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MINI-REVIEW

Biofuel production in *Escherichia coli*: the role of metabolic engineering and synthetic biology

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Abstract The microbial production of biofuels is a promising avenue for the development of viable processes for the generation of fuels from sustainable resources. In order to become cost and energy effective, these processes must utilize organisms that can be optimized to efficiently produce candidate fuels from a variety of feedstocks. *Escherichia coli* has become a promising host organism for the microbial production of biofuels in part due to the ease at which this organism can be manipulated. Advancements in metabolic engineering and synthetic biology have led to the ability to efficiently engineer *E. coli* as a biocatalyst for the production of a wide variety of potential biofuels from several biomass constituents. This review focuses on recent efforts devoted to engineering *E. coli* for the production of biofuels, with emphasis on the key aspects of both the utilization of a variety of substrates as well as the synthesis of several promising biofuels. Strategies for the efficient utilization of carbohydrates, carbohydrate mixtures, and noncarbohydrate carbon sources will be discussed along with engineering efforts for the exploitation of both fermentative and nonfermentative pathways for the production of candidate biofuels such as alcohols and higher carbon biofuels derived from fatty acid and isoprenoid pathways. Continued advancements in metabolic engineering and synthetic biology will help improve not only the titers, yields, and productivities of biofuels discussed herein, but also increase the potential range of compounds that can be produced.

Keywords Synthetic biology · Metabolic engineering · Biofuels production · *Escherichia coli* · Biofuels

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Introduction

The rising concerns related to the cost, sustained availability, and environmental impact of use of fossil fuels has led to the search for new technologies that generate alternative fuels from renewable carbon sources (Hirsch et al. 2006; Kerr 2007). In light of these concerns, interest and investment in the production of biofuels from renewable resources has grown significantly in recent years (Schubert 2006). This growth, combined with recent advancements in biotechnology, has resulted in extensive research efforts devoted to the microbial production of biofuels.

Numerous microorganisms have been found to naturally produce a wide variety of compounds with uses as fuels, chemicals, and pharmaceutical products. However in many cases, the ability to optimize the production of these compounds has been limited due to the lack of genetic tools and physiological knowledge required for the effective manipulation of the microorganism. In order to become a viable means for producing biofuels, microbial processes must utilize microorganisms that can be readily manipulated to maximize the production of candidate biofuels in large scale industrial applications. Based on this criteria, *Escherichia coli*, the workhorse of modern biotechnology, has become a promising host organism for the microbial production of biofuels. The availability of genetic tools and the large genomic, metabolic, and

physiological knowledge base for *E. coli* has facilitated the ease at which this organism can be engineered as an efficient biocatalyst. In addition, *E. coli* also possesses several advantageous traits for industrial biofuel production, including showing efficient growth at industrially relevant conditions, the ability to grow in mineral salts medium with inexpensive components and in the absence of oxygen, as well as having the capability of utilizing a wide variety of substrates including main biomass constituents such as carbohydrates, polyols, and fatty acids. High productivities and yields of biofuels can also be attained with *E. coli* in part due to its high growth and metabolic rates and tolerance to high concentrations of substrate and products. While both the number and productivity of compounds naturally produced by *E. coli* are limited, advances in synthetic biology, metabolic engineering, and systems biology have resulted in the ability to engineer *E. coli* to produce a wide variety of biofuels far beyond the scope of what any single organism can naturally produce.

Advancements in the efficient microbial production of fuels and chemicals have been shaped by the development of tools for the understanding and manipulation of cellular metabolism, including the ability to transfer genes, modulate gene expression, and engineer proteins (Stephanopoulos 2007). Increased production of useful compounds through strategies designed to engineer cells and their metabolic pathways have been used extensively over the past few decades (Bailey 1991; Stephanopoulos 2002). More recently, the emerging field of synthetic biology (Pleiss 2006) has provided key tools aiding in the design, assembly, and implementation of various synthetic pathways whose expression results in the production of novel compounds. Furthermore, the advent of a systems-level approach for analyzing and monitoring cellular metabolism (systems biology; Ideker et al. 2001) has resulted in an unprecedented understanding of complex metabolic relationships and responses that has further enabled the discovery and implementation of novel biosynthetic pathways. Combinatorial approaches utilizing the concepts and strategies of these fields have been implemented in order to develop efficient microbial platforms for the production of biofuels from a wide variety of feedstocks.

This review focuses on recent efforts for microbial biofuel production through the engineering of *E. coli* by the implementation of various metabolic engineering and synthetic biology approaches. Special efforts are also made to discuss not only novel biofuels produced via *E. coli*, but also engineering strategies to effectively utilize different feedstocks for the production of biofuels. Both engineering elements are essential for the development of viable processes for the industrial scale microbial production of biofuels.

Engineering the efficient utilization of carbon sources for the microbial production of biofuels

The availability of diverse biomass resources such as agricultural lignocellulosic residues, edible and nonedible crops, and waste streams (e.g., bagasse from sugar manufacture and industrial by-products) for conversion into generic feedstock constituents (carbohydrates, polyols, fatty acids, etc.) provides ample opportunity to employ microbial means for the conversion of a wide variety of carbon sources into valuable fuels. However, the vast array of feedstock constituents also results in the challenge of developing processes to produce biofuels from a multitude of different carbon sources which is essential for the long-term viability and practicality of the biofuel industry. From an engineering standpoint, the diversity of the chemical structure and composition of these various feedstock constituents requires knowledge of the metabolic pathways associated with the metabolism of each carbon source in order to develop and implement a variety of engineering strategies for the efficient production of fuels from each available source.

The synthesis of the majority of proposed biofuels utilizes metabolic pathways that require the generation of common metabolic intermediates such as pyruvate and acetyl-CoA. For this reason, the overall pathways for the microbial production of biofuels can be separated into two main elements. The first element encompasses the pathways required to generate these key metabolic intermediates from a chosen carbon source, while the second element includes the pathways which convert these intermediates to the fuel of interest. For the most part, the pathways leading to metabolic intermediates generate reducing equivalents which are required for fuel production, as the pathways for the synthesis of fuels result in a consumption of reducing equivalents from intermediate metabolites. The requirement of an overall redox balance within the cell is a key factor determining the overall yields and productivities that can be achieved during the synthesis of a given product from a given carbon source. Another key determinant in the microbial synthesis of a given product is the need for the formation of ATP as an energy source for many cellular functions. Therefore, the design of any metabolic engineering strategy for the production of biofuels must take into account all of these key factors in order to maximize titers, yields, and productivities of the desired product from a chosen carbon source.

E. coli is capable of utilizing a variety of carbon sources that are constituents of readily available renewable feedstocks. These include carbohydrates such as glucose and xylose, as well as noncarbohydrate carbon sources such as glycerol and fatty acids, which together encompass the main constituents of biomass. The pathways mediating the

conversion of these carbon sources into key metabolic intermediates are illustrated in Fig. 1. The following sections detail the use of these feedstock constituents as carbon sources as well as the engineering efforts to optimize their utilization by *E. coli*.

Carbohydrate-based feedstocks

Starches and simple sugars

The most commonly used feedstocks for the industrial production of biofuels are starches and simple sugars derived from sources such as sugar cane (sucrose) and corn (starch). The conversion of sucrose or starch into fuels begins with enzymatic hydrolysis, which yields glucose. Glucose is then readily utilized by *E. coli* and other organisms via uptake and catabolism through the glycolytic pathway. The production of one molecule of pyruvate from glucose through the Embden–Meyerhof–Parnas pathway results in the generation of one reducing equivalent (Fig. 1). Glucose is the preferred carbon source of *E. coli* and thus this organism has naturally evolved to maximize its utilization under many different conditions. Therefore, few engineering efforts have been needed to achieve the high utilization rates of glucose that are required for the efficient production of biofuels. Numerous metabolic engineering efforts for the production of novel biofuels have utilized glucose as a carbon source for their production. Some of these recent efforts are discussed in subsequent sections of this review. While the production of biofuels, such as ethanol, from these feedstocks is highly efficient (Fischer et al. 2008), they are expensive and nonsustainable feedstocks due to their concurrent integration as an essential part of the food–feed chain. For this reason, it is desirable to employ other feedstocks, which overcome these issues.

Lignocellulosic sugars

Lignocellulosic crops, in contrast to sugar cane and corn, have the benefit of higher productivities and their use as a feedstock for biofuel production is considered sustainable and renewable. However, their conversion into fermentative sugars is more difficult than that of corn or sugarcane due to three major factors. The first factor is presence of lignin, a highly recalcitrant network polymer of aromatic alcohols that accounts for ~25% of most common sources of

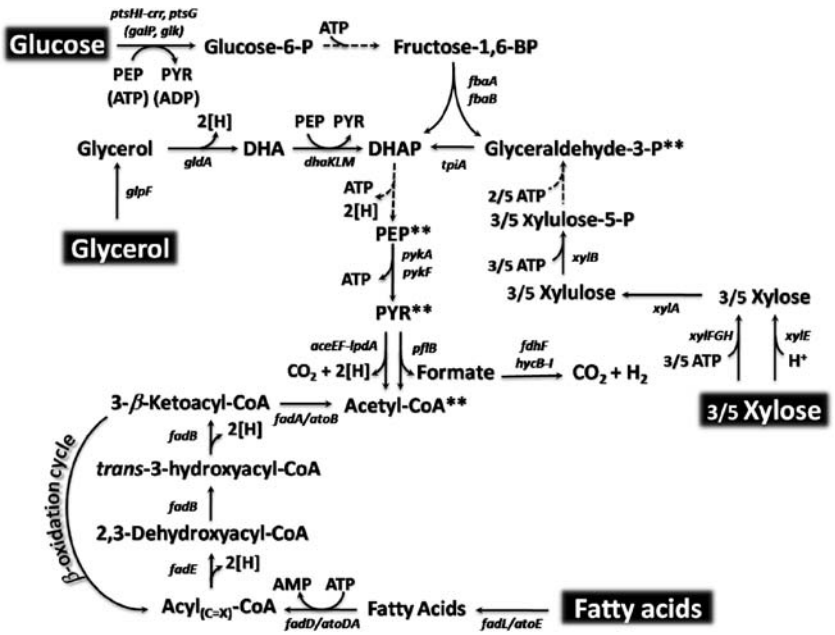

Fig. 1 Metabolic pathways for the conversion of feedstock constituents into key intermediate metabolites which are *marked* by *double asterisks*. *E. coli* is capable of utilizing a wide variety of carbon sources (shown by *black boxes*) derived from the main constituents of biomass (see text for details). *Broken lines* represent multiple reaction steps. Relevant reactions are represented by the name of the genes coding for the enzyme (all genes native to *E. coli*): *aceEF-lpdA*, pyruvate dehydrogenase multienzyme complex; *atoA*, *atoD*, acetyl-CoA:acetoacetyl-CoA transferase; *atoB*, acetyl-CoA acyltransferase; *atoE*, putative short-chain fatty acid transporter; *dhaKLM*, PEP-dependent dihydroxyacetone kinase; *fadA*, 3-ketoacyl-CoA thiolase; *fadB*, enoyl-CoA hydratase/3-hydroxyaceyl-CoA dehydrogenase; *fadD*, fatty acyl-CoA synthetase; *fadE*, acyl-CoA dehydrogenase; *fadL*, long-chain fatty acid outer membrane transporter; *fbaA*, *fbaB*, fructose-1,6-bisphosphate aldolase; *fdhF*, formate dehydrogenase; *galP*, galactose permease; *gldA*, glycerol dehydrogenase; *glk*, glucokinase; *glpF*, glycerol MIP channel; *hycB-1*, hydrogenase; *pflB*, pyruvate formate lyase; *ptsHI-crr*, *ptsG*, glucose-specific phosphoenolpyruvate phosphotransferase system; *pykA*, *pykF*, pyruvate kinase; *tpiA*, triose phosphate isomerase; *xylA*, xylose isomerase; *xylB*, xylulokinase; *xylE*, xylose MFS transporter; *xylFGH*, xylose ABC transporter. *2[H]* NAD(P)H=FADH$_2$, *DHA* dihydroxyacetone, *DHAP* dihydroxyacetone-phosphate, *Fructose-1,6-BP* fructose-1,6-bisphosphate, *PEP* phosphoenolpyruvate, *PYR* pyruvate

cellulosic biomass (Aristidou and Penttila 2000). The enzymatic digestion of lignin is far too slow to be practical on an industrial level, and therefore the cellulosic and hemicellulosic portions are typically separated from lignin in order to overcome this obstacle. Secondly, cellulose is much more resistant to enzymatic hydrolysis than starches and simple oligosaccharides. Recalcitrance of cellulosic biomass requires pretreatment to create a partially hydrolyzed product that is easier for "cellulose enzymes" to digest. However, this pretreatment often generates fermentation inhibitors. Several pretreatment variations have been investigated (Himmel et al. 2007), and this remains an active area of research in an effort to alleviate this significant obstacle. The final and most significant factor from a metabolic engineering standpoint is the presence of five carbon sugars (mainly xylose, with arabinose, mannose, and galactose additional sugar constituents), usually consisting of 10–25% of the total carbohydrates (Fischer et al. 2008). This obstacle has been overcome through extensive engineering efforts to construct genetically altered *E. coli* strains capable of efficiently utilizing sugar mixtures (Hernandez-Montalvo et al. 2001; Nichols et al. 2001; Flores et al. 1996).

When exposed to a mixture of carbon sources, wild-type *E. coli* exhibits a specific hierarchy for their utilization. As long as they are present in sufficient amounts in the growth medium, sugars such as glucose, sucrose, and fructose repress the synthesis of enzymes necessary for the transport and metabolism of less favorable carbon sources such as xylose, arabinose, maltose, and lactose. This phenomenon is known as carbon catabolite repression (CCR), a regulatory mechanism in which the expression of genes required for the transport and utilization of secondary carbon sources is prevented by the presence of a preferred carbon source (Stulke and Hillen 1999). Due to CCR, fermentation of sugar mixtures, such as those found after the breakdown of lignocellulosic materials, exhibit delayed and often incomplete carbon source utilization resulting in lower productivities and yields. Thus, the development of recombinant *E. coli* strains capable of efficiently utilizing sugar mixtures is essential for the large scale production of biofuels from lignocellulosic materials.

In *E. coli*, CCR involves both global and operon-specific regulatory mechanisms, such as inducer exclusion and inducer prevention (Goerke and Stulke 2008). The key components in the global pathway of CCR in *E. coli* are the transcription activator CRP (cyclic AMP (cAMP) receptor protein), the signal metabolite cAMP, the enzyme adenylate cyclase, and the component IIA ($EIIA^{Glc}$) of the glucose-specific phosphoenolpyruvate:carbohydrate phosphotransferase system (PTS). The phosphorylation state of $EIIA^{Glc}$ is a key component in CCR, as it binds and activates adenylate cyclase when phosphorylated, thus leading to the synthesis of cAMP. High concentrations of cAMP, in turn, lead to the formation of cAMP-CRP complexes, which bind and activate the promoters of catabolic genes. In the presence of glucose or other PTS sugars, $EIIA^{Glc}$ is mostly in its nonphosphorylated state, resulting in the inability to activate adenylate cyclase. Furthermore, nonphosphorylated $EIIA^{Glc}$ inactivates enzymes and transporters involved in the metabolism of other carbon sources. In addition to the above global regulatory mechanisms, regulatory systems such as Cra (Hardiman et al. 2007), DgsA (Decker et al. 1998; Kimata et al. 1998), and CsrA (Babitzke and Romeo 2007), among others, are also involved in regulating sugar metabolism in *E. coli*.

Due to the link between sugar transport through the PTS and CCR, one metabolic engineering strategy for the simultaneous consumption of multiple sugars is the inactivation of the PTS. CCR has been shown to be partially relieved allowing for coutilization of carbon sources during growth on sugar mixtures in *E. coli* mutants deficient in the PTS (Hernandez-Montalvo et al. 2001; Nichols et al. 2001). However, due to the impairment of the glucose transport system, these mutants exhibit decreased sugar uptake rates and slower growth rates. This has been overcome by the activation of an alternative glucose transporter (galactose permease encoded by the *galP* gene) and phoshorylation system (glucokinase encoded by the *glk* gene). Functional activation of these enzymes allowing for increased glucose uptake has been successfully implemented through either overexpression (Hernandez-Montalvo et al. 2003) or through an adaptive evolution approach in which PTS^- variants are selected for the ability to grow on glucose (Flores et al. 1996). Another approach that has been investigated to relieve CCR is the use of CRP mutants that do not require cAMP to activate the expression of catabolic genes (CRP* mutants) (Stulke and Hillen 1999). The transcriptional effects of expressing CRP* in place of wild-type CRP in *E. coli* have recently been studied through the use of microarray experiments (Khankal et al. 2009). It was found that while fewer genes show expression sensitivity to glucose in the mutant expressing CRP* compared to the wild-type, expression of CRP* did not completely eliminate glucose effects. Other strategies currently being investigated include the overexpression of cAMP-independent adenylate cyclase as well as the constitutive expression of the xylose- and arabinose-utilization operons (Gonzalez et al., unpublished data).

While the efforts to coutilize hexose and pentose sugars in *E. coli* through the inactivation of the PTS were successful, the existence of other, less understood regulatory systems that prioritize the sequential metabolism of sugar mixtures provides another avenue to engineer *E. coli* strains for the simultaneous consumption of hexose and

pentose sugars. A recent example of this is the finding that the deletion of the *mgsA* gene, encoding methylglyoxal synthase, in an ethanologenic *E. coli* strain resulted in the ability to coutilize glucose and xylose mixtures at much improved rates in simple mineral salts medium (Yomano et al. 2009). In addition, coutilization of glucose, xylose, arabinose, mannose, and galactose was also observed with this strain. During metabolic imbalance, methylglyoxal, the product of the first reaction of the methylglyoxal bypass catalyzed by methylglyoxal synthase, is believed to function as a general inhibitor of sugar metabolism (Totemeyer et al. 1998; Zhu et al. 2001). However, this is the first evidence to suggest that methylglyoxal synthase (*mgsA*) and methylglyoxal are directly involved in regulating the cometabolism of sugars in ethanologenic *E. coli*. This finding not only provides another approach for engineering strains for the simultaneous consumption of hexose and pentose sugars, but also shows that the engineering of other sugar metabolism regulatory systems may provide other means for additional improvements in sugar coutilization.

Noncarbohydrate feedstocks

While lignocellulosic biomass typically consists of carbohydrate feedstocks, oil-seed crops are mainly composed of various triacylglycerols: i.e. fatty acids (FAs) bonded to a backbone structure of glycerol (Durrett et al. 2008). In addition to oil-seed crops, several algal species have been found to produce large amounts of triacylglycerols (oleaginous algae; Hu et al. 2008) with significant investment in their future use forecasted (Service 2009). The availability of oleaginous feedstocks makes both FAs and glycerol attractive carbon sources for the production of fuels and chemicals.

Fatty acids

Fatty acids offer additional advantages for the production of fuels due to both the efficiency of their conversion to the key intermediate metabolite acetyl-CoA and the reduced nature of their carbon atoms. The synthesis of one molecule of acetyl-CoA via glycolysis from sugars, such as glucose and xylose, is accompanied by the generation of one molecule of carbon dioxide (or formate). This stoichiometry limits the carbon recovery and therefore lowers the yield of products derived from acetyl-CoA. On the other hand, the metabolism of FAs to acetyl-CoA does not utilize the glycolytic pathway and results in full carbon recovery (Fig. 1). An additional advantage over sugars is the highly reduced nature of carbon atoms in FAs molecules, enabling higher yields of fuels and reduced chemicals. These advantages are illustrated through the comparison of the maximum theoretical yields of ethanol that can be achieved from glucose and palmitic acid (a representative 16-carbon fatty acid; Dellomonaco and Gonzalez, unpublished).

$$C_6H_{12}O_6 \rightarrow 2C_2H_6O + 2CO_2$$

(Ethanol from Glucose)

$$C_{16}H_{32}O_2 \rightarrow \frac{23}{2}C_2H_6O + \frac{2}{3}CO_2$$

(Ethanol from Palmitic Acid)

As seen from these equations, the maximum theoretical yield of ethanol obtained from palmitic acid (1.38 g ethanol/g palmitic acid or 23/16 carbon mole ethanol/carbon mole palmitic acid) is significantly higher than that obtained from glucose (0.51 g ethanol/g glucose or 2/3 carbon mole ethanol/carbon mole glucose) illustrating the pronounced advantage of utilizing fatty acids as a feedstock for the production of fuels

In *E. coli*, the enzymes encoded by the *fad* regulon and the *ato* operon are responsible for the degradation of FAs and conversion into acetyl-CoA (Fig. 1). Products of the fad regulon mediate the transport, acylation, and β-oxidation of medium chain (C_7 to C_{11}) and long-chain (C_{12} to C_{18}) FAs (Fig. 1). For growth on short-chain (C_4 to C_6) FAs, two additional enzymes encoded by the *atoD-atoA* and *atoB* (part of the *atoDAEB* operon) are required. The expression of the *fad* regulon and *ato* operon is controlled by FadR (*fadR*) and AtoC (*atoC*), respectively. However, in order to utilize FAs as the sole carbon and energy source, respiratory conditions are required in order attain redox balance as none of the fermentative pathways active in *E. coli* are able to consume the amount of reducing equivalents generated by the synthesis of cell mass from FAs. The engineering of a respiro-fermentative mode to enable the use of FAs as the sole carbon source for the production of fuels and chemicals in *E. coli* has recently been investigated (Dellomonaco and Gonzalez, unpublished). Combination of this respiro-fermentative mode with *E. coli* mutants exhibiting constitutive expression of the *fad* regulon and *ato* operon resulted in the ability to efficiently and completely utilize palmitic acid as the sole carbon and energy source thus providing a platform for further engineering efforts to produce a variety of fuels and chemicals (Dellomonaco and Gonzalez, unpublished).

Glycerol

Glycerol is a polyol molecule generated as a by-product of the biodiesel, oleochemical, and bioethanol production processes. The tremendous growth of the biofuels industry has created a surplus of glycerol, resulting in a dramatic decrease in crude glycerol prices (Yazdani and Gonzalez

2007). Future opportunities for the availability of even larger amounts of glycerol also exist due to the intracellular accumulation of up to 7.8 M glycerol (equivalent to 718 g/L glycerol in water) in certain species of algae such as those in the *Dunaliella* genus (Oren 2005). In addition to the availability and low-prices of glycerol, the highly reduced nature of carbon atoms in glycerol confers an additional advantage for the production of fuels and reduced chemicals when compared to the use of sugars. The conversion of glycerol into pyruvate generates twice the number of reducing equivalents produced during the metabolism of glucose or xylose (Fig. 1). This permits, for example, the coproduction of ethanol and formic acid (or ethanol and hydrogen), the former would result in doubling the overall product yield compared with glucose fermentation to ethanol, a process in which half of the sugar in lost as CO_2 due to the oxidation state of glucose (Dharmadi et al. 2006). These advantages are illustrated by the following equations comparing the maximum production of ethanol from glycerol and glucose, considering redox balanced routes.

$$\frac{1}{2}C_6H_{12}O_6 \rightarrow C_2H_6O + CO_2$$

(Ethanol from Glucose)

$$C_3H_8O_3 \rightarrow C_2H_6O + CH_2O_2/(CO_2 + H_2)$$

(Ethanol and Formate/H_2 from Glycerol)

Until recently, it was thought that *E. coli* was only able to metabolize glycerol under respiratory conditions, minimizing its potential for the production of biofuels from glycerol. The use of glycerol as a carbon source under fermentative conditions was limited to studies in which genes encoding the glycerol dehydratase and 1,3-PDO dehydrogenase enzymes from organisms such as *Klebsiella pneumoniae* (Skraly et al. 1998) and *Citrobacter freundii* (Daniel and Gottschalk 1992) were expressed to enable glycerol utilization. However, these studies focused on the production of 1,3-propanediol (1,3-PDO), a product naturally produced in these organisms but not by *E. coli*, from glycerol through the expression of a synthetic 1,3-PDO pathway which directly converts glycerol into 1,3-PDO without entering the central carbon metabolism of *E. coli*. Therefore, additional carbon sources other than glycerol were usually required to help sustain cell growth and the production of other products from glycerol was limited.

The recent discovery that *E. coli* is able to metabolize glycerol in a fermentative manner and the identification of key pathways and environmental conditions affecting glycerol metabolism under anaerobic conditions (Dharmadi et al. 2006; Gonzalez et al. 2008; Murarka et al. 2008) has provided another platform for the use of metabolic engineering approaches to utilize *E. coli* for the efficient conversion of glycerol into fuels, namely ethanol. The conversion of glycerol into the glycolytic intermediate dihydroxyacetone-phosphate (DHAP) under fermentative conditions was found to be mediated by the action of glycerol dehydrogenase (*gldA*) and a PEP-dependent dihydroxyacetone kinase (*dhaKLM*; Fig. 1). However, this pathway appears to be expressed at low levels as inferred by the kinetics of glycerol fermentation (Dharmadi et al. 2006). A recent study focused on engineering *E. coli* for the efficient production of ethanol and coproducts formate and hydrogen from glycerol (Yazdani and Gonzalez 2008). In order to improve the rates of glycerol utilization the two genes found to mediate glycerol dissimilation (*gldA* and *dhaKLM*) were overexpressed, which accelerated the formation of DHAP from glycerol. Common glycolytic enzymes, known to be expressed at high levels, then catalyzed the formation of pyruvate which in turn can be converted into various products through native or engineered pathways.

Another study utilized the same strategy of the overexpression of glycerol dehydrogenase (*gldA*) and PEP-dependent dihydroxyacetone kinase (*dhaKLM*) to successfully engineer *E. coli* for the simultaneous onsumption of the carbon sources present in thin stillage. As a by-product generated in large amounts during commercialized ethanol production from sugars, the conversion of thin stillage, a complex mixture of chemicals including low concentrations of sugars such as glucose and maltose and up to 2% glycerol, into more valuable products is highly desirable. The overexpression of *gldA* and *dhaKLM* in a strain engineered to produce ethanol resulted in the ability to simultaneously consume glycerol and sugars in mineral salts medium with efficient ethanol production (Gonzalez et al. 2009).

Engineering fermentative and nonfermentative pathways for biofuel production

While the engineering of efficient pathways for the utilization of a variety of feedstocks in *E. coli* described in previous sections has shown great promise, engineering of production pathways converting key intermediates into biofuels of interest are needed in order to maximize yields and productivities. The fuels produced from these key intermediates can be roughly grouped into two main classes, fermentative (Fig. 2) and nonfermentative (Fig. 3), depending on the pathways from which they are derived. Fermentative pathways have been utilized to engineer *E. coli* for the production of a variety of alcohols, while several nonfermentative pathways have been engi-

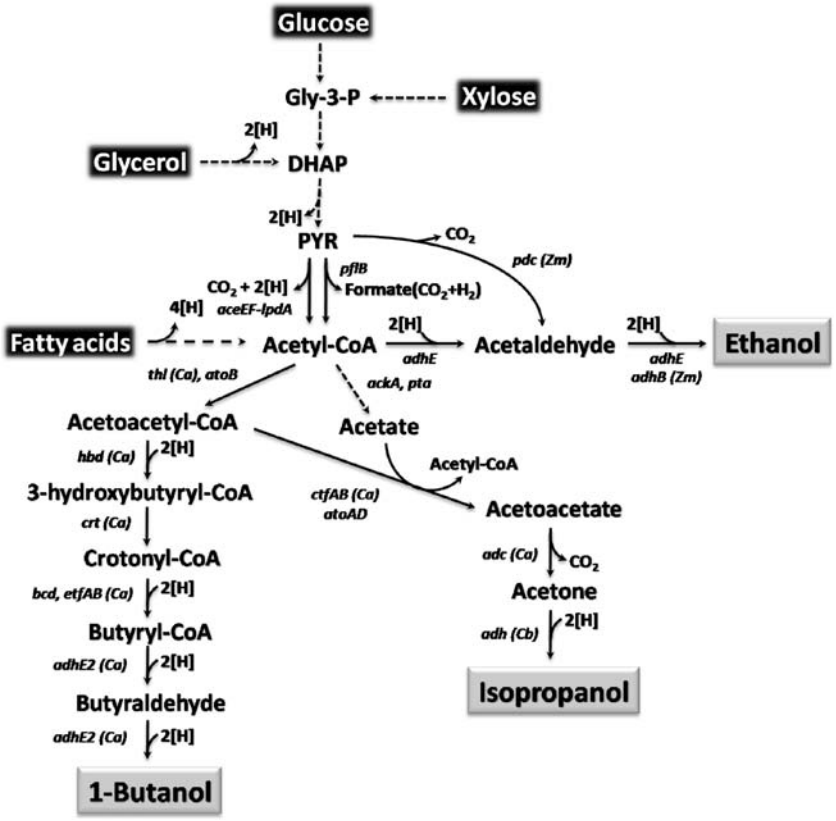

Fig. 2 Fermentative pathways for the production of biofuels in *E. coli*. Metabolic engineering and synthetic biology strategies have been utilized for the conversion of a wide variety of carbon sources (shown in *black shaded boxes*) into several candidate biofuel compounds (shown in *gray shaded boxes*). *Broken lines* represent multiple reaction steps. Relevant reactions are represented by the name of the genes coding for the enzyme (*E. coli* genes unless otherwise noted in *parenthesis* as follows: *Clostridium acetobutylicum*, *Ca*; *Clostridium beijerinckii*, *Cb*; *Zymomonas mobilis*, *Zm*): *aceEF-lpdA*, pyruvate dehydrogenase multienzyme complex; *ackA*, acetate kinase; *adc*, acetoacetate dehydrogenase (*Ca*); *adh*, secondary alcohol dehydrogenase (*Cb*); *adhB*, alcohol dehydrogense (*Zm*); *adhE*, acetaldehyde/alcohol dehydrogenase; *adhE2*, secondary alcohol dehydrogenase (*Ca*); *atoA*, *atoD*, acetyl-CoA:acetoacetyl-CoA transferase; *atoB*, acetyl-CoA acyltransferase; *bcd*, butyryl-CoA dehydrogenase (*Ca*); *crt* crotonase (*Ca*); *ctfAB*, acetoacetyl-CoA transferase (*Ca*) *etfAB*, electron transfer flavoprotein (*Ca*); *hbd*, β-hydroxybutyryl-CoA dehydrogenase (*Ca*); *pdc*, pyruvate decarboxylase (*Zm*); *pflB*, pyruvate formate lyase; *pta*, phosphate acetyltransferase; *thl*, acetyl-CoA acyltransferase (*Ca*). *2[H]* NAD(P)H=$FADH_2$, *DHAP* dihydroxyacetone-phosphate, *Gly-3-P* glyceraldehyde-3-phosphate, *PYR* pyruvate

neered to enable the production of biofuels such as higher chain alcohols from the amino acid biosynthetic pathways and novel fuels derived from the fatty acid and isoprenoid biosynthetic pathways. Each of these will be reviewed in the next sections and representative examples are summarized in Table 1.

Fermentative pathways for biofuel production

Ethanol

Under fermentative conditions, growth on simple sugars such as glucose and xylose results in the production of ethanol, currently the most widely used gasoline-supplementing biofuel. However, ethanol represents only a small fraction of the product mixture, as significant amounts of lactic, formic, succinic and acetic acids are also produced (Sawers and Clark 2004). The overlying factor controlling this mixed-acid fermentation is the requirement of maintaining an overall redox balance. The native pathway for producing ethanol in *E. coli* starts with the intermediate metabolite pyruvate, which is cleaved into acetyl-CoA and formic acid through the action of pyruvate formate lyase (PFL; Fig. 2). The two step reduction of acetyl-CoA to ethanol results in the consumption of two reducing equivalents and proceeds with acetaldehyde as the intermediate, with the enzyme AdhE serving as an acetaldehyde and alcohol dehydrogenase. Therefore, on a 3-carbon basis, the glycolytic pathway to pyruvate generates one reducing equivalent, while the synthesis of ethanol from pyruvate consumes two reducing equivalents (Fig. 2). This hinders homoethanol fermentation, as more oxidized products such as acetic acid must be produced to maintain an overall redox balance. In order to alleviate this

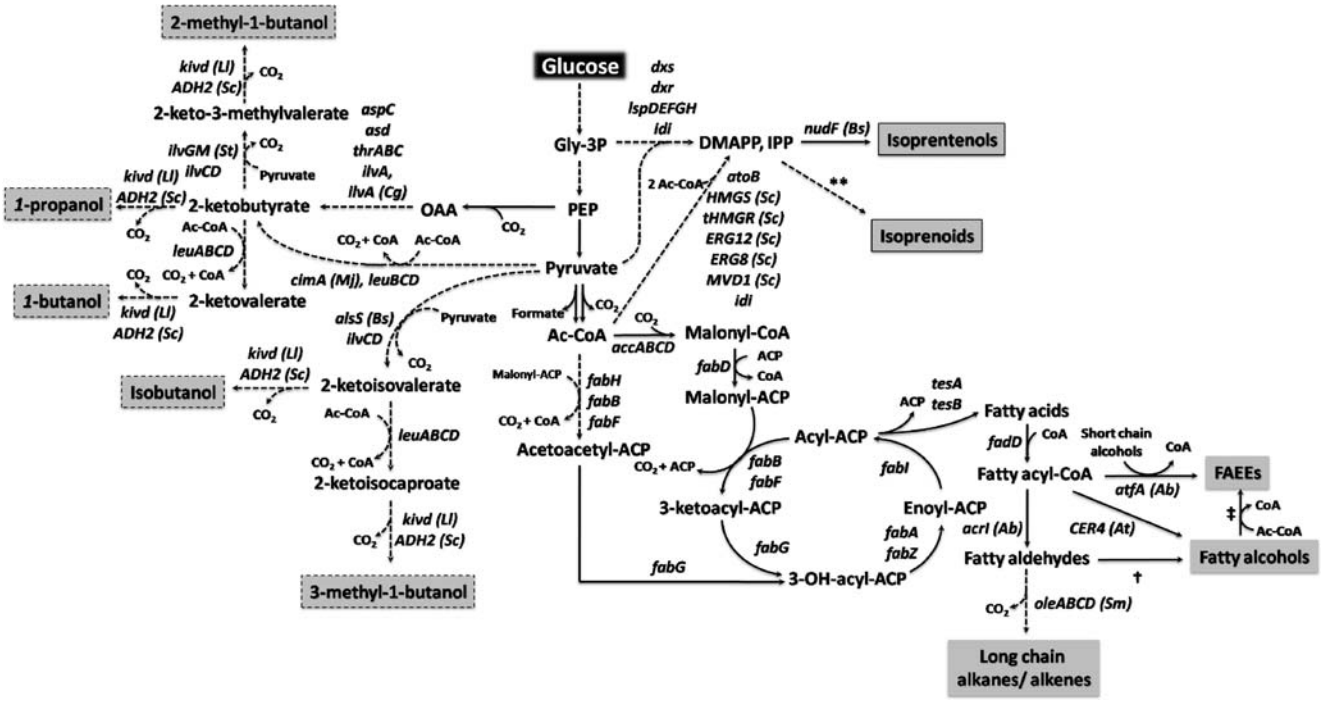

Fig. 3 Nonfermentative pathways for the production of alcohols (*dotted line box with gray shade*), fatty acid-derived biofuels (*no line box with gray shade*), and isoprenoid-derived biofuels (*solid line box with gray shade*) in *E. coli*. *Broken lines* represent multiple reaction steps. Relevant reactions are represented by the name of the genes coding for the enzyme (*E. coli* genes unless otherwise noted in *parenthesis* as follows: *Acinetobacter baylyi* ADP1, *Ab*; *Arabidopsis thaliana*, *At*; *Bacillus subtilis*, *Bs*; *Corynebacterium glutamicum*, *Cg*; *Lactococcus lactis*, *Ll*; *Methanococcus jannaschii*, *Mj*; *Saccharomyces cerevisiae*, *Sc*; *Stenotrophomonas maltophilia*, *Sm*; *Salmonella typhimurium*, *St*): *accABCD*, acetyl-CoA carboxylase; *acrI*, acyl-CoA reductase (*Ab*); *ADH2*, alcohol dehydrogenase (*Sc*); *alsS*, acetolactate synthase (*Bs*); *asd*, aspartate semialdehyde dehydrogenase; *aspC*, aspartate aminotransferase; *atfA*, acyltransferase (*Ab*); *atoB*, acetyl-CoA acyltransferase; *CER4*, fatty acyl-CoA reductase (*At*); *cimA*, citramalate synthase (*Mj*); *dxr*, 1-deoxy-D-xylulose 5-phosphate reductoisomerase; *dxs*, 1-deoxy-D-xylulose 5-phosphate synthase; *ERG12*, mevalonate kinase (*Sc*); *ERG8*, phosphomevalonate kinase (*Sc*); *fabA*, hydroxydecanoyl-ACP dehydrase; *fabB*, ketoacyl-ACP synthase I; *fabD*, malonyl-CoA-ACP transacylase; *fabF*, ketoacyl-ACP synthase II; *fabG*, ketoacyl-ACP reductase; *fabH*, ketoacyl-ACP synthase III; *fabI*, enoyl-ACP reductase; *fabZ*, hydroxyacyl-ACP dehydratase; *fadD*, acyl-CoA synthetase; *HMGS*, 3-hydroxy-3-methylglutaryl-CoA synthase (*Sc*); *idi*, isopentenylpyrophosphate isomerase; *ilvA*, threonine deaminase (either native *E. coli* or *Cg*); *ilvC*, acetohydroxy acid isomeroreductase; *ilvD*, dihydroxy acid dehydratase; *ilvGM*, acetohydroxybutanoate synthase (*St*); *ispD*, 4-diphosphocytidyl-2-methylerythritol synthase; *ispE*, 4-diphosphocytidyl-2-methylerythritol kinase; *ispF*, 2-methylerythritol 2,4-cyclodiphosphate synthase; *ispG*, 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate synthase; *ispH*, 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate reductase; *kivd*, ketoisovalerate decarboxylase (*Ll*); *leuA*, 2-isopropylmalate synthase; *leuB*, 3-isopropylmalate dehydrogenase; *leuCD*, 2-isopropylmalate isomerase; *MVD1*, mevalonate pyrophosphate decarboxylase (*Sc*); *nudF*, prenyl phosphatase (*Bs*); *tHMGR*, truncated 3-hydroxy-3-methylglutaryl-CoA reductase (*Sc*); *thrA*, aspartate kinase, homoserine dehydrogenase; *thrB*, homoserine kinase; *thrC*, threonine synthase; *oleA*, *oleB*, *oleC*, *oleD*, β-ketoacyl-ACP synthases (*Sm*); *tesA*, acyl-CoA thioesterase I; *tesB*, acyl-CoA thioesterase II. *Dagger* represents reaction with assumed alcohol dehydrogenase activity; *double dagger* represents reaction with assumed ester synthase activity; *double asterisk* represents numerous reactions leading to the formation of a wide range of isoprenoids. *Ac-CoA* acetyl-CoA, *ACP* acyl carrier protein, *CoA* coenzyme A, *DMAPP*, dimethylallyl pyrophosphate, *FAEE* fatty acid ethyl ester, *Gly-3-P* glyceraldehyde-3-phosphate, *IPP* isopentenyl pyrophosphate, *OAA* oxaloacetate, *PEP* phosphoenolpyruvate

drawback, metabolic engineering strategies have been implemented to enable higher yields of ethanol production from sugars.

In contrast to the native *E. coli* pathway, the pathway for ethanol production in *Zymomonas mobilis* is a two step process in which pyruvate decarboxylase (PDC) converts pyruvate directly to acetaldehyde and CO_2 and alcohol dehydrogense (ADH) then converts acetaldehyde into ethanol (Fig. 2; Ingram et al. 1987). The key difference from a redox balance standpoint is the fact that the conversion of pyruvate to acetaldehyde is nonoxidative and does not involve any reducing equivalents. Thus, the synthesis of ethanol from pyruvate results in the consumption of one reducing equivalent in this pathway as opposed to two in the native *E. coli* pathway. Expression of *Z. mobilis pdc* and *adhB* genes (encoding for pyruvate decarboxylase and alcohol dehydrogenase, respectively) in *E. coli*, via a plasmid bearing an artificial *pet* (production of ethanol) operon, resulted in much higher ethanol yields, with ethanol accounting for 95% of the fermentation products (Ingram et al. 1987). In order to take advantage of the strong, constitutive native *pfl* promoter, the *pet*

operon was integrated into the *pfl* locus of *E. coli*. However, the resulting recombinant bacteria had low ethanol yield attributed to low PDC expression (Ohta et al. 1991). This problem was circumvented by selection on high chloramphenicol or aldehyde indicator plates resulting in mutants with PDC expression comparable to the plasmid-bearing strain and *Z. mobilis*. Further improvement of ethanol yield was accomplished from the deletion of fumarate reductase (Δ*frdA*), which reduced succinic acid production by 95% resulting in a strain (KO11) that produced ethanol at 100% theoretical yield when grown on glucose or xylose in rich medium (Ohta et al. 1991). When grown anaerobically on xylose, KO11 exhibited 30% higher maximum growth rate and 50% higher glycolytic flux compared to the parent strain. A systems-wide analysis of gene expression using DNA microarrays revealed that this was due to higher expression of the xylose catabolite genes (Tao et al. 2001). While this increased productivity and yield provides an excellent biocatalyst for the production of ethanol, the increased levels of ethanol pose a significant problem for the industrial scale production of ethanol, where it is desirable to convert high concentrations of carbon sources to high concentrations of ethanol. In order to utilize *E. coli* for the viable commercial production of ethanol, improvements in ethanol tolerance are required, as significant growth inhibition results from ethanol concentrations upwards of 35 g/L while yeasts used to commercially produce ethanol are capable of exceeding 90 g/L ethanol (Yomano et al. 1998). Directed evolution of KO11 using an enrichment method which alternatively selected for ethanol tolerance during growth in broth and for ethanol production on solid medium, resulted in a strain (LY01) capable of fermenting xylose to 60 g/L ethanol titer with an 85% yield (Yomano et al. 1998). Further improvements were made after microarray analysis revealed increased glycine metabolism and betaine synthesis (glycine and betaine are protective osmolytes) in LY01 compared to KO11, linking ethanol tolerance to osmotic stress (Gonzalez et al. 2003). While these results represent promising strategies for the production of ethanol from sugars, one important drawback from an industrial standpoint was the fact that the above strains required rich nutrients for ethanol production. This was addressed through the re-engineering of a lactate-producing derivative of KO11 for ethanol production by deleting genes encoding for fermentative routes for NADH and randomly inserting a promoterless cassette containing the complete *Z. mobilis* ethanol pathway (*pdc*, *adhA*, *adhB*) into the chromosome (Yomano et al. 2008). Selection by fermentative growth on mineral salts medium containing xylose resulted in the isolation of a highly productive strain enabling the fermentation of 9% (*w*/*v*) xylose to 4% (*w*/*v*) ethanol in 48 h.

An *E. coli* K-12 mutant capable of the homoethanologenic fermentation of glucose or xylose with yields up to 82% was recently reported using only native *E. coli* genes (Kim et al. 2007). Essential to this approach was a mutation within the *pdh* operon (*pdhR aceEF lpd*), which encodes components of the pyruvate dehydrogenase complex (PDH), an enzyme typically only active under aerobic conditions. Anaerobic ethanol production is the result of a novel pathway combining the activities of pyruvate dehydrogenase with the fermentative alcohol dehydrogenase. By enabling the oxidative decarboxylation of pyruvate to acetyl-CoA through PDH, an extra reducing equivalent (in the form of NADH) is generated which in turn is consumed in the formation of ethanol (Fig. 2). This extra reducing equivalent, which would be contained in formate and dissipated as hydrogen if conversion of pyruvate to acetyl-CoA was mediated by the PFL complex (typically active under anaerobic conditions), enables redox balance to be maintained in a homoethanol pathway from glucose or xylose.

Another recent study developed a minimal *E. coli* cell for efficient ethanol production from hexoses and pentoses using elementary mode analysis to dissect the metabolic network into its basic building blocks (Trinh et al. 2008). Analysis and construction of a minimal *E. coli* cell expressing the *Z. mobilis pdc* and *adhB* genes, resulted in the conversion of glucose or xylose to ethanol at theoretical yields. Remarkably, catabolite repression was absent during anaerobic growth with this strain, enabling the simultaneous utilization of glucose and xylose.

The engineering efforts discussed above mainly focused on increasing ethanol yields from sugars by circumventing the redox imbalance caused by the differences in oxidation states of ethanol and sugars (glucose or xylose). However, as previously discussed, the reduced nature of carbon atoms in glycerol provide significant advantages for the production of reduced fuels, such as ethanol, compared to production from sugars. Under fermentative conditions, the synthesis of ethanol is more preferable than other fermentation products due to the fact that it represents a redox-balanced pathway from glycerol that produces ATP via substrate level phosphorylation. However, recent studies have found that succinate and acetate were also produced during glycerol fermentation and therefore represented competing by-products whose synthesis decreases the yield of ethanol (Dharmadi et al. 2006; Murarka et al. 2008). Therefore, metabolic engineering strategies were implemented in order to minimize by-product formation through the disruption the genes encoding fumarate reductase (*frdA*) and phosphotransacetylase (*pta*), key enzymes in the synthesis of succinate and acetate, respectively (Yazdani and Gonzalez 2008). This strategy resulted in an engineered strain that was able to coproduce ethanol and hydrogen in

Table 1 Select examples of engineering *E. coli* for biofuel production

Biofuel	Strategy[a]	Titer	Reference
Fermentative pathways			
Ethanol	Expression of *Zymomonas mobilis* ethanol production pathway; deletion of competing pathways	40 g/L	Yomano et al. (2008)
	Ethanol production from glycerol; overexpression of anaerobic glycerol dissimilation pathway; deletion of competing pathways	21.3 g/L	Durnin et al. (2009)
Isopropanol	Expression of various gene combinations of synthetic isopropanol pathway modeled after native *Clostridium* pathway	4.9 g/L	Hanai et al. (2007)
1-Butanol	Expression of synthetic 1-butanol pathway modeled after native *Clostridium* pathway	1.2 g/L	Inui et al. (2008)
Nonfermentative pathways			
Isobutanol	Overexpression of valine biosynthetic genes to generate 2-ketoisovalerate; expression of KDC and ADH to produce isobutanol from 2-ketoisovalerate; deletion of competing pathways	22 g/L	Atsumi et al. (2008b)
1-propanol	Expression of the citramalate pathway for 2-ketobutyrate synthesis; expression of KDC and ADH to produce 1-propanol from 2-ketobutyrate; deletion of competing pathways	3.5 g/L	Atsumi and Liao (2008a)
1-butanol	Expression of the citramalate pathway and LeuABCD to generate 2-ketovalerate; expression of KDC and ADH to produce 1-butanol from 2-ketovalerate; deletion of competing pathways	0.5 g/L	Atsumi and Liao (2008a)
2-methyl-1-butanol	Overexpression of a combination of threonine and isoleucine biosynthetic pathways for 2-keto-3-methylvalerate synthesis; expression of KDC and ADH to produce 2 MB from 2-keto-3-methylvalerate; deletion of competing pathways	1.25 g/L	Cann and Liao (2008)
3-methyl-1-butanol	Overexpression of a combination of valine and leucine biosynthetic pathways to generate 2-ketoisocaproate; expression of KDC and ADH to produce 3 MB from 2-ketoisocaproate; deletion of competing pathways	1.28 g/L	Connor and Liao (2008)
Isopentenol	Expression of isopentenol biosynthetic gene *nudF* from *Bacillus subtilis*; overexpression of mevalonate pathway	0.11 g/L	Withers et al. (2007)
Fatty alcohols	Overexpression of acyl-CoA reductase from *A. baylyi* and acyl-CoA synthetase from *E. coli*; disruption of *fadE* gene to inhibit fatty acid degradation; overexpression of key genes for fatty acid/fatty acyl-CoA generation	~0.01 g/L	Keasling et al. (2007)
Olefins	Identification and expression of genes from *Stenotrophomonas maltophilia* involved in the biosynthesis of hydrocarbons; overexpression of key genes for fatty acid/fatty acyl-CoA generation	0.032 g/L	Friedman and Rude (2008)

[a] All examples utilized glucose as the substrate for biofuel production unless otherwise noted in strategy

nearly equimolar amounts approaching the maximum theoretical yield of 1 mol each of ethanol and hydrogen per mole of glycerol fermented with an ethanol production of 4.6 mmol/L/h. A similar strategy was used for the coproduction of ethanol and formate, with the additional disruption of a component of the formate-hydrogen lyase (encoded by the *fdhF* gene), the enzyme responsible for the oxidation of formate into H_2 and CO_2 (Yazdani and Gonzalez 2008). This strain was able to produce ethanol and formate at maximum volumetric rates of 3.58 and 3.18 mmol/L/h, respectively. Despite the promising results obtained in this study, one drawback from an industrial standpoint was the requirement of rich supplementation in the form of tryptone. This requirement was alleviated through the use of microaerobic conditions as a means of eliminating the need for rich nutrients while still taking advantage of the higher degree of reduction of glycerol for the efficient coproduction of ethanol and hydrogen (Durnin et al. 2009). Experimental analysis of the role of both respiratory and fermentative pathways of glycerol utilization under microaerobic conditions were used as the foundation to develop a metabolic engineering strategy to efficiently convert high concentrations of glycerol (up to 60 g/L) into ethanol and hydrogen and ethanol and formate.

Another recent study utilized elementary mode analysis to design an *E. coli* strain for the conversion of glycerol into ethanol (Trinh and Srienc 2009). The functional space of central carbon metabolism was reduced to a total of 28 glycerol utilizing pathways which supported cell function, of which two main competing pathways (one for biomass formation and one for ethanol production) were regulated by the oxygen availability. This coupling between cell growth and ethanol production enabled metabolic evolution of the designed strain which resulted in an evolved strain

capable of converting 40 g/L glycerol to ethanol at 90% of the theoretical yield.

E. coli has recently been engineered for the production of ethanol from fatty acid feedstocks, resulting in ethanol yields much higher than the theoretical maximum obtained from sugars (Dellomonaco and Gonzalez, unpublished). In order to produce ethanol under the respiro-fermentative conditions required for fatty acid utilization, protein engineering was used to construct an aerotolerant mutant of alcohol dehydrogenase (encoded by *adhE*), an oxygen sensitive enzyme whose expression is very low in the presence of oxygen. An aerobically functional AdhE (AdhE*) was obtained by site-directed mutagenesis of AdhE to substitute the residue at position 568 (Glu568Lys). Overexpression of AdhE* from an oxygen-independent promoter in an *E. coli* strain engineered for efficient and complete degradation of fatty acids resulted in the production of ethanol with yields (1.08 g ethanol/g FAs) two-times greater than the theoretical maximum value than can be achieved with the use of lignocellulosic sugars (0.51 g ethanol/g sugar; Dellomonaco and Gonzalez, unpublished).

Higher chain alcohols

While ethanol currently represents the first model biofuel to reach commercial production, its high hygroscopicity and corrosiveness result in incompatibility with current fuel infrastructure posing a potential problem for its wide scale use. For this reason, traditional fermentative pathways for the production of higher chain alcohols have also been examined to enable the production of compounds such as isopropanol and 1-butanol. *E. coli* does not naturally produce either of these compounds, however they are natively produced by a number of *Clostridium* strains (Chen and Hiu 1986; Jones and Woods 1986). The maximum production of isopropanol from evaluation of several *Clostridium* strains was found to be 30 mM (Chen and Hiu 1986), while 1-butanol concentrations ranging from 11.9 to 14.3 g/L produced by *Clostridium beijerinckii* BA101 have been reported (Ezeji et al. 2005). In addition, natural 1-butanol producing *Clostridium* strains have been shown to suffer butanol toxcitiy and exhibit limited growth in 2% butanol, an amount that is similar to that at which *E. coli* has been shown to exhibit butanol toxicity (Knoshaug and Zhang 2009). Additional improvements on yields and titers required for industrial production of these compounds with these organisms is limited due to the lack of genetic capabilities available for the optimization of their productivity. The use of synthetic biology and metabolic engineering strategies for the production of these compounds in *E. coli* has recently been investigated with promising results.

Isopropanol production in *E. coli* was accomplished through the expression of the acetone production pathway of *Clostridium acetobutylicum* along with a secondary alcohol dehydrogenase to convert acetone into isopropanol (Fig. 2). The acetone production pathway consists of four genes, *thl*, *ctfAB*, and *adc*, encoding acetyl-CoA acetyltransferase, acetoacetyl-CoA acetyltransferase, and acetoacetate decarboxylase (Bermejo et al. 1998). The expression of this synthetic pathway in *E. coli* resulted in a maximum production of 4.9 g/L isopropanol from glucose, which represents production levels of isopropanol higher than any seen in naturally producing organisms (Hanai et al. 2007). *E. coli* has also been engineered to produce isopropanol from fatty acids through the expression of a similar synthetic pathway. In this study, the expression of the *C. acetobutylicum* acetone pathway converting acetyl-CoA to acetone and a secondary alcohol dehydrogenase (*adh*) from *C. beijerinckii* for the conversion of acetone to isopropanol along with the overexpression of the *E. coli* pathway that converts acetyl-CoA to acetate (*ackA*, *pta*) in *E. coli* strains engineered for efficient degradation of FAs resulted in isopropanol production (Dellomonaco and Gonzalez, unpublished).

The engineered fermentative route for 1-butanol production in *E. coli* follows a similar strategy to that of isopropanol. The native pathway for 1-butanol production in *C. acetobutylicum* was transferred into *E. coli* to enable the conversion of acetyl-CoA to butanol (Fig. 2). The expression of all the necessary genes for butanol production from acetyl-CoA (*thl*, *hbd*, *crt*, *bcd*, *etfAB*, and *adhE2*) in *E. coli* resulted in the production of 14 mg/L of butanol from glucose (Atsumi et al. 2008a). In an attempt to optimize this pathway for 1-butanol production alternative enzymes from other organisms were expressed, including native *E. coli* AtoB (*atoB*). In addition, the native pathways competing with the butanol pathway for acetyl-CoA and NADH were disrupted to increase butanol yields, which involved the deletion of *ldhA*, *adhE*, *frdBC*, *pta*, and *fur*. The best combination of gene expression and competing pathway disruption lead to a maximum butanol titer of 550 mg/L (Atsumi et al. 2008a). In another study, the expression of the entire heterologous pathway from a single plasmid resulted in the production of 1.2 g/L of 1-butanol after 60 h (Inui et al. 2008). The effects of individual expression, opposed to expression of polycistronic constructs, of key genes in the butanol pathway (*thl*, *hbd*, *crt*, *bcd*, *etfAB*, and *adhE1*) has also recently been investigated (Nielsen et al. 2009). Expression of these genes under a T7*lac* promoter and ribosomal binding site in *E. coli* resulted in a fivefold improvement over the butanol production from the expression of polycistronic construct containing the genes. Further increases in butanol production were achieved through the expression of formate

dehydrogenase (*fdh1*) from *Saccharomyces cerevisiae*, which increases the availability of reducing equivalents through the conversion of formate into CO_2 with the generation of a NADH, and glyceraldehyde 3-phosphate (*gapA*) from *E. coli* to increase glycolytic flux, resulting in the production of 580 mg/L of butanol (Nielsen et al. 2009).

The production of butanol from fatty acids has also been recently investigated through the expression of a similar butanol pathway (*E. coli atoB* with *C. acetobutylicum hbd*, *crt*, *bcd*, *etfAB*, and *adhE2*) in an *E. coli* strain engineered for efficient and complete fatty acid degradation resulting in the production of 2.05 g/L butanol which represents titers and yields between two- and threefold higher than those discussed above for its production from sugars in engineered *E. coli* (Dellomonaco and Gonzalez, unpublished). While these results demonstrate the feasibility of such an approach for the production of 1-butanol in *E. coli*, higher levels of optimization and productivity are required for a viable industrial process.

Nonfermentative pathways for biofuel production

The traditional fermentative pathways exploited for biofuel production have led to the synthesis of a variety of alcohols viewed as candidate fuels. However, recent efforts utilizing synthetic nonfermentative pathways have yielded molecules closer in composition and structure to those currently found in gasoline, which opens the door for the continued use of current gasoline infrastructure. These include higher carbon biofuels derived from fatty acids and isoprenoid pathways, in addition to higher chain alcohols derived from ketoacid pathways (Fig. 3).

Alcohols via nonfermentative pathways

In contrast to the fermentative pathways for alcohol production, which take advantage of naturally occurring pathways in *E. coli* and other organisms, the nonfermentative route to similar products is not naturally occurring. This approach exploits the amino acid biosynthetic pathways in *E. coli* to generate 2-ketoacid precursors for higher chain alcohol production through the last two steps of the Ehrlich degradation pathway (Atsumi and Liao 2008b). The Ehrlich degradation pathway consists of the two-step conversion of 2-ketoacids to an alcohol, with an aldehyde as an intermediate, through the action of a 2-ketoacid decarboxylase (KDC) and an alcohol dehydrogenase (ADH; Fig. 3). The 2-ketoacid decarboxylase from *Lactococcus lactis* and the alcohol dehydrogenase Adh2 from *S. cerevisiae* where shown to have broad substrate activity toward and variety of 2-ketoacids and their expression in *E. coli* resulted in the production of 1-propanol, isobutanol, 1-butanol, 2-methyl-1-butanol, 3-methyl-1-butanol, and 2-phenylethanol were produced (Atsumi et al. 2008b; Fig. 3). In order to improve yields and productivity during the production of these compounds, efforts to increase the production of the 2-ketoacid intermediates were undertaken. For isobutanol production, the expression of *alsS* (*Bacillus subtilis*) and *ilvCD* (*E. coli*) to generate 2-ketoisovalerate was combined with the expression of the aforementioned KDC and ADH (Fig. 3). The engineered strain was able to produce ~22 g/L of isobutanol after 110 h at a yield of 86% of the theoretical maximum (Atsumi et al. 2008b). The efficiency of isobutanol production through the ketoacid pathway in *E. coli* opened the possibility of similar efficiency for the production of higher alcohols. The production of 1-propanol and 1-butanol, which share the common intermediate threonine, was investigated in *E. coli* by utilizing norvaline biosynthesis with the leucine biosynthesis operon (*leuABCD*) for the production of 1-butanol (Fig. 3). It was demonstrated that threonine production was the bottleneck for 1-propanol and 1-butanol production, and the increased production of threonine through the expression of a feedback resistant $thrA^{fbr}BC$ mutant and the deletion of competing pathways resulted in a total alcohol titer of 1.8 g/L with nearly a 1:1 ratio of 1-propanol and 1-butanol (Shen and Liao 2008). An alternative route for the production of 2-ketobutyrate (2-KB), a precursor for both 1-propanol and 1-butanol produced from threonine in the previous strategy, provided by the citramalate pathway was also recently investigated (Atsumi and Liao 2008a). In *E. coli*, the production of 2-KB takes place through the deamination of threonine, which is produced from oxaloacetate in six enzymatic steps. The citramalate pathway provides a much shorter route to 2-KB, with the expression of citramalate synthase from *Methanococcus jannaschii* resulting in the ability synthesize citramalate directly from pyruvate and acetyl-CoA. Citramalate can then be converted into 2-KB in two enzymatic steps catalyzed by LeuBCD from *E. coli*. Directed evolution of citramalate synthase was used to develop a mutant with higher catalytic activity and insensitivity to isoleucine feedback inhibition, which resulted in a mutant showing a 9-fold (1-propanol) and 22-fold (1-butanol) increase in production over the wild-type CimA after 40 h. Using this strategy, the maximum production of 3.5 g/L of 1-propanol and ~500 mg/L of 1-butanol after 92 h was observed (Atsumi and Liao 2008a).

These natural amino acid pathways were also utilized to produce the 5-carbon alcohols, 2-methyl-1-butanol (2-MB), and 3-methyl-1-butanol (3-MB; Fig. 3). The pathway for 2-MB production begins with the synthesis of 2-keto-3-methylvalerate (KMV) from a combination of the threonine

(*aspC-asd-thrABC*) and isoleucine (*ilvGMCD*) biosynthetic pathways. KMV synthesis begins with the carboligation of pyruvate and 2-ketobutyrate catalyzed by acetohydroxyacid synthase (AHAS) (Connor and Liao 2009). Evaluation of select isozymes for the production of KMV from threonine yielded an AHAS II encoded by *Salmonella typhimurium ilvGM* and a threonine deaminase encoded by *Corynebacterium glutamicum ilvA* that improved 2-MB production the most. Increasing threonine production by overexpression *thrABC* along with deletion of *metA* and *tdh* to minimize the consumption of precursors led to the production of 1.25 g/L of 2-MB in 24 h (Cann and Liao 2008). Alternatively, the pathway for the production of 3-MB makes use of the valine (*ilvIHCD*), which generates the valine and isobutanol precursor 2-ketoisovalerate (KIV), and leucine (*leuABCD*) biosynthetic pathways, which converts KIV into 2-ketoisocaproate (KIC) with the addition of acetyl-CoA. Initial 3-MB production with the expression of these genes was low due to the low activity of isoproylmalate synthase (IPMS, encoded by *leuA*). Elimination of feedback inhibition on IPMS and expression of a feedback resistant mutant of LeuA resulted in the production of 1.28 g/L 3-MB in 28 h (Connor and Liao 2008).

In addition, the amino acid biosynthetic pathways for the production of alcohols has been expanded to produce nonnatural five to eight carbon higher alcohols derived from isoleucine biosynthesis. Protein engineering was used to expand the product chain length of LeuA and substrate chain length of 2-ketoacid decarboxylase (KIVD) resulting in higher chain length nonnatural ketoacids that were subsequently converted into higher chain alcohols such as 1-pentanol, 1-hexanol, (S)-3-methyl-1-pentanol, 4-methyl-1-pentanol, (S)-4-methyl-1-hexanol, and (S)-5-methyl-heptanol (Zhang et al. 2008).

Isoprenoid-derived biofuels

Isoprenoids, a group of compounds synthesized naturally in plants, animals, and bacteria, have been produced for many years for their pharmaceutical and nutritional value (Rude and Schirmer 2009). In recent years however, there has been an interest in producing isoprenoid-derived biofuels. Isoprenoids are synthesized from the precursor metabolites isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP), for which two different pathways have been utilized to over produce them (Fig. 3). The mevalonate pathway produces these precursors using acetyl-CoA, while they are produced from glyceraldehyde-3-phosphate and pyruvate by the methyl-erythritol phosphate pathway (Dewick 2002). IPP and DMAPP are then linked to one another to form the activated monoterpene (C10), sesquiterpene (C15), and diterpene precursors geranyl pyrophosphate (GPP), farnesyl phosphate (FPP), and geranylgeranyl pyrophosphate (GGPP; Rude and Schirmer 2009). Fuel-like molecules such as isoprenoid alcohols and olefins can then be synthesized from IPP, GPP, FPP, or GGPP in just one step through the actions of phosphatases, pyrophosphatases, and terpene synthases (Christianson 2008; Rude and Schirmer 2009). Isopentenol has recently been produced in an *E. coli* strain engineered to overproduce IPP and DMAPP via the mevalonate pathway (Martin et al. 2003; Withers et al. 2007). Key to this pathway was the discovery of the isopentenol biosynthetic genes *yhfR* and *nudF* from *B. subtilis* through screening a genomic library and enriching the clones with a restored growth phenotype (Withers et al. 2007). The production of 112 mg/L of isopentenol after 43 h was observed upon expression of the *B. subtilis nudF* gene along with the full mevalonate pathway from acetyl-CoA to IPP and GMAPP (Withers et al. 2007). Although these engineering efforts are still in their early stage, these compounds than can potentially be produced in *E. coli* represent a promising avenue for the production of precursor components of diesel fuel or next generation jet fuels (Fortman et al. 2008; Lee et al. 2008).

Fatty acid-derived biofuels

The high energy content and wide array of chain length and degree of saturation of microbially produced fatty acids provide the potential to produce numerous candidate biofuels similar in structure and composition to molecules currently found in petroleum-based fuels. Fatty acids activated as thioesters with coenzyme A (fatty acyl-CoAs) or acyl carrier proteins (fatty acyl-ACPs) are key intermediates in the biosynthesis of fatty acid-derived fuels (Fig. 3). The most common biofuel belonging to this class of fuels is biodiesel, which consists of fatty acid methyl esters and fatty acid ethyl esters (FAEEs) generally produced from renewable biomass through the alkali-catalyzed transesterification of triacylglycerols from plant oils (Ma and Hanna 1999). However, biodiesel production via this route faces several problems including limited supply and land yield and challenging economics due to the generation of by-products and the cost of separation. While biodiesel produced from microalgal oil (Chisti 2007) and the microbial production of fuels and chemicals from by-products formed during the transesterification process (Yazdani and Gonzalez 2007) have been proposed as potential solutions to some of these issues, metabolic engineering and synthetic biology approaches have also been utilized to provide another route to biodiesel production which could alleviate these problems. A recent report demonstrates one strategy to engineer *E. coli* for the microbial production of fatty acid ethyl esters utilizing the esterification of acyl moieties with ethanol produced from

glucose (Kalscheuer et al. 2006). Key to this strategy is the expression of the ethanol production pathway from *Z. mobilis* (pyruvate decarboxylase (*pdc*) and alcohol dehydrogenase (*adh*)) in combination with a broad substrate range acyltransferase (*atfA*) from *Acinetobacter baylyi*. The resulting *E. coli* strain was able to produce 1.3 g/L of FAEE, consisting mostly of ethyl oleate, through the esterification of acyl moieties generated from supplied oleic acid with ethanol produced from glucose (Kalscheuer et al. 2006). This type of approach could potentially be combined with engineering efforts to overproduce free fatty acids in *E. coli* (Lu et al. 2008) to eliminate the requirement for externally supplied fatty acids and increase FAEE yields.

While microbial esterification represents one method of converting fatty acids into usable fuels, several other pathways for the synthesis of fatty acid derivatives have also been investigated as a means of producing biofuels such as fatty alcohols and olefins (Fig. 3). For example, the expression of several different *Arabidopsis* fatty acyl-CoA reductases in *E. coli* resulted in the synthesis of low titers of fatty alcohols from endogenous *E. coli* fatty acids (Doan et al. 2009). The production of fatty acid derivatives as biofuels has also been reported in recent patents. Keasling et al. (2007) harnessed the existing fatty acid synthesis pathways in *E. coli* as a means of producing fatty alcohols. Disruption of the *fadE* gene resulted in a strain not capable of degrading fatty acids and fatty acyl-CoAs. Small amounts of fatty alcohols (0.2–0.5 mg/L) were produced in the constructed strain, which was increased through the overexpression of the acyl-CoA reductase gene *acrI* from *A. baylyi* ADPI and the acyl-CoA synthetase gene *fadD* from *E. coli*. Coexpression of *E. coli accABCD* and *tesA* (acetyl-CoA carboxylase and thioesterase, respectively) resulted in an additional increase of fatty alcohol production. In addition, pathways for the production of fatty acid derivatives with defined carbon chain length, saturation points, and branch points were proposed through the selection of various thioesterases and modulation of fatty acid biosynthesis. Friedman and Rude (2008) report the production of olefins after the identification of four genes (*oleA*, *oleB*, *oleC*, and *oleD*) from *Stenotrophomonas maltophilia* encoding proteins involved in the biosynthesis of hydrocarbons by shunting fatty acid biosynthesis. Overexpression of these genes in *E. coli* resulted in 7.5 mg/L olefin production ranging in carbon chain length from 27 to 31. The titer was increased to 32 mg/L with the overexpression of *fadD* and *tesA* and the deletion of *fadE*. While the production levels of these fatty acid-derived biofuels are well below the level required for viable industrial production, the feasibility of these approaches represents an exciting prospect for the synthesis of advanced biofuels from renewable feedstocks.

Conclusions and future directions

As the above examples show, engineering efforts employing *E. coli* as the host organism have enabled the production of a wide variety of candidate biofuel compounds while also highlighting the vast array of renewable feedstock constituents that can be utilized by *E. coli* for the production of these fuels. While many of these results simply demonstrate the feasibility of approach for the production of fuels from diverse starting materials, further improvements of both areas (feedstock/carbon-source utilization and biofuel synthesis/biosynthesis pathways) should facilitate the development of efficient, energy-effective, and cost-effective processes viable for the industrial production of biofuel compounds. Key to these current and future developments is the large knowledge base of the metabolism, physiology, and genetics of *E. coli* resulting in an assortment of tools available for the effective manipulation and engineering of *E. coli*. Advancements in the fields of metabolic engineering, synthetic biology, and systems biology have further increased the ability to successfully implement and analyze different strategies to engineer *E. coli* for the production of a broad range of novel biofuels through the exploitation of various metabolic pathways. The continued development of synthetic biology tools that both reduce the time required to make genetic constructs as well as increasing their predictability and reliability should greatly improve metabolic engineering techniques for the effective production of a wide variety of fuels (Lee et al. 2008) and chemicals (Carothers et al. 2009). In addition, the use of system biology tools such as genomics, transcriptomics, proteomics, metabolomics, and fluxomics will help facilitate the design, characterization, and integration of new metabolic pathways for biofuel production (Rodriguez-Moya and Gonzalez 2009; Mukhopadhyay et al. 2008). The continued use and development of strategies and tools from these fields can only serve to further increase the yield, titer, and productivity of an even greater variety of biofuels from various feedstocks representing a promising path for the viable industrial production of renewable fuels critical to lessening our dependence on fossil fuels.

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References

Aristidou A, Penttila M (2000) Metabolic engineering applications to renewable resource utilization. Curr Opin Biotechnol 11:187–198

Atsumi S, Cann AF, Connor MR, Shen CR, Smith KM, Brynildsen MP, Chou KJY, Hanai T, Liao JC (2008a) Metabolic engineering of *Escherichia coli* for 1-butanol production. Metab Eng 10:305–311

Atsumi S, Hanai T, Liao JC (2008b) Non-fermentative pathways for synthesis of branched-chain higher alcohols as biofuels. Nature 451:86–89

Atsumi S, Liao JC (2008a) Directed evolution of *Methanococcus jannaschii* citramalate synthase for biosynthesis of 1-propanol and 1-butanol by *Escherichia coli*. Appl Environ Microbiol 74:7802–7808

Atsumi S, Liao JC (2008b) Metabolic engineering for advanced biofuels production from *Escherichia coli*. Curr Opin Biotechnol 19:414–419

Babitzke P, Romeo T (2007) CsrB sRNA family: sequestration of RNA-binding regulatory proteins. Curr Opin Microbiol 10:156–163

Bailey JE (1991) Toward a Science of Metabolic Engineering. Science 252:1668–1675

Bermejo LL, Welker NE, Papoutsakis ET (1998) Expression of *Clostridium acetobutylicum* ATCC 824 genes in *Escherichia coli* for acetone production and acetate detoxification. Appl Environ Microbiol 64:1079–1085

Cann AF, Liao JC (2008) Production of 2-methyl-1-butanol in engineered *Escherichia coli*. Appl Microbiol Biotechnol 81:89–98

Carothers JM, Goler JA, Keasling JD (2009) Chemical synthesis using synthetic biology. Curr Opin Biotechnol 20:498–503

Chen JS, Hiu SF (1986) Acetone butanol isopropanol production by *Clostridium beijerinckii*. Biotechnol Lett 8:371–376

Chisti Y (2007) Biodiesel from microalgae. Biotechnol Adv 25:294–306

Christianson DW (2008) Unearthing the roots of the terpenome. Curr Opin Chem Biol 12:141–150

Connor MR, Liao JC (2008) Engineering of an *Escherichia coli* strain for the production of 3-methyl-1-butanol. Appl Environ Microbiol 74:5769–5775

Connor MR, Liao JC (2009) Microbial production of advanced transportation fuels in non-natural hosts. Curr Opin Biotechnol 20:307–315

Daniel R, Gottschalk G (1992) Growth temperature-dependent acitivity of glycerol dehydratase in *Escherichia coli* expressing the *Citrobacter freundii* dha Regulon. FEMS Microbiol Lett 100:281–285

Decker K, Plumbridge J, Boos W (1998) Negative transcriptional regulation of a positive regulator: the expression of malT, encoding the transcriptional activator of the maltose regulon of *Escherichia coli*, is negatively controlled by Mlc. Mol Microbiol 27:381–390

Dewick PM (2002) The biosynthesis of C-5-C-25 terpenoid compounds. Nat Prod Rep 19:181–222

Dharmadi Y, Murarka A, Gonzalez R (2006) Anaerobic fermentation of glycerol by *Escherichia coli*: a new platform for metabolic engineering. Biotechnol Bioeng 94:821–829

Doan TTP, Carlsson AS, Hamberg M, Bulow L, Stymne S, Olsson P (2009) Functional expression of five *Arabidopsis* fatty acyl-CoA reductase genes in *Escherichia coli*. J Plant Physiol 166:787–796

Durnin G, Clomburg J, Yeates Z, Alvarez PJJ, Zygourakis K, Campbell P, Gonzalez R (2009) Understanding and harnessing the microaerobic metabolism of glycerol in *Escherichia coli*. Biotechnol Bioeng 103:148–161

Durrett TP, Benning C, Ohlrogge J (2008) Plant triacylglycerols as feedstocks for the production of biofuels. Plant J 54:593–607

Ezeji TC, Qureshi N, Blaschek HP (2005) Continuous butanol fermentation and feed starch retrogradation: butanol fermentation sustainability using *Clostridium beijerinckii* BA101. J Biotechnol 115:179–187

Fischer CR, Klein-Marcuschamer D, Stephanopoulos G (2008) Selection and optimization of microbial hosts for biofuels production. Metab Eng 10:295–304

Flores N, Xiao J, Berry A, Bolivar F, Valle F (1996) Pathway engineering for the production of aromatic compounds in *Escherichia coli*. Nat Biotechnol 14:620–623

Fortman JL, Chhabra S, Mukhopadhyay A, Chou H, Lee TS, Steen E, Keasling JD (2008) Biofuel alternatives to ethanol: pumping the microbial well. Trends Biotechnol 26:375–381

Friedman L, Rude M (2008) Process for producing low molecular weight hydrocarbons from renewable resources. WO/2008/113041

Goerke B, Stulke J (2008) Carbon catabolite repression in bacteria: many ways to make the most out of nutrients. Nat Rev Microbiol 6:613–624

Gonzalez R, Campbell P, Wong M (2009) Production of ethanol from thin stillage by metabolically engineered *Escherichia coli*. Biotechnol Lett 32. doi:10.1007/s10529-009-0159-2

Gonzalez R, Murarka A, Dharmadi Y, Yazdani SS (2008) A new model for the anaerobic fermentation of glycerol in enteric bacteria: trunk and auxiliary pathways in *Escherichia coli*. Metab Eng 10:234–245

Gonzalez R, Tao H, Purvis JE, York SW, Shanmugam KT, Ingram LO (2003) Gene array-based identification of changes that contribute to ethanol tolerance in ethanologenic *Escherichia coli*: Comparison of KO11 (Parent) to LY01 (resistant mutant). Biotechnol Prog 19:612–623

Hanai T, Atsumi S, Liao JC (2007) Engineered synthetic pathway for isopropanol production in *Escherichia coli*. Appl Environ Microbiol 73:7814–7818

Hardiman T, Lemuth K, Keller MA, Reuss M, Siemann-Herzberg M (2007) Topology of the global regulatory network of carbon limitation in *Escherichia coli*. J Biotechnol 132:359–374

Hernandez-Montalvo V, Martinez A, Hernandez-Chavez G, Bolivar F, Valle F, Gosset G (2003) Expression of *galP* and *glk* in a *Escherichia coli* PTS mutant restores glucose transport and increases glycolytic flux to fermentation products. Biotechnol Bioeng 83:687–694

Hernandez-Montalvo V, Valle F, Bolivar F, Gosset G (2001) Characterization of sugar mixtures utilization by an *Escherichia coli* mutant devoid of the phosphotransferase system. Appl Microbiol Biotechnol 57:186–191

Himmel ME, Ding SY, Johnson DK, Adney WS, Nimlos MR, Brady JW, Foust TD (2007) Biomass recalcitrance: engineering plants and enzymes for biofuels production. Science 315:804–807

Hirsch RL, Bezdek R, Wendling R (2006) Peaking of world oil production and its mitigation. AIChE J 52:2–8

Hu Q, Sommerfeld M, Jarvis E, Ghirardi M, Posewitz M, Seibert M, Darzins A (2008) Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances. Plant J 54:621–639

Ideker T, Galitski T, Hood L (2001) A new approach to decoding life: systems biology. Annu Rev Genomics Hum Genet 2:343–372

Ingram LO, Conway T, Clark DP, Sewell GW, Preston JF (1987) Genetic engineering of ethanol production in *Escherichia coli*. Appl Environ Microbiol 53:2420–2425

Inui M, Suda M, Kimura S, Yasuda K, Suzuki H, Toda H, Yamamoto S, Okino S, Suzuki N, Yukawa H (2008) Expression of *Clostridium acetobutylicum* butanol synthetic genes in *Escherichia coli*. Appl Microbiol Biotechnol 77:1305–1316

Jones DT, Woods DR (1986) Acetone-butanol fermentation revisited. Microbiol Rev 50:484–524

Kalscheuer R, Stolting T, Steinbuchel A (2006) Microdiesel: *Escherichia coli* engineered for fuel production. Microbiology 152:2529–2536

Keasling JD, Hu Z, Somerville C, Church G, Berry D, Friedman L, Schirmer A, Brubaker S, Del Cardayre SB (2007) Production of fatty acids and derivatives thereof. WO/2007/136762

Kerr RA (2007) Global warming is changing the world. Science 316:188–190

Khankal R, Chin JW, Ghosh D, Cirino PC (2009) Transcriptional effects of CRP* expression in *Escherichia coli*. J Biol Eng 3:13

Kim Y, Ingram LO, Shanmugam KT (2007) Construction of an *Escherichia coli* K-12 mutant for homoethanologenic fermentation of glucose or xylose without foreign genes. Appl Environ Microbiol 73:1766–1771

Kimata K, Inada T, Tagami H, Aiba H (1998) A global repressor (MIc) is involved in glucose induction of the *ptsG* gene encoding major glucose transporter in *Escherichia coli*. Mol Microbiol 29:1509–1519

Knoshaug EP, Zhang M (2009) Butanol tolerance in a selection of microorganisms. Appl Biochem Biotechnol 153:13–20

Lee SK, Chou H, Ham TS, Lee TS, Keasling JD (2008) Metabolic engineering of microorganisms for biofuels production: from bugs to synthetic biology to fuels. Curr Opin Biotechnol 19:556–563

Lu XF, Vora H, Khosla C (2008) Overproduction of free fatty acids in *E. coli*: implications for biodiesel production. Metab Eng 10:333–339

Ma FR, Hanna MA (1999) Biodiesel production: a review. Bioresour Technol 70:1–15

Martin VJJ, Pitera DJ, Withers ST, Newman JD, Keasling JD (2003) Engineering a mevalonate pathway in *Escherichia coli* for production of terpenoids. Nat Biotechnol 21:796–802

Mukhopadhyay A, Redding AM, Rutherford BJ, Keasling JD (2008) Importance of systems biology in engineering microbes for biofuel production. Curr Opin Biotechnol 19:228–234

Murarka A, Dharmadi Y, Yazdani SS, Gonzalez R (2008) Fermentative utilization of glycerol by *Escherichia coli* and its implications for the production of fuels and chemicals. Appl Environ Microbiol 74:1124–1135

Nichols NN, Dien BS, Bothast RJ (2001) Use of catabolite repression mutants for fermentation of sugar mixtures to ethanol. Appl Microbiol Biotechnol 56:120–125

Nielsen DR, Leonard E, Yoon SH, Tseng HC, Yuan C, Jones Prather KL (2009) Engineering alternative butanol production platforms in heterologous bacteria. Metab Eng 11:262–273

Ohta K, Beall DS, Mejia JP, Shanmugam KT, Ingram LO (1991) Genetic improvement of *Escherichia coli* for ehtnao, production: chromosomal integration of *Zymomonas mobilis* genes enconding pyruvate decarboxylase and alcohol dehydrogenase II. Appl Environ Microbiol 57:893–900

Oren A (2005) A hundred years of *Dunaliella* research: 1905–2005. Saline Syst 1:2

Pleiss J (2006) The promise of synthetic biology. Appl Microbiol Biotechnol 73:735–739

Rude MA, Schirmer A (2009) New microbial fuels: a biotech perspective. Curr Opin Microbiol 12:274–281

Rodriguez-Moya M, Gonzalez R (2009) Systems biology approaches for the microbial production of biofuels. Biofuels 1. doi:10.4155/BFS.10.5

Sawers RG, Clark DP (2004) Fermentative pyruvate and acetyl-CoA metabolism. In: Neidhardt FC, Curtiss R III, Ingraham JL, Lin ECC, Low KB, Magasanik B, Reznikoff WS, Riley M, Schaechter M, Umbarger HE (eds) *Escherichia coli* and *Salmonella*: cellular and molecular biology, Webth edn. ASM Press, Washington

Schubert C (2006) Can biofuels finally take center stage? Nat Biotechnol 24:777–784

Service RF (2009) Biofuels: ExxonMobil fuels venter's efforts to run vehicles on algae-based oil. Science 325:379–379

Shen CR, Liao JC (2008) Metabolic engineering of *Escherichia coli* for 1-butanol and 1-propanol production via the keto-acid pathways. Metab Eng 10:312–320

Skraly FA, Lytle BL, Cameron DC (1998) Construction and characterization of a 1,3-propanediol operon. Appl Environ Microbiol 64:98–105

Stephanopoulos G (2002) Metabolic engineering: perspective of a chemical engineer. AIChE J 48:920–926

Stephanopoulos G (2007) Challenges in engineering microbes for biofuels production. Science 315:801–804

Stulke J, Hillen W (1999) Carbon catabolite repression in bacteria. Curr Opin Microbiol 2:195–201

Tao H, Gonzalez R, Martinez A, Rodriguez M, Ingram LO, Preston JF, Shanmugam KT (2001) Engineering a homo-ethanol pathway in *Escherichia coli*: Increased glycolytic flux and levels of expression of glycolytic genes during xylose fermentation. J Bacteriol 183:2979–2988

Totemeyer S, Booth NA, Nichols WW, Dunbar B, Booth IR (1998) From famine to feast: the role of methylglyoxal production in *Escherichia coli*. Mol Microbiol 27:553–562

Trinh CT, Srienc F (2009) Metabolic Engineering of *Escherichia coli* for Efficient Conversion of Glycerol to Ethanol. Appl Environ Microbiol 75:6696–6705

Trinh CT, Unrean P, Srienc F (2008) Minimal *Escherichia coli* cell for the most efficient production of ethanol from hexoses and pentoses. Appl Environ Microbiol 74:3634–3643

Withers ST, Gottlieb SS, Lieu B, Newman JD, Keasling JD (2007) Identification of isopentenol biosynthetic genes from *Bacillus subtilis* by a screening method based on isoprenoid precursor toxicity. Appl Environ Microbiol 73:6277–6283

Yazdani SS, Gonzalez R (2007) Anaerobic fermentation of glycerol: a path to economic viability for the biofuels industry. Curr Opin Biotechnol 18:213–219

Yazdani SS, Gonzalez R (2008) Engineering *Escherichia coli* for the efficient conversion of glycerol to ethanol and co-products. Metab Eng 10:340–351

Yomano LP, York SW, Ingram LO (1998) Isolation and characterization of ethanol-tolerant mutants of *Escherichia coli* KO11 for fuel ethanol production. J Ind Microbiol Biotech 20:132–138

Yomano LP, York SW, Shanmugam KT, Ingram LO (2009) Deletion of methylglyoxal synthase gene (*mgsA*) increased sugar co-metabolism in ethanol-producing *Escherichia coli*. Biotechnol Lett 31:1389–1398

Yomano LP, York SW, Zhou S, Shanmugam KT, Ingram LO (2008) Re-engineering *Escherichia coli* for ethanol production. Biotechnol Lett 30:2097–2103

Zhang KC, Sawaya MR, Eisenberg DS, Liao JC (2008) Expanding metabolism for biosynthesis of nonnatural alcohols. Proc Natl Acad Sci USA 105:20653–20658

Zhu MM, Skraly FA, Cameron DC (2001) Accumulation of methylglyoxal in anaerobically grown *Escherichia coli* and its detoxification by expression of the *Pseudomonas putida* glyoxalase I gene. Metab Eng 3:218–225