate	Anaerobic, batch	116	0.98	2.29	[22]
Xylitol	Aerobic, fed-batch	38	1.40	0.81	[26]
L-alanine	Anaerobic, batch	114	0.95	2.38	[27]

[(1)] All fermentations were done in mineral salts medium with glucose, except for the ethanol fermentations which used xylose.

colonize the human gut [34]. Although K-12 is the most characterized and widely used strain, *E. coli* W (ATCC# 9637) and C (ATCC# 8739) have proven to be better chassis for synthesizing fuels and chemicals. For example, K-12-derived strains were unable to completely ferment 10% (w/v) glucose in either complex or mineral salts medium [1, 35], while derivatives of strains W or C can completely ferment more than 10% (w/v) of glucose with higher cell growth and sugar utilization rates than K-12. Additionally, *E. coli* W strains have the native ability to ferment sucrose [1, 36].

Foreign genes may be unstable in host cells due to recombination facilitated by mobile DNA elements, and thus the mobile DNA elements in *E. coli* K-12 strain have been deleted [37]. This minimal genome construction strategy is an excellent approach to improve this chassis for the production of fuels and chemicals.

2.2. Systems Biology Tools

2.2.1. Genome-Scale Models and In Silico Simulation. Given the rational basis of metabolic engineering and synthetic biology, models and simulations are critical predictive and tools. Genome sequencing and automatic annotation tools have enabled construction of genome-scale metabolic models of nearly 20 microorganisms [38]. These constraint-based models and *in silico* simulations can be used to predict metabolic flux redistribution after genetic manipulation, or to predict other cellular functions, such as substrate preference, outcomes of adaptive evolution and shifts in expression profiles [39]. They can also aid in pathway design to obtain desired phenotypes [40–42]. For example, the *E. coli* *i*JE660a GSM model was used to successfully simulate single- and multiple-gene knockouts to improve lycopene production [42]. The computational framework, Optknock, was developed to identify gene deletion targets for system optimization [41], and simulation results for gene deletions for succinate, lactate, and 1,3-propanediol production were in agreement with experimental data. Another simulation program, OptStrain, was developed to guide metabolic pathway modification for target compound production, through both the addition of heterologous metabolic reactions and deletion of native reactions [40]. However, most of the current models only have stoichiometric information, while kinetic and regulatory effects are not included [38, 39]. Integration of kinetic and regulatory information will improve the accuracy and predictive power of these models.

2.2.2. High-Throughput Omics Analysis. High-throughput omics analysis, such as transcriptome, proteome, metabolome, and fluxome [43–45], aids in characterization of cellular function on multiple levels, and therefore provide a "debugging" capability for system optimization [12, 45].

Genetic manipulations can disturb the metabolic balance or impair cell growth due to depletion of important precursors [46, 47], accumulation of toxic intermediates [48], or redox imbalance [1]. For example, high NADH levels in *E. coli* reengineered for ethanol production inhibited citrate synthase activity, thereby limiting cell growth by lowering production of the critical metabolite 2-ketoglutarate [49]. Metabolome and fluxome analysis can quickly identify the limiting metabolites or altered metabolic flux distribution, providing the basis for problem solving [45, 50]. For example, metabolite measurements of *Aspergillus terreus* were implemented in the rational metabolic redesign for increased production of lovastatin [45, 50]. Changes of mRNA and protein profiles can be identified by transcriptome and proteome analysis, providing gene targets for further engineering [46, 47]. The work of Choi et al. demonstrate this concept: transcriptome analysis of *E. coli* producing the human insulin-like growth factor I fusion protein aided in selection for targets for gene deletion. The resulting redesigned strain showed a greater than 2-fold increase in product titer and volumetric productivity [46, 47]. Additionally, comparative genome sequence analysis facilitates identification of mutated genes or regulators during evolution, and these mutations can be used to redesign the systems for better synthetic capability. For example, in an effort described as "genome-based strain reconstruction", evolved strains of *Corneybacterium glutamicum* selected for L-lysine production were compared to the parental strain, and mutations were found that were proposed as beneficial to L-lysine production. Three of these mutations were introduced into the parent strain and enabled production of up to 3.0 g/L/hr L-lysine [51].

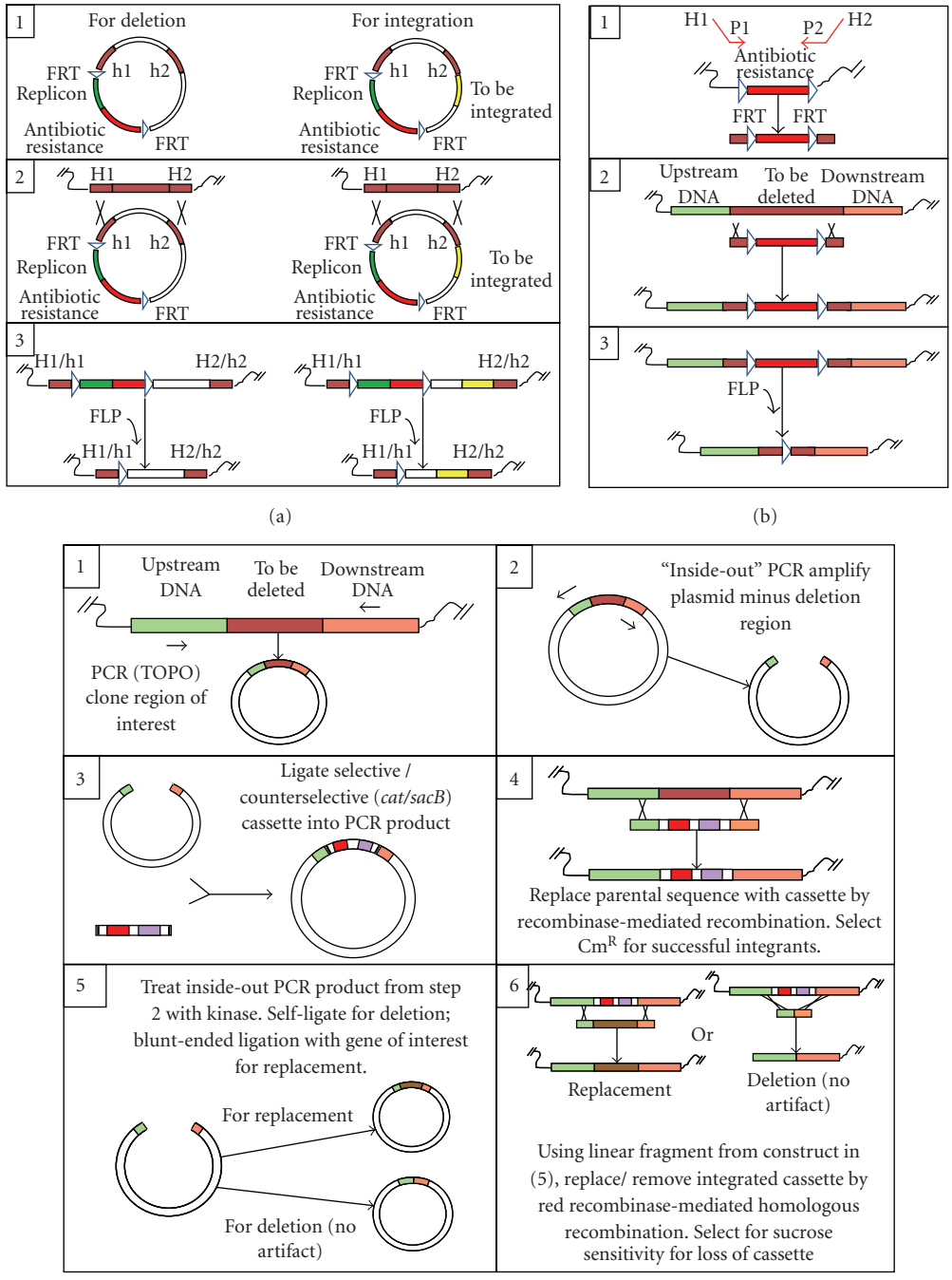


Figure 2: Comparison of three-gene deletion methods in *E. coli*. These methods can also be used in other enteric bacteria. The first and third methods can also be used for gene integration into the chromosome and promoter replacement for tuning gene expression. 2(a) *plasmid-based method*. Step 1 is construction of the deletion plasmid containing DNA fragments homologous to the target gene (h1 and h2), a selectable marker, and either a temperature sensitive or conditional replicon. Step 2 is double-crossover recombination; the plasmid cannot replicate in the host strain, and antibiotic-resistant colonies are selected. In step 3, the FRT, replicon, and antibiotic resistance marker are removed by FLP. 2(b) *Linear DNA-based method*. Step 1 is construction of the linear DNA fragment by PCR (H1-P1 and H2-P2 as primers). H1 and H2 refer to short DNA fragments homologous to target gene. Step 2 is replacement of the target gene with the antibiotic resistance gene through crossover recombination with the help of Red recombinase. Step 3 is removal of FRT and antibiotic marker by FLP. 2(c) *Two-stage recombination-based method developed in our lab*. Steps 1, 2, 3, and 5 describe construction of the plasmids and linear DNA fragments for the two-stage recombinations. Step 4 describes the first recombination step, in which the *cat, sacB* cassette is inserted into the target gene. Step 6 is the second recombination step, in which the *cat, sacB* cassette is removed by selection on sucrose.

2.3. Genetic Manipulation Tools

2.3.1. Gene Deletion. Gene deletion can redistribute carbon flux toward the target product by deleting genes critical to competing metabolic pathways and, thus, is widely used in metabolic redesign strategies. Homologous recombination is the most frequently used strategy for gene-deletion (Figure 2). Historically, plasmids containing a selectable marker flanked by DNA fragments homologous to the target gene and either temperature sensitive or conditional replicons were needed for efficient gene deletion in bacteria [52] (Figure 2(a)). In contrast, genes can be directly disrupted in yeast by linear PCR fragments with short flanking DNA fragments homologous to chromosomal DNA. Linear DNA is not as easy to transform into *E. coli* because of the intracellular exonuclease system and low recombination efficiency. Gene deletion systems based on bacteriophage λ Red recombinase facilitate chromosomal gene deletion using a linear PCR fragment [53]. In this method, the chromosomal gene is replaced by the selectable marker flanked by two FRT (FLP recognition target) fragments (Figure 2(b)) and then the marker can be removed by the FLP recombinase [54]. However, this method leaves a 68bp-FRT scar on the chromosome after each excision [52], reducing further gene deletion efficiency. Repeated use of this FRT/FLP system for specific gene deletions has the potential to generate large unintended chromosomal deletions.

To facilitate sequential gene deletions, our lab has developed a two-stage recombination strategy (Figure 2(c)), using the sensitivity of *E. coli* to sucrose when *Bacillus subtilis* levansucrase (*sacB*) is expressed [24, 27, 55]. Gene deletions created by this method do not leave foreign DNA, antibiotic resistance markers, or scar sequences at the site of deletion. In the first recombination, part of the target gene is replaced by a DNA cassette containing a chloramphenicol resistance gene (*cat*) and levansucrase gene (*sacB*). In the second recombination, the *cat, sacB* cassette is removed by selection for resistance to sucrose. Cells containing the *sacB* gene accumulate levan during incubation with sucrose and are killed [55]. Surviving recombinants are highly enriched for loss of the *cat, sacB* cassette [24, 27].

2.3.2. Gene Expression Tuning. Like gene deletions, plasmid-based expression systems are ubiquitous to metabolic redesign. However, plasmid-based systems have several disadvantages. (1) Plasmid maintenance is a metabolic burden on the host cell, especially for high-copy number plasmids [56]. Note that high copy numbers are not essential, considering that most central metabolic enzymes are encoded by a single gene; (2) plasmid-based expression is dependent on plasmid stability, with only few natural unit-copy plasmids having the desired stability [12]; (3) only low-copy number plasmids have replication that is timed with the cell cycle, and thus maintaining a consistent copy number in all cells is challenging [12]; (4) metabolic redesign can require construction of a complex heterologous pathway, and thus several genes, encoded in large pieces of DNA, need to be incorporated. Most commercial plasmids have difficulties carrying large DNA fragments.

Chromosomal integration of the target genes followed by fine-tuning their expression could eliminate these plasmid-associated problems. The abovementioned two-step recombination strategy for gene deletion can also be used for gene integration or promoter replacement (Figure 2).

Gene expression in prokaryotes is mainly controlled at the transcriptional level, and therefore the promoter is the most tunable element. While inducible promoters, such as *lac* and *ara*, have been traditionally used to modulate gene expression, large-scale inducer use is cost prohibitive for production of fuels and bulk chemicals. However, several strategies have been developed to construct constitutive promoter libraries for fine-tuning gene expression. Some methods rely on the use of natural promoters. For example, *Zymomonas mobilis* genomic DNA was used to construct a promoter library for screening optimal expression of *Erwinia chrysanthemi* endoglucanase genes (*celY* and *celZ*) in *Klebsiella oxytoca* P2 in order to improve ethanol production from cellulose [57]. Other methods rely on random modification of existing promoters, such as the randomization of the spacer sequences between the consensus sequences [58], or mutagenesis of a constitutive promoter [59]. This promoter modification method was used to assess the impact of phosphoenolpyruvate carboxylase levels on cell yield and deoxy-xylulose-P synthase levels on lycopene production, and the optimal expression levels of these genes were identified for maximal desired phenotype [59]. These synthetic promoter libraries could also be integrated into the chromosome directly, which could facilitate expression modulation of chromosomal genes [60, 61].

The fine-tuning methods described above rely on the selection of the best natural promoter or random alteration of existing promoters. One of the goals of synthetic biology is construction of standard parts, and posttranscriptional processes, such as transcriptional termination, mRNA degradation, and translation initiation, have been engineered with this goal in mind. Examples include construction of a synthetic library of 5' secondary structures to successfully manipulate mRNA stability [62], and modulation of the ribosome binding site (RBS) as well as Shine-Dalgarno (SD) and AU-rich sequences to tune gene expression at the translation initiation process [60, 63]. Riboregulators were also developed to tune gene expression via RNA-RNA interactions [64]. A final method of fine-tuning gene expression is codon optimization, which can improve translation of foreign genes [65]. These optimized gene sequences often do not exist in nature and must be generated using DNA synthesis techniques.

In many cases, more than one gene needs to be introduced into the chassis and expression of these genes needs to be coordinated to attain desired biocatalyst performance. One such method is modulation of the expression of each individual gene via its own promoter. However, it is difficult to predict the appropriate expression level of each gene. Another option is to combine multiple genes into a synthetic operon with a single promoter, and fine-tune expression of each gene through posttranscriptional processes [12] with tunable control elements (such as mRNA secondary structure, RNase cleavage sites, ribosome binding sites, and

sequestering sequences) at intergenic regions. Libraries of tunable intergenic regions (TIGRs) were generated and screened to tune expression of several genes in an operon [48]. This method was used to coordinate expression of three genes in an operon that encodes a heterologous mevalonate biosynthetic pathway, improving mevalonate production by 7-fold [48]. Another method to control expression of more than one gene is to engineer global transcription machinery by random mutagenesis of transcription factors [66, 67]. This method was shown to efficiently improve tolerance to toxic compounds and production of metabolites, and to alter phenotypes [66, 67].

2.3.3. Protein Engineering. Natural proteins may not meet the required criteria for specific and efficient system performance, and thus alteration for a specific application may be needed. Directed evolution of proteins offers a way to rapidly optimize enzymes, even in the absence of structural or mechanistic information [68]. For directed evolution, a protein library is usually generated by random mutagenesis [68], recombination of a target gene [69], or a family of related genes [70] and then the library is analyzed by high-throughput screening. This method has been used to successfully increase enzyme activity [71, 72], increase protein solubility and expression, invert enantioselectivity, and increase stability and activity in unusual environments [68]. For example, a mutation library of the gene-encoding geranylgeranyl diphosphate synthase of *Archaeoglobus fulgidus* was generated to screen for mutants with higher activity, enabling lycopene production in *E. coli*. Screening of more than 2,000 variants identified eight with increased activity; one of which increased lycopene production by 100% [71].

Of particular relevance to the field of synthetic biology is the creation of novel enzymatic activity through protein engineering [73, 74]. For example, the unnatural isomerization of α-alanine to β-alanine was attained by evolving a lysine 2,3-aminomutase to expand its substrate specificity to include α-alanine [73].

Rational design is another powerful tool to increase protein properties, especially with the aid of computational analysis [75, 76]. Based on knowledge of protein structure and function, one can predict which amino acid(s) to change in order to obtain the desired function. In the redesign of *Lactobacillus brevis* for the production of secondary alcohols, it was desired to change the cofactor preference of the R-specific alcohol dehydrogenase from NADPH to NADH. A structure-based computational model was used to identify potentially beneficial amino acid substitutions and one of these changes increased NADH-dependent activity four-fold [77].

While these examples demonstrate the power of rational enzyme (re-)design, this approach requires detailed information about the protein structure and mechanism, while random mutagenesis does not. Recent advances have combined directed evolution and rational design in a so-called "semi-rational" approach to successfully improve enzyme activity when only limited information is available [78, 79]. When the mutagenesis is limited to specific residues, as chosen from existing structural or functional knowledge, these "smart" libraries are more likely to yield positive results [79]. For example, the catalytic activity of pyranose-2-oxidase was improved by mutagenesis of the known active site [80].

While the 20 natural amino acids supply enzymes with a wide range of possible activity, this range can be expanded even further by the use of unnatural amino acids (UAAs). There are more than 40 UAAs available at this time and they have been used to probe protein function, photocage critical residues, and alter metalloprotein properties [81, 82]. While this technology is still in the developmental stage, at least one study has shown an improvement in enzyme activity following insertion of UAAs. Site 124 of *E. coli*'s nitroreductase was replaced with a variety of natural and unnatural amino acids and certain UAA variants had a greater than 2-fold increase in activity over the best natural amino acid variant [83]. This biomimetic approach has been expanded to other metabolites, such as carbohydrates [84] and lipids [85].

2.4. Evolution. As described above, a robust biocatalyst with high yield, titer, and productivity is critical for a fermentation process to compete with petrochemical-based production. Current models and simulation tools provide a framework given the constraints of known protein functions. But the many reactions and enzymes that remain uncharacterized cannot be included in this theoretical analysis. Therefore rational design methods often result in a biocatalyst that performs poorly relative to the model. Metabolic evolution provides a complementary approach to improve biocatalyst productivity and robustness, dependent upon the design of an appropriate selection pressure. Where feasible, synthesis of the target compound can be coupled to the production of ATP, redox balance, or key metabolites that are essential for growth, and selection for improvements in growth during metabolic evolution (serial transfers) can be used to coselect for higher rates or titers of target compounds (Figure 3). Both redox balance and net ATP production in such a synthetic system are requisites for successful evolution.

We have used this metabolic evolution strategy to optimize biocatalysts redesigned for production of several fermentation products [1], including ethanol, D-lactate, L-lactate, L-alanine (Figure 3), and succinate, as described in more detail below. A frequently-used design scheme is to couple synthesis of the target product to growth by inactivating competing NADH-consuming pathways. Thus, the only way for cells to regenerate NAD^+ for glycolysis is to produce the target compound. Increased cell growth, supported by higher ATP production rate during glycolysis, is coupled with higher NADH oxidization rate, and thus tightly coupled with synthesis of target product. This evolution strategy has been shown to increase productivity by up to two orders of magnitude.

Computational frameworks based on genome-scale metabolic models have been used to construct biocatalysts that couple biomass formation with chemical production [40, 41], and therefore provide a basis for selective pressure for high productivity. For example, Optknock identified gene deletion targets for the construction of lactate-producing

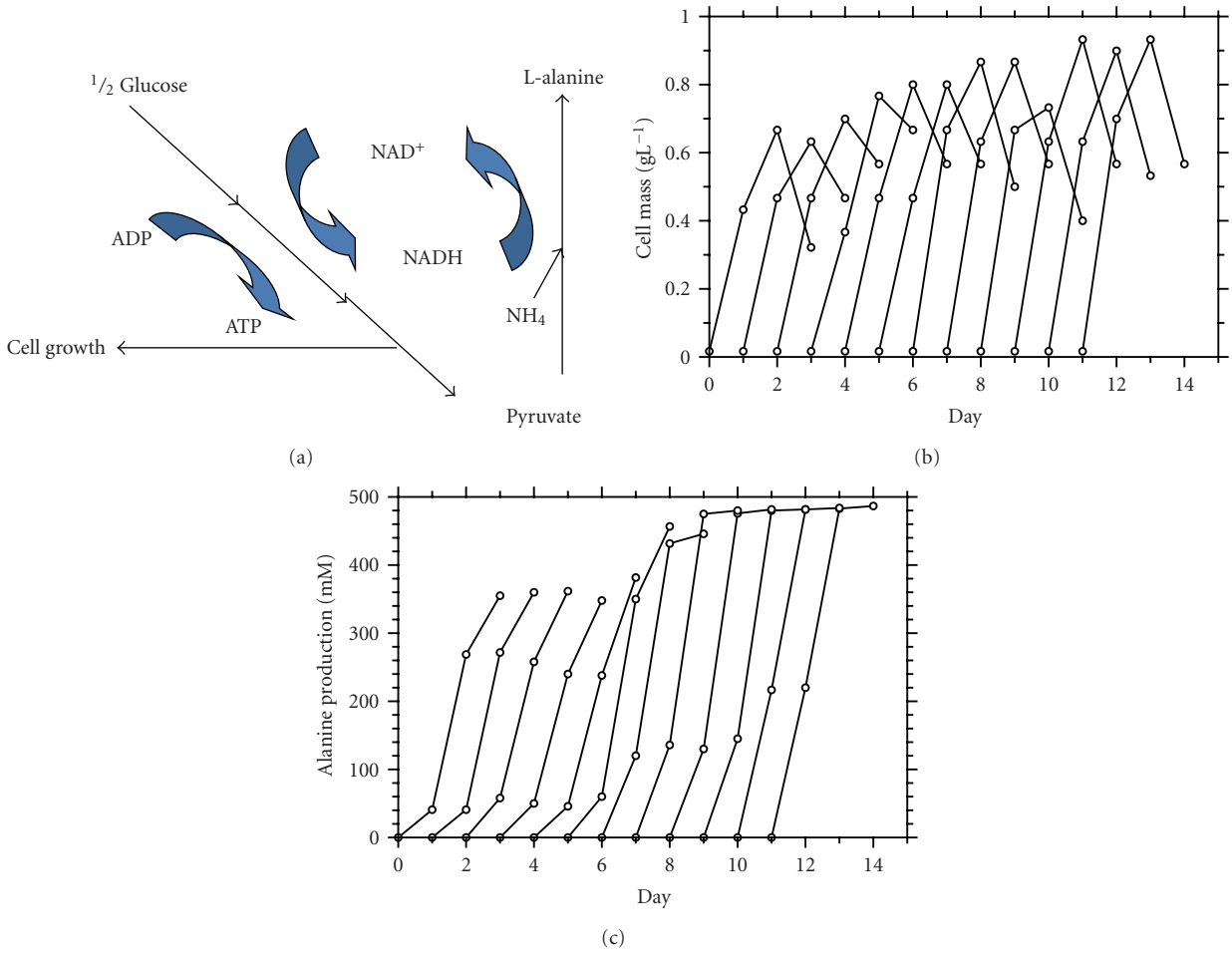


Figure 3: Metabolic evolution for improving L-alanine production in *E. coli* [27]. 3(a) Redesigned metabolic pathway for L-alanine production: ATP production and cell growth is coupled to NADH oxidation and L-alanine production. 3(b) Directed evolution improves cell growth. Parental strain XZ112 reaches a maximum cell mass of 0.7 gL^{-1} after 48 hours of fermentation; evolved strain XZ113 attains 0.7 gL^{-1} after 24 hours and a maximum of 0.9 gL^{-1} after 48 hours; 3(c) metabolic evolution to improve cell growth also improves alanine production. Parental strain XZ112 produces 355 mM alanine after 72 hours of fermentation; evolved strain XZ113 produces 484 mM in 48 hours.

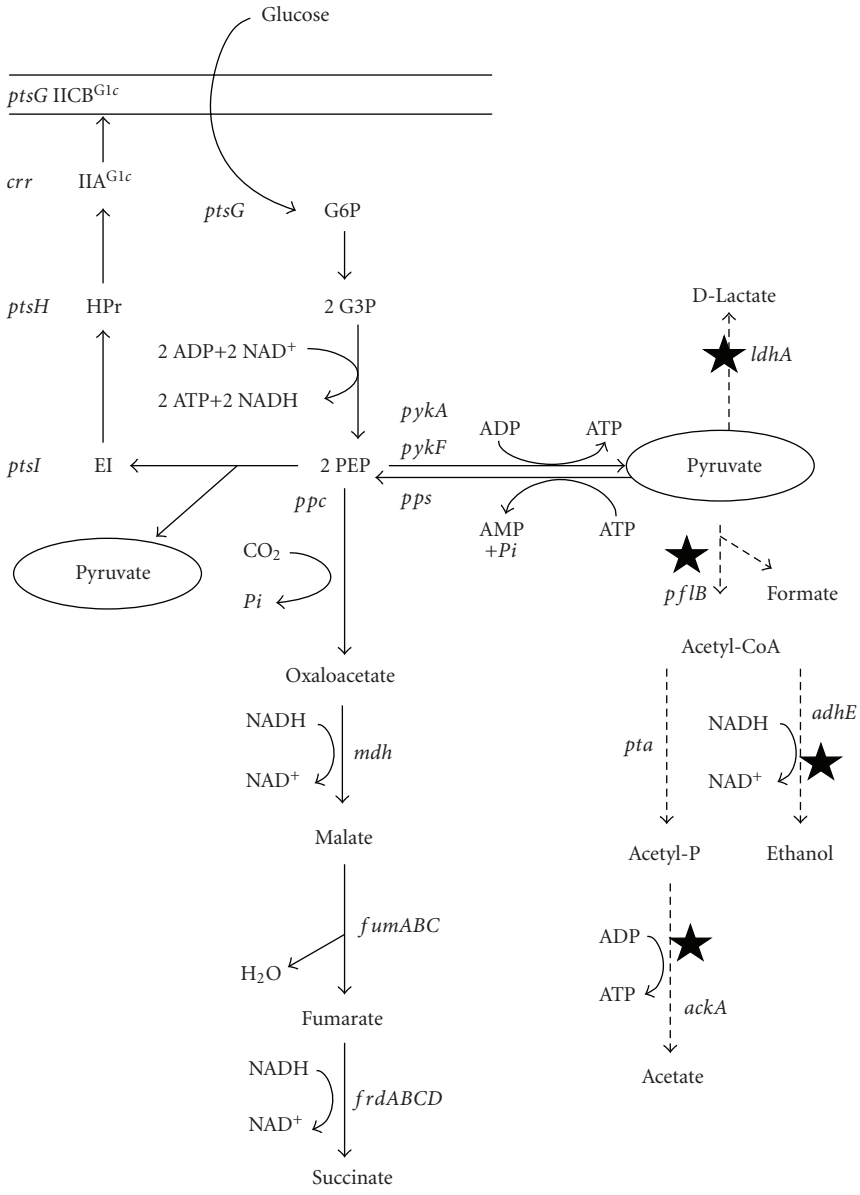
E. coli, and then directed evolution improved production capability [86]. Although rational design of metabolic pathways based on current metabolic models is a common method for maximizing yield of the target compound, this method is not always the best strategy, due to our limited understanding of the complicated metabolic network and dynamic kinetics of each reaction. Metabolic evolution provides an excellent alternative method for strain improvement, through which reactions that are not currently predictable would be selected to improve biocatalyst performance [87]. As our knowledge of biocatalyst behavior and metabolism improves, predictive models will become even more powerful.

3. Redesign through Modification of Existing Pathways

In this section, we highlight projects that have redesigned a chassis to produce target compounds at high yield and titer without the introduction of foreign pathways. In the next section, we describe biocatalyst redesigns which used foreign or nonnatural pathways.

3.1. Succinate. Succinate, a four-carbon dicarboxylic acid, is currently used as a specialty chemical in food, agricultural, and pharmaceutical industries [88] but can also serve as a starting point for the synthesis of commodity chemicals used in plastics and solvents, with a potential global market of $15 billion [89]. Succinate is primarily produced from petroleum and there is considerable interest in the fermentative production of succinate from sugars [89].

Several rumen bacteria can produce succinate from sugars with a high yield and productivity [90–92], but require complex nutrients. Alternatively, native strains of *E. coli* ferment glucose effectively in simple mineral salts medium but produce succinate only as a minor product [93]. Therefore *E. coli* strain C (ATCC 8739) was redesigned for



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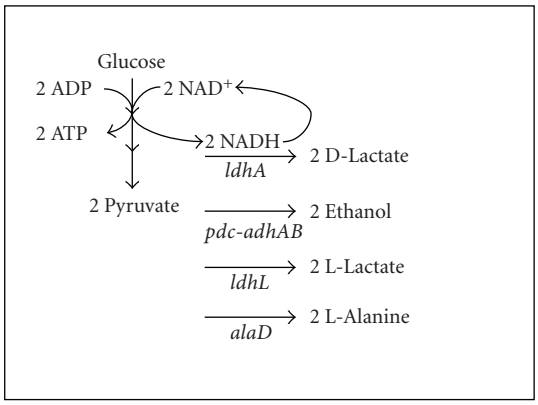

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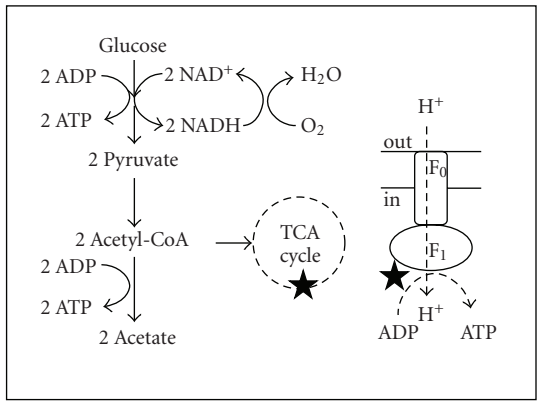

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Figure 4: Continued.

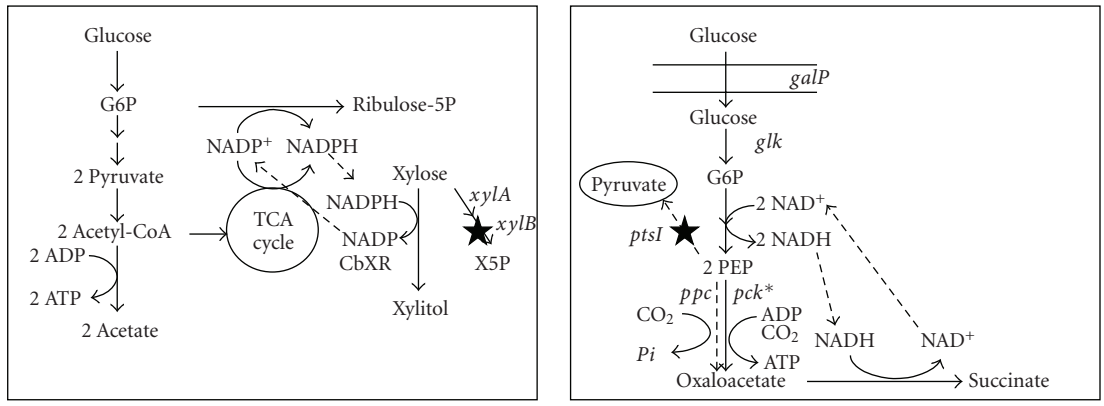

Figure 4: Synthetic pathways of *E. coli* for production of fuels and chemicals in our lab: 4(a) Native metabolic pathways of glucose fermentation in *E. coli*; 4(b) synthetic pathways for production of D-lactate, ethanol, L-lactate and L-alanine; 4(c) synthetic pathways for production of pyruvate and acetate; 4(d) synthetic pathway for production of xylitol, 4(e) synthetic pathway for production of succinate. ★ indicate gene deletion. Genes and enzymes: *ackA*, acetate kinase; *adhAB*, alcohol dehydrogenase (*Z. mobilis*); *adhE*, alcohol/aldehyde dehydrogenase; *alaD*, L-alanine dehydrogenase (*G. stearothermophilus*); *crr*, glucose-specific phosphotransferase enzyme IIA component; *frd*, fumarate reductase; *fum*, fumarase; *galP*, galactose-proton symporter (glucose permease); *glk*, glucokinase; *ldhA*, D-lactate dehydrogenase; *ldhL*, L-lactate dehydrogenase (*P. acidilactici*); *mdh*, malate dehydrogenase; *pdc*, pyruvate decarboxylase; *pflB*, pyruvate formate-lyase; *ppc*, phosphoenolpyruvate carboxylase; *pta*, phosphate acetyltransferase; *ptsG*, PTS system glucose-specific EIICB component; *ptsH*, phosphocarrier protein HPr; *ptsI*, phosphoenolpyruvate-protein phosphotransferase (Phosphotransferase system, enzyme I); *pyk*, pyruvate kinase; *xrd*, xylose reductase (*C. boidinii*); *xylB*, xylulokinase. Metabolites: G6P, glucose-6-phosphate; G3P, glycerol-3-phosphate; PEP, phosphoenol pyruvate; X5P, D-xylulose-5-phosphate.

succinate production at high yield, titer, and productivity [94].

The initial redesign strategy focused on inactivation of competitive pathways, specifically deletion of lactate dehydrogenase (*ldhA*), alcohol/aldehyde dehydrogenase (*adhE*), and acetate kinase (*ackA*). However, the resulting strain grew poorly in mineral salts medium under anaerobic condition and accumulated only trace amounts of succinate. Because NADH oxidization is coupled to succinate synthesis in this strain, metabolic evolution was used to improve both the cell growth and succinate production. After inactivation of pyruvate formate-lyase and methylglyoxal synthase to eliminate formate and lactate production, the final strain, KJ073, produced near 670 mM succinate (80 g/L) in mineral salts medium with a high yield (1.2 mol/mol glucose) and high productivity (0.82 g/L/h) [94]. Inactivation of threonine decarboxylase (*tdcD*), 2-ketobutyrate formate-lyase (*tdcE*), and aspartate aminotransferase (*aspC*) further increased succinate yield (1.5 mol/mol glucose), titer (700 mM), and productivity (0.9 g/L/h) [24].

Despite its power in improving biocatalyst performance, metabolic evolution has the undesirable property of being a black box; evolved strains show the desired biocatalyst properties, but the metabolic evolution process does not improve our understanding of the biocatalyst. Therefore, reverse engineering of evolved strains can help us identify key mutations that can then be rationally applied to other biocatalysts. Reverse engineering of the succinate-producing strain revealed two significant changes in cellular metabolism that increased energy efficiency [87]. The first change is that PEP carboxykinase (*pck*), which normally functions in gluconeogenesis during the oxidative metabolism of organic acids [90, 95, 96], became the major carboxylation pathway for succinate production. High-level expression of PCK dominated CO_2 fixation and increased ATP yield (1 ATP per oxaloacetate produced). The second change is that the native phosphoenolpyruvate- (PEP-) dependent phosphotransferase system for glucose uptake was inactivated and replaced by an alternative glucose uptake pathway: GalP permease (*galP*) and glucokinase (*glk*). These changes increased the pool of PEP available for maintaining redox balance, as well as increasing energy efficiency by eliminating the need to produce additional PEP from pyruvate, a reaction that requires two ATP equivalents [97].

While rational design based on current metabolic understanding is a key component of metabolic engineering and synthetic biology, our limited understanding of the complicated metabolic network and dynamic kinetics of each reaction can lead to failure of predictive models. In this example, metabolic evolution was demonstrated as an excellent alternative method for strain improvement, through which currently unpredictable reactions would be selected to expand cellular metabolic capability [87]. By understanding the mutations that enabled desirable performance of the succinate-producing strain, we have more options available for the redesign of future systems. To demonstrate this, *E. coli* was again redesigned based on the findings from the evolved strain [98]. This time, the design strategy shifted from inactivating competitive fermentation pathways to recruiting energy conserving pathways for efficient succinate production (Figure 4(e)). After increasing *pck* gene expression and inactivating the native glucose PTS system, the

native *E. coli* metabolic system was converted to an efficient succinate synthetic system, equivalent to the native pathway of succinate-producing rumen bacteria [98].

3.2. D-Lactate. D-lactate is widely used as a specialty chemical in the food and pharmaceutical industry. It can also be combined with L-lactate for the production of polylactic acid (PLA), an increasingly popular biorenewable and biodegradable plastic [99, 100] whose commercial success obviously depends on the production cost. Although glucose is the current substrate for fermentative production of lactate, it is desirable to produce this commodity chemical from lignocellulosic feedstock, which contains a mixture of sugars. Some lactic acid bacteria have the desirable native ability to produce large amount of D-lactate under low pH condition, where the low pH reduces the process cost [101, 102]. However, these lactic acid bacteria require complex nutrients, and many of them lack the ability to ferment pentose sugars. The lactic acid bacteria that do ferment pentose sugars unfortunately produce a mixture of lactate and acetate and, thus, are not a good chassis for commercial production of D-lactate. While *E. coli* can ferment many sugars effectively in a simple mineral salts medium, inherent D-lactate productivity is low and other undesirable metabolites are also produced [103]. Therefore, the *E. coli* metabolic system was redesigned to attain the desired properties of high yield and productivity of D-lactate.

E. coli strain W3110 was used as the chassis for D-lactate production with a redesign strategy that focused on inactivation of competitive fermentation pathways [104]. After deleting the genes encoding fumarate reductase (*frdABCD*), alcohol/aldehyde dehydrogenase (*adhE*), pyruvate formate lyase (*pflB*), and acetate kinase (*ackA*), the resulting strain, SZ63, can only oxidize NADH via D-lactate synthesis (Figure 4(b)). Although this strain could completely utilize 5% (w/v) glucose in a mineral salts medium with a yield near theoretical maximum (96%), the volumetric D-lactate productivity of 0.42 g/L/h was relatively low compared with lactic acid bacteria [35]. In addition, this strain can neither utilize sucrose nor completely utilize 10% (w/v) sugar [35]. Therefore, an *E. coli* W derivative strain was chosen as chassis for more robust D-lactate production [35, 105]. After redesigning central metabolism so that D-lactate production was the sole means of oxidizing NADH, metabolic evolution was used to further improve cell growth and D-lactate productivity. The resulting strain, SZ194, efficiently consumed 12% (w/v) glucose in mineral salts medium and produced 110 g/L D-lactate [105] with a volumetric productivity of 2.14 g/L/h, a 5-fold increase over the W3110 derivative. The biocatalyst was further optimized by deleting methylglyoxal synthase gene (*mgsA*) to eliminate L-lactate production, and by metabolic evolution to increase yield and productivity. The final D-lactate producing strain, TG114, could convert 12% (w/v) glucose to 118 g/L D-lactate with an excellent yield (98%) and productivity (2.88 g/L/h) [22].

3.3. Acetate. Acetate is a commodity chemical with 2001 worldwide production estimated at 6.8 million metric tons [23]. Biological production of acetate accounts for only 10% of world production, mainly in the form of vinegar, with the remainder of production through petrochemical routes [106–108]. Biological production of commodity chemicals has historically focused on anaerobic production of reduced products, since substrate loss as cell mass and CO_2 is minimal and product yields are high. Contrastingly, acetate is an oxidized chemical, and traditional biological production involves a complex two-stage process: fermentation of sugars to ethanol by *Saccharomyces*, followed by aerobic oxidation of ethanol to acetate by *Acetobacter* [106–108]. To enable microbial production of redox-neutral or oxidized products at high yield, the biocatalyst metabolism needs to be redesigned to combine attributes of both fermentative and oxidative metabolisms.

Redesign of *E. coli* W3110 metabolism for acetate production focused on three major pathways: fermentative metabolism, oxidative metabolism, and energy supply (Figure 4(c)) [23]. The competitive fermentation pathways (*pflB, ldhA, frd, adhE*) were inactivated to prevent the consumption of common precursor pyruvate, and the oxidative tricarboxylic acid (TCA) cycle was interrupted to reduce the carbon loss as CO_2. Finally, oxidative phosphorylation was disrupted (*atpFH*) to reduce ATP production while maintaining the ability to oxidize NADH by the electron transport system, thus increasing the glycolytic flux for more ATP production through substrate-level phosphorylation. Although rationally designed, the resulting strain, TC32, had an undesirable auxotrophic requirement for succinate during growth in glucose-minimal medium. Evolution was used to eliminate this auxotrophy and the final strain, TC36, produced 878 mM acetate (53 g/L) in mineral salts medium with 75% of the maximal theoretical yield. Although this is a lower titer than acetate produced from ethanol oxidation by *Acetobacter*, TC36 has a two-fold higher production rate, requires only mineral salts medium, and can metabolize a wide range of carbon sources in a simple one-step process [23].

3.4. Others. Butanol is an excellent alternative transportation fuel with several advantages compared to ethanol, including higher-energy content, lower volatility, less hydroscopicity, and less corrosivity [109]. Redesign of *E. coli* for butanol production is discussed below. *C. acetobutylicum* ATCC 824 naturally produces butanol and was redesigned to increase butanol production and decrease coproduct accumulation. Metabolic engineering-type modifications, such as overexpression of the acetone formation pathway to increase formation of butanol precursor butyryl-CoA, inactivation of the transcriptional repressor SolR, and overexpression of alcohol/aldehyde dehydrogenase all increased butanol production [110–112]. In an excellent example of synthetic biology-type applications, expression of the butyrate kinase gene was fine-tuned by a rationally designed antisense RNA to increase butanol production [113].

1,2-propanediol (1,2-PD) is a major commodity chemical currently derived from propylene. *E. coli* naturally produces low amounts of 1,2-PD, and therefore its metabolism was redesigned to produce 1,2-PD at high yield and titer

from glucose this was achieved by inactivation of competing pathways (lactate dehydrogenase and glyoxalase I), and overexpression of essential genes of 1,2-PD synthetic pathway (methylglyoxal synthase, glycerol dehydrogenase, and 1,2-PD oxidoreductase) [114]. Evolution was also used in combination with rational design for increased 1,2-PD production [115].

L-valine, an essential hydrophobic and branched-chain amino acid, is used in cosmetics, pharmaceuticals, and animal feed additives [116]. *E. coli* was redesigned for L-valine production at high yield and titer from glucose through a combination of traditional metabolic engineering and synthetic biology. Traditional metabolic engineering was used to inactivate competing pathways and overexpress acetohydroxy acid synthase I (*ilvBN*), part of the valine biosynthesis pathway. Unfortunately, the *E. coli* chassis has regulatory elements that tightly control L-valine biosynthesis, making production of valine at high yield and titer difficult. Feedback inhibition was eliminated by rational site-directed mutagenesis of acetohydroxy acid synthase III. In an excellent demonstration of the gene expression tuning techniques discussed above, transcriptional attenuation of valine biosynthesis genes *ilvGMEDA* was eliminated by replacing the attenuator leader region with the constitutive *tac* promoter. Transcriptome analysis and *in silico* simulation guided selection of additional target genes for amplification and deletion, and the final biocatalyst produced 0.378 g L-valine per g glucose, giving a titer of 7.55 g/L valine from 20%(w/v) glucose [116]. A similar strategy was also used for L-threonine production [117].

4. Redesign through Introduction of Foreign or Nonnatural Pathways

4.1. Foreign Pathways

4.1.1. Ethanol. Ethanol is a renewable transportation fuel. Replacement of gasoline with ethanol would significantly reduce US import oil dependency, increase the national security, and reduce environmental pollution [118]. However, only 9 billion gallons of ethanol were produced in 2008, and all were from corn-based production. Lignocellulose is generally regarded as an excellent source of sugars for conversion into fuel ethanol. It is, thus, desirable to design or obtain biocatalysts that can utilize all the sugar components in lignocellulose and convert them to ethanol with high yield and productivity in mineral salts medium. Native *S. cerevisiae* and *Z. mobilis* strains can efficiently convert glucose to ethanol, but cannot utilize pentose sugars. In contrast, *E. coli* strains can utilize all the sugar components of lignocelluloses but ethanol is only a minor fermentation product, with mixed acids accumulating as the major fermentation product [103]. While recent advances have been made engineering the native *E. coli* metabolic pathways for ethanol production [119], the most successful example used a foreign metabolic pathway to enable ethanol production from *E. coli* strain W (ATCC# 9637) [1].

Redesign for ethanol production was decoupled to three parts: construction of a metabolic pathway for production of ethanol as the major fermentation product, elimination of competitive NADH oxidization pathways, and disruption of side-product formation. The *Z. mobilis* homoethanol pathway (pyruvate decarboxylase and alcohol dehydrogenase) was introduced as a foreign pathway, enabling redox-balanced production of ethanol at high yield [120] (Figure 4(b)). Then fumarate reductase (*frd*) was disrupted to increase ethanol yield. The resulting strain, KO11, produced ethanol at a yield of 95% in a complex medium [121]. This strain was developed at the dawn of metabolic engineering and has been used to produce ethanol from a variety of lignocellulosic materials, as reviewed in [1].

Although the ethanol production rate of KO11 was as high as yeast, the ethanol tolerance and performance in minimal medium did not meet the desired standards. Therefore strain SZ110, a derivative of KO11 modified for lactate production in mineral salts media [35], was redesigned for ethanol production [122]. As with the design of KO11, redesign of SZ110 was decoupled to construction of an ethanol synthetic pathway, elimination of competitive NADH oxidization pathways, and blockage of side-product formation. However, this redesign strategy also included the acceleration of mixed sugar co-utilization. The lactate producing pathway was disrupted and the *Z. mobilis* homoethanol pathway was integrated into the chromosome by random insertion to select for optimal expression. The *Pseudomonas putida* short-chain esterase (*estZ*) [123] was introduced to decrease ethyl acetate levels in the fermentation broth and decrease the downstream purification cost. In addition, methylglyoxal synthase (*mgsA*) was inactivated, resulting in co-metabolism of glucose and xylose, and accelerated the metabolism of a 5-sugar mixture (mannose, glucose, arabinose, xylose, and galactose) to ethanol [25]. After using evolution to increase cell growth and production, the final strain, LY168, could concurrently metabolize a complex combination of the five principal sugars present in lignocellulosic biomass with a high yield and productivity in mineral salts medium [25].

4.1.2. L-Lactate. As described above, L-lactate is the major component of the biodegradable plastic PLA. Although many lactic acid bacteria produce L-lactate with high yield and productivity [124], they usually require complex nutrients. *E. coli* does not have a native pathway for L-lactate production, and therefore introduction of a foreign pathway was necessary.

The strategy for redesigning *E. coli* W3110 for L-lactate production was to eliminate competitive NADH oxidization pathways and then construct the desired L-lactate synthetic pathway (Figure 4(b)) [125]. The L-lactate production pathway, L-lactate dehydrogenase (*ldhL*) from *Pediococcus acidilactici,* was used and its coding region and terminator were integrated into the *E. coli* chromosome at the *ldhA* site, so that *ldhL* could be expressed under the native *ldhA* promoter. In addition, since the *ldhL* gene contains a weak ribosomal-binding region, this region was rationally replaced with *ldhA*'s RBS [125]. Following a period of metabolic evolution, the resulting strain, SZ85, synthesized 45 g/L L-lactate in a mineral salts medium with yield near theoretical

maximum (94%). However, this strain was a K-12 derivative and displayed the same problems seen with the K12-based D-lactate-producing strain described above, meaning that it was unable to completely ferment high sugar concentrations and had a low productivity (0.65 g/L/h). Therefore, the same design strategy was implemented in an *E. coli* W (ATCC# 9637) derivative. After further deleting *mgsA* gene to improve chiral purity and using metabolic evolution to improve cell growth and productivity, the final L-lactate-producing strain, TG108, could convert 12% glucose to 116 g/L L-lactate with an excellent yield (98%) and productivity (2.29 g/L/h) [22].

4.1.3. Xylitol. The pentahydroxy sugar alcohol xylitol is commonly used to replace sucrose in food and as a natural, non-nutritive sweetener that inhibits dental caries [126]. Xylitol can also be used as a building block for synthesizing new polymers [127]. Current xylitol commercial production involves hydrogenation of hemicellulose-derived xylose with an active metal catalyst [127]. Biological-based processes have also recently been developed, but although high xylitol titer was achieved by some yeast, the process requires complex medium with numerous expensive vitamin supplements [128]. While *E. coli* does not have the native capability to synthesize xylitol, a redesign strategy for strain W3110 was proposed involving a foreign metabolic pathway [26]. In the proposed redesign, glucose would support cell growth and provide reducing equivalents, while xylose would be used as substrate for xylitol synthesis (Figure 4(d)). The design strategy consisted of three major components: enabling co-utilization of glucose and xylose, separation of xylose metabolism from central metabolism, and construction of a xylitol production pathway (Figure 4(d)). In order to enable co-utilization of glucose and xylose, glucose-mediated repression of xylose metabolism was eliminated by replacing the native *crp* gene with a cAMP-independent mutant (CRP*). Xylose metabolism was separated from central metabolism by deleting the xylulokinase (*xylB*) gene, preventing the loss of xylose carbon to central metabolism. Finally, xylose reductase and xylitol dehydrogenase from several microorganisms were tested for xylitol synthetic capability, and the NADPH-dependent xylose reductase from *C. boidinii* (CbXR) was found to support optimal xylitol production. The final strain, PC09 (CbXR), could produce 250 mM (38 g/L) xylitol in mineral salts medium. The yield was 1.7 mol xylitol per mol glucose consumed, which was improved to 4.7 mol/mol by using resting cells. It was proposed that xylitol production could be further improved by increasing supply of reducing equivalents [129].

4.1.4. L-Alanine. L-alanine can be used with other L-amino acids as a pre- and postoperative nutrition therapy in pharmaceutical and veterinary applications [130]. It is also used as a food additive because of its sweet taste. The annual worldwide production of L-alanine is around 500 tons [131], and this market is currently limited by production costs. The current commercial production process converts aspartate to alanine via aspartate decarboxylase, where aspartate is produced from fumarate by aspartate ammonia-lyase catalysis [27]. An efficient fermentative process with a renewable feedstock such as glucose offers the potential to reduce L-alanine cost and facilitate a broad expansion of the alanine market into other products.

SZ194, a derivative of *E. coli* W (ATCC# 9637) that was previously engineered for D-lactate production, was used as the chassis for L-alanine production [27] (Figure 4(b)). Alanine production in the native strain uses glutamate- and NADPH-dependent glutamate-pyruvate aminotransferase. It is preferable to produce L-alanine directly from pyruvate and ammonia using an NADH-dependent enzyme, and therefore L-alanine dehydrogenase (*alaD*) of *Geobacillus stearothermophilus* was employed. The native ribosome binding site, coding region, and terminator of *alaD* gene were integrated into the *E. coli* chromosome at the *ldhA* site, so that expression of *alaD* could be controlled by the native promoter of *ldhA*, a promoter that has worked well for production of D- and L-lactate, as described above. Further redesign focused on elimination of trace amounts of lactate and increasing the L-alanine chiral purity by deleting *mgsA* and the major alanine racemase gene (*dadX*). Metabolic evolution increased the final titer and productivity by 15- and 30-fold, respectively (Figure 3). The latest L-alanine producing strain, XZ132, converted 12% glucose to 114 g/L L-alanine with a 95% yield and the excellent volumetric productivity of 2.38 g/L/h [27].

4.1.5. Combining Multiple Foreign Pathways in a Single Chassis. Although the work described above relied on the introduction of a single foreign pathway, there are other excellent examples that employ pathways from more than one organism in a single host.

E. coli was redesigned for 1,3-propanediol production using *S. cerevisiae* pathway to convert glucose to glycerol and a *K. pneumonia* pathway to convert glycerol to 1,3-propanediol [132]. *E. coli* was also redesigned for isopropanol production by combining acetyl CoA acetyltransferase (*thl*) and acetoacetate decarboxylase (*adc*) from *C. acetobutylicum* with the second alcohol dehydrogenase (*adh*) from *C. beijerinckii* and *E. coli*'s own acetoacetyl-CoA transferase (*atoAD*) [133]. Artemisinic acid, a precursor of antimalarial drug artemisin, was produced by *E. coli* following the combination of a mevalonate pathway from *S. cerevisiae* and *E. coli*, amorphadiene synthase, and a novel cytochrome P450 monooxygenase (CYP71AV1) from *Artemisia annua* [12, 134].

S. cerevisiae was redesigned for flavanone production by combining *Arabidopsis thaliana* cinnamate 4-hydroxylase (C4H), *Petroselinum crispum* 4-coumaroyl: CoA-ligase (4CL), and *Petunia* chalcone synthase (CHS), *Petunia* chalcone isomerase (CHI) [135]. A similar synthetic system producing hydroxylated flavonols was also constructed in *E. coli* with additional amplification of *C. roseus* P450 flavonoid 3′, 5′-hydroxylase (F3′5′H) fused with P450 reductase, *Malus domestica* flavanone 3β-hydroxylase (FHT), and *Arabidopsis thaliana* flavonol synthase (FLS) [136]. The

flavonoid production was significantly increased through further redesigning of the central metabolic system of *E. coli* to increase precursor (*Malonyl-CoA*) supply [137].

4.2. Modification of Natural Pathways for Production of Unnatural Compounds. One of the goals of synthetic biology is to design or construct new genetic circuits. In the examples given thus far, existing biological parts have been reassembled to engineer a biocatalyst that efficiently produces a product that already exists in nature. However, metabolic pathways can also be constructed to produce unnatural compounds.

As discussed above, directed evolution of proteins can modify their activity such that new substrates are recognized or new products are formed [138]. For example, novel carotenoid compounds were generated by evolution of two key carotenoid synthetic enzymes, phytoene desaturase, and lycopene cyclase [139]. Additionally, combinatorial biosynthesis, which combines genes from different organisms into a heterologous host, can also generate new products [140]. For example, four previously unknown carotenoids were produced by combinatorial biosynthesis in *E. coli* [141].

4.3. De Novo Pathway Design. In order to broaden the available biosynthesis space, it is essential to go beyond the natural pathways and design pathways *de novo* [142]. Although this exciting design strategy still has many challenges, several successful examples have been reported.

For example, a synthetic pathway for 3-hydroxypropionic acid (3-HP) production was designed involving the unnatural isomerization of α-alanine to β-alanine, as mentioned above. In this example the researchers used directed evolution to expand the substrate specificity of lysine 2,3-aminomutase to include α-alanine [73]. The resulting β-alanine can then be converted to 3-HP through existing metabolic pathways.

Unnatural pathways for higher alcohol production in *E. coli* were designed by combining the native amino acid synthetic pathways with a 2-keto acid decarboxylase from *Lactococcus lactis* and alcohol dehydrogenase from *S. cerevisiae* [143]. The 2-keto acid intermediates in amino acid biosynthesis pathways were redirected from amino acid production to alcohol production, enabling production of 3-methyl-1-pentanol. This pathway was then expanded for production of unnatural alcohols by rational redesign of two enzymes, with the resulting biocatalysts having the ability to synthesize various unnatural alcohols ranging in length from five to eight carbons [144].

4.4. Engineering Tolerance to Inhibitory Compounds. As our repertoire of biologically-produced compounds increases, tolerance to high product titers becomes more important. Biofuels, such as ethanol and butanol, can inhibit biocatalyst growth, and therefore the tolerance of the biocatalyst needs to be improved [145–147]. As described above, our goal is to use lignocellulosic biomass as a substrate for production of commodity fuels and chemicals. Unfortunately, the processes used to convert biomass to soluble sugars also produce a mixture of minor products, such as furfural and acetic acid, that inhibit biocatalyst metabolism [148]. Although most of these inhibitors could be removed by detoxification [149], this additional process would increase operational cost. It is, thus, desirable to obtain microorganisms that are tolerant to these inhibitors and can directly ferment hemicellulose hydrolysate.

One approach to increasing tolerance is to understand the mechanism of inhibition. Transcriptome analysis has been used to probe the response to ethanol [145, 150], furfural [151], and butanol [147]. Another approach is to use directed evolution, as highlighted by the following example. Ethanologenic *E. coli* strain LY180 (a derivative of LY168 with restored lactose utilization and integration of an endoglucanase, and cellobiose utilization) was used as the chassis to select for furfural resistance through evolution [148]. The evolved strain, EMFR9, had significantly increased furfural resistance. Reverse engineering efforts, including transcriptome analysis, attributed furfural resistance to the silencing expression of several oxidoreductases. These oxidoreductases use NADPH for furfural reduction, depleting the available pools for biosynthesis. Thus furfural-mediated growth inhibition can be attributed to NADPH depletion [148], an insight that can be applied to other biocatalyst design projects.

5. Perspectives

Although many biocatalysts have been successfully redesigned for production of industrially important fuels and chemicals through traditional metabolic engineering, we are just beginning to see the potential of synthetic biology in this area. One of the foremost goals in our lab is the improvement of biocatalysts for biomass utilization. To attain this goal, tolerance to hydrolysate-derived inhibitors needs to be improved. For all applications, tolerance to high substrate and product titers is also important. This goal of redesigning a biocatalyst's phenotype, that is, tolerance, is not as clear as redesigning metabolism and a rational redesign strategy is particularly difficult when the mechanism of inhibition is not known.

As the understanding of our biocatalysts improves, particularly through reverse engineering of evolved strains, genome-scale models can be improved. Inclusion of kinetic and regulatory effects will also improve the accuracy and predictive power of these models. Note that some models have recently been developed that bypass the need for kinetic data, though [152]. Since enzymes are the major functional part performing the metabolic synthesis, improved protein engineering tools and new protein catalytic capability will aid in advancement of this field. It is important to generate high-quality protein mutagenesis libraries (relatively small libraries with a high diversity of enzymes) to facilitate efficient screening efforts [138]. Direct screening from metagenomic libraries of environmental samples can aid in isolation of enzymes with new functions, which cannot be obtained by the traditional strain isolation methods [153]. Enzymes can even be synthesized from scratch by

a rational design strategy with computational aid [154]. Finally, new tools for better *de novo* design of synthetic pathways need to be developed. Several databases, such as BNICE (Biochemical Network Integrated Computational Explorer) [155] and ReBiT (Retro-Biosynthesis Tool) [142], have already been established to facilitate identification of enzymes to construct a complete synthetic pathway for producing target compounds. It is important to establish guidelines, such as redox balance, energy production, and thermodynamic feasibility, to screen among these enormous pathways for the optimal routes.

By including synthetic biology tools in metabolic engineering projects, and vice versa, these two fields can significantly advance the replacement of petroleum-derived commodity products with those produced from biorenewable carbon and energy.

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