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REVIEW ARTICLE

Metabolic engineering: Use of system-level approaches and application to fuel production in *Escherichia coli*

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Abstract

Metabolic engineering was formally defined more than two decades ago (Bailey, 1991) and it is now an established discipline. Metabolic engineering is generally defined as the directed improvement of product formation or cellular properties through the modification of specific biochemical reactions or the introduction of new ones with the use of recombinant DNA technology (Bailey, 1991; Stephanopoulos et al. 1998). Therefore, the analysis and engineering/synthesis of metabolic pathways is of central importance to metabolic engineering. The analytical part uses a number of experimental and modeling techniques for the systematic study of cellular responses (in terms of RNA, protein and metabolite levels, metabolic fluxes, etc.) to genetic and environmental perturbations. This facilitates a rational design of metabolic modifications, which are implemented using recombinant DNA technology. Both, the analysis and the synthesis of metabolic pathways will be covered in this review. Recent efforts on the engineering of fermentative and biosynthetic pathways for biofuel production in *Escherichia coli*, as well as those enabling the utilization of novel feedstocks, will be highlighted.

Keywords: biofuels, *Escherichia coli*, metabolic engineering, systems biology.

SYSTEM-LEVEL ANALYTICAL AND SYNTHETIC TOOLS IN THE METABOLIC ENGINEERING TOOLBOX

Given the emphasis that metabolic engineering places on a system-level approach to analyze and engineer metabolism, several genome-wide techniques have been extensively used to analyze the metabolism of *Escherichia coli* and support engineering efforts (Figure 1). Below some of these analytical and synthetic tools in the metabolic engineering toolbox are presented.

Metabolic engineering undoubtedly benefits from traditional techniques that enable engineering and de novo synthesis of promoters, ribosome binding sites and entire coding regions, thus allowing modulation of both expression level and activity of target proteins/enzymes. However, new tools that enable genome engineering and system-wide identification of genes that confer target traits are very useful (Boyle and Gill, 2012). These emerging techniques include multiscale analysis of library enrichment (SCALEs) (Lynch et al. 2007), trackable multiplex engineering (TRMR) (Warner et al. 2010), coexisting/coexpressing genomic libraries (CoGeL) (Nicolaou et al. 2011), genome-scale analysis of library sorting (GALibSo) (Stadlmayr et al. 2010), multiplex automated genome engineering (MAGE) (Wang et al. 2009), conjugative assembly genome engineering (CAGE) (Isaacs et al. 2011), and global transcription machinery engineering (gTME) (Alper and Stephanopoulos, 2007). SCALEs are libraries that provide quantification of the effects that the expression of a specific gene or gene

fragment has on a specific phenotypic trait (Lynch et al. 2007). Bonomo et al. (2008) used the SCALES method to quantify several genome-wide growth phenotypes for *E. coli* libraries cultured under inhibitory levels of different substances that targeted metabolic pathways concerning aspartate. Also, recently developed TRMR can be used to construct and map genome-wide promoter mutations in *E. coli* and evaluate them simultaneously. On the other hand, gTME enables the creation and isolation of polygenic mutants under several conditions (Alper and Stephanopoulos, 2007; Tyo et al. 2007), thus facilitating phenotypes that would be difficult to obtain with conventional gene modification techniques. This technique has been used to improve *E. coli* tolerance to ethanol (Alper and Stephanopoulos, 2007) and shown to be superior to traditional random mutagenesis and selection methods. One of the applications investigated in the study was increasing *E. coli* tolerance to ethanol. The acquired values for the doubling time of the strains transformed by gTME were 13-44% lower than those previously obtained by serial sub-culturing (Yomano et al. 1998).

METABOLIC FLUX ANALYSIS

Metabolic fluxes are probably the best metric to capture the metabolic state of a cell. Flux quantification can be performed using a number of approaches, which can be broadly classified into *in silico*- and *in vivo*-based metabolic flux analysis (isMFA and ivMFA, respectively). One of the most representative examples of isMFA is flux balance analysis (Raman and Chandra, 2009), a technique used to construct genome-scale metabolic models and assess the metabolic capabilities of the organism of interest. For example, Feist et al. (2010) reported the use of isMFA to evaluate the production potential for growth-coupled products of *E. coli*. A recent development in this area is the introduction of the OptForce procedure (Ranganathan et al. 2010), which identifies all possible engineering interventions by classifying reactions in the metabolic model depending upon whether their flux values must increase, decrease or become equal to zero to meet a pre-specified overproduction target. The OptForce framework was validated by identifying non-intuitive engineering strategies for succinate production in *E. coli*. An updated genome-scale network reconstruction of *E. coli* was also recently reported, demonstrating how it can be used to discover non-annotated genes in all strains sequenced to date (Orth et al. 2011).

One of the most widely used versions of ivMFA is a technique known as metabolite balancing, in which intracellular fluxes are estimated based on a stoichiometric model and extracellular measurements (Stephanopoulos et al. 1998; Klapa et al. 1999). This technique is based on mass balances around intracellular metabolites (which are considered in pseudo-steady-state) with the measurements of extracellular fluxes acting as constraints for flux calculation.

In combination with isMFA, metabolite balancing can provide significant insights into microbial metabolism (Murarka et al. 2010a). However, given the small number of extracellular measurements, when compared to the number of intracellular fluxes, this technique relies on the incorporation of redox (NADH/NADPH) and/or ATP balances as part of the stoichiometric model. This, in turn, can lead to incorrect flux estimation due to the incomplete knowledge about pathways involving NADH/NADPH and ATP. Another drawback of metabolite balancing is its inability to account for parallel metabolic pathways, metabolic cycles, and reversible or bidirectional reactions (Wiechert et al. 2001).

The use of isotopically labeled substrates (usually with ^{13}C) provides additional constraints to the stoichiometric equations used in metabolite balancing, avoiding assumptions about redox and energy balances and potentially accounting for parallel pathways, cycles, and reversibility (Klapa et al. 1999). This approach is based on feeding an organism with a mixture of a specifically ^{13}C -labeled and a naturally abundant version of the same substrate and measuring the ^{13}C enrichments or isotopomer distributions in the carbon atoms of different metabolites. The most common metabolites used for this purpose are proteinogenic amino acids because they are abundant, stable, and their labeling pattern reflects that of precursors metabolites generated in central metabolism. In one approach called metabolic flux ratio (MetaFoR) analysis relative local fluxes around a node are obtained from the abundances of intact carbon fragments in metabolites (calculated from the 2-D [^{13}C , ^1H] NMR data) (Fischer and Sauer, 2003; Fischer and Sauer, 2005). Comprehensive ^{13}C -based metabolic flux analysis (^{13}C -MFA), on the other hand, is achieved through a modeling approach that requires information on the metabolic network, labeling patterns of amino acids, and extracellular fluxes. This information is combined in an error function that accounts for the average difference between measured and simulated labeling patterns. An optimization procedure is then followed to solve for intracellular fluxes that minimize this error function (Klapa et al. 1999; Tang et al. 2009). ^{13}C -MFA has

been extensively used to characterize the fermentative metabolism of *E. coli* (Murarka et al. 2010b; Choudhary et al. 2011).

An excellent example of the value of different MFA approaches to the design and implementation of ME strategies was recently reported on an integrated computational and experimental study for overproducing fatty acids in *E. coli* (Ranganathan et al. 2012). The OptForce procedure was used for suggesting and prioritizing genetic manipulations that could support overproduction of fatty acids of different chain lengths (C₆-C₁₆) starting with wild-type *E. coli*. When interventions suggested by OptForce were implemented, the engineered strain produced 1.70 g/L of C₍₁₄₋₁₆₎ fatty acids in minimal medium at a yield of 0.14 g fatty acid/g glucose.

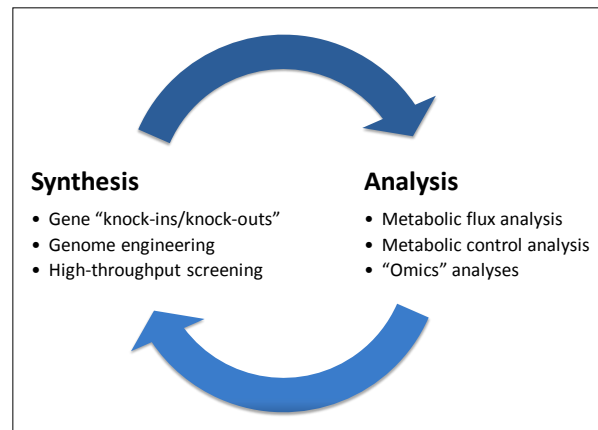


Fig. 1 Interplay between *synthesis* and *analysis* of metabolism, the key components of the metabolic engineering cycle. Representative approaches for synthesis and analysis are shown.

Metabolic control analysis

The identification of metabolic bottlenecks and rate-limiting steps has always been of paramount importance for metabolic engineering and Metabolic Control Analysis (MCA) has for long provided a framework to conducting such analyses. MCA can be used to elucidate the control structure of a metabolic network (Fell and Sauro, 1985). This is achieved through the calculation of flux and concentration control coefficients, the former being of special importance because they indicate how a relative change in the activity of an enzyme affects the flux of a pathway (Stephanopoulos et al. 1998). Such an analysis can identify reactions that control the carbon flux through the pathway and hence genes/proteins that need to be manipulated to increase the flux towards the desired product. The MCA approach was recently used to conduct a quantitative analysis of the fermentative metabolism of glycerol in *E. coli*, gain a better understanding of glycerol fermentation and identify key targets for genetic manipulation that could enhance product synthesis (Cintolesi et al. 2012). The calculated flux control coefficients predicted that the glycolytic flux during glycerol fermentation is almost exclusively controlled by the enzymes glycerol dehydrogenase (encoded by *gldA*) and dihydroxyacetone kinase (encoded by *dhaKLM*). In agreement with the MCA findings, overexpression of *gldA* and *dhaKLM* led to significant increase in glycerol utilization and ethanol synthesis fluxes.

An MCA-based method, called ORACLE (optimization and risk analysis of complex living entities), has also been proposed to calculate control coefficients without the need for a kinetic model (Miskovic and Hatzimanikatis, 2010).

“Omics” analyses

Advances of the genomic revolution have opened new opportunities for the challenging task of assigning biological function to each gene and elucidating the metabolic and regulatory networks that operate in a biological system. Functional genomics has then emerged as a new discipline that relates

to “the development and application of high-throughput global (genome-wide or system-wide) experimental approaches to assess gene function by making use of the information and reagents provided by structural genomics” (Hieter and Boguski, 1997). The field of functional genomics has grown exponentially in the last decades, embracing areas such as transcriptomics (mRNAs), proteomics (proteins), metabolomics (metabolites), and fluxomics (fluxes), among others. This systems-level approach to understand complex metabolic and regulatory networks enables the design of more efficient microorganisms for the production of valuable molecules, including biofuels.

Significant progress in the development of functional genomic tools in recent years has powered the elucidation of complex phenotypes and the engineering of new ones for the development of promising industrial strains (Stephanopoulos et al. 2004; Zhang et al. 2006). The use of transcriptional profiling tools, such as DNA microarrays, allows the study of gene expression by identifying differentially expressed genes under different experimental conditions or resulting from certain genetic perturbations. However, other functional genomics tools are required to complement transcriptomic studies. Proteomics is of special importance because most of the cellular activities are mediated by proteins, and is the primary tool used to describe cellular interactions beyond mRNA level (Graham et al. 2007). The large-scale quantification and identification of proteins can be achieved by one- or two-dimensional gel electrophoresis, followed by software aided quantification tools and mass spectrometry techniques (Watt et al. 2003), or by gel-free techniques such as liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) and protein isotope labeling (Gevaert et al. 2007). Comparative proteomic analyses detect differentially expressed proteins by variations in the intensities of labeled protein spots in the gel, under different genetic or environmental conditions. Similar to proteins, metabolites are also context-dependent entities whose concentration is directly related to the physiological state of the cell (Raamsdonk et al. 2001). Metabolomics makes use of high-throughput analyses like Nuclear Magnetic Resonance (NMR), and Gas or Liquid Chromatography coupled to Mass Spectrometry (GC-MS, and LC-MS) for the quantification of intra- and extra-cellular small-molecular-weight metabolites (Buchholz et al. 2002). Metabolic studies also serve as a screening tool to investigate the unknown effect of a specific genetic modification on the phenotype of the organism, and as a means to describe metabolism kinetics by performing pulse experiments (Buchholz et al. 2002).

ENGINEERING OF FERMENTATIVE AND NON-FERMENTATIVE PATHWAYS FOR FUEL PRODUCTION IN *E. COLI*

The engineering of pathways that convert key intermediates into the fuel of interest is typically the first step taken to ensure product synthesis at maximum yield and rate. Both fermentative and biosynthetic pathways have been engineered in *E. coli* for the production of a variety of alcohols, fatty acids and a host of novel fuels derived from the fatty acid, polyketide, and isoprenoid biosynthesis pathways, as well as engineered ketoacid and reversal of the β -oxidation pathways, among others.

Engineering of fermentative pathways

One of the first applications of metabolic engineering was reported about 25 years ago, when the laboratory of Lonnie Ingram engineered *E. coli* to produce ethanol as the primary product of glucose fermentation (Ingram et al. 1987). Under fermentative conditions, growth of wild-type *E. coli* on simple sugars, such as glucose and xylose, results in the production of acids (lactic, formic, succinic and acetic) and small amounts of ethanol (Sawers and Clark, 2004). To engineer a homoethanogenic pathway, Ingram and co-workers (Ingram et al. 1987) transferred the ethanol synthesis pathway from *Zymomonas mobilis* (a two step pathway in which pyruvate decarboxylase (PDC) converts pyruvate directly to acetaldehyde and CO₂ and alcohol dehydrogenase (ADH) then converts acetaldehyde into ethanol: Figure 2) to a strain of *E. coli* containing a mutation in the *frd* operon (encoding the enzyme fumarate reductase, which is responsible for the conversion of fumarate to succinate). These two sets of genetic manipulations led to a complete shift of the fermentative metabolism of *E. coli* from mixed acid to homoethanogenic fermentation (Ingram et al. 1987; Ohta et al. 1991) and paved the way for the metabolic engineering of *E. coli* to efficiently synthesize ethanol and a host of fuel molecules.

While ethanol was the first biofuel to reach commercial production, its incompatibility with current fuel infrastructure poses a significant challenge for its widespread adoption. Higher-chain alcohols, on the other hand, represent a potential alternative and hence their biological production has been explored.

Some of these alcohols are natively produced by a number of *Clostridium* strains (Chen and Hiu, 1986; Jones and Woods, 1986). However, given the lack of genetic capabilities available for the optimization of these organisms, the engineering of *E. coli* was recently investigated with promising results. For example, *E. coli* was engineered to produce isopropanol at levels higher than any seen in naturally producing organisms (4.9 g/L) by the expression of the acetone production pathway of *Clostridium acetobutylicum* along with a secondary alcohol dehydrogenase to convert acetone into isopropanol (Figure 2) (Hanai et al. 2007).

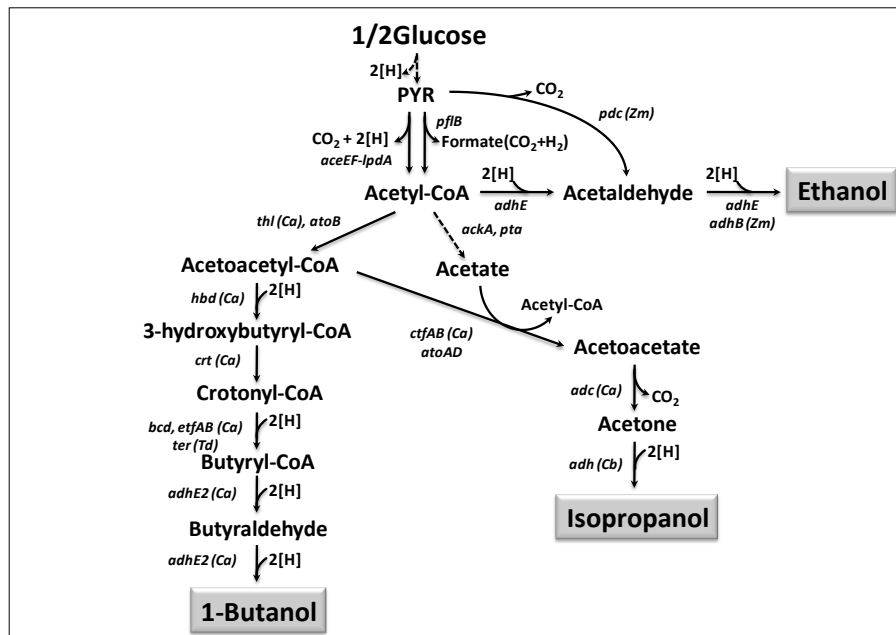


Fig. 2 Pathways engineered in *E. coli* for the fermentative production of ethanol, isopropanol, and 1-butanol (shown in grey shaded boxes). Broken lines represent multiple reaction steps. Relevant reactions are represented by the name of the genes coding for the enzyme. Non-*E. coli* genes are noted in parenthesis using the following abbreviations: *Clostridium acetobutylicum* (Ca); *Clostridium beijerinckii* (Cb); *Treponema denticola* (Td); *Zymomonas mobilis* (Zm). *aceEF-tpdA*, pyruvate dehydrogenase multienzyme complex; *ackA*, acetate kinase; *adc*, acetoacetate dehydrogenase (Ca); *adh*, secondary alcohol dehydrogenase (Cb); *adhB*, alcohol dehydrogenase (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); *ter*, transenoyl-CoA reductase (Td). Abbreviations: 2[H] = NAD(P)H = FADH₂; DHAP, dihydroxyacetone-phosphate; Gly-3-P, glyceraldehyde-3-phosphate; PYR, pyruvate.

The synthesis of 1-butanol via engineered fermentative pathways has also been reported in *E. coli*. Initial work focused on the transfer of the 1-butanol synthesis portion of the Clostridial Acetone-Butanol-Ethanol (ABE) pathway into strains of *E. coli* devoid of native fermentative pathways (Atsumi et al. 2008a; Inui et al. 2008) (Figure 2). Expression of native *E. coli* AtoB (*atoB*), along with all the necessary genes for butanol production from acetyl-CoA (*thl*, *hbd*, *crt*, *bcd*, *etfAB*, and *adhE2*), and disruption of native pathways competing with the butanol pathway for acetyl-CoA and NADH (deletion of *ldhA*, *adhE*, *frdBC*, *pta*, and *fur*) led to a maximum butanol titer of 550 mg/L (Atsumi et al. 2008a). Similar levels of 1-butanol production were achieved upon expression of the same pathway from polycistronic constructs, along with the expression of formate dehydrogenase (*fdh1*) from *Saccharomyces cerevisiae*, which increases the availability of reducing equivalents, and glyceraldehyde 3-phosphate (*gapA*) from *E. coli* to increase glycolytic flux (Nielsen et al. 2009). Another study reported the production of 1.2 g/L of 1-butanol after 60 hrs upon expression of the entire heterologous pathway from a single plasmid (Inui et al. 2008).

While the success of the above-mentioned efforts was limited by the low activity of the foreign pathway in *E. coli*, identification of key limiting steps and use of genes/enzymes not involved in the native 1-butanol synthesis pathway has recently enabled the efficient synthesis of 1-butanol (Bond-Watts et al. 2011; Shen et al. 2011). Of particular relevance was the use of a transenoyl-CoA reductase from *Treponema denticola* to replace the clostridial butyryl-CoA dehydrogenase in the conversion of crotonyl-CoA to butyryl-CoA (Bond-Watts et al. 2011; Shen et al. 2011). Process-based improvements, such as the removal of 1-butanol from the fermentation broth, led to the synthesis of 30 g/L of 1-butanol (Shen et al. 2011).

Engineering of non-fermentative pathways

While fermentative pathways can be exploited for the synthesis of valuable fuel candidates, the use of non-fermentative pathways represents an invaluable opportunity to generate “designer” fuels with properties that make them compatible with current infrastructure for fuel transportation, storage and use. Such pathways include the fatty acid, polyketide, and isoprenoid biosynthesis pathways, as well as engineered keto-acid and reversal of the β -oxidation pathways (Figure 3).

Fuels derived from the fatty acid biosynthesis pathway

The high-energy content and wide array of chain length and degree of saturation of fatty acids could support the synthesis of molecules similar in structure and composition to those currently found in petroleum-based fuels. The activated form of fatty acids, thioesters with coenzyme A (fatty acyl-CoAs) or acyl carrier protein (fatty acyl-ACPs), are key intermediates in the biosynthesis of fatty acid derived fuels (Figure 3). A number of recent reports have shown the potential of engineering the native fatty acid biosynthesis pathway in *E. coli* to produce fatty acids (Lennen et al. 2010; Steen et al. 2010; Zhang et al. 2011), fatty esters (Steen et al. 2010), long-chain alcohols (Steen et al. 2010), waxes (Steen et al. 2010), hydrocarbons (Lennen et al. 2010; Schirmer et al. 2010), methyl ketones (Park et al. 2012), and biodiesel (Steen et al. 2010).

In an early study, *E. coli* was engineered to produce fatty esters (biodiesel), fatty alcohols, and waxes directly from simple sugars or hemicellulose (Steen et al. 2010). Production of free fatty acids and acyl-CoAs through the native fatty acid biosynthesis pathway was achieved by eliminating β -oxidation, and by overexpressing thioesterases and acyl-CoA ligases. The synthesis of biodiesel, alcohols and wax esters was achieved by overexpression of non-native pathways. For example, alcohols were produced from fatty acyl-CoAs by overexpressing fatty acyl-CoA reductases, esters by expressing an acyltransferase in conjunction with an alcohol-forming pathway, and biodiesel by introducing the ethanol pathway shown in Figure 2.

The synthesis of alkanes, on the other hand, was facilitated by the discovery of an alkane biosynthesis pathway from cyanobacteria that consists of an acyl-acyl carrier protein reductase that reduces acyl-ACPs to fatty aldehydes, which are then decarboxylated to odd-chain alkanes and alkenes by an aldehyde decarbonylase (Schirmer et al. 2010). Although the mechanism employed by the decarbonylase is still under investigation (Das et al. 2011; Li et al. 2011), the expression of the two-step pathway in *E. coli* produced C₁₃-C₁₇ mixtures of alkanes and alkenes (Schirmer et al. 2010).

Fuels derived from the isoprenoid biosynthesis pathway

While isoprenoids are naturally synthesized by plants, animals, and bacteria, and used for their pharmaceutical and nutritional value (Rude and Schirmer, 2009), only recently there has been an interest in producing isoprenoid-derived biofuels. Two different pathways have been utilized to overproduce isoprenoids from the precursor metabolites isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) (Figure 3). The mevalonate pathway generates the aforementioned precursors using acetyl-CoA, while the methylerythritol phosphate pathway produces them from glyceraldehyde-3-phosphate (G3P) and pyruvate (Dewick, 2002). Diterpene precursors geranyl pyrophosphate (GPP), farnesyl phosphate (FPP), and geranylgeranyl pyrophosphate (GGPP) are then generated from IPP and DMAPP through the action of prenyltransferases (Rude and Schirmer, 2009). Isoprenoid alcohols and olefins, which are fuel molecules, can be synthesized from IPP, GPP, FPP, or GGPP through the action of phosphatases, pyrophosphatases, and terpene synthases (Christianson, 2008; Rude and Schirmer, 2009). For example, isopentenol was produced in an *E. coli* strain engineered to overproduce IPP and DMAPP via the mevalonate pathway, in

combination with the isopentenol biosynthetic pathway from *Bacillus subtilis* (*yhfR* and *nudF*) (Martin et al. 2003; Withers et al. 2007).

More recently, *E. coli* has been engineered for the production of farnesol (Wang et al. 2010), farnesene (Renninger and McPhee, 2008), pinene (Reiling et al. 2004), limonene (Carter et al. 2003), and bisabolene (Peralta-Yahya et al. 2011). In the case of farnesol, heterologous mevalonate pathways and FPP synthase were over expressed in combination with endogenous phosphatases for FPP hydrolysis (Wang et al. 2010). Bisabolene, on the other hand, was produced at levels approaching 1 g/L by expressing in *E. coli* a codon-optimized bisabolene synthase from *Abies grandis* in combination with an optimized heterologous mevalonate pathway (Peralta-Yahya et al. 2011).

Fuels derived from amino acid/ketoacid biosynthesis pathways

The native amino acid biosynthetic pathways in *E. coli* can be harnessed to generate 2-ketoacids, which serve as precursors for higher chain alcohol production through a two step synthetic pathway that mimics the Ehrlich degradation pathway: *i.e.* 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) (Figure 3) (Atsumi and Liao, 2008b). A 2-ketoacid decarboxylase from *Lactococcus lactis* and an alcohol dehydrogenase Adh2 from *S. cerevisiae* exhibited broad substrate activity toward a number of 2-ketoacids and enabled the production of 1-propanol, isobutanol, 1-butanol, 2-methyl-1-butanol, 3-methyl-1-butanol, and 2-phenylethanol in an engineered *E. coli* (Atsumi et al. 2008b) (Figure 3). The yield and productivity of these alcohols was improved by increasing the production of the 2-ketoacid intermediates using different strategies. For example, expression of *als* (*B. subtilis*) and *ilvCD* (*E. coli*) to generate 2-ketoisovalerate, combined with the expression of the aforementioned KDC and ADH, led to an engineered strain able to produce ~22 g/L of isobutanol after 110 hrs at a yield of 86% of the theoretical maximum (Atsumi et al. 2008b). Similar strategies were used to demonstrate the efficient synthesis of 1-propanol and 1-butanol, observing the production of 3.5 g/L of 1-propanol and ~500 mg/L of 1-butanol after 92 hrs (Atsumi and Liao, 2008a; Shen and Liao, 2008). These natural amino acid pathways were also utilized to produce the 5-carbon alcohols, 2-methyl-1-butanol (2MB) and 3-methyl-1-butanol (3MB) (Figure 3) (Cann and Liao 2008; Connor and Liao 2008; Connor and Liao, 2009).

The above strategy, based on the use of the amino acid biosynthetic pathways, has also been exploited to produce non-natural 5 to 8 carbon higher alcohols derived from isoleucine biosynthesis. To this end, the product chain length of LeuA and substrate chain length of 2-ketoacid decarboxylase (KIVD) was expanded to enable the synthesis of non-natural ketoacids, which were then converted to 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).

Fuels derived from an engineered β -oxidation pathway

A variation of the β -oxidation cycle has been engineered to work in the reverse biosynthetic direction (as opposed to its natural catabolic direction) in order to produce fuels and chemicals from different carbon sources (Figure 3) (Dellomonaco et al. 2011; Clomburg et al. 2012). In the earlier report (Dellomonaco et al. 2011), a system-level approach was used in which several points for regulation of the β -oxidation cycle, namely *fad*, *ato*, *crp*, and *arcA*, were engineered. These modifications, in combination with the overexpression of endogenous dehydrogenases and thioesterases, enabled the synthesis of *n*-alcohols, FAs, and carboxylic 3-hydroxy-, 3-keto-, and trans- Δ^2 -carboxylic acids (Dellomonaco et al. 2011). In addition, the deletion of the competing pathways for ethanol (*adhE*), acetate (*pta*), and succinate (*frdA*) helped minimizing the formation of by-products, while the expression of selected termination enzymes enabled the production of a variety of β -oxidation cycle intermediates. The introduction of the engineered reversal of the β -oxidation cycle opened many opportunities for engineering *E. coli* metabolism for the production of fuels and chemicals in an ATP-independent and efficient manner, but given the implications of modifying major regulators in the cell it could be difficult to completely elucidate and understand the responsible genes/proteins. To address these issues, a more recent study presented *in vitro* kinetic characterization of the functional units in the pathway and used this information to guide a synthetic biology approach to assemble the pre-defined components of the reversed β -oxidation pathway *in vivo* conditions and to enable production of a variety of carboxylic acids with the combination of different thioesterases (Clomburg et al. 2012). A

functional equivalent of the β -oxidation reversal that does not rely on β -oxidation enzymes, has also been reported (Dekishima et al. 2011; Machado et al. 2012).

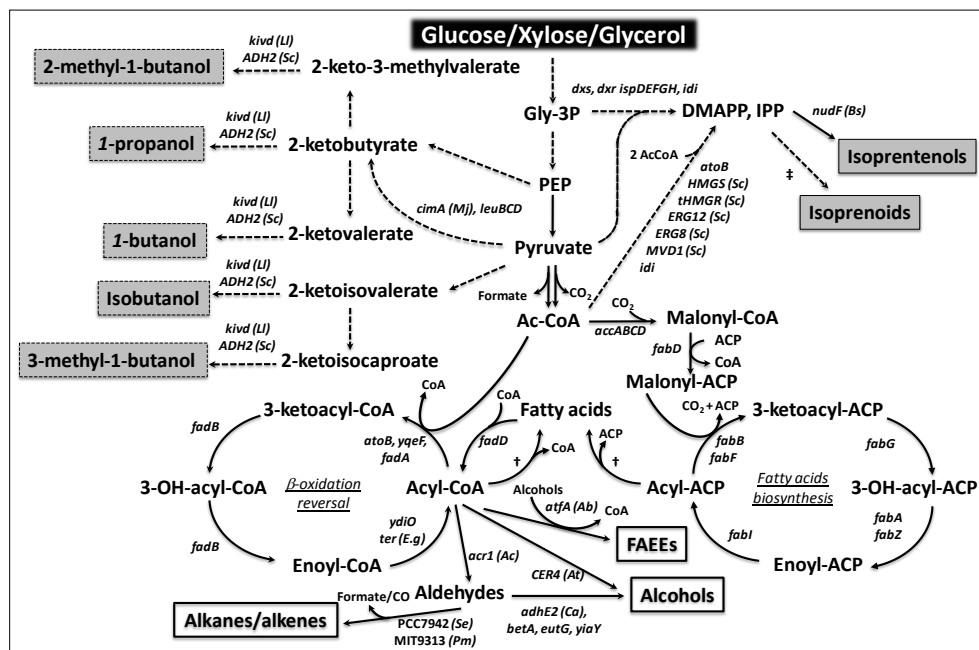


Fig. 3 Non-fermentative routes for the production of alcohols via the ketoacid pathway (dotted line box with grey shade), fatty acid- and β -oxidation reversal-derived fuels (solid line box with no shade), and isoprenoid-derived biofuels (solid line box with grey shade) in *E. coli*. Broken lines represent multiple reaction steps. Relevant reactions are represented by the name of the genes coding for the enzyme. Non-*E. coli* genes are noted in parenthesis using the following abbreviations: *Acinetobacter calcoaceticus* (*Ac*); *Arabidopsis thaliana* (*At*); *Bacillus subtilis* (*Bs*); *Clostridium acetobutylicum* (*Ca*); *Corynebacterium glutamicum* (*Cg*); *Euglena gracilis* (*Eg*); *Lactococcus lactis* (*Ll*); *Methanococcus jannaschii* (*Mj*); *Saccharomyces cerevisiae* (*Sc*); *Stenotrophomonas maltophilia*, (*Sm*). *accABCD*, acetyl-CoA carboxylase; *acr1*, acyl-CoA reductase (*Ac*); *ADH2*, alcohol dehydrogenase (*Sc*); *adhE2*, alcohol dehydrogenase (*Ca*); *atfA*, acyltransferase (*Ab*); *atoB/yqeF/fadA*, thiolase; *betA/eutG/yiaY*, alcohol dehydrogenase; *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 dehydratase; *fabB*, ketoacyl-ACP synthase I; *fabD*, malonyl-CoA-ACP transacylase; *fabF*, ketoacyl-ACP synthase II; *fabG*, ketoacyl-ACP reductase; *fabI*, enoyl-ACP reductase; *fabZ*, hydroxyacyl-ACP dehydratase; *fadD*, acyl-CoA synthetase; *fadB*, hydroxyacyl-CoA dehydrogenase & enoyl-CoA hydratase; *HMGs*, 3-hydroxy-3-methylglutaryl-CoA synthase (*Sc*); *idi*, isopentenylpyrophosphate isomerase; *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*); *MVD1*, mevalonate pyrophosphate decarboxylase (*Sc*); *nudF*, prenyl phosphatase (*Bs*); *ter*, transenoyl-CoA reductase (*Eg*); *tHMGR*, truncated 3-hydroxy-3-methylglutaryl-CoA reductase (*Sc*); *ydiO*, enoyl-CoA reductase; † represents a number of endogenous acyl-CoA and acyl-ACP thioesterases; ‡ represents numerous reactions leading to the formation of a wide range of isoprenoids. Abbreviations: 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; PEP, phosphoenolpyruvate.

The β -oxidation pathway has also been exploited for the production of methyl ketones in *E. coli* (Goh et al. 2012). The authors used a strategy in which synthesis of methyl-ketones is achieved by overexpression of acyl-CoA oxidase to generate β -ketoacyl-CoA, which is then hydrolyzed by an endogenous thioesterase (*FadM*) and the resulting ketoacid decarboxylated to C₁₁-C₁₅ methyl ketones at a titer of 380 mg/L and 58% of the maximum theoretical yield.

ENGINEERED PATHWAYS THAT ENABLE THE USE OF ALTERNATIVE FEEDSTOCKS FOR FUEL PRODUCTION IN *E. COLI*

The engineering efforts discussed above mainly focused on engineering pathways that convert metabolites in central metabolic pathways to the target fuel molecule. Another important consideration is the use of more efficient substrates that can lead to higher product yields. Two abundant substrates, glycerol and fatty acids, can be considered in this category.

The reduced nature of carbon atoms in glycerol and fatty acids provides significant advantages for the production of fuels, such as alcohols, alkanes, and others, when compared to their production from sugars (Clomburg and González, 2013). For example, fermentative metabolism of glycerol leads to the synthesis of ethanol as the preferable product due to the fact that ethanol synthesis from glycerol represents an NADH balanced pathway that produces ATP via substrate level phosphorylation. Metabolic engineering strategies have been implemented to minimize by-product formation through the disruption of genes encoding fumarate reductase (*frdA*) and phosphotransacetylase (*pta*), key enzymes in the synthesis of succinate and acetate, respectively (Yazdani and González, 2008). The resulting strain co-produced ethanol and hydrogen in nearly equimolar amounts approaching the maximum theoretical yield of 1 mol each of ethanol and hydrogen per mol of glycerol fermented. Using a similar strategy, with the additional disruption of the enzyme responsible for the oxidation of formate into H₂ and CO₂, the efficient co-production of ethanol and formate was achieved (Yazdani and González, 2008). While promising, strains reported in that study (Yazdani and González, 2008) required rich supplementation in the form of tryptone to be able to produce ethanol and co-products formate or hydrogen. Microaerobic conditions were then used as a means to eliminate the need for rich nutrients while still taking advantage of the higher degree of reduction of glycerol for product synthesis (Durnin et al. 2009). In another study, elementary mode analysis was used to design an *E. coli* strain for the conversion of glycerol into ethanol (Trinh and Sreenc, 2009). A coupling between cell growth and ethanol production enabled metabolic evolution of the designed strain to convert 40 g/L glycerol to ethanol at 90% of the theoretical yield.

E. coli has also been engineered for the production of ethanol, isopropanol, and 1-butanol from fatty acid feedstocks, resulting in product yields much higher than the theoretical maximum obtained from sugars (Dellomonaco et al. 2010). In order to produce ethanol under the respiro-fermentative conditions required for fatty acid utilization, an aerotolerant mutant of alcohol dehydrogenase (encoded by *adhE*) was constructed. Overexpression of AdhE* from an oxygen-independent promoter in a strain engineered for efficient and complete degradation of fatty acids resulted in the production of ethanol with yields two-times greater than the theoretical maximum value than can be achieved with the use of lignocellulosic sugars (Dellomonaco et al. 2010). In the same study, *E. coli* was also engineered to produce isopropanol and 1-butanol from fatty acids through the expression of the fermentative pathways for the synthesis of these molecules from acetyl-CoA shown in Figure 2 and described in previous sections.

CONCLUDING REMARKS

As the above sections show, metabolic engineering has become an established discipline and enabled the development of microbial systems for fuel production from a number of renewable feedstocks. Advancements in the field of synthetic biology are key to current and future success. In addition, the use of system biology tools such as genomics, transcriptomics, proteomics, metabolomics, and fluxomics will facilitate the design, characterization and integration of new metabolic pathways for biofuel production. The development of new strategies and tools from these fields will support further increases in yield, titer, and productivity of existing and new biofuel molecules from various feedstocks. This, in turn, represents a promising avenue for the viable industrial production of renewable fuels.

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REFERENCES

- ALPER, H. and STEPHANOPOULOS, G. (2007). Global transcription machinery engineering: A new approach for improving cellular phenotype. *Metabolic Engineering*, vol. 9, no. 3, p. 258-267. [\[CrossRef\]](#)
- ATSUMI, S. and LIAO, J.C. (2008a). Directed evolution of *Methanococcus jannaschii* citramalate synthase for biosynthesis of 1-propanol and 1-butanol by *Escherichia coli*. *Applied and Environmental Microbiology*, vol. 74, no. 24, p. 7802-7808. [\[CrossRef\]](#)
- ATSUMI, S. and LIAO, J.C. (2008b). Metabolic engineering for advanced biofuels production from *Escherichia coli*. *Current Opinion in Biotechnology*, vol. 19, no. 5, p. 414-419. [\[CrossRef\]](#)
- ATSUMI, S.; CANN, A.F.; CONNOR, M.R.; SHEN, C.R.; SMITH, K.M.; BRYNILDSEN, M.P.; CHOU, K.J.Y.; HANAI, T. and LIAO, J.C. (2008a). Metabolic engineering of *Escherichia coli* for 1-butanol production. *Metabolic Engineering*, vol. 10, no. 6, p. 305-311. [\[CrossRef\]](#)
- ATSUMI, S.; HANAI, T. and LIAO, J.C. (2008b). Non-fermentative pathways for synthesis of branched-chain higher alcohols as biofuels. *Nature*, vol. 451, no. 7174, p. 86-89. [\[CrossRef\]](#)
- BAILEY, J.E. (1991). Toward a science of metabolic engineering. *Science*, vol. 252, no. 5013, p. 1668-1675. [\[CrossRef\]](#)
- BOND-WATTS, B.B.; BELLERSE, R.J. and CHANG, M.C. (2011). Enzyme mechanism as a kinetic control element for designing synthetic biofuel pathways. *Nature Chemical Biology*, vol. 7, no. 4, p. 222-227. [\[CrossRef\]](#)
- BONOMO, J.; LYNCH, M.D.; WARNECKE, T.; PRICE, J.V. and GILL, R.T. (2008). Genome-scale analysis of anti-metabolite directed strain engineering. *Metabolic Engineering*, vol. 10, no. 2, p. 109-120. [\[CrossRef\]](#)
- BOYLE, N.R. and GILL, R.T. (2012). Tools for genome-wide strain design and construction. *Current Opinion in Biotechnology*, vol. 23, no. 5, p. 666-671. [\[CrossRef\]](#)
- BUCHHOLZ, A.; HURLEBAUS, J.; WANDREY, C. and TAKORS, R. (2002). Metabolomics: Quantification of intracellular metabolite dynamics. *Biomolecular Engineering*, vol. 19, no. 1, p. 5-15. [\[CrossRef\]](#)
- CANN, A.F. and LIAO, J.C. (2008). Production of 2-methyl-1-butanol in engineered *Escherichia coli*. *Applied Microbiology and Biotechnology*, vol. 81, no. 1, p. 89-98. [\[CrossRef\]](#)
- CARTER, O.A.; PETERS, R.J. and CROTEAU, R. (2003). Monoterpene biosynthesis pathway construction in *Escherichia coli*. *Phytochemistry*, vol. 64, no. 2, p. 425-433. [\[CrossRef\]](#)
- CHEN, J.S. and HIU, S.F. (1986). Acetone-butanol-isopropanol production by *Clostridium beijerinckii* (synonym, *Clostridium butylicum*). *Biotechnology Letters*, vol. 8, no. 5, p. 371-376. [\[CrossRef\]](#)
- CHOUDHARY, M.K.; YOON, J.M.; GONZALEZ, R. and SHANKS, J.V. (2011). Re-examination of metabolic fluxes in *Escherichia coli* during anaerobic fermentation of glucose using ¹³C labeling experiments and 2-dimensional nuclear magnetic resonance (NMR) spectroscopy. *Biotechnology and Bioengineering*, vol. 16, no. 3, p. 419-437. [\[CrossRef\]](#)
- CHRISTIANSON, D.W. (2008). Unearthing the roots of the terpenome. *Current Opinion in Chemical Biology*, vol. 12, no. 2, p. 141-150. [\[CrossRef\]](#)
- CINTOLESI, A.; CLOMBURG, J.M.; RIGOU, V.; ZYGOURAKIS, K. and GONZÁLEZ, R. (2012). Quantitative analysis of the fermentative metabolism of glycerol in *Escherichia coli*. *Biotechnology and Bioengineering*, vol. 109, no. 1, p. 187-198. [\[CrossRef\]](#)
- CLOMBURG, J.M.; VICK, J.E.; BLANKSCHEN, M.D.; RODRÍGUEZ-MOYA, M. and GONZÁLEZ, R. (2012). A synthetic biology approach to engineer a functional reversal of the β -oxidation cycle. *ACS Synthetic Biology*, vol. 1, no. 11, p. 541-554. [\[CrossRef\]](#)
- CLOMBURG, J.M. and GONZÁLEZ, R. (2013). Anaerobic fermentation of glycerol: A platform for renewable fuels and chemicals. *Trends in Biotechnology*, vol. 31, no. 1, p. 20-28. [\[CrossRef\]](#)
- CONNOR, M.R. and LIAO, J.C. (2008). Engineering of an *Escherichia coli* strain for the production of 3-methyl-1-butanol. *Applied and Environmental Microbiology*, vol. 74, no. 18, p. 5769-5775. [\[CrossRef\]](#)
- CONNOR, M.R. and LIAO, J.C. (2009). Microbial production of advanced transportation fuels in non-natural hosts. *Current Opinion in Biotechnology*, vol. 20, no. 3, p. 307-315. [\[CrossRef\]](#)
- DAS, D.; ESER, B.E.; HAN, J.; SCIORE, A. and MARSH, E.N.G. (2011). Oxygen-independent decarbonylation of aldehydes by cyanobacterial aldehyde decarbonylase: A new reaction of diiron enzymes. *Angewandte Chemie International Edition*, vol. 50, no. 31, p. 7148-7152. [\[CrossRef\]](#)
- DEKISHIMA, Y.; LAN, E.I.; SHEN, C.R.; CHO, K.M. and LIAO J.C. (2011). Extending carbon chain length of 1-butanol pathway for 1-hexanol synthesis from glucose by engineered *Escherichia coli*. *Journal of the American Chemical Society*, vol. 133, no. 30, p. 11399-11401. [\[CrossRef\]](#)
- DELLOMONACO, C.; RIVERA, C.; CAMPBELL, P. and GONZÁLEZ, R. (2010). Engineered respiro-fermentative metabolism for the production of biofuels and biochemicals from fatty acid-rich feedstocks. *Applied and Environmental Microbiology*, vol. 76, no. 15, p. 5067-5078. [\[CrossRef\]](#)
- DELLOMONACO, C.; CLOMBURG, J.M.; MILLER, E.N. and GONZÁLEZ, R. (2011). Engineered reversal of the β -oxidation cycle for the synthesis of fuels and chemicals. *Nature*, vol. 476, no. 7360, p. 355-359. [\[CrossRef\]](#)
- DEWICK, P.M. (2002). The biosynthesis of C₅-C₂₅ terpenoid compounds. *Natural Product Reports*, vol. 19, no. 2, p. 181-222. [\[CrossRef\]](#)
- DURNIN, G.; CLOMBURG, J.; YEATES, Z.; ÁLVAREZ, P.J.J.; ZYGOURAKIS, K.; CAMPBELL, P. and GONZÁLEZ, R. (2009). Understanding and harnessing the microaerobic metabolism of glycerol in *Escherichia coli*. *Biotechnology and Bioengineering*, vol. 103, no. 1, p. 148-161. [\[CrossRef\]](#)

- FEIST, A.M.; ZIELINSKI, D.C.; ORTH, J.D.; SCHELLENBERGER, J.; HERRGARD, M.J. and PALSSON, B.O. (2010). Model-driven evaluation of the production potential for growth-coupled products of *Escherichia coli*. *Metabolic Engineering*, vol. 12, no. 3, p. 173-186. [\[CrossRef\]](#)
- FELL, D.A. and SAURO, H.M. (1985). Metabolic control and its analysis. Additional relationships between elasticities and control coefficients. *European Journal of Biochemistry*, vol. 148, no. 3, p. 555-561. [\[CrossRef\]](#)
- FISCHER, E. and SAUER, U. (2003). Metabolic flux profiling of *Escherichia coli* mutants in central carbon metabolism using GC-MS. *European Journal of Biochemistry*, vol. 270, no. 5, p. 880-891. [\[CrossRef\]](#)
- FISCHER, E. and SAUER, U. (2005). Large-scale *in vivo* flux analysis shows rigidity and suboptimal performance of *Bacillus subtilis* metabolism. *Nature Genetics*, vol. 37, no. 6, p. 636-640. [\[CrossRef\]](#)
- GEVAERT, K.; VAN DAMME, P.; GHESQUIÈRE, B.; IMPENS, F.; MARTENS, L.; HELSENS, K. and VANDEKERCKHOVE, J. (2007). A la carte proteomics with an emphasis on gel-free techniques. *Proteomics*, vol. 7, no. 16, p. 2698-2718. [\[CrossRef\]](#)
- GOH, E.B.; BAIDOO, E.E.K.; KEASLING, J.D. and BELLER, H.R. (2012). Engineering of bacterial methyl ketone synthesis for biofuels. *Applied and Environmental Microbiology*, vol. 78, no. 1, p. 70-80. [\[CrossRef\]](#)
- GRAHAM, R.L.J.; GRAHAM, C. and MCMULLAN, G. (2007). Microbial proteomics: A mass spectrometry primer for biologists. *Microbial Cell Factories*, vol. 6, no. 1, p. 26. [\[CrossRef\]](#)
- HANAI, T.; ATSUMI, S. and LIAO, J.C. (2007) Engineered synthetic pathway for isopropanol production in *Escherichia coli*. *Applied and Environmental Microbiology*, vol. 73, no. 24, p. 7814-7818. [\[CrossRef\]](#)
- HIETER, P. and BOGUSKI, M. (1997). Functional genomics: It's all how you read it. *Science*, vol. 278, no. 5338, p. 601-602. [\[CrossRef\]](#)
- INGRAM, L.O.; CONWAY, T.; CLARK, D.P.; SEWELL, G.W. and PRESTON, J.F. (1987). Genetic engineering of ethanol production in *Escherichia coli*. *Applied and Environmental Microbiology*, vol. 53, no. 10, p. 2420-2425.
- INUI, M.; SUDA, M.; KIMURA, S.; YASUDA, K.; SUZUKI, H.; TODA, H.; YAMAMOTO, S.; OKINO, S.; SUZUKI, N. and YUKAWA, H. (2008). Expression of *Clostridium acetobutylicum* butanol synthetic genes in *Escherichia coli*. *Applied Microbiology and Biotechnology*, vol. 77, no. 6, p. 1305-1316. [\[CrossRef\]](#)
- ISAACS, F.J.; CARR, P.A.; WANG, H.H.; LAJOIE, M.J.; STERLING, B.; KRAAL, L.; TOLONEN, A.C.; GIANOULIS, T.A.; GOODMAN, D.B.; REPPAS, N.B.; EMIG, C.J.; BANG, D.; HWANG, S.J.; JEWETT, M.C.; JACOBSON, J.M. and CHURCH, G.M. (2011). Precise manipulation of chromosomes *in vivo* enables genome-wide codon replacement. *Science*, vol. 333, no. 6040, p. 348-353. [\[CrossRef\]](#)
- JONES, D.T. and WOODS, D.R. (1986). Acetone-butanol fermentation revisited. *Microbiology Reviews*, vol. 50, no. 4, p. 484-524.
- KLAPA, M.I.; PARK, S.M.; SINSKEY, A.J.; and STEPHANOPOULOS, G. (1999). Metabolite and isotopomer balancing in the analysis of metabolic cycles: I. Theory. *Biotechnology and Bioengineering*, vol. 62, no. 4, p. 375-391. [\[CrossRef\]](#)
- LENNEN, R.M.; BRADEN, D.J.; WEST, R.M.; DUMESIC, J.A. and PFLEGER, B.F. (2010). A process for microbial hydrocarbon synthesis: Overproduction of fatty acids in *Escherichia coli* and catalytic conversion to alkanes. *Biotechnology and Bioengineering*, vol. 106, no. 2, p. 193-202. [\[CrossRef\]](#)
- LI, N.; NORGAARD, H.; WARUI, D.M.; BOOKER, S.J.; KREBS, C. and BOLLINGER, J.M. (2011). Conversion of fatty aldehydes to alka(e)nes and formate by a cyanobacterial aldehyde decarbonylase: Cryptic redox by an unusual dimetal oxygenase. *Journal of the American Chemical Society*, vol. 133, no. 16, p. 6158-6161. [\[CrossRef\]](#)
- LYNCH, M.D.; WARNECKE, T. and GILL, R.T. (2007). SCALEs: Multiscale analysis of library enrichment. *Nature Methods*, vol. 4, no. 1, p. 87-93. [\[CrossRef\]](#)
- MACHADO, H.B.; DEKISHIMA, Y.; LUO, H.; LAN, E.I. and LIAO, J.C. (2012). A selection platform for carbon chain elongation using the CoA-dependent pathway to produce linear higher alcohols. *Metabolic Engineering*, vol. 14, no. 5, p. 504-511. [\[CrossRef\]](#)
- MARTIN, V.J.J.; PITERA, D.J.; WITHERS, S.T.; NEWMAN, J.D. and KEASLING, J.D. (2003). Engineering a mevalonate pathway in *Escherichia coli* for production of terpenoids. *Nature Biotechnology*, vol. 21, no. 7, p. 796-802. [\[CrossRef\]](#)
- MISKOVIC, L. and HATZIMANIKATIS, V. (2010). Production of biofuels and biochemicals: In need of an ORACLE. *Trends in Biotechnology*, vol. 28, no. 8, p. 391-397. [\[CrossRef\]](#)
- MURARKA, A.; CLOMBURG, J.M. and GONZÁLEZ, R. (2010a). Metabolic flux analysis of wild-type *Escherichia coli* and mutants deficient in pyruvate-dissimilating enzymes during the fermentative metabolism of glucuronate. *Microbiology-SGM*, vol. 156, no. 6, p. 1860-1872. [\[CrossRef\]](#)
- MURARKA, A.; CLOMBURG, J.M.; MORAN, S.; SHANKS, J.V. and GONZÁLEZ, R. (2010b). Metabolic analysis of wild-type *Escherichia coli* and a pyruvate dehydrogenase complex (PDHC)-deficient derivative reveals the role of PDHC in the fermentative metabolism of glucose. *The Journal of Biological Chemistry*, vol. 285, no. 41, p. 31548-31558. [\[CrossRef\]](#)
- NICOLAOU, S.A.; GAIDA, S.M. and PAPOUTSAKIS, E.T. (2011). Coexisting/Coexpressing genomic libraries (CoGeL) identify interactions among distantly located genetic loci for developing complex microbial phenotypes. *Nucleic Acids Research*, vol. 39, no. 22, p. 152. [\[CrossRef\]](#)
- NIELSEN, D.R.; LEONARD, E.; YOON, S.H.; TSENG, H.C.; YUAN, C. and JONES-PRATHER, K.L. (2009). Engineering alternative butanol production platforms in heterologous bacteria. *Metabolic Engineering*, vol. 11, no. 4-5, p. 262-273. [\[CrossRef\]](#)
- OHTA, K.; BEALL, D.S.; MEJIA, J.P.; SHANMUGAM, K.T. and INGRAM, L.O. (1991). Genetic improvement of *Escherichia coli* for ethanol production: Chromosomal integration of *Zymomonas mobilis* genes encoding pyruvate decarboxylase and alcohol dehydrogenase II. *Applied and Environmental Microbiology*, vol. 57, no. 4, p. 893-900.

- ORTH, J.D.; CONRAD, T.M.; NA, J.; LERMAN, J.A.; NAM, H.; FEIST, A.M. and PALSSON, B.O. (2011). A comprehensive genome-scale reconstruction of *Escherichia coli* metabolism - 2011. *Molecular Systems Biology*, vol. 7, no. 535. [\[CrossRef\]](#)
- PARK, J.; RODRÍGUEZ-MOYA, M.; LI, M.; PICHERSKY, E.; SAN, K.Y. and GONZÁLEZ, R. (2012). Synthesis of methyl ketones by metabolically engineered *Escherichia coli*. *Journal of Industrial Microbiology & Biotechnology*, vol. 39, no 11, p. 1703-1712. [\[CrossRef\]](#)
- PERALTA-YAHYA, P.P.; OUELLET, M.; CHAN, R.; MUKHOPADHYAY, A.; KEASLING, J.D. and LEE, T.S. (2011). Identification and microbial production of a terpene-based advanced biofuel. *Nature Communications*, vol. 2, no. 483. [\[CrossRef\]](#)
- RAAMSDONK, L.M.; TEUSINK, B.; BROADHURST, D.; ZHANG, N.; HAYES, A.; WALSH, M.C.; BERDEN, J.A.; BRINDLE, K.M.; KELL, D.B.; ROWLAND, J.J.; WESTERHOFF, H.V.; VAN DAM, K. and OLIVER, S.G. (2001). A functional genomics strategy that uses metabolome data to reveal the phenotype of silent mutations. *Nature Biotechnology*, vol. 19, no. 1, p. 45-50. [\[CrossRef\]](#)
- RAMAN, K. and CHANDRA, N. (2009). Flux balance analysis of biological systems: Applications and challenges. *Briefings in Bioinformatics*, vol. 10, no. 4, p. 435-449. [\[CrossRef\]](#)
- RANGANATHAN, S.; SUTHERS, P.F. and MARANAS, C.D. (2010). OptForce: An optimization procedure for identifying all genetic manipulations leading to targeted overproductions. *PLoS Computational Biology*, vol. 6, no. 4, p. e1000744. [\[CrossRef\]](#)
- RANGANATHAN, S.; TEE, T.W.; CHOWDHURY, A.; ZOMORRODI, A.R.; YOON, J.M.; FU, Y.; SHANKS, J.V. and MARANAS, C.D. (2012). An integrated computational and experimental study for overproducing fatty acids in *Escherichia coli*. *Metabolic Engineering*, vol. 14, no. 6, p. 687-704. [\[CrossRef\]](#)
- REILING, K.K.; YOSHIKUNI, Y.; MARTIN, V.J.J.; NEWMAN, J.; BOHLMANN, J. and KEASLING, J.D. (2004). Mono and diterpene production in *Escherichia coli*. *Biotechnology and Bioengineering*, vol. 87, no. 2, p. 200-212. [\[CrossRef\]](#)
- RENNINGER, N.S. and MCPHEE, D.J. (2008). Fuel compositions comprising farnesane and farnesane derivatives and method of making and using same, US patent 7399323.
- RUDE, M.A. and SCHIRMER, A. (2009). New microbial fuels: A biotech perspective. *Current Opinion in Microbiology*, vol. 12, no. 3, p. 274-281. [\[CrossRef\]](#)
- SAWERS, R.G. and CLARK, D.P. (2004). Fermentative pyruvate and acetyl-CoA metabolism. In: NEIDHARDT, F.C.; CURTISS III, R.; INGRAHAM, J.L.; LIN, E.C.C.; LOW, K.B.; MAGASANIK, B.; REZNIKOFF, W.S.; RILEY, M.; SCHAECHTER, M. and UMBARGER, H.E. eds. *Escherichia coli and Salmonella: Cellular and molecular biology*. Washington, DC, ASM Press, Web edn. p. 1-43.
- SCHIRMER, A.; RUDE, M.A.; LI, X.; POPOVA, E. and DEL CARDAYRE, S.B. (2010). Microbial biosynthesis of alkanes. *Science*, vol. 329, no. 5991, p. 559-562. [\[CrossRef\]](#)
- SHEN, C.R. and LIAO, J.C. (2008). Metabolic engineering of *Escherichia coli* for 1-butanol and 1-propanol production via the keto-acid pathways. *Metabolic Engineering*, vol. 10, no. 6, p. 312-320. [\[CrossRef\]](#)
- SHEN, C.R.; LAN, E.I.; DEKISHIMA, Y.; BÁEZ, A.; CHO, K.M. and LIAO, J.C. (2011). Driving forces enable high-titer anaerobic 1-butanol synthesis in *Escherichia coli*. *Applied and Environmental Microbiology*, vol. 77, no. 9, p. 2905-2915. [\[CrossRef\]](#)
- STADLMAYR, G.; BENAKOVITSCH, K.; GASSER, B.; MATTANOVICH, D. and SAUER, M. (2010). Genome-scale analysis of library sorting (GALibSo): Isolation of secretion enhancing factors for recombinant protein production in *Pichia pastoris*. *Biotechnology and Bioengineering*, vol. 105, no. 3, p. 543-555. [\[CrossRef\]](#)
- STEEN, E.J.; KANG, Y.; BOKINSKY, G.; HU, Z.; SCHIRMER, A.; MCCLURE, A.; DEL CARDAYRE, S.B. and KEASLING, J.D. (2010). Microbial production of fatty-acid-derived fuels and chemicals from plant biomass. *Nature*, vol. 463, no. 7280, p. 559-562. [\[CrossRef\]](#)
- STEPHANOPOULOS, G.; ARISTIDOU, A.A. and NIELSEN, J. (1998). *Metabolic engineering-principles and methodologies*. San Diego, CA, Academic Press, 663 p. ISBN-13: 978-0126662603.
- STEPHANOPOULOS, G.; ALPER, H. and MOXLEY, J. (2004). Exploiting biological complexity for strain improvement through systems biology. *Nature Biotechnology*, vol. 22, no. 10, p. 1261-1267. [\[CrossRef\]](#)
- TANG, Y.J.; GARCÍA MARTÍN, H.; MYERS, S.; RODRÍGUEZ, S.; BAIDOO, E.K. and KEASLING, J.D. (2009). Advances in analysis of microbial metabolic fluxes via ¹³C isotopic labeling. *Mass Spectrometry Reviews*, vol. 28, no. 2, p. 362-375. [\[CrossRef\]](#)
- TRINH, C.T. and SRIENC, F. (2009). Metabolic engineering of *Escherichia coli* for efficient conversion of glycerol to ethanol. *Applied and Environmental Microbiology*, vol. 75, no. 21, p. 6696-6705. [\[CrossRef\]](#)
- TYO, K.E.; ALPER, H.S. and STEPHANOPOULOS, G.N. (2007). Expanding the metabolic engineering toolbox: More options to engineer cells. *Trends in Biotechnology*, vol. 25, no. 3, p. 132-137. [\[CrossRef\]](#)
- WANG, H.H.; ISAACS, F.J.; CARR, P.A.; SUN, Z.Z.; XU, G.; FOREST, C.R. and CHURCH, G.M. (2009). Programming cells by multiplex genome engineering and accelerated evolution. *Nature*, vol. 460, no. 7257, p. 894-898. [\[CrossRef\]](#)
- WANG, C.; YOON, S.H.; SHAH, A.A.; CHUNG, Y.R.; KIM, J.Y.; CHOI, E.S.; KEASLING, J.D. and KIM, S.W. (2010). Farnesol production from *Escherichia coli* by harnessing the exogenous mevalonate pathway. *Biotechnology and Bioengineering*, vol. 107, no. 3, p. 421-429. [\[CrossRef\]](#)
- WARNER, J.R.; REEDER, P.J.; KARIMPOUR-FARD, A.; WOODRUFF, L.B.A. and GILL, R.T. (2010). Rapid profiling of a microbial genome using mixtures of barcoded oligonucleotides. *Nature Biotechnology*, vol. 28, no. 8, p. 856-862. [\[CrossRef\]](#)
- WATT, S.A.; PATSCHKOWSKI, T.; KALINOWSKI, J. and NIEHAUS, K. (2003). Qualitative and quantitative proteomics by two-dimensional gel electrophoresis, peptide mass fingerprint and a chemically-coded affinity tag (CCAT). *Journal of Biotechnology*, vol. 106, no. 2-3, p. 287-300. [\[CrossRef\]](#)

- WITHERS, S.T.; GOTTLIEB, S.S.; LIEU, B.; NEWMAN, J.D. and KEASLING, J.D. (2007). Identification of isopentenol biosynthetic genes from *Bacillus subtilis* by a screening method based on isoprenoid precursor toxicity. *Applied and Environmental Microbiology*, vol. 73, no. 19, p. 6277-6283. [\[CrossRef\]](#)
- WIECHERT, W.; MÖLLNEY, M.; PETERSEN, S. and DE GRAAF, A.A. (2001). A universal framework for 13C metabolic flux analysis. *Metabolic Engineering*, vol. 3, no. 3, p. 265-283. [\[CrossRef\]](#)
- YAZDANI, S.S. and GONZÁLEZ, R. (2008). Engineering *Escherichia coli* for the efficient conversion of glycerol to ethanol and co-products. *Metabolic Engineering*, vol. 10, no. 6, p. 340-351. [\[CrossRef\]](#)
- YOMANO, L.P.; YORK, S.W. and INGRAM, L.O. (1998). Isolation and characterization of ethanol-tolerant mutants of *Escherichia coli* KO11 for fuel ethanol production. *Journal of Industrial Microbiology & Biotechnology*, vol. 20, no. 2, p. 132-138. [\[CrossRef\]](#)
- ZHANG, S.; YE, B.C.; CHU, J.; ZHUANG, Y.P. and GUO, M.J. (2006). From multi-scale methodology to systems biology: To integrate strain improvement and fermentation optimization. *Journal of Chemical Technology and Biotechnology*, vol. 81, no. 5, p. 734-745. [\[CrossRef\]](#)
- ZHANG, K.C.; SAWAYA, M.R.; EISENBERG, D.S. and LIAO, J.C. (2008). Expanding metabolism for biosynthesis of nonnatural alcohols. *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 52, p. 20653-20658. [\[CrossRef\]](#)
- ZHANG, X.; LI, M.; AGRAWAL, A. and SAN, K.Y. (2011) Efficient free fatty acid production in *Escherichia coli* using plant acyl-ACP thioesterases. *Metabolic Engineering*, vol. 13, no. 6, p. 713-722. [\[CrossRef\]](#)

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