Review

Bioengineering of microorganisms for C_3 to C_5 alcohols production

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Production of renewable fuels and chemicals is an absolute requirement for the sustainability of societies. This fact has been neglected during the past century as cheap and abundant, yet not renewable, sources of hydrocarbons were available. Since fossil fuel availability is decreasing, biological production of fuels and chemicals has been proposed to be a potential alternative to fossil sources. Higher alcohols (from C_3 to C_5) are useful substitutes for gasoline because of their high energy density and low hygroscopicity and are important feedstocks for other chemicals. Some *Clostridia* species are known to naturally ferment sugars to isopropanol and 1-butanol. However, other C_3 to C_5 alcohols are not produced in large quantities by natural microorganisms. A non-fermentative strategy to produce a broad range of higher alcohols has been devised using the ubiquitous keto acid biosynthetic pathways. This review provides a current overview of these different strategies.

Keywords: Biofuels · Higher alcohols · Metabolic engineering

1 Introduction

Around the world, transportation as well as many industrial processes rely on engines designed to derive mechanical energy from combustion of hydrocarbon fuels. For two centuries, these were mostly *fossil* fuels, made over a hundred million years of fossilization processes that involve tremendous pressure and heat, from the remains of organisms. However, these resources are finite, and their availability presents great geographical disparities. In addition, their combustion releases net CO_2 in the atmosphere, which is implicated to contribute significantly to global climate change. Lately, the uncertainty for petroleum supply and its ability to meet the growing demand resulted in fluctuations in the oil price. All of these concerns have led, during the last few years, to unprecedented research efforts toward the synthesis of renewable and efficient fuels. To be renewable, the carbon of these biofuels must be originated from the CO_2 produced by their combustion. In addition, the energy used for the reduction of inorganic carbon should be inexhaustible.

Among the considered alternatives to fossil fuels, ethanol (two carbons alcohol) derived from carbohydrates was the first to reach industrial production. This rapid success has been made possible by the fact that ethanol produced by fermentation of sugars is a process that has been of premium importance to humans dated several thousand years back. Therefore, ethanol producing strains and underlying biochemistry are thoroughly characterized, and ethanol fermentation optimization is a well developed art [1]. However, the low energy density and the hygroscopicity of ethanol itself are

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Abbreviations: ABE, acetone-butanol-ethanol; **ADH,** alcohol dehydrogenase; **AHAS,** acetohydroxy-acid synthase; **CCR,** carbon catabolite repression; **CoA,** coenzyme-A; **IPMS,** 2-isopropylmalate synthase; **2KB,** 2-keto-butyrate; **KDC,** keto acid decarboxylase; **2KIV,** 2-keto-isovalerate; **2K3MV,** 2-keto-3-methyl-valerate; **2K4MV,** 2-keto-4-methyl-valerate; **2KV,** 2-keto-valerate; **2MB,** 2-methyl-1-butanol; **3MB,** 3-methyl-1-butanol; **NADH,** nicotinamide adenine dinucleotide

Table 1. Comparision of common fuel properties with C_3 to C_5 higher alcohols.

	Vapor pressure of gasoline-alcohol blend	Energy density (MJ/kg)	Avg Octane Number[a)]	Hygroscopicity	Compatible with current infrastructure
Ethanol	High	29.7	116	High	No
Isopropanol	n/a	33.6	n/a	n/a	n/a
Isobutanol	Low	36.1	110	Low	Yes
n-Pentanol	Low	37.7	n/a	Low	Yes
Alkanes (gasoline)	–	47	90	Very low	Yes

a) Average of research octane number (RON) and motor octane number (MON). n/a, not available.

major drawbacks, and alternative biofuels are desirable.

Higher alcohols (three carbon chain and longer), besides their industrial usefulness as chemical feedstocks, display attractive properties regarding these latter problems when used as fuels (Table 1). These compounds have a higher energy density and are less hygroscopic than ethanol. During the last decade, dramatic advances have been made in order to engineer microorganisms to produce these advanced biofuels. Several reviews summarizing these recent progresses came out in the last 2 years [2–5]. The present paper focuses on higher alcohols ranging from C_3 to C_5 (propanols, butanols, and pentanols), and details the various metabolic engineering strategies leading to their production by microorganisms.

2 Higher alcohol production by native producers

Historically, the first organisms to be used for higher alcohol production were native producers, such as a number of *Clostridia*. These anaerobic Gram-positive bacteria naturally ferment a variety of sugars to acetone, 1-butanol, and ethanol (acetone-butanol-ethanol (ABE) fermentation) as well as isopropanol for some strains [6, 7]. The *Clostridia* pathway to produce 1-butanol or isopropanol is coenzyme-A (CoA) dependent (Fig. 1). The first step, catalyzed by an acetyl-CoA acetyltransferase (Thl), is the condensation of acetate and acetyl-CoA, leading to acetoacetyl-CoA, a four carbon metabolite. Then, acetoacetyl-CoA undergoes several rounds of reduction leading to 1-butanol, or can be decarboxylated to acetone (three carbons) and subsequently reduced to isopropanol. Until the mid-20th century, *Clostridia*-based fermentation was the main 1-butanol production process [8]. This renewable way to produce 1-butanol gradually declined as the chemical synthesis process and fossil feedstocks became more competitive. Today, ABE fermentation is regaining attention as the petroleum resources are declining. However, *Clostridia*'s complex life cycle, susceptibility to high 1-butanol titers, and difficulty in genetic engineering, hinder substantially the research on solvent production using this organism. The genetic tools and engineering strategies available for 1-butanol production in *Clostridia* have been thoroughly reviewed recently [2, 9, 10]. Notably, a convenient gene knock-out system for *Clostridia* remains to be developed, and it is still difficult to disrupt the side-reactions that consume the desired precursors. However, some gene knock-outs in *Clostridia* have been reported, and have led to significant improvements in 1-butanol production (Table 2). The removal of the *solR* region, which is suspected to encode a transcriptional repressor of the solvento-

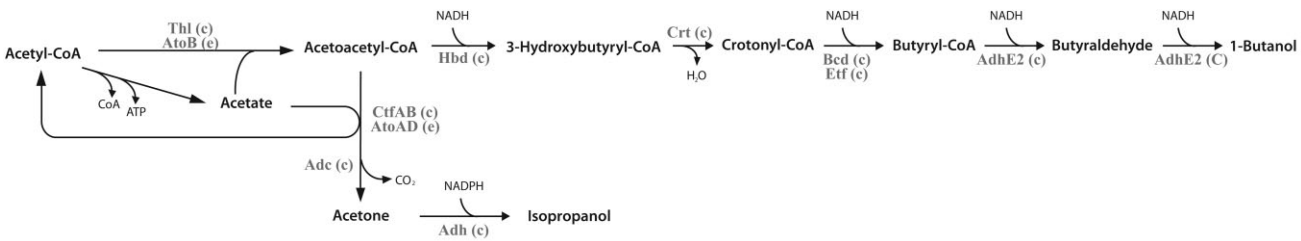

Figure 1. CoA dependent higher alcohol biosynthesis. (c), Natural *Clostridium* enzymes; (e), *E. coli* enzymes that have been used in the transplantation of the pathway in non-native hosts; AtoB, acetyl-CoA acetyltransferase; Thl, acetoacetyl-CoA thiolase; Hbd, 3-hydroxybutyryl-CoA dehydrogenase; Crt, crotonase; Bcd, butyryl-CoA dehydrogenase; Etf, electron transfer flavoprotein; AdhE2, aldehyde/alcohol dehydrogenase; CftAB/AtoAD, acetoacetyl-CoA transferase; Adc, acetoacetate decarboxylase; Adh, secondary alcohol dehydrogenase.

genic genes, increased the 1-butanol titer to more than 300% to that of the wild type with glucose as a substrate (17.8 g/L [11]). Harris *et al.* [12] deleted the *buk* locus, which encodes a butyrate kinase, in order to favor solventogenesis over acidogenesis. The Δ*buk* mutant strain 1-butanol production was increased by 42% in comparison to the wild type at pH > 5 [12].

The use of glucose for fuel production is controversial because of the excessive arable land needed for its production. Lignocellulose is a much more abundant and inexpensive feedstock, but its hydrolysis is difficult and results in a complex blend of sugars that are challenging to use for many bacteria because of the carbon catabolite repression (CCR) regulatory mechanism [13]. Efficient fermentation of lignocellulosic hydrolysates by *Clostridium acetobutylicum* for 1-butanol production has been recently achieved by disrupting the CcpA pleiotropic regulator responsible for the CCR [14]. The Δ*ccpA* mutant strain gained the ability to use D-xylose even in the presence of the preferred substrate glucose in the medium. Acetone and ethanol are byproducts intrinsically produced in ABE fermentation. These byproducts hinder maximal yield of 1-butanol. Several successful attempts have been made to increase the 1-butanol: byproducts ratio by downregulating or disrupting side reaction [15, 16]. Unfortunately, these approaches did not lead to significantly higher 1-butanol titers.

Intensive research is going on for developing new engineering tools for *Clostridia*, and innovative transformation or genome mutagenesis procedures have been recently reported [17–20]. Also, comprehensive analysis of *Clostridia* genome [21], transcriptome [22–24], and proteome [25, 26] will substantially accelerate the future biotechnological improvements of solvent production by this organism.

3 Transplantation of the CoA-dependent pathway in non-native producers

Given the difficulty of *Clostridia*, an alternative is to transfer the pathway of interest into user-friendly tractable organisms such as *Escherichia coli* or *Saccharomyces cerevisiae*. Hanai *et al.* [27] transferred the four genes (codon optimized) required for isopropanol biosynthesis from *Clostridium* to *E. coli*. Without any further engineering, the modified strains were able to produce up to ~5 g/L isopropanol. Interestingly, it has been found that overexpressing the acetoacetyl-CoA transferase of *E. coli* (AtoAD) led to higher isopropanol titers than overexpressing the same enzyme of *C. acetobutylicum* (CtfAB), whose Km for acetate is higher [27] (Fig. 1). This approach led to a glucose-to-isopropanol weight yield of 73.5% of the theoretical maximum, which by far out-competed the highest reported productivity of native producers [27].

Similarly, the pathway for 1-butanol biosynthesis has been transferred to *E. coli* [28, 29]. However, without optimization, only expressing the *Clostridium* 1-butanol pathway in *E. coli* led to a much lower titer than the native host: 14 and 34 mg/L [28, 29]. Nonetheless, working with such a user-friendly organism allows a variety of manipulations to optimize the desired product formation. Indeed, three-fold higher 1-butanol titers were obtained when the *E. coli* acetyl-CoA acetyltransferase coding gene (*atoB*) was overexpressed instead of the *C. acetobutylicum* gene (*thl*) [28] (Fig. 1). Moreover, overexpression of the individual genes of the butanol pathway led to a fivefold increase in 1-butanol production in comparison to the whole operon overexpression (0.2 and 0.034 g/L, respectively) [29]. Furthermore, unlike *Clostridium*, *E. coli* can be grown aerobically, and moderately oxygenated cultures have also proven to increase 1-butanol production [28]. Also, the extensive knowledge of *E. coli* metabolism and underlying genes, together with its unrivaled tractability, allow the researcher to test numerous gene deletions/overexpression in order to prevent the substrate consumption by side-reactions, and/or to improve co-factor supply. Deletions of only five genes (*adh*, *ldh*, *fdr*, *fnr*, *pta*) increased the 1-butanol titer in *E. coli* cultures by 250% [28]. Another successful strategy to improve nicotinamide adenine dinucleotide (NADH) supply has been to overexpress *S. cerevisiae* formate dehydrogenase and supplement the growth medium with formate (+87% 1-butanol titer increase) [29]. Overexpression of glyceraldehyde 3-phosphate dehydrogenase, by stimulating glycolytic flux, also resulted in higher 1-butanol titers (+150% 1-butanol titer increase) [29]. Finally, culture medium optimization has also contributed to major increase in solvent production. "Terrific Broth" medium supplemented with 2% glycerol increased the 1-butanol production by 500% compared with M9 supplemented with 2% glucose [28]. Taken together, all these improvements resulted in 1-butanol titers of 0.5 g/L, which is 20- to 40-fold the initial productivity of the 1-butanol producing *E. coli* [28, 29]. Even if the non-native 1-butanol producer *E. coli* is still less efficient than the native producer, these works highlight the potential of using non-native but user-friendly organisms. Indeed, the pathway has also been transferred in *Bacillus subtilis* and *Pseudomonas putida*, which are known to better tolerate 1-butanol in the growth medium [29]. With-

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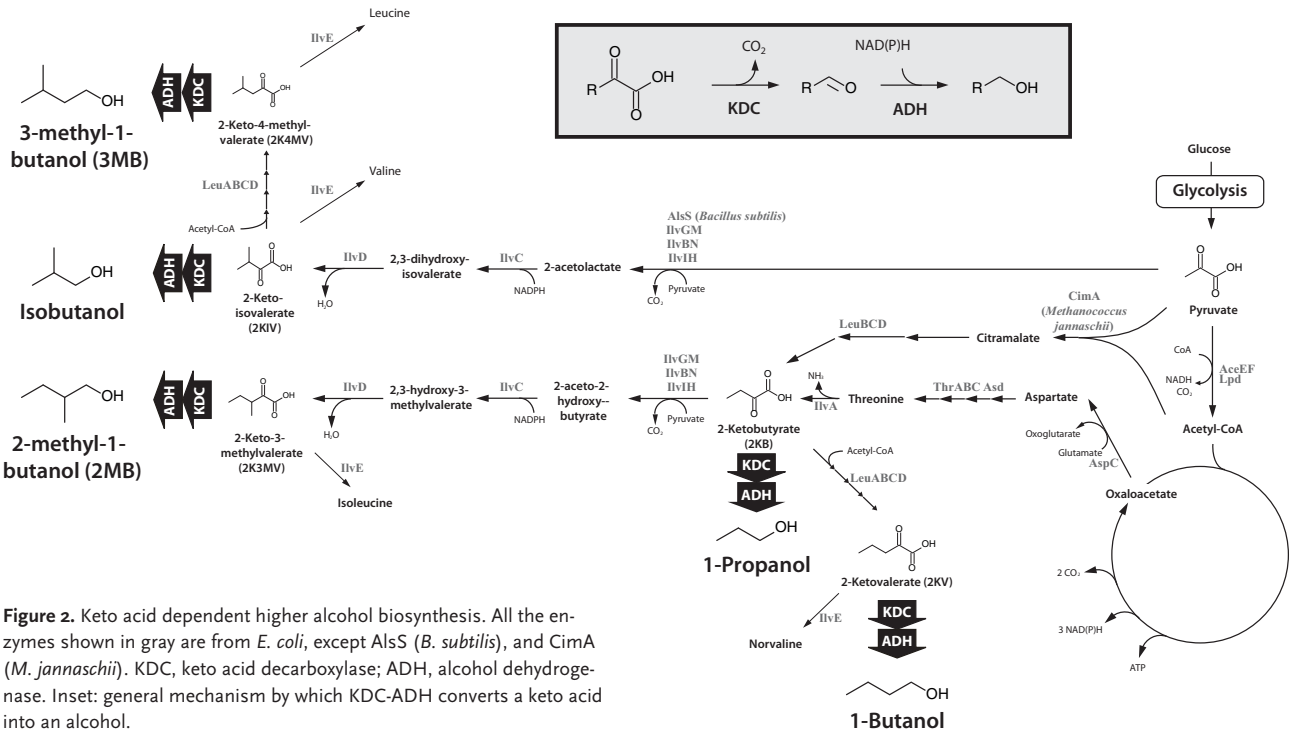

Figure 2. Keto acid dependent higher alcohol biosynthesis. All the enzymes shown in gray are from *E. coli*, except AlsS (*B. subtilis*), and CimA (*M. jannaschii*). KDC, keto acid decarboxylase; ADH, alcohol dehydrogenase. Inset: general mechanism by which KDC-ADH converts a keto acid into an alcohol.

out further improvements, the introduction of the 1-butanol pathway in *P. putida* led to higher solvent titer than its *E. coli* counterpart (0.12 and 0.034 g/L, respectively). However, under the same condition, *B. subtilis* was only able to produce 24 mg/L 1-butanol.

Transfer of the *Clostridia* 1-butanol pathway has also been carried out in *S. cerevisiae*, the current workhorse of ethanol fermentation [30]. Besides the *C. beijerinckii* pathway, ortholog genes from *S. cerevisiae*, *E. coli*, *Ralstonia eutropha*, and *Streptomyces collinus* have also been tested for various steps in the 1-butanol pathway. However, the best enzyme combination led only to a 2.5 mg/L 1-butanol titer [30]. Like *E. coli,* the genetics and metabolism of *S. cerevisiae* are well-known and easy to manipulate. Engineering the genetic background would undoubtedly improve the productivity of this organism for 1-butanol production.

4 Keto acid pathways for higher alcohol production

4.1 Principle

The pathways described above involve the oxidative decarboxylation of pyruvate leading to acetyl-CoA (Fig. 1). However, the most efficient biofuel production pathway known thus far is the ethanol production by *S. cerevisiae* or *Zymomonas mobilis*, which uses non-oxidative decarboxylation of pyruvate, and does not require CoA [31]. This reaction, catalyzed by pyruvate decarboxylase, leads to acetaldehyde, which is further reduced to ethanol in a NADH dependent manner by an alcohol dehydrogenase (ADH). Therefore, the case of ethanol exemplifies that a keto acid with *n* carbon can be very efficiently converted to an alcohol with *n*–1 carbon by the successive action of a keto acid decarboxylase (KDC) and an ADH (Fig. 2, insert). 2-keto acids (a.k.a. α-keto acids) are organic acids that also carry a ketone functional group on the second carbon (a.k.a α-carbon). They are naturally found in all organisms as intermediates in a number of amino-acid biosynthesis pathways, and their degradation into alcohols is known as the Ehrlich pathway. It is then tempting to take advantage of these compounds and to try to divert them toward higher alcohol formation using the KDC-ADH two-step reaction. In this scenario, many valuable higher alcohols could be produced from naturally occurring keto acids (Fig. 2). In the isoleucine biosynthetic pathway, 1-propanol could be produced from 2-keto-butyrate (2KB) and 2-methyl-1-butanol (2MB) could be produced from 2-keto-3-methyl-valerate (2K3MV). In the valine/leucine biosynthetic pathway, isobutanol could be produced from

Table 2. C_3 to C_5 alcohol production in engineered microorganisms: significant contributions.

	Pathway	Ref.	Organism	Titer (g/L)	Yield	Overexpressed genes	Deleted genes
1-Propanol	Keto acids	[32]	*E. coli* JCL16[b)]	0.031	nd	*KivD* (Ll), *ADH2* (Sc)	no
		[34]	*E. coli* JCL16[b)]	0.8	nd	*thrA*^{FBR}*BC* (Ec), *ilvA* (Ec), *leuABCD* (Ec), *KivD* (Ll), *ADH2* (Sc)	*metA*, *tdh*, *ilvB*, *ilvI*, *adhE*
		[37]	*E. coli* JCL16[b)]	3.6	nd	*CimA* (Mj), *leuABCD* (Ec), KivD (Ll), *ADH2* (Sc)	*ilvB*, *ilvI*
Isopropanol	Coenzyme A	[27]	*E. coli* ATCC11303	4.9	43.5%[a)]	*thl* (Ca), *atoAD* (Ec), *adc* (Ca), *adh* (Cb)	no
Isobutanol	Keto acids	[32]	*E. coli* J CL16[b)]	22	86%[a)]	*alsS* (Bs), *ilvCD* (Ec), *KivD* (Ll), *ADH2* (Sc)	*adhE*, *ldhA*, *frdAB*, *fnr*, *pta*, *pfl*
		[41]	*S. elongatus* PCC7942	0.45	nd	*alsS* (Bs), *ilvCD* (Ec), *KivD* (Ll), *rbcLS* (Se), *yqhD* (Ec)	no
		[39]	*C. glutamicum* ATCC13032	4.9	23%[a)]	*alsS* (Bs), *ilvCD* (Cg), *KivD* (Ll), *adhA* (Cg)	*pyc*, *ldh*
1-Butanol	Coenzyme A	[61]	*C. acetobutylicum* ATCC 824	13	nd	*ctfAB* (Ca), *adc* (Ca)	no
		[11]	*C. acetobutylicum* ATCC 824	17.8	54%[a)]	no	*solR*
		[12]	*C. acetobutylicum* ATCC 824	16.6	nd	*aad* (Ca)	*buk*
		[28]	*E. coli* JCL16[b)]	0,55	nd	*atoB* (Ec), *crt* (Ca), *bcd* (Ca), *etfAB* (Ca), *hbd* (Ca), *adhE2* (Ca)	*adh, ldh, frd, fnr, pta*
		[29]	*E. coli* BL21Star(DE3)	0.58[c)] 0.52[d)]	nd	*thl* (Ca), *crt* (Ca), *bcd* (Ca), *etfAB* (Ca), *hbd* (Ca), *adhE2* (Ca) [c]*gapA* (Ec), [d]*fdh1* (Sc)	no
		[29]	*P. putida* S12	0.12	nd	*thl* (Ca), *crt* (Ca), *bcd* (Ca), *etfAB* (Ca), *hbd* (Ca), *adhE1* (Ca)	no
		[29]	*B. subtilis* KS438	0.024	nd	*thl* (Ca), *crt* (Ca), *bcd* (Ca), *etfAB* (Ca), *hbd* (Ca), *adhE2* (Ca)	*amyE thrC pyrD*
		[30]	*S. cerevisiae* BY4742	0.0025	nd	*ERG10* (Sc), *crt* (Ca), *ccr* (Sco), *hbd* (Ca), *adhE2* (Ca)	no
	Keto acids	[32]	*E. coli* JCL16[b)]	0.066	nd	*ilvA* (Ec), *leuABCD* (Ec), *KivD* (Ll), *ADH2* (Sc)	*ilvD*
		[34]	*E. coli* JCL16[b)]	0.8	nd	*thrA*^{FBR}*BC* (Ec), *IlvA* (Ec), *leuABCD* (Ec), *KivD* (Ll), *ADH2* (Sc)	*metA*, *tdh*, i*lvB*, *ilvI*, *adhE*
		[37]	*E. coli* JCL16[b)]	0.52	nd	*cimA* (Mj), *leuABCD* (Ec), *KivD* (Ll), *ADH2* (Sc)	*ilvB*, *ilvI*
2-Methyl-1-butanol	Keto acids	[32]	*E. coli* JCL16[b)]	0.067	nd	*KivD* (Ll), *ADH2* (Sc)	no
		[43]	*E. coli* JCL16[b)]	1.25	44%[a)]	*ilvGM* (St), *ilvA* (Cg), *thrABC* (Ec), *KivD* (Sc), *ADH2* (Sc)	*metA*, *tdh*

Table 2. Continued

3-Methyl-1-butanol	Keto acids	[32]	*E. coli* JCL16[b)]	0.13	nd	*KivD* (Ll), *ADH2* (Sc)	no
		[44]	*E. coli* JCL16[b)]	1.28	0.11 g/g glucose	*alsS* (Bs), *ilvCD* (Ec), *leuA*^{FBR}*BCD* (Ec), *KivD* (Ll), *ADH2* (Sc)	*adhE*, *frdBC*, *ldhA*, *pta*, *fnr*, *pflB*, *ilvE*, *tyrB*
		[45]	*E. coli* JCL16[b)]	9,5	0.11g/g glucose	*alsS* (Bs), *ilvCD* (Ec), *leuA*^{FBR}*BCD* (Ec), *KivD* (Ll), *ADH2* (Sc)	no
Phenylethanol	Keto acids	[32]	*E. coli* JCL16[b)]	0.057	nd	*ARO10* (Sc), *ADH2* (Sc)	no

a) Percentage of the theoretical maximum mol/mol glucose conversion.
b) The JCL16 strain is BW25113 (*rrnB*$_{T14}$ Δ*lacZ*$_{WJ16}$ *hsdR514* Δ*araBAD*$_{AH33}$ Δ*rhaBAD*$_{LD78}$).
Initials of original organism are in parenthesis. Bs, *B. subtilis*; Ca, *C. acetobutylicum*; Cb, *C. beijerinckii*; Cg, *C. glutamicum*; Ec, *E. coli*; Ll, *L. lactis*; Mj, *M. jannaschii*; Sc, *S. cerevisiae*; Sco, *S. collinus*; St, *S. typhimurium*; nd, not determined.

2-keto-isovalerate (2KIV), and 3-methyl-1-butanol (3MB) could be produced from 2-keto-4-methyl-valerate (2K4MV). Norvaline biosynthesis, a minor side-reaction of leucine biosynthesis, includes the precursor 2-keto-valerate (2KV), which can be rerouted toward 1-butanol. Finally, in the phenylalanine pathway, phenylpyruvate can be converted to phenylethanol.

Many of the above mentioned keto acids are not naturally subjected to decarboxylation and reduction by KDCs and ADHs, meaning that no natural highly specific KDC or ADH can be used to carry out every single desired reaction. A prerequisite for this approach would be to identify, or engineer, a KDC-ADH pair of enzymes with a substrate range broad enough to perform non-natural conversions of keto acids to higher alcohols. In their pioneer work, Atsumi *et al*. [32] expressed in *E. coli* five KDCs from various organisms and kingdoms, together with Adh2 from *S. cerevisiae*. When the KDCs Aro10 (*S. cerevisiae*) or Kivd (*Lactococcus lactis*) were expressed, all of the above mentioned higher alcohols could be detected, Kivd being more active than Aro10 for the production of all the alcohols except phenylethanol. This demonstrates that Kivd substrate range include all the desired keto acids. Moreover, the aldehyde intermediates between keto acids and alcohols were only detected in trace amounts, indicating that Adh2 also has a broad enough substrate range and high enough activity to reduce the KDC products. Atsumi *et al*. [33] later found that *E. coli* YqhD and *L. lactis* AdhA work even better than yeast Adh2. Therefore, several pairs of enzymes seem to be suitable for the production of five highly valuable higher alcohols.

It is desirable to engineer strains highly efficient for the production of one specific compound. To drive metabolic flux toward the production of one higher alcohol, it would be necessary to (i) increase the amount of the desired keto acid and decrease that of the unwanted, and/or (ii) increase the specificity of KDC toward the desired keto acid. The different approaches that have been reported to efficiently produce higher alcohols from keto acids are detailed in the following section, and summarized in Table 2.

4.2 1-Propanol

In the isoleucine biosynthetic pathway, threonine is synthesized from aspartate by the *thrABC* gene products, and then deaminated to 2KB by the threonine deaminase *ilvA*, leading to the keto-acid 2KB. Naturally, 2KB is metabolized into 2-aceto-2-hydroxy-butyrate by an acetohydroxy-acid synthase (AHAS, *ilvIH*/*ilvBN*, Fig. 2). However, it has been proven that the Kivd/ADH2 was able to convert 2KB to the corresponding higher alcohol, 1-propanol [32]. Together with Kivd/ADH2, overexpression of *ilvA* and a *thrA*^{FBR}*BC* operon (where a single mutation abolishes ThrA susceptibility to threonine feedback inhibition) led to a three- to fourfold increase in 1-propanol production [34]. These authors also realized several gene deletions that enhanced 1-propanol production. To disrupt methionine and 2-amino-3-ketobutyrate synthesis from 2KB precursors, *metA* and *tdh* were deleted. Δ*adhE* knock-out resulted also in higher 1-propanol titers, by preventing ethanologenic fermentation (1.01 g/L *vs*. 0.148 g/L with the wild type). Surprisingly, deletion of *ilvI* and *ilvB*, the catalytic subunits of AHASI and AHASIII respectively, which abolish competitive substrate consumption by the natural isoleucine biosynthesis pathway, had no effect on 1-propanol titer. It should be noted that a pathway for 1-butanol production, competing for 2KB, was simultaneously expressed (*cf*. 1-butanol section below). Therefore, the nice 1.01 g/L 1-propanol titer reported by Shen and Liao [34]

should be certainly ameliorated if desired, by not expressing the 1-butanol pathway.

Another attractive pathway leading to 2KB has been described in some bacteria such as *Leptospira interrogans* [35] and *Methanococcus jannaschii* [36]. This pathway, which does not have threonine as an intermediate, is more direct than the one based on threonine because it does not involve the successive ammonia-lyase and deaminase reactions. The first committed step in this alternative route is catalyzed by the citramalate synthase CimA, which condenses pyruvate and acetyl-CoA into (R)-citramalate (Fig. 2). This latter product is then converted to 2KB by *leuBCD* gene products. *cimA* from the thermophilic bacterium *M. jannaschii* has been expressed in *E. coli* together with the *leuABCD* operon and Kivd/ADH2 [37]. After several rounds of directed evolution to improve CimA activity at 37°C in *E. coli*, 1-propanol titer as high as 3.6 g/L has been obtained.

4.3 1-Butanol

The keto acid leading to 1-butanol through the action of Kivd and ADH2 is 2KV. It is formed in minute quantities in nature by leucine biosynthetic *leuABCD* gene products using 2KB instead of 2KIV as a substrate, leading ultimately to the synthesis of the nonnatural amino-acid L-norvaline [38] (Fig. 2). As 2KV can be metabolized by Kivd/ADH2 to 1-butanol [32], attempts to produce high titers of 1-butanol using this pathway have been carried out recently [32, 34, 37]. To drive metabolic flux toward 2KV, the 2KB production pathway has been overexpressed along with a *leuABCD* operon. To enhance further 1-butanol formation, *ilvI* and *ilvB* [34] or *ilvD* [32] have been deleted. These deletions impede the synthesis of 2KIV, the natural substrate of LeuABCD, they also prevent the synthesis of other keto acids that could compete with 2KV for Kivd substrate, and reduce 2KB consumption by the natural isoleucine biosynthesis. In a comparable manner to 1-propanol production, Δ*metA*, Δ*tdh*, and Δ *adhE* deletions resulted in more than eightfold 1-butanol production improvements (0.89 g/L *versus* 0.084 g/L with the wild type) [34].

Even if leading to nice 1-butanol titers, an intrinsic property of this pathway is to produce 1-propanol as a byproduct. Indeed, there is no easy way to produce 2KV without producing KB as an intermediate. Directed evolution of Kivd to increase specificity toward 2KV could be envisaged, but the structural relatedness of 2KB and 2KV could make this task arduous. Channeling 2KB to LeuA, by fusing IlvA and LeuA, might also increase the 1-butanol/1-propanol ratio.

4.4 Isobutanol

The keto acid required for isobutanol production through the Ehrlich pathway is 2KIV. It is an intermediate in valine and leucine biosynthesis. 2KIV is formed from pyruvate by the products of *ilvIH* (AHAS), *ilvC* (an NADPH ketol acid reductoisomerase), and *ilvD* (a 2,3-dihydroxy-acid hydrolyase). 2KIV natural fates are either to be transaminated by IlvE to form valine, or to be metabolized by LeuABCD to form 2-keto-4-methylvalerate (2KMV), the precursor of leucine (Fig. 2). High level production of isobutanol from 2KIV using the Ehrlich pathway has been first achieved in *E. coli* [32]. To enhance 2KIV production, these authors overexpressed AlsS, an AHAS enzyme from *B. subltilis*, together with IlvCD. Indeed, AlsS prefers pyruvate as a substrate, whereas IlvIH prefers 2KB. Also, to increase further the yields of isobutanol production, multiple genes involved in pyruvate consumption were deleted (*adhE*, *ldhA*, *frdAB*, *fnr*, *pta*, *pfl*). The combined effects of these gene overexpressions and deletions led to the highest isobutanol titer reported to date: 22 g/L [32]. A similar approach has been recently carried out in *Corynebacterium glutamicum* [39], the workhorse of amino-acid production [40]. As the keto acid target for isobutanol production is part of valine/leucine biosynthetic pathway, such an amino-acid overproducer is attractive to receive the isobutanol production pathway. Moreover, *C. glutamicum* is more resistant than *E. coli* to high isobutanol concentrations in the medium [39]. Overexpression of *alsS*, *ilvCD*, *Kivd* and the endogenous ADH *adhA*, together with the deletion of *pyc* and *ldh* to prevent pyruvate consumption, led to a maximal 4.9 g/L isobutanol titer [39].

As mentioned earlier, glucose is expensive and somehow controversial as a renewable feedstock for biofuel production. To tackle this issue, the isobutanol production pathway has also been transferred into the cyanobacterium *Synechococcus elongatus* [41]. This organism can grow photoautotrophically with carbon dioxide as the sole carbon source. Therefore, isobutanol produced by *S. elongatus* grown autotrophically would come directly from the photosynthetic recycling of atmospheric CO_2. High titer isobutanol production by *S. elongatus* has been achieved by overexpressing *alsS*, *ilvCD*, *Kivd*, the *E. coli* ADH *yqhD*, as well as the *S. elongatus* RuBisCo coding genes *rbcLS* [41]. Productivity as high as 6.2 mg/L/h, with final titer of 1.1 g/L, was obtained for isobutyraldehyde production with sodium bicarbonate as the sole carbon source. With the addition of the ADH, isobutanol productivity was 3 mg/L/h, with a final titer of

0.45 g/L. This work marks the first high-level production of fuels and chemicals directly from CO_2, and the productivity in the laboratory conditions is already higher than the overall productivity of the current corn ethanol, cellulosic ethanol, and algal lipid technologies [42].

Further improvements in keto acid based isobutanol production rely on the accurate selection of the enzymes that are part of the pathway, with regard to their substrate specificity and activity. In that direction, three ADH from *S. cerevisiae* (ADH2), *E. coli* (YqhD), and *L. lactis* (AdhA) have been compared for their isobutanol production when expressed with Kivd in *E. coli* [33]. This study shows that in *E. coli*, the NADH dependent *L. lactis adhA* is the best candidate to convert isobutyraldehyde to isobutanol.

4.5 2-Methyl-1-butanol and 3-methyl-1-butanol

The keto acid required for the synthesis of C_5 alcohol 2MB is 2K3MV, the immediate precursor of isoleucine (Fig. 2). Similarly to 2KV, biosynthesis of 2K3MV involves the keto acid intermediate 2KB, and both 2K3MV and 2KB are Kivd substrates [32]. Therefore, the AHAS that metabolizes 2KB toward 2K3MV production has to be carefully chosen to reduce as much as possible 1-propanol byproduct formation. Cann and Liao [43] compared six AHAS isoenzymes for their 2MB production capability. The best enzyme was *Salmonella typhimurium* AHASII encoded by *ilvGM*, which was the only one to lead to a 2MB:1-propanol ratio >1. However, in this study, the authors had to cope with the production of another byproduct, isobutanol. 50% reduction of isobutanol titer has been achieved by enhancing threonine production and deamination, thus providing more 2KB and 2K3MV substrates for Kivd than 2KIV [43].

To efficiently produce 3MB using the Ehrlich pathway, metabolic flux toward keto acid 2K4MV has to be optimized (Fig. 2). 2K4MV, the immediate precursor of leucine, is formed from 2KIV (*cf.* isobutanol production), through the reactions catalized by *leuABCD* gene products. *leuA* encodes a 2-isopropylmalate synthase (IPMS) that elongates the carbon chain of KIV using acetyl-CoA. When the genes for the whole 3MB pathway are overexpressed in *E. coli*, *i.e.*, *alsS ilvCD leuABCD kivd adh2*, isobutanol is produced in much higher amount than 3MB (2 and 0.067 g/L, respectively) [44]. This was due to a low expression of *leuABCD*, since changing the ribosome binding site (RBS) of *leuA* improved significantly 3MB production. A feedback-resistant LeuA has also been expressed to prevent eventual product feedback inhibition. This resulted in a dramatic shift in keto acid production, with 2K4MV production being almost ten times higher than that of 2KIV. When combined with several gene deletion to prevent metabolic intermediates consumption, this approach led to 1.28 g/L 3MB production, with only negligible isobutanol byproduct formation [44].

Recently, directed evolution has been performed on *E. coli* in order to enhance leucine biosynthesis, and therefore 3MB production [45]. The strategy used was to randomly mutagenize the bacteria and to select mutants resistant to 4-aza-D,L-leucine (AZL), a toxic leucine analog. Among the multiple ways for an organism to acquire resistance to a toxic amino-acid, one is to overproduce the natural amino acid, which would out-compete with its toxic counterpart. Because several other ways could lead to similar AZL resistance, the selected mutants were transformed with the 3MB pathway and screened for enhanced 3MB production. After two successive rounds of selection, 2.8 g/L 3MB titer was measured, which is more than sixfold higher than the production of the wild-type transformed with the same 3MB pathway. The authors also noticed that the 3MB was toxic to *E. coli* at the concentration where it was produced, with an LD_{50} around 2 g/L. Therefore, two-phase fermentation has been tested to extract 3MB as it is produced. This dramatically improved the 3MB production, with 3MB titer reaching 9.5 g/L [45].

4.6 Higher alcohols production from non-natural keto acids

The higher alcohols whose production is described above take advantage of the naturally occurring keto acids, which are limited in number and in carbon length. Higher alcohols >C_5 do not have naturally occurring keto acid counterparts. To overcome this limitation, Zhang *et al.* [46] have taken advantage of IPMS-based keto acid elongation. *leuABCD* gene products perform such a metabolic process, naturally elongating KIV to produce the C + 1 keto acid 2K4MV (Fig. 2). In order to broaden the substrate specificity of LeuA and Kivd, structure-based directed mutagenesis has been carried out. Mutations were chosen so that their substrate binding pocket was enlarged. As a result, engineered IPMS were able to perform several rounds of keto acid elongation, leading to the production of various C_6 to C_{10} non-natural keto acids that could be converted to C_5 to C_9 alcohols by the optimized Kivd [46].

5 Efforts to improve producers' tolerance to solvent

Engineering an efficient pathway for higher alcohol production will result in titers that are limited by the tolerance of the producer toward high concentrations of the desired or by-products metabolites. Indeed, higher chain alcohols are toxic beyond a concentration that is compound- and strain-dependent. Therefore, to increase the final titers of a given compound, the tolerance of the strain toward the produced higher alcohol has to be enhanced. Intensive research is done toward this goal, and the the topic has been recently reviewed [47, 48]. The earliest efforts in this direction have been to isolate strains naturally tolerant to organic solvents that could be used as a producer, or help in deciphering the mechanisms that mediate this tolerance. Such a *C. acetobutylicum* mutant strain has been obtained by Lin *et al*. [49], after several rounds of culture transfer with increasing 1-butanol concentration. The resulting SA-1 strain was able to grow in 20 g/L 1-butanol. The same approach has been successfully carried out with *E. coli* to increase its tolerance to isobutanol [32]. Organic solvents are thought to be toxic to bacteria mainly by damaging the cell membrane, thus impairing its vital functions (*e.g*., loss of ions, dissipation of the pH gradient, inactivation of membrane proteins) [50]. Indeed, most of the bacteria that display naturally high solvent tolerance adapt their membrane composition according to the solvent challenge [51]. Rational genetic engineering of *C. acetobutylicum* for a different membrane composition was attempted by overexpressing the *cfa* gene coding a cyclopropane fatty acid synthase [52]. The resulting strain did tolerate higher solvent titers in the medium, and was able to grow in a medium containing 6.6 g/L 1-butanol. However, its solvent productivity was significantly lower (–24%) than the parent strain. Tomas *et al*. [53] successfully improved *Clostridium* solvent tolerance by overexpressing *groESL* class I heat shock proteins. The resulting solvant-tolerant strain produced 17.1 g/L 1-butanol, representing a 30% increase compared to the strain carrying the empty vector. In addition, the active (1-butanol producing) phase lasted longer in the *groESL* overexpressor than in the control.

Recently, comprehensive analysis of *Clostridium* transcriptomic response to solvent exposure has provided interesting insights into the cellular modifications triggered by this stress [22]. However, as mentioned by the authors, the next challenge will be to determine which gene expression deregulation participates to the solvent resistance and which one participate to deleterious effects. As described previously, *E. coli* is also a promising biocatalyst for solvents production. A similar overview of 1-butanol exposure responses at the transcript and at the protein level have been made available in *E. coli* [54]. The authors also took advantage of the Keio collection of single-gene deletion *E. coli* mutants [55] to test several gene knock-outs. Eleven candidate genes were chosen based on expression changes in response to 1-butanol stress. Among the corresponding knock-out strains tested, three were consistently more solvent resistant than the parent strain at the two time points investigated (Δ*cpxP*, Δ *envY*, Δ *sodC*). This shows the potential of systematical approaches to discover candidate genes to engineer. Indeed, Brynildsen and Liao [56] used a systems biology approach to unravel the underlying regulation networks behind *E. coli* response to isobutanol, 1-butanol, and ethanol stress. This study confirmed that plasma membrane is the primary target for these alcohols, although the cell response to ethanol exposure is significantly different to that of *n*-butanol. Specifically, *n*-butanol mediated quinone malfunction have been shown to trigger an important part of the disorders caused by the solvent [56]. A simple outcome of this discovery would be to engineer a strain to be less dependent on quinone for carbon utilization, in order to limit the deleterious effects of *n*-butanol.

6 Conclusion and overall design issues

As discussed above, significant progress has been made in the past few years in the production of higher alcohols using native and non-native pathways using metabolically engineered microbes. A variety of higher alcohols have been successfully produced, using CoA- or keto acids-dependent pathways, in a number of micro-organisms. Therefore, a number of choices are possible, and the theoretical efficiency of each design has to be considered. Li *et al*. [57] recently examined thoroughly these different production designs and their respective theoretical yields. Data for higher alcohols is provided in Table 3. Another useful consideration is the redox balance of each pathway (*i.e*., reduced co-factors produced versus oxidized ones). In this regard, it is noteworthy that biosynthesis of alcohols with even numbers of carbons have redox factors completely balanced from glucose, whereas ones with odd numbers of carbons do not. Whether such redox balance affects the actual productivity or yield remains unclear.

Table 3. Energy density and biosynthetic yields of common fuels and higher alcohols (from [57]).

Fuel	Energy density (MJ/kg)	Pathway stochiometric yield (g/g glucose)	Energy yield from glucose (%)[a)]
Gasoline	42.7	–	–
Ethanol	29.7	0.511	97.6
n-Propanol	33.6	0.444	95.9
n-Butanol	36.1	0.411	95.4
1-Pentanol	37.7	0.326	79.0
3-Methylpentanol	37.7	0.326	79.0
2-Methylpentanol	37.7	0.391	94.8

a) Glucose energy density is 15.6 MJ/kg.

In the design of production strains, it is common to assume that the cell growth is necessary for product formation. In industrial fermentations, both growth-associated and non-growth associated product formation have been observed, with primary metabolites typically classified as growth-associated and secondary metabolites as non-growth associated. However, metabolic and genetic manipulations have significantly changed this classification, and the distinction between primary and secondary metabolites becomes blurred for non-native metabolites. For example, isobutanol is a non-native metabolite for *E. coli*, and its production mainly occurs in the non-growth phase [32]. This phenomenon challenges the common growth-associated design principle. The ideal situation is to separate the control for growth and product formation. Theoretical and experimental studies show that product formation yield (amount of product formed *per* substrate consumed) is necessarily decreased if the growth rate and growth yield increase [58–60]. This situation is a direct consequence of mass balance: production of extra compounds decreases the resource available for growth. Thus, given the fact that substrate uptake rate cannot be proportionally increased, high growth yield decreases the resource available for product formation. Consequently, during the growth phase, the best objective is to increase the growth rate without much product formation. After the cell density achieves the desired level, the cell can be switched to the production mode without growth. Such a strategy is theoretically possible but difficult to achieve. Isobutanol production in *E. coli* comes close to this situation, which may be the reason for the high yield production much beyond the product toxicity level.

In conclusion, higher-alcohol production using metabolically engineered microorganisms is a promising source of renewable fuels. Although some production systems are already very efficient [11, 32, 41] there is still extensive room for improvements, which ensure even better-performing production designs to come in the near future. Isobutanol is currently the most efficiently higher alcohol produced, probably because it has received the most attention to date. To our knowledge, there is no apparent reason why all the higher alcohols described herein cannot be produced at least as efficiently as isobutanol.

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J. C. L. has financial interest in companies commercializing the technologies discussed in this article.

7 References

[1] Bai, F. W., Anderson, W. A., Moo-Young, M., Ethanol fermentation technologies from sugar and starch feedstocks. *Biotechnol. Adv.* 2008, *26*, 89–105.

[2] Huang, H., Liu, H., Gan, Y., Genetic modification of critical enzymes and involved genes in butanol biosynthesis from biomass. *Biotechnol. Adv.* 2010, *28*, 651–657.

[3] Yan, Y., Liao, J. C., Engineering metabolic systems for production of advanced fuels. *J. Ind. Microbiol. Biotechnol.* 2009, *36*, 471–479.

[4] Cann, A. F., Liao, J. C., Pentanol isomer synthesis in engineered microorganisms. *Appl. Microbiol. Biotechnol.* 2010, *85*, 893–899.

[5] Connor, M. R., Liao, J. C., Microbial production of advanced transportation fuels in non-natural hosts. *Curr. Opin. Biotechnol.* 2009, *20*, 307–315.

[6] George, H. A., Johnson, J. L., Moore, W. E., Holdeman, L. V., Chen, J. S., Acetone, isopropanol, and butanol production by *Clostridium beijerinckii* (syn. *Clostridium butylicum*) and *Clostridium aurantibutyricum*. *Appl. Environ. Microbiol.* 1983, *45*, 1160–1163.

[7] Häggström, L., Acetone-butanol fermentation and its variants. *Biotechnol. Adv.* 1985, *3*, 13–28.

[8] Jones, D. T., Woods, D. R., Acetone-butanol fermentation revisited. *Microbiol. Rev.* 1986, *50*, 484–524.

[9] Papoutsakis, E. T., Engineering solventogenic clostridia. *Curr. Opin. Biotechnol.* 2008, *19*, 420–429.

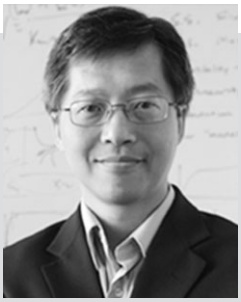
[10] Lee, S. Y., Park, J. H., Jang, S. H., Nielsen, L. K. *et al.*, Fermentative butanol production by Clostridia. *Biotechnol. Bioeng.* 2008, *101*, 209–228.

[11] Nair, R. V., Green, E. M., Watson, D. E., Bennett, G. N., Papoutsakis, E. T., Regulation of the sol locus genes for butanol and acetone formation in *Clostridium acetobutylicum* ATCC 824 by a putative transcriptional repressor. *J. Bacteriol.* 1999, *181*, 319–330.

[12] Harris, L. M., Desai, R. P., Welker, N. E., Papoutsakis, E. T., Characterization of recombinant strains of the *Clostridium acetobutylicum* butyrate kinase inactivation mutant: need for new phenomenological models for solventogenesis and butanol inhibition? *Biotechnol. Bioeng.* 2000, *67*, 1–11.

[13] Görke B., Stülke, J., Carbon catabolite repression in bacteria: Many ways to make the most out of nutrients. *Nat. Rev. Microbiol.* 2008, *6*, 613–624.

[14] Ren, C., Gu, Y., Hu, S., Wu, Y. *et al.*, Identification and inactivation of pleiotropic regulator CcpA to eliminate glucose repression of xylose utilization in *Clostridium acetobutylicum*. *Metab. Eng.* 2010, *12*, 446–454.
[15] Tummala, S. B., Welker, N. E., Papoutsakis, E. T., Design of antisense RNA constructs for downregulation of the acetone formation pathway of *Clostridium acetobutylicum*. *J. Bacteriol.* 2003, *185*, 1923–1934.
[16] Jiang, Y., Xu, C., Dong, F., Yang, Y. *et al.*, Disruption of the acetoacetate decarboxylase gene in solvent-producing *Clostridium acetobutylicum* increases the butanol ratio. *Metab. Eng.* 2009, *11*, 284–291.
[17] Dong, H., Zhang, Y., Dai, Z., Li, Y., Engineering clostridium strain to accept unmethylated DNA. *PloS One* 2010, *5*, e9038.
[18] Blouzard, J., Valette, O., Tardif, C., De Philip, P., Random mutagenesis of *Clostridium cellulolyticum* by using a Tn1545 derivative. *Appl. Environ. Microbiol.* 2010, *76*, 4546–4549.
[19] Heap, J. T., Cartman, S. T., Kuehne, S. A., Cooksley, C., Minton, N. P., ClosTron-targeted mutagenesis. *Methods Mol. Biol.* 2010, *646*, 165–182.
[20] Heap, J. T., Pennington, O. J., Cartman, S. T., Carter, G. P., Minton, N. P., The ClosTron: A universal gene knock-out system for the genus *Clostridium*. *J. Microbiol. Meth.* 2007, *70*, 452–464.
[21] Nölling, J., Breton, G., Omelchenko, M. V., Makarova, K. S. *et al.*, Genome sequence and comparative analysis of the solvent-producing bacterium *Clostridium acetobutylicum*. *J. Bacteriol.* 2001, *183*, 4823–4838.
[22] Alsaker, K. V., Paredes, C., Papoutsakis, E. T., Metabolite stress and tolerance in the production of biofuels and chemicals: Gene-expression-based systems analysis of butanol, butyrate, and acetate stresses in the anaerobe *Clostridium acetobutylicum*. *Biotechnol. Bioeng.* 2010, *105*, 1131–1147.
[23] Jones, S. W., Paredes, C. J., Tracy, B., Cheng, N. *et al.*, The transcriptional program underlying the physiology of clostridial sporulation. *Genome Biol.* 2008, *9*, R114.
[24] Alsaker, K. V., Papoutsakis, E. T., Transcriptional program of early sporulation and stationary-phase events in *Clostridium acetobutylicum*. *J. Bacteriol.* 2005, *187*, 7103–7118.
[25] Mao, S., Luo, Y., Zhang, T., Li, J. *et al.*, Proteome reference map and comparative proteomic analysis between a wild type *Clostridium acetobutylicum* DSM 1731 and its mutant with enhanced butanol tolerance and butanol yield. *J. Proteome Res.* 2010, *4*, 3046–3061.
[26] Sullivan, L., Bennett, G. N., Proteome analysis and comparison of *Clostridium acetobutylicum* ATCC 824 and Spo0A strain variants. *J. Ind. Microbiol. Biotechnol.* 2006, *33*, 298–308.
[27] Hanai, T., Atsumi, S., Liao, J. C., Engineered synthetic pathway for isopropanol production in *Escherichia coli*. *Appl. Environ. Microbiol.* 2007, *73*, 7814–7818.
[28] Atsumi, S., Cann, A. F., Connor, M. R., Shen, C. R. *et al.*, Metabolic engineering of *Escherichia coli* for 1-butanol production. *Metab. Eng.* 2008, *10*, 305–311.
[29] Nielsen, D. R., Leonard, E., Yoon, S., Tseng, H. *et al.*, Engineering alternative butanol production platforms in heterologous bacteria. *Metab. Eng.* 2009, *11*, 262–273.
[30] Steen, E. J., Chan, R., Prasad, N., Myers, S. *et al.*, Metabolic engineering of *Saccharomyces cerevisiae* for the production of n-butanol. *Microb. Cell Fact.* 2008, *7*, 36–43.



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[31] Yomano, L. P., York, S. W., Zhou, S., Shanmugam, K. T., Ingram, L.O., Re-engineering *Escherichia coli* for ethanol production. *Biotechnol. Lett.* 2008, *30*, 2097–2103.
[32] Atsumi, S., Hanai, T., Liao, J. C., Non-fermentative pathways for synthesis of branched-chain higher alcohols as biofuels. *Nature* 2008, *451*, 86–89.
[33] Atsumi, S., Wu, T., Eckl, E., Hawkins, S. D. *et al.*, Engineering the isobutanol biosynthetic pathway in *Escherichia coli* by comparison of three aldehyde reductase/alcohol dehydrogenase genes. *Appl. Microbiol. Biotechnol.* 2010, *85*, 651–657.
[34] Shen, C. R., Liao, J. C., Metabolic engineering of *Escherichia coli* for 1-butanol and 1-propanol production via the keto-acid pathways. *Metab. Eng.* 2008, *10*, 312–320.

[35] Xu, H., Zhang, Y., Guo, X., Ren, S. *et al*., Isoleucine biosynthesis in *Leptospira interrogans* serotype lai strain 56601 proceeds via a threonine-independent pathway. *J. Bacteriol.* 2004, *186*, 5400–5409.

[36] Howell, D. M., Xu, H., White, R. H., (R)-citramalate synthase in methanogenic archaea. *J. Bacteriol.* 1999, *181*, 331–333.

[37] Atsumi S., Liao, J. C., Directed evolution of *Methanococcus jannaschii* citramalate synthase for biosynthesis of 1-propanol and 1-butanol by *Escherichia coli*. *Appl. Environ. Microbiol.* 2008, *74*, 7802–7808.

[38] Kisumi, M., Sugiura, M., Chibata, I., Biosynthesis of norvaline, norleucine, and homoisoleucine in *Serratia marcescens*. *J. Biochem.* 1976, *80*, 333–339.

[39] Smith, K. M., Cho, K., Liao, J. C., Engineering *Corynebacterium glutamicum* for isobutanol production. *Appl. Microbiol. Biotechnol.* 2010, *87*, 1045–1055.

[40] Leuchtenberger, W., Huthmacher, K., Drauz, K., Biotechnological production of amino acids and derivatives: Current status and prospects, *Appl. Microbiol. Biotechnol.* 2005, *69*, 1–8.

[41] Atsumi, S., Higashide, W., Liao, J. C., Direct photosynthetic recycling of carbon dioxide to isobutyraldehyde. *Nat. Biotechnol.* 2009, *27*, 1177–1180.

[42] Sheehan, J., Engineering direct conversion of CO2 to biofuel. *Nat. Biotechnol.* 2009, *27*, 1128–1129.

[43] Cann, A. F., Liao, J. C., Production of 2-methyl-1-butanol in engineered *Escherichia coli*. *Appl. Microbiol. Biotechnol.* 2008, *81*, 89–98.

[44] Connor, M. R., Liao, J. C., Engineering of an *Escherichia coli* strain for the production of 3-methyl-1-butanol. *Appl. Environ. Microbiol.* 2008, *74*, 5769–5775.

[45] Connor, M. R., Cann, A. F., Liao, J. C., 3-Methyl-1-butanol production in *Escherichia coli*: Random mutagenesis and two-phase fermentation. *Appl. Microbiol. Biotechnol.* 2010, *86*, 1155–1164.

[46] Zhang, K., Sawaya, M. R., Eisenberg, D. S., Liao, J. C., Expanding metabolism for biosynthesis of nonnatural alcohols. *Proc. Natl. Acad. Sci. U. S. A.* 2008, *105*, 20653–20658.

[47] Liu S., Qureshi, N., How microbes tolerate ethanol and butanol. *New Biotechnol.* 2009, *26*, 117–121.

[48] Ezeji, T., Milne, C., Price, N. D., Blaschek, H. P., Achievements and perspectives to overcome the poor solvent resistance in acetone and butanol-producing microorganisms. *Appl. Microbiol. Biotechnol.* 2010, *85*, 1697–1712.

[49] Lin, Y. L., Blaschek, H. P., Butanol production by a butanol-tolerant strain of *Clostridium acetobutylicum* in Extruded Corn Broth. *Appl. Environ. Microbiol.* 1983, *45*, 966–973.

[50] Ingram, L. O., Microbial tolerance to alcohols: Role of the cell membrane. *Trends Biotechnol.* 1986, *4*, 40–44.

[51] Isken, S., de Bont, J. A., Bacteria tolerant to organic solvents. *Extremophiles* 1998, *2*, 229–238.

[52] Zhao, Y., Hindorff, L. A., Chuang, A., Monroe-Augustus, M. *et al*., Expression of a cloned cyclopropane fatty acid synthase gene reduces solvent formation in *Clostridium acetobutylicum* ATCC 824. *Appl. Environ. Microbiol.* 2003, *69*, 2831–2841.

[53] Tomas, C. A., Welker, N. E., Papoutsakis, E. T., Overexpression of groESL in *Clostridium acetobutylicum* results in increased solvent production and tolerance, prolonged metabolism, and changes in the cell's transcriptional program. *Appl. Environ. Microbiol.* 2003, *69*, 4951–4965.

[54] Rutherford, B. J., Dahl, R. H., Price, R. E., Szmidt, H. L. *et al*., Functional genomic study of exogenous n-butanol stress in *Escherichia coli*. *Appl. Environ. Microbiol.* 2010, *76*, 1935–1945.

[55] Baba, T., Ara, T., Hasegawa, M., Takai, Y. *et al*., Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: The Keio collection, *Mol. Syst. Biol.* 2006, *2*, 2006.0008.

[56] Brynildsen, M. P., Liao, J. C., An integrated network approach identifies the isobutanol response network of *Escherichia coli*. *Mol. Syst. Biol.* 2009, *5*, 277.

[57] Li, H., Cann, A. F., Liao, J. C., Biofuels: Biomolecular engineering fundamentals and advances. *Ann. Rev. Chem. Biomol. Eng.* 2010, *1*, 19–26.

[58] Wong, W. W., Tran, L. M., Liao, J. C., A hidden square-root boundary between growth rate and biomass yield. *Biotechnol. Bioeng.* 2009, *102*, 73–80.

[59] Wong, W. W., Liao, J. C., Microbial maximal specific growth rate as a square-root function of biomass yield and two kinetic parameters. *Metabol. Eng.* 2009, *11*, 409–414.

[60] Chao, Y. P., Liao, J. C., Alteration of growth yield by overexpression of phosphoenolpyruvate carboxylase and phosphoenolpyruvate carboxykinase in *Escherichia coli*. *Appl. Environ. Microbiol.* 1993, *59*, 4261–4265.

[61] Mermelstein, L. D., Papoutsakis, E. T., Petersen, D. J., Bennett, G. N., Metabolic engineering of *Clostridium acetobutylicum* ATCC 824 for increased solvent production by enhancement of acetone formation enzyme activities using a synthetic acetone operon. *Biotechnol. Bioeng.* 1993, *42*, 1053–1060.

