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Reassessing *Escherichia coli* as a cell factory for biofuel production

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Via metabolic engineering, industrial microorganisms have the potential to convert renewable substrates into a wide range of biofuels that can address energy security and environmental challenges associated with current fossil fuels. The user-friendly bacterium, *Escherichia coli*, remains one of the most frequently used hosts for demonstrating production of biofuel candidates including alcohol-, fatty acid- and terpenoid-based biofuels. In this review, we summarize the metabolic pathways for synthesis of these biofuels and assess enabling technologies that assist in regulating biofuel synthesis pathways and rapidly assembling novel *E. coli* strains. These advances maintain *E. coli*'s position as a prominent host for developing cell factories for biofuel production.

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Introduction

The development of biofuel-producing microbes is motivated by a desire to find sustainable alternatives to fossil fuels and to reduce the net amount of greenhouse gases released through the combustion of transportation fuels [1,2]. Microbial metabolism has evolved to comprise pathways for synthesizing a variety of metabolites with fuel-compatible properties including linear, branched and cyclic alcohols, alkanes, alkenes, esters and aromatics. Microbes isolated from environmental samples often possess these metabolic pathways for biofuel synthesis [3,4]; however, they rarely synthesize biofuels in the elevated quantities needed for economically-viable processes. That said, there exist several natural isolates that produce relevant quantities of fuels, for example, efficient fermentation of sugar to ethanol by yeast or to butanol by *Clostridium* species [5], but the probability of identifying a natural isolate capable of producing other advanced biofuels is infinitesimally small. Furthermore, natural hosts rarely possess the complete suite of traits required for commercial biofuel production. Engineering native isolates into robust workhorses often requires overcoming limitations such as low growth rates, limited catabolic capabilities, poor tolerance to substrates, products, and desirable environmental conditions. For this reason, the alternative approach of engineering a model, genetically-tractable host is a common strategy for accessing the repertoire of natural and synthetic biofuel-producing pathways.

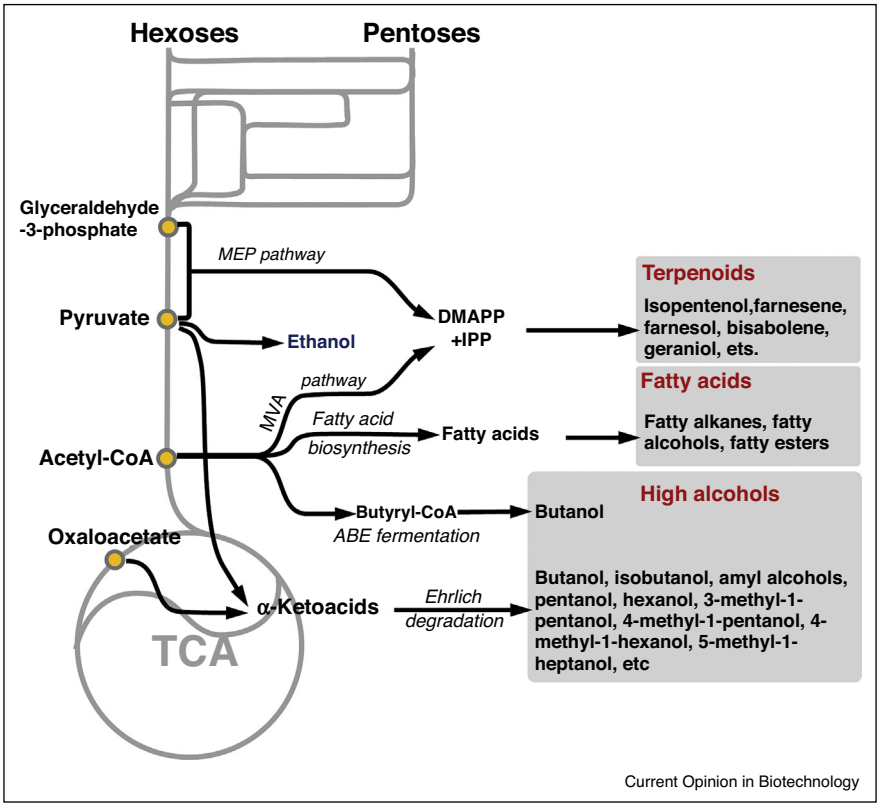
With the advent of recombinant DNA technology, harnessing a user-friendly microorganism such as *Escherichia coli* for biofuel production could be an attractive alternative to the natural isolates [6–8]. *E. coli* as a host has several advantages over other industrial microbes for biofuel production (Table 1). The advantages are as follows: (i) both aerobic and anaerobic growth using various carbon sources in defined salt media; (ii) possession of high growth and metabolic rates; (iii) a vast knowledgebase of genetic, metabolic and physiological traits; and (iv) a plethora of genetic tools available for performing metabolic engineering. These benefits encourage metabolic engineers to construct novel pathways to synthesize desired biofuel products in the genetically tractable *E. coli* host. Enormous genomic sequences have been disclosed for new metabolic reactions and are available in public databases, which provides a wealth of gene variants to construct novel and efficient pathways for biofuels production in *E. coli*. In fact, several biofuel candidates with great potential to replace traditional fuels have been investigated for production in *E. coli* over the past two decades. Such an engineering process relies on introduction of one or more genes encoding the enzymes of the pathway, which are orchestrated by tools of systems biology, metabolic engineering, and synthetic biology to effectively convert intracellular metabolites into the target products [9,10]. The pathway enzymes therefore need to be expressed in *E. coli* at a level that provides sufficient pathway activity but prevents wasteful usage of cellular resources needed elsewhere in metabolism. Similar

Table 1

Comparison of *Escherichia coli* with other industrial strains for biofuel synthesis

Strains	Biofuel types			Available genetic tools	Biofuel tolerance
	Higher alcohols	Fatty acids	Terpenoids		
Escherichia coli	• Non-native producer • Engineered for production of many higher alcohols • Titer:143 g/L isopropanol [27], 50.9 g/L isobutanol [28], 30 g/L 1-butanol [29], and so on.	• Non-native producer • Engineered for production of many fatty acid derivatives • Titer: 1.95 g/L fatty alcohols [45•], 0.6 g/L fatty alkanes [46], 1.1 g/L fatty ester [97••], and so on.	• Non-native producer • Engineered for production of many terpenoids via MEP or MVA pathway • Titer: 2.2 g/L isopentenol [61•], 0.53 g/L farnesol [63•], 1.1 g/L bisabolene [91], > 60 g/L isoprene [69], and so on.	Many	• Low tolerance to most biofuels • Many studies on tolerance engineering
Corynebacterium glutamicum	• Non-native producer • Engineered for production of few higher alcohols • Titer: 4.0 g/L isobutanol [113], 2.8 g/L 3-methyl-1-butanol, and 0.37 g/L 2-methyl-1-butanol [114]	• Non-native producer	• Non-native producer • Engineered for valencene production via MEP pathway • Titer: 2.4 mg/L valencene [115]	Not many	• Not clear for biofuel tolerance • Few studies on tolerance engineering
Saccharomyces cerevisiae	• Native ethanol producer • Engineered for isobutanol production • Titer: 1.62 g/L isobutantol [31]	• Non-native producer • Engineered for production of many fatty acid derivatives • Titer: 0.1 g/L fatty alkanes [47], 1.1 g/L hexadecanol [48], and so on.	• Non-native producer • Engineered for production of many terpenoids via MVA pathway • Titer: 0.1 g/L farnesol [64], 1.0 g/L bisabolene [68], >100 g/L farnesene [71], and so on.	Many	• High tolerance to ethanol • Not many studies on tolerance engineering
Clostridium acetobutylicum	• Native butanol producer • Engineered for high butanol production • Titer: 130 g/L butanol [30]	• Non-native producer	• Non-native producer	Few	• High tolerance to butanol • Not many studies on tolerance engineering
Pseudomonas putida	• Non-native producer • Engineered for butanol production • Titer range: 0.12 g/L butanol [21]	• Non-native producer	• Non-native producer • Engineered for geranic acid production via MVA pathway • Titer: 0.19 g/L geranic acid [116]	Not many	• High tolerance to many organic solvents • Few studies on tolerance engineering
Bacillus subtilis	• Non-native producer • Engineered for production of few higher alcohols • Titer: 24 mg/L butanol [21] and 6.1 g/L isobutanol [33]	• Non-native producer	• Native isoprene producer • Engineered for isoprene production via MEP pathway	Not many	• Not clear for biofuel tolerance • No study on tolerance engineering
Yarrowia lipolytica	• Non-native producer	• Native producer • Engineered for production of fatty acid derivatives • Titer: 0.5 g/L decanol [53], 4.98 mg/L pentane [54], and 0.64 g/L hexadecanol [55]	• Non-native producer • Engineered for α-farnesene production via MVA pathway • Titer: 0.26 g/L α-farnesene [117]	Not many	• Not clear for biofuel tolerance • No study on tolerance engineering

Figure 1



Scheme of engineered metabolic pathways to produce candidate biofuels in *E. coli*.
Introduction of the heterologous metabolic pathways (black arrows) enables recombinant *E. coli* to synthesize various candidate biofuels from the central metabolites (gray arrows) of *E. coli*. These candidates were categorized into three types: high alcohols, fatty acids and terpenoids.

engineering efforts are required to optimize the expression of genes that encode other critical traits, for example, product tolerance, without reducing flux to biofuel or depleting cellular resources.

Therefore, the essences in development of *E. coli* as a cell factory for biofuel production are the construction of novel biosynthesis pathways, the sophisticated regulation of pathways and cellular metabolism, and the manipulation of host tolerance to biofuel products. Other aspects including breakdown of biomass into usable carbohydrates are beyond the scope of this review and have been discussed elsewhere [11,12]. In this review, we will mainly assess the recent metabolic engineering achievements and enabling technologies, and highlight their great application potential to realize overproduction of biofuels in *E. coli* in the future.

Construction of metabolic pathway leading to biofuels in *E. coli*

Pioneering work on the metabolic engineering for biofuels could be traced back to the import of the ethanologenic pathway of *Zymomonas mobilis* into *E. coli* [13]. Heterologous expression of pyruvate decarboxylase and alcohol dehydrogenase results in conversion of pyruvate to ethanol in *E. coli*. However, ethanol as the first generation biofuel has lower energy density of around 30% than gasoline, and is incompatible with current fuel infrastructure owing to its highly hygroscopic and corrosive drawbacks [14,15]. To circumvent this problem, many ingenious biosynthesis pathways have been developed in *E. coli* to generate a variety of fuel-like molecules from the general metabolites (Figure 1) [16,17]. Collectively, these Biofuel candidates can be categorized into three types: alcohol-derived (larger than two-carbon), fatty acid-derived, and terpenoid-derived biofuels.

One of the most recognized and promising biofuel candidates is 1-butanol, which is naturally produced from some *Clostridium* species (*e.g.*, *C. acetobutylium*) via the typical acetone-butanol-ethanol (ABE) fermentation pathway [5,18]. The fermentative butanol synthesis is initiated with acetyl-CoAs, which are catalyzed consecutively by thiolase, 3-hydroxylbutyryl-CoA dehydrogenase, crotonase and butyryl-CoA dehydrogenase for synthesis of butyryl-CoA, and finally converted to 1-butanol

by aldehyde/alcohol dehydrogenase. This pathway has been reconstructed in *E. coli* by several research groups for non-native production of butanol [19–21]. Amino acid metabolism leads to the synthesis of many interesting small organic acids including α-ketoacids that can be converted to alcohols with promising fuel properties. Production of C_3–C_5 alcohols was demonstrated in *E. coli* using the Ehrlich degradation pathway from yeast; the pathway consists of a promiscuous decarboxylase converting α-ketoacids to aldehydes and a dehydrogenase converting aldehydes to alcohols [22]. Alcohol mixtures including propanol, butanol, isobutanol, and amyl alcohols were produced from the amino acid precursors of various α-ketoacids in *E. coli* [23]. Engineering of enzymes involved in leucine synthesis pathway enabled recursive elongation of α-ketobutyrate into long-chain α-ketoacids, and resulted in production of longer chain (C_5-C_8) alcohols including pentanol, hexanol, 3-methyl-1-pentanol, 4-methyl-1-pentanol, 4-methyl-1-hexanol and 5-methyl-1-heptanol [24,25]. In addition, overexpression of *O*-acyltransferase allowed the recombinant *E. coli* to produce various acetate esters using acetyl-CoA as an acyl donor [26]. Other types of esters such as butyrate and isobutyrate esters could be also produced by combination of diverse acyl-CoA synthesis with alcohols production. In addition to serving as a demonstration host for the function of biofuel pathways, *E. coli* has been successfully engineered to increase the performance (TRY: titer, rate, and yield) of alcohol (1-propanol, isopropanol, 1-butanol, isobutanol, *etc.*) production beyond other species (Table 1). Engineered *E. coli* strains produced isopropanol of 143 g/L, 1-butanol of 30 g/L, and isobutanol of 50.9 g/L, respectively [27–29]. *C. acetobutylicum* as a native butanol producer produced 1-butanol up to 130 g/L [30], but failed in the production of other higher alcohols. *Saccharomyces cerevisiae* was engineered to produce isobutanol at a titer of 1.62 g/L only [31]. *Corynebacterium glutamicum*, a well-known amino acid producer, was also engineered to produce isobutanol based on its effective L-valine synthesis pathway. The productivity obtained from the engineered *C. glutamicum* is 13 g/L of titer, 0.32 g/L/h of rate, and 0.20 g/g glucose of yield, which is however much lower than the productivity (titer, 50.9 g/L; rate, 0.70 g/L/h; and yield, 0.29 g/g glucose) obtained from an engineered *E. coli* [27,32]. *Bacillus subtilis* was made to produce butanol and isobutanol albeit a quiet low in titer [21,33], but the conversion of protein waste to biofuels was a benefit of using *B. subtilis* [34].

Fatty acids are natural precursors to many classes of compounds found in current fuels (*e.g.*, alcohols, alkanes, and esters) but are more commonly thought of as the main component of naturally occurring lipids, such as phosphoglycerides of cell membrane constituents and triglycerides of energy storage materials. Biosynthesis of fatty acids is an iterative carbon-chain elongation process using acetyl-CoA as a primer, and malonyl-CoA or methylmalonyl-CoA as extending molecules by type I fatty acid synthase (FAS) in animals and fungi, or type II FAS in most bacteria. Fatty acids can be converted to fatty acid esters by promiscuous acyltransferases, to fatty alcohols by reductase and alkanes by decarboxylases [35–37,38•]. For example, the aldehyde reductase YbbO of *E. coli* has been re-illuminated to reduce fatty aldehyde to fatty alcohol production [38•]. The major concern for fatty acid-derived biofuels in terms of fuel properties is to synthesize various fatty acids with different chain lengths or branched chains that alter their physical properties (*e.g.*, cloud point). Chain length can be controlled by thioesterases expressed in many bacteria and plants and the introduction of these thioesterases in *E. coli* enabled production of fatty acids with different chain lengths [39,40]. The 1,4-dihydroxy-2-naphthoyl-CoA thioesterase YdiI of *E. coli* could function as a cycle-terminating enzymes to hydrolyze acyl-CoAs and produce corresponding short- and medium-chain α,β-unsaturated carboxylic acids [41]. The unexpected role of YdiI could be assessed in *E. coli* with an engineered reversal of the β-oxidation. Replacement of a straight chain acyl-CoA-specific β-ketoacyl-ACP synthase (straight FabH) with a branched-chain-acyl-CoA-specific homolog (branch FabH) led to the synthesis of branched chain fatty acids [42]. Complete lipoylation of 2-oxoacid dehydrogenase by engineering of protein lipoylation pathways increased the percentage of the branched chain fatty acids to 77% of total fatty acid production [43]. The fatty acid β-oxidation cycle was reversed to increase the carbon and energy efficiency of acyl-CoAs synthesis by using acetyl-CoA instead of malonyl-CoA for the acyl-chain elongation, which resulted in higher production of fatty alcohols [44]. *E. coli* and *S. cerevisiae* have been engineered to produce fatty alcohols and fatty alkanes, albeit relatively low in the production titers caused by a low efficiency of the relevant enzymes. *E. coli* produced 1.95 g/L fatty alcohols and 0.58 g/L fatty alkanes [45•,46], whereas *S. cerevisiae* produced 1.1 g/L of fatty alcohols and 0.1 g/L of fatty alkanes, respectively [47,48]. *E. coli* was superior to *S. cerevisiae* in production of both fatty alcohols and alkanes. Oleaginous microorganisms such as *Yarrowia lipolytica*, which are likely to accumulate lipids in a high content, can be a good host for production of fatty acid-derived biofuels (Table 1) [49,50]. An engineered *Y. lipolytica* expressing acetyl-CoA carboxylase (ACC) and diacylglycerol acyltransferase (DGAT) presented the overall yield of 0.195 g lipids/g glucose, an equivalent to 61% of the theoretical maximum yield [51]. The further overexpression of Δ-9 stearoyl-CoA desaturase (SCD) improved the production yield to 0.234 g/g glucose (85% of the theoretical maximum yield) [52]. However, metabolic engineering of *Y. lipolytica* for fatty acid-derived biofuels is still in its infancy and remains to be developed by more studies [53–56].

Terpenoids are a large family of natural products with great pharmaceutical and nutritional interests. They have been also considered as ideal drop-in biofuel molecules because of their long chain hydrocarbon structure. Biosynthesis of terpenoids is initiated from two universal C_5 units, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), which can be condensed by a head-to-tail manner into a series of isoprenyl diphosphate: geranyl diphosphate (GPP, C_{10}), farnesyl diphosphate (FPP, C_{15}) and geranylgeranyl diphosphate (GGPP, C_{20}). Metabolic engineering of mevalonate (MVA) pathway and 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway for the synthesis of IPP and DMAPP has been reviewed elsewhere [57,58]. Terpenoids with simple olefins structure render promising physical and chemical traits as biofuels, which can be produced by simple dephosphorylation of the isoprenyl diphosphate such as IPP, DMAPP, GPP and FPP. As terpene synthases and phosphatases mediating the dephosphorylation serve a critical role in production of terpenoid-derived biofuels, a challenge is to secure an appropriate enzyme from either plants or bacteria converting isoprenyl diphosphates to terpenoid biofuels. Production of isopentenols was reported only from *E. coli*. Screening of *Bacillus subtilis* genomic DNA library revealed the Nudix hydrolase NudF hydrolyzing DMAPP and IPP to prenol and isoprenol [59], which are collectively called isopentenols. The investigation of Nudix (Nucleoside diphosphate linked to X) family members from *E. coli* demonstrated another good candidate, Nudix hydrolase NudB [60]. Both NudF and NudB have been used for isopentenol production in *E. coli*, and 2.23 g/L of isopentenol (70% of apparent theoretical yield) was produced in the engineered *E. coli* overexpressing NudB [61•,62]. *E. coli* undecaprenyl diphosphate phosphatases, YbjG and PgpB, have been recently identified to hydrolyze FPP to farnesol, and successfully applied for farnesol production of 526.1 mg/L in a shake flask [63•], which was significantly higher than the farnesol production (145.7 mg/L) of *S. cerevisiae* in a bioreactor [64]. Thus, some promiscuous phosphatases have been re-evaluated as important participants in production of terpenoid alcohols. To date, the advances in engineering of terpene synthases have also proceeded production of pinene, geraniol, limonene, farnesene and bisabolene [65–69], although the reported production titers have been limited at a scale of mg/L in a shake flask culture. Terpenoid molecules are promising alternatives to diesel and jet fuels caused by their branched hydrocarbon chains and various ring structures, which lower their freezing temperature [68]. However, production of these molecules has required significant effort and successful demonstrations are being reported (*e.g.*, isoprene (C_5, hemiterpene) production in *E. coli* and farnesene (C_{15}, sesquiterpene) production in *S. cerevisiae*) [70,71]. By optimizing fermentation conditions, an engineered *E. coli* strain produced more than 60 g/L of isoprene with volumetric productivity of 2 g/L/h, and an engineered *S. cerevisiae* produced more than 100 g/L of farnesene with volumetric productivity of up to 2.24 g/L/h in a bioreactor. The significant production of isoprene and farnesene encourages us to improve productivity of terpenoid biofuels by a creative engineering of host strains and a fermentation optimization in bioreactor (Table 2).

New enabling technologies for biofuel production in *E. coli*

To build a synthetic metabolic pathway in *c* one typically collects genes from various organisms and combines them with gene expression signals (*e.g.*, promoter, terminator, ribosome binding site—RBS) in expression vectors. Genes are often selected based on their performance in their native host or in *in vitro* assays. Transplantation of genes between hosts is not always able to recapitulate these advantages. The performance of the heterologous pathway can suffer from incorrect or poor expression of proteins, promiscuous activity of enzymes on substrates not found in the native host, accumulation of toxic intermediates, and cross-talk with native host metabolism. Subcellular localization of certain eukaryotic enzymes of terpenes synthesis is also a challenge in their implementation in *E. coli* owing to lack of organelles in the host. For these reasons, metabolic engineers have developed a wide array of tools for optimizing functional expression of heterologous pathways. Common approaches include re-coding of genes to optimize codon usage, overexpression of rate-determining enzymes, titrating gene expression with plasmid copy number, promoter strength and RBS strength, and screening combinatorial libraries of genes and control elements [72,73]. In addition, numerous synthetic biology techniques have been developed to enhance the rate of strain construction and to increase the complexity of gene regulation [74,75].

The bacteria adaptive immunity system CRISPR (clustered, regularly interspaced, short palindromic repeats) technology provides a powerful genetic tool for genome editing and gene silencing [76••,77••]. The endonuclease Cas9 with guide RNA (gRNA) can specifically recognizes and cleaves target DNAs, which facilitates generation of mutations, deletions or insertions for engineering of host strain (Figure 2a). A large DNA fragment encoding an isobutanol synthesis pathway was effectively integrated into *E. coli* genome by using a Cas9/CRISPR-based one-step approach [78]. A Cas9 mutant, which has lost the endonuclease activity, has been used to block transcriptions through its binding to target genes, which allows a simultaneous repression of multiple genes by using multiple gRNAs (Figure 2a). Such a CRISPR interference (CRISPRi) technology was used to repress the transcription factor FadR, which resulted in an increase in fatty acid synthesis and a reduction in β-oxidation in *E. coli* [79]. Kim *et al.* used the same approach to regulate a heterologous MVA pathway for production of bisabolol as well as to

Table 2

Enzymes involved in metabolic engineering of *E. coli* for biofuel synthesis

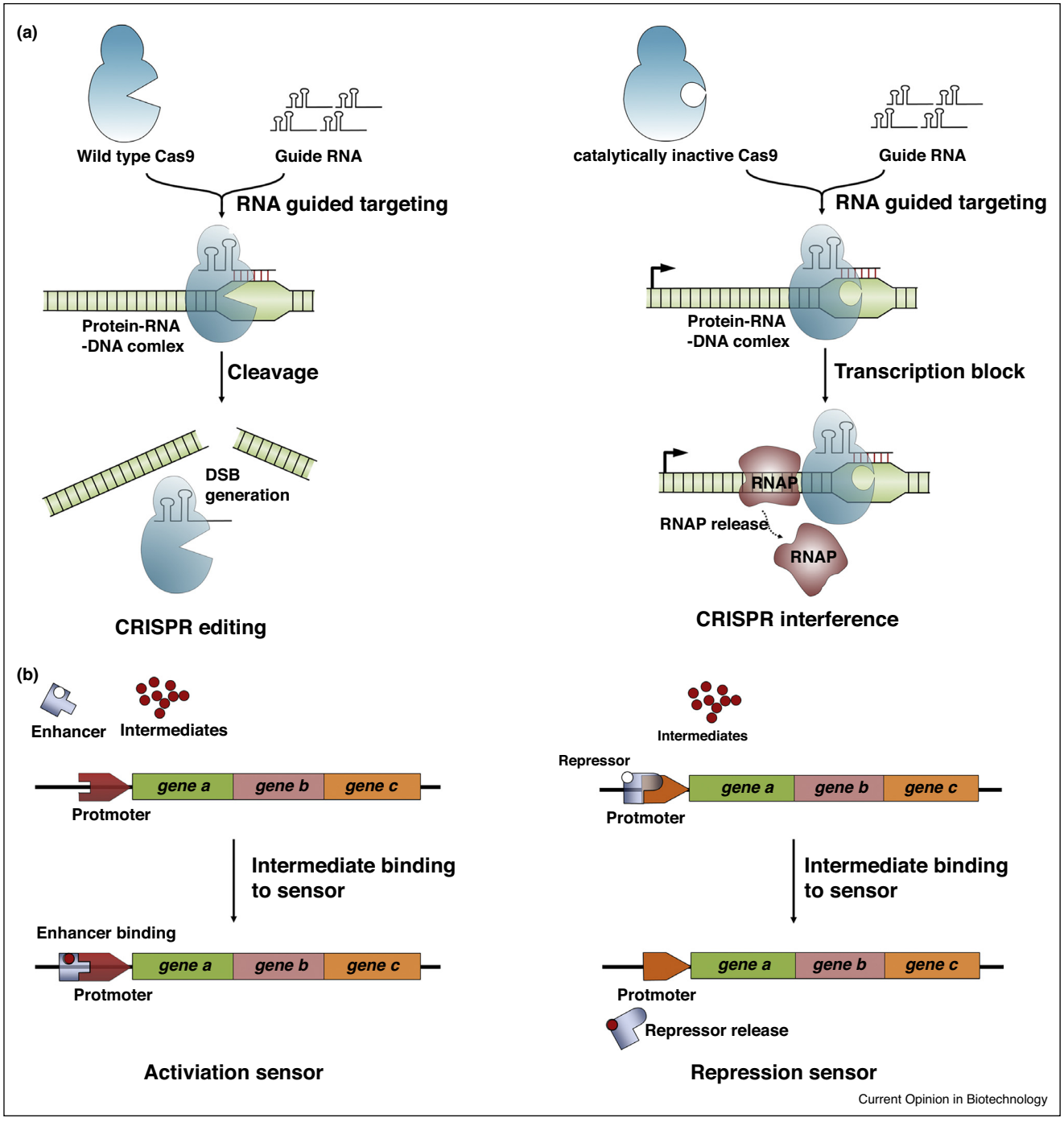
Pathway types		Engineered enzymes	Products
Alcohols	Ethanologenesis	Pyruvate decarboxylase; Alcohol dehydrogenase	Ethanol
	Ehrilich degradation	α-Ketoacids decarboxylase; Aldehyde dehydrogenase	Butanol, Isobutanol, Amyl alcohols, Pentanols, Hexanol, 3-Methyl-1-pentanol, 4-Methyl-1-hexanol, 5-Methyl-1-heptanol, and so on.
	ABE fermentation	Thiolase; 3-Hydroxylbutyryl-CoA dehydrogenase; Crotonase; Butyryl-CoA dehydrogenase Aldehyde/alcohol dehydrogenase	Butanol, Ethanol
Fatty acids	Fatty acid synthesis	Acetyl-CoA carboxylase; Fatty acid synthases (*e.g.*, FabH); Thioesterase; Promiscuous acyltransferase, reductase or decarboxylase	Fatty alcohols; Fatty esters; Fatty alkanes
	Reversal of β-oxidation	AtoB (acetyl-CoA acetyltransferase); Aldehyde/alcohol dehydrogenase; Thioesterase; YdiO (acyl-CoA dehydrogenase/enoyl-CoA reductase); FadB (3-hydroxyacyl-CoA dehydrogenase and enoyl-CoA hydratase; FadA (3-ketoacyl-CoA thiolase)	Butanol; Other fatty alcohols
Terpenoids	MEP pathway	MEP pathway enzymes Isoprenyl diphosphate synthase Terpene synthases	Isopentenol, Geraniol, Farnesol, Farnesene, Bisabolene, and so on.
	MVA pathway	MVA pathway enzymes Isoprenyl diphosphate synthase Terpene synthases Promiscuous phosphatases	

repress a chromosomal *ispA* gene for production of isoprene [80]. Antisense RNAs (asRNAs), which are able to complementarily bind to 5′-untranslated region (5′-UTR) of their cognate transcripts, have been created and applied for precise control of gene expression [81]. The tailored asRNAs targeting *fabB* and *fabF* effectively repressed malonyl-CoA synthesis in *E. coli* [82]. Small regulatory RNAs (sRNAs) can also target their complementary mRNAs, which recruits host factor Hfq and subsequently induce the mRNA degradation [83]. Inspired from this, artificial sRNAs have been designed with the predictable repression efficiency to improve production of tyrosine and 1,3-diaminopropane [84,85].

Prokaryotic *E. coli* allows expression of a synthetic pathway in a single operon, which is convenient for transcriptional control of multi-genes, but ineffective for modulation of expression level of each gene independently. The expression level of each gene in an operon can be modulated by rational design of the ribosomal binding site (RBS) to have a desired translational initiation rate (TIR). *In silico* program is available to guide the RBS design [86], which has been validated in *E. coli* by many examples such as production of geraniol and amorphadiene [73,87]. Single stranded mRNA easily forms secondary structures affecting the translational efficiency, which is worse in lengthy ploycistronic transcripts. CRISPR-based mRNA processing has been developed for physical separation of each cistron to minimize the translational interference caused by the formation of secondary structures of ploycistronic transcripts [88]. An entire synthetic pathway can also be divided into several modules and the modules are combinatorically combined and modulated to maximize the pathway efficiency, which is called multivariate modular metabolic engineering (MMME) [89]. Besides, the advance of Systems biology is helping us to mitigate bottleneck of the engineered pathway and to understand the complex crosstalk that occurs between the pathway and the native metabolic network. Targeted proteomics via selected-reaction monitoring (SRM) mass spectrometry was used to optimize the amorphadiene synthetic pathway in *E. coli* [90]. A computational tool termed as principal component analysis of proteomics (PCAP) was consequently developed to mine a quantitative data set of targeted proteomics and to guide metabolic engineering for production of limonene and bisabolene [91]. A recent report described a workflow that integrates metabolomics, proteomics and genome-scale models of *E. coli* metabolism to tailor the host to be suitable for biofuel production [92].

Living organisms have evolved bifunctional enzymes such as MvaE with dual roles of acetoacetyl-CoA synthase and HMG-CoA reductase in the MVA pathway of *Enterococcus faecalis* or organized consecutive enzymes of a metabolic pathway in a complex such as pyruvate dehydrogenase complex to reduce intermediate accumulation and improve the pathway efficiency [93,94]. Artificial fusion of FPP synthase and farnesene synthase significantly improved farnesene production through the

Figure 2



Schemes of CRISPR technology for gene editing and silencing **(a)**, and biosensor modes for pathway regulation **(b)**.

directed flow of IPP and DMAPP to farnesene [67]. Artificial protein scaffolds could spatially organize pathway enzymes in a desired stoichiometric ratio for optimal metabolic flux in the synthetic pathway. Organization of the MVA pathway by protein scaffolds showed a great promise for mevalonate production [95]. Beyond these static regulation approaches of gene expression, the application of biosensors opens up a new avenue to dynamic control of metabolic pathways and cellular behaviors. Biosensors have been devised to positively or negatively respond to accumulation of metabolites (Figure 2b). FPP responsive promoter was screened by comparative

transcriptomics of *E. coli* with accumulation and no accumulation of FPP [96]. The FPP responsive promoter-based biosensor could sense the FPP accumulation in cells, which is applied to dynamic regulation of expression of sesquiterpene synthases. The FPP biosensor showed a great advantage over the inducible *trc* promoter for amorphadiene production in *E. coli* by dynamic expressional regulation of amorphadiene synthase in response to FPP accumulation [96]. FadR-based dynamic sensor-regulation system (DSRS) was developed to sense fatty acyl-CoA, which thereby regulated biosynthesis of fatty acid ethyl ester [97••]. Malonyl-CoA responsive FapR was rewired to dynamically control acetyl-CoA carboxylase and fatty acid synthase involved in malonyl-CoA generation and consumption, respectively [98].

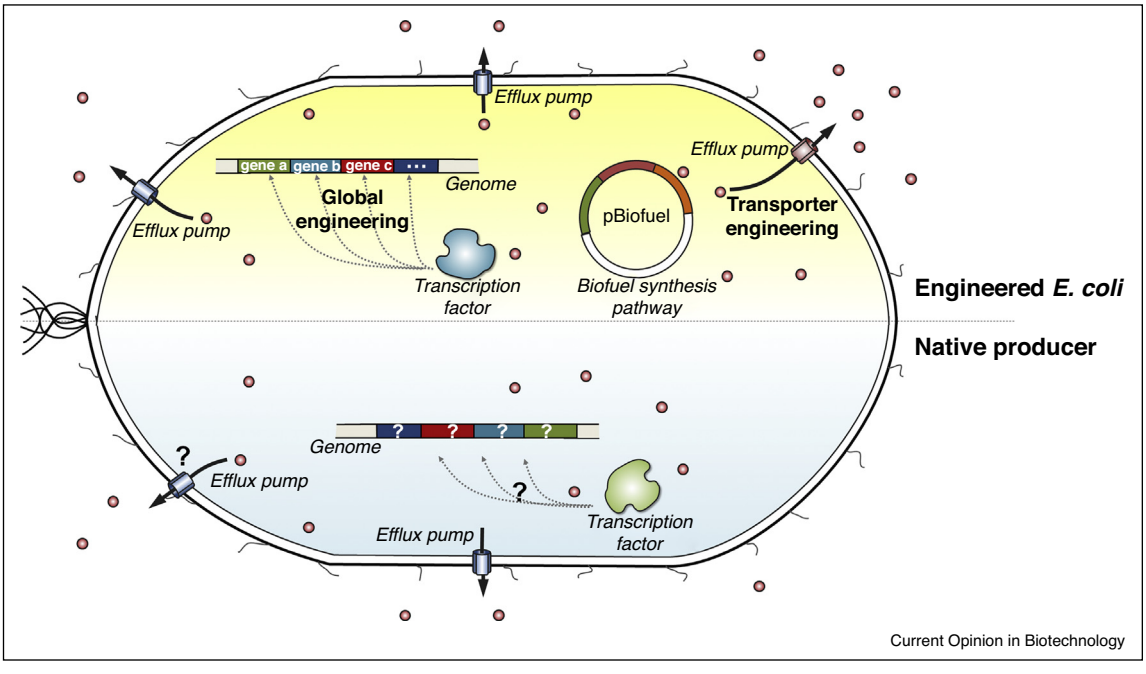
Another important concern in construction of a synthetic pathway is substrate stero-specificities of the enzyme components. The inappropriate assembly of butanol synthetic pathway disregarding the substrate stereo-specificity of crotonases severely impaired the pathway efficiency, although crotonase homologs have similar kinetic behavior to their preferred 3-hydroxybutyl-CoA steroisomers [99].The inherent enzyme mechanism affords a powerful regulatory strategy called kinetic control element or driving forces to improve the efficiency of the entire pathway. The crotonyl-CoA reductase can serve as a regulatory valve to direct butanol synthesis [29,99].

Tolerance engineering of *E. coli* host against biofuels

E. coli is a non-native biofuel strain from the viewpoint to *S. cerevisiae* and *C. acetobutylicum* naturally producing fuel-like ethanol and butanol. Thanks to advances in synthetic biology, *E. coli* can be drastically engineered to produce drop-in biofuels, while the accumulated organic solvents are generally toxic to *E. coli* cells. Many strains such as *Psedumonas putida* possess their own built-in tolerance mechanisms against toxic organic solvents, whereas *E. coli* is not competent to deal with toxicity of the biofuels produced in it. A prerequisite to create ideal *E. coli* host for biofuel production is the tolerance engineering and development of associated technologies (Figure 3).

Living organisms employ various strategies against cytotoxicity of organic solvents, such as change of membrane composition, activation of stress response genes and expression of efflux pumps, which could be applied to manipulation of *E. coli* against toxic biofuels. Expression of *Geobacillus sp.* thioesterase, which primarily targets unsaturated medium chain length acyl-ACPs, restored a normal membrane lipids saturation level and improved tolerance towards high production of free fatty acids

Figure 3



Engineering schemes of efflux pump and transcription factor for tolerance improvement.
Introduction of biofuel synthesis pathway (pBiofuel) in *E. coli* leads to the accumulation of biofuel products, which would cause cytotoxicity in the host cell (upper panel). An engineered efflux pump transports the biofuel products out of cell to reduce the toxicity, and an engineering of transcription factor can regulate global gene expression of the host to improve the biofuel tolerance (upper panel). In a native biofuel producer (lower panel), the tolerance engineering has been restricted caused by a limited information on efflux pump and transcription factor of the host.

[100]. Overexpression of MarA involved in multiple antibiotic resistances could improve *E. coli* tolerance to geraniol [101], which was probably caused by the MarA-mediated regulation of many stress responsible genes and ArcAB efflux pump. Engineering of efflux pump to expel biofuels out of cells would be the most promising strategy to improve host strain tolerance [102]. Several heterologous efflux pumps were evaluated to secrete terpenoid-based biofuels and found to improve biofuels yields [103,104]. The availability of outer membrane protein TolC to its inner membrane partners to form tripartite efflux channel was elucidated to play an important role in the host tolerance to medium-chain (C_4–C_7) alcohols [105•]. Through knock-out of the major efflux pump AcrAB, the increased TolC availability to the relevant inner membrane efflux pumps significantly improved the *E. coli* tolerance to medium-chain alcohols including 1-butanol and isopentenol. These rational approaches weigh on prior knowledges on regulator mechanism and efflux pump preference; however, the advances in "Omics" technology provide a random approach to elucidate new tolerance mechanism and engineering rationales. It is possible to identify the sense mutations accumulated in a long-term adaptive evolution and to intensify the understanding of the physiological response network of cells to biofuels [106–108], which would contribute to tolerance engineering of the host with desired phenotypes.

Transcription initiation factors, the so-called sigma factors, such as RpoD can regulate a set of genes and change global metabolism to combat exotic stresses in *E. coli* [109]. Thus, the engineering of the sigma factors, which is called global transcription machinery engineering (gTME), offers another convenient and efficient method to improve host tolerance, because the sophisticated works of tolerance manipulation can be simplified to the level of engineering a single endogenous transcription factor. The error-prone RCR-based gTME has been developed to improve *E. coli* tolerance to biofuels and exposed several potential engineering targets for further strain improvements [110]. Biofuel tolerance is a complicated multi-genic phenotype involving up-regulation of heat shock proteins, induction of efflux pumps, modifications of cell membrane and envelope, modifications of energy generation and utilization, and other unknown tolerance mechanisms [111,112]. It is difficult to achieve the enhanced biofuel tolerance traits without deep understanding of genetic, metabolic, and physiological characteristics of host strains. Thus, the most of successful engineering work on biofuel tolerance has been reported in *E. coli*.

Conclusions

During the past decade, a diverse array of natural and artificial biofuel synthesis pathways have been demonstrated in the genetically tractable bacterium, *E. coli*. Given the speed with which *E. coli* can be engineered, many demonstrations have advanced to near-commercial levels of biofuel production. While many targets are not yet produced in sufficient quantities, new synthetic and systems biology tools are enabling the creation of sophisticated regulatory and pathway assembly strategies. Similar efforts will be used to enhance other traits critical to industrial biofuel synthesis. In our opinion, these endeavours will keep *E. coli* as a preferred host when developing microbial cell factories for biofuel production

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