Metabolic engineering of *Escherichia coli* for biofuel production

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Global energy demand and environmental concerns have stimulated increased efforts to develop more sustainable and cost-effective fuels with economical production processes that would make it feasible to replace petroleum-based fuels. The biological synthesis of such fuels relies on the exploitation of the diverse metabolic pathways leading to fuel-like biomolecules and has further opened up the possibility of synthesizing biofuels other than those naturally produced through fermentative pathways. This is because, as a framework, metabolic engineering has made it possible to reconstruct and assemble biosynthetic pathways in user-friendly microorganisms for *de novo* synthesis of fuel molecules. To highlight the advancements and the tremendous potential that exists, we review the recent progress in engineering of *Escherichia coli* for biofuel production.

**Biofuels as an alternative to petroleum fuels**

Petroleum-based fuels have provided the means to achieve the economic growth and life improvements experienced since the 19th Century. However, an array of political and environmental concerns have recently arisen, related to energy security and climate change issues. In an effort to address these issues, governments and leading oil companies are now investing in big shifts in the use of energy, with billions in funding aimed towards developing renewable alternative sources that would turn simple and complex sugars into biofuels with the use of engineered microorganisms. Compared with other energy forms (i.e., solar, wind and nuclear energy), biological fuels are more desirable as they have a more favorable carbon footprint and are compatible with current fuel infrastructure as far as storage, distribution and engine compressibility is concerned [1].

Having evolved for millions of years, microorganisms can produce a variety of chemicals such as alcohols, hydrocarbons and esters, which could serve as an inexhaustible pool for fuel substitutes. However, fuel-like compounds cannot be efficiently synthesized from native organisms for cost-effective application, except for ethanol, which has been produced traditionally from yeast. For this reason, a variety of new concepts and approaches must be introduced to make biofuels a sustainable alternative.

**Metabolic engineering** has emerged as a powerful technological platform for improving the cellular phenotype, such as production ability [2]. Metabolic engineering can be defined as the design of native or entirely new metabolic pathways in a cell. It provides an integrative method to manipulate the metabolic pathway within the context of the whole metabolism rather than a single reaction step (or a single gene) [3]. The user-friendly host, *Escherichia coli*, has been given preference for metabolic pathway manipulation in the biotechnological realm due to its substrate suitability, extremely fast growth rate, tractable genetic system, as well as our extensive knowledge of its overall physiology [4]. In this review, we briefly summarize some recent advances associated with metabolic pathway modifications in *E. coli* for production of biofuels and the challenges involved thereafter. Prospective biofuels such as ethanol, butanol, higher chain alcohols, biodiesel and isoprenoids and the corresponding biosynthetic pathways are emphasized in this article.

**Ethanol production in *E. coli* using the fermentative pathway**

Ethanol, currently the most prevalent form of biofuel, has gained tremendous success in the last three decades. According to the Renewable Fuels Association, more than 100 biofuel factories in the USA produced,

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6.5 billion gallons of bioethanol in 2007. To date, the majority of ethanol is produced from corn starch by yeast. However, corn-derived ethanol has several problems, with the most alarming being the synergistic effect of its production with a dramatic increase in food prices. The International Organization for Economic Cooperation and Development (OECD) has warned that the “rapid growth of the biofuels industry” could bring about fundamental shifts in agricultural markets and could even “cause food shortages”. Furthermore, corn-derived ethanol may not be as energy efficient as required. This is because a significant amount of energy is required both for growing the corn and running fermentation facilities. In fact, estimates suggest that only 25% more energy is obtained from corn-derived ethanol than is used to produce it. Finally, the availability of arable land is another significant constraint. According to some estimates, even if all the corn planted in the USA were used for ethanol, only approximately 12% of gasoline would be displaced. For all these reasons, the development of cost-efficient microbial systems for conversion of cellulosic material (wood, agricultural waste and grasses) to ethanol has been under the spotlight around the world [5]. Besides relying on a vast supply of feedstock, the growth of cellulosic material requires far less energy than corn. The US Department of Energy has established the goal to replace approximately 30% of petroleum-based fuels with cellulosic ethanol by 2030.

Besides issues related to the carbon source, large-scale replacement of gasoline with ethanol is hampered by other roadblocks; primarily the current producer organism (Saccharomyces cerevisiae) cannot efficiently metabolize pentoses, the main components of lignocellulose hydrolysates after pretreatment, to fermentable sugars [6,7]. As such, a significant amount of effort has been devoted towards engineering pentose metabolism in yeast for hemicellulosic ethanol production, which has been covered in other reviews [8,9]. Unlike yeast, E. coli is naturally able to convert a number of pentose and hexose sugars into a mixture of acids (succinic and acetic acid) and ethanol via the heteroethanologenic pathway. One disadvantage is that this pathway can only produce 1 mole of ethanol from 1 mole of glucose, which is relatively inefficient as opposed to the homoethanologenic pathway, the Embden–Meyerhof–Parnas (EMP) pathway by Saccharomyces species and the Entner–Doudoroff (ED) pathway by Zymomonas species. Consequently, significant effort has been devoted into engineering the existing E. coli pathways for the purpose of efficient production of ethanol.

In heteroethanologenic organisms such as E. coli, ethanol is generated from pyruvate, a reaction catalyzed by pyruvate formate lyase (PFL). At the same time, the cofactor NADPH must be regenerated by reducing the oxidative intermediates (i.e., lactate and fumarate), a process that is known as mixed acid fermentation. For homoethanologenic organisms (e.g., yeast and Zymomonas mobilis), ethanol is generated as the sole product from pyruvate decarboxylase (PDC). Engineering of the homoethanologenic pathway into hexose- and pentose-fermenting E. coli has been reported by Ingram et al. [10]. Briefly, genes encoding pyruvate decarboxylase (pdc) and alcohol dehydrogenase (ADH II [adhII]) from Z. mobilis have been combined into a portable ethanol production cassette under the control of an artificial per operon and integrated into the chromosome of E. coli B at the pyruvate formate lyase (pfl) locus to generate E. coli strain KO11. The fumarate reductase gene (fdr) and lactate dehydrogenase gene (ldh) were also knocked out to minimize succinate and lactate production (Figure 1). The resulting KO11 strain was able to effectively direct carbon flux to higher ethanol production. One explanation for this result is that the introduced PDC from Z. mobilis has much higher affinity to pyruvate (low K_m value), therefore eliminating or attenuating the competing pathway for consumption of pyruvate and leading to the exclusive production of ethanol.

Based on transcriptional profiling analysis, the same authors investigated the expression levels of 30 genes involved in xylose catabolism in the parental (B) and engineered (KO11) strains; increased glycolytic flux and expression levels of glycolytic genes were demonstrated in the strain KO11 during xylose fermentation [11]. Recently, a novel mutant of E. coli KO11 (E. coli SE2378) that relies only on native enzymes has been constructed for homoethanologenic fermentation, which could effectively ferment both hexose and xylose to ethanol. This strain has a mutation within the pdb operon (pdbR aceEF ldpl), which encodes components of the pyruvate dehydrogenase complex. A novel anaerobic pathway that involved pdb was also proposed to explain the homoethanologenic mechanism [12].

E. coli strain KO11 can achieve high ethanol titer in rich media; however, this strain performs poorly in minimal media. The low ethanol production by KO11...
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Review

Metabolic network redundancy evolved by natural selection has allowed organisms to survive in diverse environments. The resulting biological complexity is manifested (among others) by an intricate metabolic network comprised of highly interconnected reactions; however, it was recently shown that this network can be reduced to uniquely organized pathways with minimal metabolic functionality, through which a minimal cell that is committed to produce a certain compound, such as ethanol, in the most efficient way can be constructed [17]. More specifically, based on the nongrowth-associated production and balanced redox state assumption, the authors employed an iterative algorithm to optimize the functionality of the central metabolic network, resulting in a reduced network that consists of six pathways (four for substrate decomposition and two for cell anaerobic growth) from over 15,000 possible combinations. The constructed strain is free from catabolite repression and able to simultaneously convert pentoses and hexoses to ethanol at the theoretical yield. Based on elementary mode analysis, an E. coli strain with nine gene knockouts for efficient production was identified to be due to the suboptimal partitioning of pyruvate for biosynthesis and the corresponding inhibitory effect of NADH on citrate synthase [13,14]; therefore, the biomass yield and ethanol titer could be partially restored by addition of pyruvate or ketoglutarate to oxidize the excess NADH. This hypothesis has been further verified by expression of a NADH-insensitive citrate synthase (NADH feedback-resistant mutant of citrate synthase) from Bacillus in E. coli.

For mixed-substrate fermentation, organisms will utilize glucose in preference to other sugars, which is a global regulatory mechanism and well known as the catabolite repressor. Relieved or eliminated catabolite repression would be beneficial for primary-product formation in mixed-substrate fermentation. Accordingly, a catabolite repression-deficient strain that can simultaneously utilize glucose, arabinose and xylose was constructed by introducing a mutation to the phosphoenol pyruvate–glucose phosphotransferase system (ptsG-) in E. coli [15]. This 6-pyruvoyl-tetrahydropterin synthase-deficient strain was able to efficiently convert complex substrate mixtures to ethanol. The relaxed control of the cAMP-CRP regulatory system over transcription was also confirmed by global gene expression (DNA array) analysis in the engineered xylose-grown strain [16]; and the measured mRNA levels of many genes showed good consistency with the calculated flux distribution.

Figure 1. Homoethanologenic pathway in engineered Escherichia coli. The black arrows represent the native pathway; dash lines indicate multi-enzymatic steps; the underlined italicized gene names represent the pathway that has been overexpressed and gene names with strikethrough lines represent pathways that have been knocked out.

AK: Acetylphosphate kinase; EMP: Embden–Meyerhof–Parnas; GAPD: Glyceraldehyde-3-phosphate dehydrogenase; HK: Hexose kinase; PEP: Phosphoenolpyruvate; PEPC: Phosphoenolpyruvate carboxylase; PFL: Pyruvate formate lyase; XK: Xylulose kinase.
conversion of glycerol to ethanol was designed [18]. In defined medium, the evolved strain could convert 40 g/l of glycerol to ethanol in 48 h with 90% of the theoretical ethanol yield, which demonstrated the usefulness of this analytical tool.

Final ethanol titer is an important determinant of the overall cost because it significantly affects the downstream process (distillation), another factor for competitive production of ethanol [2]. Low ethanol titer could be caused by many factors, such as limited ethanol-tolerance capability and the inhibitory effect of feedstock hydrolysates (i.e., aromatics, furfurals, furan derivatives and phenolics) on cell growth. To address this issue, several attempts have been made to develop ethanol-tolerant and byproduct-resistant strains. The ethanol-tolerant strain E. coli LY01, which was obtained by directed evolution of homoethanologenic E. coli KO11, could produce up to 60 g/l ethanol [19]. Gene arrays were used to identify the expression differences between strain LY01 and KO11 and indicated that increased tolerance was related to higher expression of transcriptional regulator gene (fnr) and osmolyte synthesis gene (gcv and betIBA) [20]. Accumulating evidence has suggested that a complex phenotype such as the ethanol-tolerant trait could be elicited by a handful of genes rather than a single gene. As such, Alper and Stephanopoulos demonstrated the feasibility of using global transcription machinery engineering (gTME) to optimize the complex phenotype of producer organism [21,22]; specifically, random mutations were introduced to the components of transcriptional regulatory machinery (i.e., σ70, a 70-KDa transcription initiation factor), mutations that would not have been attainable through rational design and traditional strain improvement. Essentially, this gTME paradigm allows for a combination of metabolic engineering and protein evolution approaches to improve the cellular phenotypes such as ethanol tolerance and metabolite overproduction. Other studies that will not be covered in this review have demonstrated that immobilization of recombinant cells in continuous fluidized bed culture can increase the ethanol tolerance and phenotypic stability [23]. Inhibitory effects of toxic byproducts (i.e., furfural) in the hemicellulose hydrolysates on cell growth are another barrier for high ethanol production. Recently, Miller et al. demonstrated that elimination of the competing pathway for the consumption of NADPH could endow the E. coli strain EMFR 9 with increased furfural-resistant capability, simply by silencing the genes yqhD and dkgA, involved in the reduction step of furfural degradation [24].

Despite its great promise, the exuberance over ethanol has significantly diminished in the past few years due to its several disadvantages [25]. To start with, ethanol was never designed to be a biofuel: a two-carbon molecule, ethanol has only two-thirds the energy content of gasoline. Since ethanol mixes with water, a costly distillation step is required at the end of the fermentation process. Finally, because ethanol is more easily contaminated with water than regular hydrocarbons, it cannot be transferred using the current petroleum pipelines but instead must be shipped in specialized trucks. For these reasons, the production of longer chain molecules has been pursued, which could perhaps circumvent some of ethanol’s drawbacks.

**Butanol & isopropanol production in E. coli using the CoA-dependent pathway**

Butanol is an alcohol with thermodynamic and physicochemical properties strongly resembling those of gasoline. It has more energy than ethanol, with a gallon of butanol containing approximately 90% as much energy as a gallon of gasoline. Butanol can be shipped in unmodified gasoline pipelines and it can be blended with gasoline in higher percentages than ethanol without requiring modifications to engines. Biobutanol production studied to date is carried out exclusively by a number of *Clostridia* species through acetone–butanol–ethanol (ABE) pathway. ABE fermentation was originally developed for solvent production in the 1920s and currently has attracted particular interest in the scientific community and industrial field for its biobutanol production potential. Most notably, the *Clostridium* species can produce a variety of cellulases that can be used directly for conversion of cellulosic feedstocks to butanol and ethanol. Recently, Cobalt Biofuels has invented a high-throughput screening technique for identifying high-butanol producers by expressing a luminescent protein in *Clostridia* bacteria [101]; a startup company, Qteros, has claimed the discovery of a *Clostridium* bacterium capable of converting plant biomass to ethanol at 7%, which is “more economical than any other process to date” [102]. Such recent efforts in engineering *Clostridium* species for butanol production have been covered in other reviews [26].

Nevertheless, *Clostridia* are not ideal producers, due to relatively poor characterization of their genetic systems, lack of suitable tools to manipulate their metabolism and their sensitivity to butanol toxicity [27]. Consequently, it is possible to reconstruct the butanologenic pathway in a user-friendly host and facilitate the homofermentative production of butanol [28]. For example, Inui et al. have introduced a synthetic butanol pathway into the *E. coli* system, composed of *Clostridium* acetyl-CoA acetyltransferase, β-hydroxybutyryl-CoA dehydrogenase, 3-hydroxybutyryl-CoA dehydratase, butyryl-CoA dehydrogenase, butyraldehyde dehydrogenase and butanol dehydrogenase [29]. To further improve the butanol yield, the
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The author proposed to delete the native pathway competing for both carbon flux and reducing power, including *ldhA*, *fdhF* and *frdABCD* genes (Figure 2). Finally, the engineered *E. coli* BUT 2 strain could produce up to 16.2 mM (1200 mg/l) of butanol using glucose as the sole carbon source.

In *Clostridium acetobutylicum*, the genes (*crt_, _bcd_, _erfAB_ and _hbd_) encoding for enzymatic reactions for sequential conversion of acet-acetyl-CoA to butyryl-CoA (catalyzed by crotonase, butyryl-CoA dehydrogenase, electron transfer proteins and 3-hydroxybutyryl-CoA dehydrogenase, respectively) have been assembled together in the polycistronic BCS operon [30]. Recently, Nielsen et al. reported that the polycistronic expression of butanol pathway genes in *E. coli* could lead to the production of 34 mg/l butanol, whereas individual expression of the pathway genes improved titers to 200 mg/l. It is possible that this improvement could be attributed to the coordinated functionality of the individual enzymes. To further improve butanol titers, formate dehydrogenase (*fdhF*) from *Saccharomyces cerevisiae* and the native glyceraldehyde 3-phosphate dehydrogenase (*gapA*) were over-expressed to regenerate NADH and enhance the glycolytic flux, which could elevate final titers to 580 mg/l [31]. They also demonstrated the applicability of engineering butanol pathway in microorganisms other than *E. coli* and yeast, by engineering the pathway in *Pseudomonas putida* and *Bacillus subtilis*.

Isopropanol is a secondary alcohol that can be naturally synthesized in large quantities by some microbes via threonine catabolism. Isopropanol is also used instead of methanol or ethanol to esterify various fatty acids to produce a diesel with a higher freezing point [32]. *E. coli* expressing the combination of *C. acetobutylicum* _bhl_, *E. coli* _atoAD_, *C. acetobutylicum* _adc_ and *C. beijerinckii* _adb_ resulted in an isopropanol production of 81.6 mM [33]. Jojima reported that genetically engineered *E. coli* harboring the isopropanol-producing pathway consisting of four genes from *Clostridium acetobutylicum* and one primary—secondary alcohol dehydrogenase from *C. beijerinckii*, could produce up to 227 mM of isopropanol from glucose under aerobic fed batch fermentation, which is the highest isopropanol titer ever reported [33]. Nevertheless, this does not exclude the possibility of other gene combinations that might improve isopropanol production further.

**Higher carbon chain alcohol production in *E. coli* using the keto acid pathway**

In contrast to the fermentative pathways, the non-fermentative keto acid pathways for production of higher chain alcohols has attracted particular attention recently. Higher chain alcohols, however, are not
commonly synthesized in large quantities by naturally occurring organisms, with the exception of n-butanol. To address this problem, Liao and co-workers at the University of California, CA, USA have successfully demonstrated the feasibility of constructing synthetic pathways that allow production of an array of higher alcohols with carbon number ranging from four to six (i.e., isobutanol, 1-butanol, 2-methyl-1-butanol, 3-methyl-1-butanol, 2-phenylethanol and 3-methyl-1-pentanol) \([34,35]\), which could be used as fuel alternatives. In this work, non-natural metabolism for conversion of keto acids to higher chain alcohols has been achieved by heterologous expression of keto acid decarboxylase (KDC) and ADH in \(E. coli\) \([34]\). In the branched-chain amino acid pathway (also known as the Ehrlich pathway), various keto acid intermediates (i.e., 2-ketobutyrate, 2-keto-3-methyl-valerate, 2-keto-isovalerate, 2-keto-4-methyl-pentanoate and phenylpyruvate) are generated within the cell. These could be exploited as a direct precursor for fuel biosynthesis. The foreign pathway, oxidative decarboxylation of keto acids to the corresponding carboxylic acids by KDC and the sequential reduction of carboxylic acids to various alcohols by ADH, was successfully constructed to accomplish this goal (Figure 3). Most distinctly, this engineered biosynthetic route for biofuel production circumvents the need to involve CoA-dependent intermediates, which could avoid withdrawal of CoA that is essential for protein synthesis and cell metabolism \([36]\). To select the most powerful and versatile KDC, a range of KDCs from different organisms were tested for their capability to metabolize the endogenous keto acids in \(E. coli\). Production levels of isobutanol that could be achieved by this strategy were as high as 5.3 mM when \(Lactococcus\) \(Kvd\) was employed \([34]\).

One possible strategy in metabolic engineering is to increase the precursor availability through overexpression of the upstream genes and/or deletion of the competitive pathways. Enhanced precursor availability will make the reaction kinetically favorable and could possibly lead to large amounts of desired products. Indeed, the wild-type \(E. coli\) only produces trace amounts of keto acid intermediates. For efficient production of higher chain alcohols, the existing \(E. coli\) metabolic pathways were genetically modified to increase the pool of the specific keto acids. For example, the authors overexpressed an array of genes, including \(a d h S\) from \(Bacillus\), the native genes \(i l vA\) and \(l e n A B C D\), which could lead to an approximately fivefold production increase over the strain without gene expression. On the other hand, genes responsible for competitive pathways were knocked out to eliminate byproduct formation, including \(i d hA\), \(f r dA B\), \(p t a\) and \(p f B\) (Figure 3), which could further increase the isobutanol titer to 300 mM, approximately 86% of the theoretical maximum. In this synthetic approach, most of the carbon flux is diverted into the final product of interest, which would be efficient and economic for industrial applications \([34]\).

Directed evolution, together with metabolic engineering, has created a new frontier for the discovery and production of useful compounds, which is far beyond the conventional concept of natural selection. As evident in a recent article by Zhang et al., the authors were able to extend the branched-chain amino acid pathways to produce longer chain keto acids and alcohols by using a non-natural metabolic approach. By comparing the conformation of the substrate-binding region of \(Lactococcus\) \(kvd\) ketoisovalerate decarboxylase (KVID), \(Zymomonas\) \(mobilis\) pyruvate decarboxylase (ZmPDC) and \(Enterobacter\) \(cloacae\) indolepyruvate decarboxylase (IPDC) using a homology model, the authors concluded that the bulkier size of a side chain can account for the wide substrate spectrum of these KDCs and suggested that substitution of related amino acids with smaller hydrophobic side chains might be able to result in higher substrate specificity. Using this rational design, they successfully obtained a KVID variant, with a specificity constant 40-fold higher than that of the wild-type KVID. Analogous to this strategy, they also engineered the chain elongation activity of 2-isopropylmalate synthase by enlarging the substrate binding pocket of LeuA to relieve the steric hindrance. The resulting mutant was shown to be more active on longer-chain substrates. By this combinatorial approach, non-natural alcohols such as 3-methyl-1-pentanol could be efficiently produced for the first time at 794 mg/l \([35]\).

Instead of using the naturally evolved fermentative pathways for alcohol production in microbes, n-butanol and isopropanol could also be synthesized by expanding the keto acid pathway in \(E. coli\) \([37]\). Upon overexpression of \(k i d (L.\ lactis)\) and \(a d h2\) (\(S\)accharomyces\ \(cerevisiae\)) and the \(E.\ coli ilvA\), \(l e n A B C D\), \(t h r A^{bc}\)\(B C\), the engineered \(E.\ coli\) strain achieved co-production of butanol and propanol. Downregulation of the threonine biosynthesis and removal of the diverging pathways catalyzed by \(M\)et\(A\) (homoserine \(O\)-succinyltransferase) and threonine dehydrogenase (\(T\)dh) could further improve isopropanol production up to 2 g/l. However, elimination of feedback inhibition of end products on LeuA failed to increase the production, presumably because the intracellular leucine level is insufficient to cause an adverse effect on LeuA \([37]\). An alternative enzyme, citramalate synthase (\(C\)im\(A\) from \(Methanococcus\) \(jannaschii\)) which can directly convert pyruvate to 2-ketobutyrate, has recently been engineered for producing propanol and butanol \([38]\). Using a growth-based evolutionary
strategy, the best CimA variant, with a truncated C-terminal domain, exhibited both improved catalytic activity and insensitivity to feedback inhibition by isoleucine, which enabled 9.2- and 21.9-fold higher production of propanol and butanol compared with the strain expressing the wild-type CimA. With this effort, a production level of 3.5 g/l isopropanol and 524 mg/l butanol was achieved.

Other alcohol derivatives such as 2-methyl-1-butanol (2MB) and 3-methyl-1-butanol (3MB) could also be synthesized by harnessing the keto acid biosynthetic pathway [39,40]. Cann and Liao investigated the specificity and diversity of enzymes catalyzing key parts of the isoleucine biosynthetic pathway, indicating that ilvGM from Salmonella typhimurium and ilvA from Corynebacterium glutamicum improved 2MB production, which is the highest production reported in E. coli. Further strain improvement included overexpression of the native threonine biosynthetic operon (thrABC) without the transcription regulatory element and knockout of the competing pathways upstream of threonine production (ΔmetA and Δtdh), which led to an ultimate titer of 1.25 g/l 2MB in 24 h [39]. For 3MB, plasmid-based expression of valine and leucine biosynthetic pathway genes could lead to a production of 67 mg/l. It has also been demonstrated that

Figure 3. Synthetic networks for the nonfermentative alcohol production in engineered Escherichia coli. The black arrows represent the native pathway; dash lines indicate multienzymatic steps; the underlined italicized gene names represent the pathway that has been overexpressed and gene names with strikethrough lines represent pathways that have been knocked out.
the main bottleneck to 3MB production in *E. coli* is due to feedback inhibition of leuA gene, caused by free leucine. In addition, 3MB and isobutanol compete for the same precursor, 2-keto-isovalerate (KIV). In this regard, it is necessary to eliminate the feedback inhibition and competing pathways and increase the specificity of LeuA on KIV. When these approaches were combined, the engineered *E. coli* could achieve a final titer of 1.28 g/l 3MB in 28 h [40].

Through a combination of synthetic pathway and directed evolution, the previously reviewed work has opened up an unexplored frontier for production of an array of higher chain alcohols, which could hold a greater promise for gasoline alternatives than ethanol. One of the hallmarks of this research is that the intermediary metabolism of user-friendly strain *E. coli* was expanded and high production of this fuel-like alcohol was achieved for the first time.

### Biodiesel production in *E. coli* using the fatty acids biosynthesis pathway

Biodiesel is defined as a fuel comprised of mono-alkyl esters of long-chain fatty acids derived from vegetable oils or animal fats. It is a possible substrate for petroleum-based fuel and is produced from plant oils through transesterification of short-chain alcohols, primarily methanol and ethanol. The resulting products are also known as fatty acid methyl esters and fatty acid ethyl esters (FAEEs) [41]. Despite its several advantages, including biodegradability, low toxicity, reduced emission of carbon monoxide and attractive fuel-like properties, large-scale application of biodiesel is limited by the availability of vegetable oil feedstocks and geographical and seasonal restrictions, resulting in a conflict between its use as a food component and its use as a biofuel, similar to the conflict previously described for corn-derived ethanol. Biotechnological approaches for the production of biodiesel, in addition to chemical and biochemical methodologies, can potentially ease this conflict. Many autotrophic microalgae, microorganisms such as *Cryptococcus albidus*, *Lipomyces lipofera*, *Lipomyces starkeyi*, *Rhodosporidium toruloïdes*, *Rhodotorula glutinis*, *Trichosporon pullulan*, *Yarrowia lipolytica* and bacteria belonging to the *Gordonia*, *Rhodococcus* and *Nocardia* sp. are able to accumulate oils [42,43], with a wide range of molecules utilized by these microorganisms as carbon sources. The utilization of such substrates makes the process very attractive and possibly economically acceptable.

In addition, extensive efforts have been made in order to engineer the efficient production of biodiesel in genetically tractable hosts such as *E. coli*. Kalscheuer et al. achieved the biosynthesis of FAEEs in metabolically engineered *E. coli*. For achieving this, the homoethanologenic pathway of *Zymomonas mobilis*, consisting of pyruvate decarboxylase and alcohol dehydrogenase, was introduced into *E. coli* to produce ethanol. This resulting ethanol was further esterified to FAEEs by heterologous expression of unspecific acyltransferase from *Acinetobacter baylyi* strain ADP1 (Figure 4). *E. coli* expressing these enzymes reached a production titer of 1.28 g/l, representing 26% of cellular dry mass by using glucose and oleic acid as substrates. By co-expression of an alcohol-producing bifunctional acyl-CoA reductase from the jojoba plant and a bacterial wax ester synthase from *A. baylyi* strain ADP1, the engineered *E. coli* was unexpectedly found to produce fatty acids butyl esters in the presence of olate [44].

An enhanced fatty acid pool would be beneficial for biodiesel production. Acetyl-CoA carboxylase (ACC), which catalyzes the reaction from acetyl-CoA to malonyl-CoA (the main fatty acid precursor), has been assumed to be the major limiting step for the synthesis of an array of economically important compounds such as fatty acids, polyketides and flavonoids. For example, overexpression of ACC has proven effective for increasing the fatty acid biosynthesis rate in *E. coli* [45]. Augmentation of the intracellular malonyl-CoA pool through the coordinated overexpression of four ACC subunits (accBCAD) from *Photorhabdus luminescens* could result in an increase in flavanone production up to 576% [46]. By knocking out *fabD* and overexpression of *acc* and a plant-derived thioesterase, Lu et al. have achieved efficient production of fatty acids (2.5 g/l) in an engineered *E. coli* [47], which could be readily used for biodiesel production. Recently, a recombinant *E. coli* expressing the *Proteus sp. lipK* (a novel alkaline lipase) has been constructed as a whole-cell biocatalyst for conversion of methanol and olive oil to biodiesel [48].

### Isoprenoid production in *E. coli* using the mevalonate pathway

Isoprenoids are a diverse family of metabolites synthesized from the C5 units isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate [49]. An array of natural products such as monoterpen (C10), sesquiterpene (C15), diterpene (C20) and triterpene (C30) are derived from this pathway. Most notably, these compounds have been known for their pharmaceutical or nutritional value. Engineered microbes have been developed for overproduction of artemisinic acid [49], β-carotene [50], lycopene [51] and terpenoids [52]. These strains are now being adapted for biosynthesis of isoprenoid-based fuels, such as cyclic alkanes, alkenes and alcohols (Figure 5). For example, introduction of *nudF* into engineered *E. coli* with
mevalonate-based isopentenyl pyrophosphate biosynthetic pathway resulted in the production of isopentenol at 110 mg/l [53]. Through the upregulation of HMG-CoA reductase activity, Pitera et al. successfully rebalanced the carbon flux through the heterologous pathway and, at the same time, achieved increased mevalonate production [54]. Although the production level is low, the longer cyclic or branched-chain isoprenoids may be suitable diesel and jet fuel substitutes in the long run, due to their high energy density and low freezing point.

**Future perspective in engineering E. coli for biofuel production**

The targeted manipulation of metabolic pathways has made it possible for genetically tractable microorganisms, such as *E. coli*, to become promising producers of desired fuel alternatives. However, several challenges stand in the way of making such biofuel production by recombinant *E. coli* economically feasible, the most important of which is achieving high production titers. Great strides have been made towards this direction, with strategies that involved both the manipulation of the native regulatory network of intermediary metabolism as well as the production pathways of interest themselves. In light of the complexities of metabolism, it is becoming increasingly important to use such hybrid approaches in order to not only improve productivity but also to produce novel compounds of potentially superior value as biofuel molecules [55,56]. It is important, however, to note that the use of more global approaches, such as *systems biology*, has hardly been demonstrated in the case of biofuel production. Such approaches could potentially lead to the development of strains with higher titers by targeting enzymes and pathways that are seemingly unrelated to the metabolic pathways of interest. It has recently been demonstrated, for example, how stoichiometric modeling of the genome-wide *E. coli* metabolism and flux balance analysis resulted in significant increases in the intracellular flux towards malonyl-CoA, a key precursor for biodiesel production [57]. Such systems approaches could also help develop strategies for satisfying cellular energetic and cofactor requirements in the case of biofuel production [58]. Such requirements arise from the fact that cells will not waste energy and resources to overproduce a certain substance; in addition, introduction and expression of non-native pathways will result in metabolic imbalance. For these reasons, regeneration of reducing power (NADH or NADPH) and coenzymes is crucial for product formation [25,59]. In this regard, constraint-based flux balance analysis can result in a genome-wide rewiring of cellular metabolism, in order to construct the most efficient cellular phenotypes. Such rewiring can be coupled with recently developed metabolic engineering tools, such as promoter evolution [60], which would allow the fine-tuning of mRNA levels as well as tools that allow better post-transcriptional control (synthetic red blood cells and tunable intergenic regions) in order to finally achieve even better biofuel titers [61].

**Conclusion**

Metabolic engineering provides an important platform for improving the cell’s capability to produce fuel alternatives and several examples have demonstrated this fact in the case of *E. coli*. Despite these
Glycolysis (EMP) pathway

D-Glucose

Acetyl-CoA

Thiolase

HMG-CoA synthase

3-hydroxy-3-methylglutaryl-CoA reductase

Mevalonate kinase

Mevalonate-5-phosphate

Mevalonate-5-pyrophosphate decarboxylase

Isopentenyl-5-pyrophosphate (IPP)

Isopentenyl-PP isomerase

Dimethylallyl-PP (DMPP)

3-methyl-2-buten-1-ol

Isopentenol

Figure 5. Representation of isopentenol production in engineered Escherichia coli. The black arrows represent the native pathway; dash lines indicate multienzymatic steps and the underlined italicized gene names represent the pathway that has been overexpressed. EMP: Embden–Meyerhof–Parnas.

accomplishments, substantial challenges remain in order to make E. coli-based processes economically feasible. It is expected that most of these challenges will be addressed in the future through a combination of system biology and metabolic pathway evolution strategies. However, the reliance on either corn- or cellulose-derived sugars as carbon sources for E. coli growth and production could potentially be the one key limitation in the industrial application of such technologies. In addition, the sharp increase in global temperatures that has been linked to greenhouse gases has generated an urgent need to use processes that rely on CO₂ sequestration. To date, biotic solutions for CO₂ sequestration have emphasized photosynthetic systems, using algae or photosynthetic microorganisms, but nonphotosynthetic, microbial CO₂ fixation from recombinant E. coli could also be a possibility in the immediate future. Such nonphotosynthetic CO₂ sequestration can utilize autotrophic carbon fixation pathways such as the 3-hydroxypropionate/4-hydroxybutyrate cycle and the propionyl-CoA carboxylase cycle that have been identified in archaea [62–64]. Because of the great limitations in employing the native archaea organisms, we expect that metabolic engineering will play a key role in the near future towards the development of recombinant E. coli and even other tractable microorganisms that would be able to functionally express the PCC and 4-hydroxybutyrate/4-hydroxybutyrate cycles and as such perform such conversions.

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Executive summary

- Biofuels are becoming increasingly attractive as an alternative to petroleum-based fuels. Breakthroughs in metabolic engineering have made it possible to manipulate the diverse metabolic pathway and develop more efficient processes for biofuel production.
- Tremendous work has been made to engineer the fermentative pathway of Escherichia coli for ethanol production. Although ethanol appears to be promising, there are some inherent problems with its use owing to its physicochemical properties (low energy content and high hygroscopicity).
- Biofuels with higher carbon chains are emerging as the next generation molecules of energy, which may find broader application compared with ethanol. Pathway engineering of E. coli to synthesize butanol, isopropanol, biodiesel and isoprenoids has demonstrated this potential.
- Expanding the keto acids pathway to produce non-natural higher chain alcohols in E. coli represents a remarkable success of metabolic pathway evolution. Through a combination of protein rational design and metabolic engineering, the authors achieved the efficient production of an array of novel alcohols, which holds great promise for future applications.
- Most of the challenges that stand in way to make biofuel economically feasible will be addressed through a combination of systems biology and directed evolution.
Bibliography

Papers of special note have been highlighted as:

- of interest
- of considerable interest

8. Comprehensive review of the efforts in engineering bacteria for ethanol production. Strategies such as expression of different heterologous gene combinations, deletion of competing pathway and increased NADH availability in host cell are emphasized.
18. Based on elementary mode analysis, the minimal metabolic network that links the desired phenotype and the corresponding genotype was identified for efficient production of ethanol.
35. Shows a synthetic pathway for production of an array of higher chain alcohols in *Escherichia coli*. Through the expanded
pathway, the endogenous 2-keto acid intermediates were successfully diverted to alcohol biosynthesis, which represents a new route for large-scale production of biofuels.

- Demonstrated the construction of an artificial metabolic network by expanding the branched-chain amino acid pathway. Through a combination of protein rational design and metabolic engineering, they successfully achieved the novel alcohol (3-methyl-1-pentanol) production.

- Provides a comprehensive review of the current state of biofuels and the involved pathways.


- Discusses different types of biofuels and how synthetic biology will affect metabolic engineering.


- **Websites**


  - Technology review: potential energy [www.technologyreview.com/blog/energy/23892](http://www.technologyreview.com/blog/energy/23892)