LETTERS

Non-fermentative pathways for synthesis of branched-chain higher alcohols as biofuels

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Global energy and environmental problems have stimulated increased efforts towards synthesizing biofuels from renewable resources[1–3]. Compared to the traditional biofuel, ethanol, higher alcohols offer advantages as gasoline substitutes because of their higher energy density and lower hygroscopicity. In addition, branched-chain alcohols have higher octane numbers compared with their straight-chain counterparts. However, these alcohols cannot be synthesized economically using native organisms. Here we present a metabolic engineering approach using *Escherichia coli* to produce higher alcohols including isobutanol, 1-butanol, 2-methyl-1-butanol, 3-methyl-1-butanol and 2-phenylethanol from glucose, a renewable carbon source. This strategy uses the host's highly active amino acid biosynthetic pathway and diverts its 2-keto acid intermediates for alcohol synthesis. In particular, we have achieved high-yield, high-specificity production of isobutanol from glucose. The strategy enables the exploration of biofuels beyond those naturally accumulated to high quantities in microbial fermentation.

Ethanol is not an ideal fuel because it has a lower energy density than gasoline, and its hygroscopicity poses a problem for storage and distribution. Higher alcohols (C4 and C5), on the other hand, have energy densities closer to gasoline, are not hygroscopic, and are less volatile compared with ethanol. Except for 1-butanol[1,2], none of the C4 and C5 alcohols has been produced from a renewable source in a yield high enough to be considered as a gasoline substitute. No microorganisms have been identified to produce, from glucose, higher alcohols such as isobutanol, 2-methyl-1-butanol or 3-methyl-1-butanol to industrially relevant quantities, although small amounts have been identified as microbial by-products[4–8].

Here, we devised a synthetic approach to produce the above-mentioned longer chain alcohols as next-generation biofuels. This strategy was implemented in *E. coli*, although other user friendly hosts such as *Saccharomyces cerevisiae* are readily applicable. These host organisms have fast growth rates and are facultative anaerobes, allowing for a flexible and economical process design for large-scale production[3,9,10]. However, importing and the expression of non-native pathways may lead to metabolic imbalance, whereas the accumulation of the heterologous metabolites may cause cytotoxicity[11–13]. To achieve high productivity of the target foreign products, it is desirable to seek pathways that are compatible to the host. Therefore, we took advantage of the existing metabolic capability in *E. coli* and the broad substrate range of the last two steps in the Ehrlich pathway[14] for 2-keto acid degradation from other organisms.

2-Keto acids are intermediates in amino acid biosynthesis pathways. These metabolites can be converted to aldehydes by broad-substrate-range 2-keto-acid decarboxylases (KDCs) and then to alcohols by alcohol dehydrogenases (ADHs). Using this strategy, only two non-native steps were needed to produce biofuels by shunting intermediates from amino acid biosynthesis pathways to alcohol production (Fig. 1a). Amino acid biosynthesis pathways produce various 2-keto acids (Fig. 1b). In this work, six different 2-keto acids for alcohol production were used. The isoleucine biosynthesis pathway generates 2-ketobutyrate and 2-keto-3-methyl-valerate, which can be converted to 1-propanol and 2-methyl-1-butanol, respectively. The valine biosynthesis pathway produces 2-keto-isovalerate, which is the precursor for isobutanol. The leucine biosynthesis pathway generates 2-keto-4-methyl-pentanoate, which is the substrate for 3-methyl-1-butanol. The phenylalanine biosynthesis pathway produces phenylpyruvate, which can lead to 2-phenylethanol. The norvaline biosynthesis pathway, which is a side-reaction of the leucine biosynthesis, produces a substrate for 1-butanol, 2-ketovalerate.

A critical enzyme in this alcohol production strategy is KDC, which is common in plants, yeasts and fungi but less so in bacteria[15]. The aldehydes produced can then be converted to alcohols by an ADH, which is commonly found in many organisms. Some of the KDCs have broad substrate ranges, whereas others are more specific. To test the capability of the endogenous 2-keto acids as a substrate for KDC in *E. coli*, five KDCs (Pdc6 (ref. 16), Aro10 (ref. 17), Thi3 (ref. 5) from *S. cerevisiae*, Kivd from *Lactococcus lactis*[18], and Pdc from *Clostridium acetobutylicum*) with alcohol dehydrogenase 2 (Adh2) of *S. cerevisiae*[19] were overexpressed. *E. coli* cultures expressing these foreign genes were grown in a minimal media with 0.2 M glucose. Gas chromatography–mass spectrometry (GC–MS) analysis (Table 1) revealed that the strains expressing either Kivd or Aro10 produced all of the expected alcohols. *S. cerevisiae* Pdc6 and *C. acetobutylicum* Pdc were not as versatile, whereas *S. cerevisiae* Thi3 did not have any expected activity. In all of these cases, aldehydes were detected only in trace amounts, indicating sufficient activity of Adh2. These results demonstrate that Kivd is the most active and versatile decarboxylase tested and, therefore, suited for our objectives. Furthermore, addition of various 2-keto acids (Table 2) to the *E. coli* culture expressing Kivd confirmed the specific production of the corresponding alcohols by 2- to 23-fold. The supply of 2-keto acids also decreased the production of the other alcohols markedly. These results indicate that increasing the flux to the 2-keto acids could improve both the productivity and specificity of production of the alcohols.

The existing *E. coli* metabolic pathways were then genetically modified to increase the production of the specific 2-keto acid so that the desired alcohol could be produced. To produce isobutanol, the *ilvIHCD* genes under the control of the $P_L lacO_1$ (ref. 20) promoter on a plasmid were overexpressed to enhance 2-ketoisovalerate biosynthesis (Fig. 1b). The amplified *ilv* pathway was then combined with the alcohol producing pathway (Kivd and Adh2) to achieve isobutanol production. As a result of the *ilvIHCD* overexpression, the strain produced 23 mM isobutanol, which is a ~5-fold increase over the strain without *ilvIHCD* overexpression (Fig. 2a and

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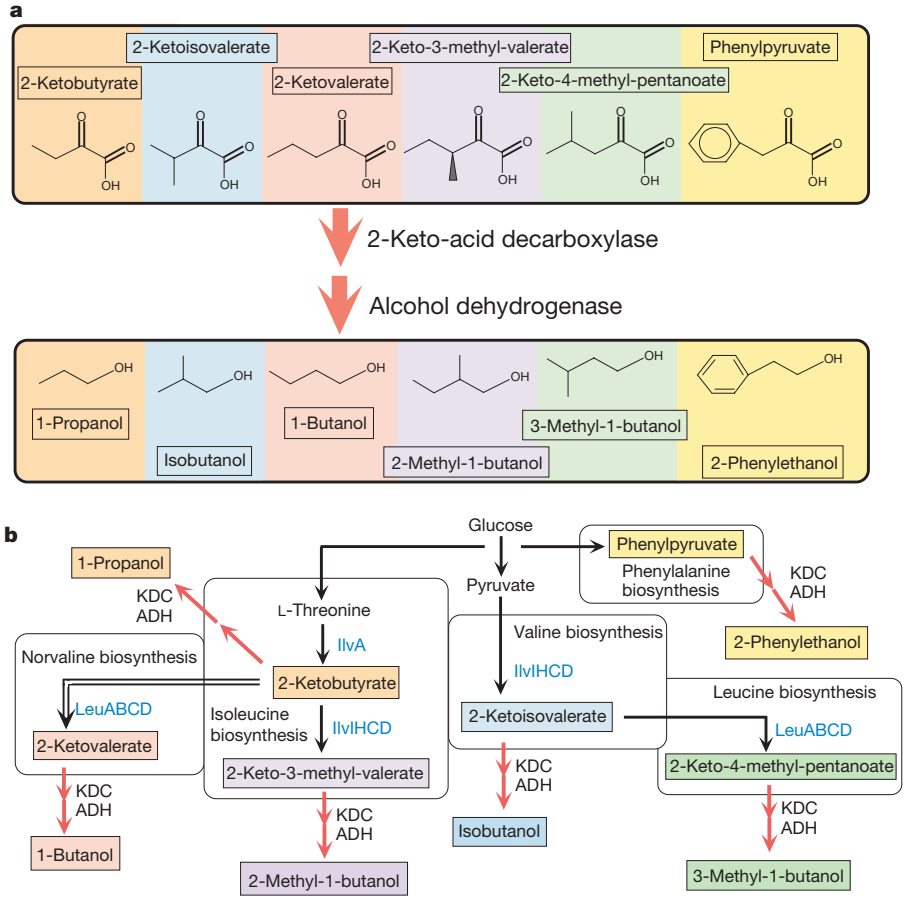

Figure 1 | Production of higher alcohols through the synthetic non-fermentative pathways. a, Various 2-keto acid precursors lead to corresponding alcohols through 2-ketoacid decarboxylase and alcohol dehydrogenase. **b**, The synthetic networks for the non-fermentative alcohol production in engineered *E. coli*. Red arrows represent the 2-keto acid decarboxylation and reduction pathway. Blue enzyme names represent amino acid biosynthesis pathways. The double lines represent a side pathway leading to norvaline and 1-butanol biosynthesis.

Supplementary Table 3). These results demonstrate that the synthetic pathway was functional and capable of supplying the 2-ketoisovalerate required for the efficient production of isobutanol. To increase further the isobutanol production, genes that contribute to by-product formation, including *adhE*, *ldhA*, *frdAB*, *fnr* and *pta*, were deleted. These deletions could increase the level of pyruvate available for the *ilvIHCD* pathway. Indeed, this strain produced 30 mM isobutanol, indicating that these deletions were beneficial for isobutanol production. In addition, this strain converted glucose to isobutanol with a yield of 0.21 g of isobutanol per gram of glucose between 16 h and 24 h (Fig. 2a, right panel).

To improve isobutanol production further, the *alsS* gene from *Bacillus subtilis* was used instead of *ilvIH* of *E. coli*. AlsS of *B. subtilis* has high affinity for pyruvate, whereas *E. coli* IlvIH has higher preference for 2-ketobutyrate[21]. The strain with the *alsS* pathway produced ~50 mM of isobutanol, which is a ~1.7-fold increase over the strain using IlvIH (Supplementary Fig. 1). In addition, *pflB* was deleted to decrease further the competition for pyruvate. The combined effects of these manipulations led to ~300 mM (22 g l^{-1}) of isobutanol under micro-aerobic conditions (Fig. 2b, left panel and Supplementary Fig. 2). In this experiment, 0.5% yeast extract was supplied in the medium to obtain higher cell density. As a control, this strain produced a negligible amount of isobutanol with 0.5% yeast extract without glucose (Supplementary Fig. 3). The yield reached 0.35 (g isobutanol per g glucose) between 40 h and 112 h (Fig. 2b, right panel), which is 86% of the theoretical maximum. This result demonstrates the potential of this strategy, as high-yield production was achieved even without detailed optimization of the pathways and production conditions.

To demonstrate the generality of this approach, the same strategy was also applied to 1-butanol production. Some clostridial species produce 1-butanol during fermentative growth and many of the enzymes in this pathway are oxygen-sensitive and CoA-dependent[22]. We found that by overexpressing Kivd or Aro10 in *E. coli*, which does not have the

Table 1 | Alcohol production with KDC and ADH in *E. coli*

Product (μM)	KDC/plasmid				
	Kivd/pSA55	Aro10/pSA56	Pdc6/pSA49	Thi3/pSA57	Pdc (*C. acetobutylicum*)/pSA58
1-Propanol	520	290	125	ND	ND
Isobutanol	5,242	2,094	260	ND	75
1-Butanol	220	95	ND	ND	ND
2-Methyl-1-butanol	766	652	56	ND	ND
3-Methyl-1-butanol	1,495	1,099	92	ND	ND
2-Phenylethanol	324	469	ND	ND	175

The strain was JCL16 with various *kdc* genes and *S. cerevisiae ADH2* expressed from plasmids. Culture was grown in M9 medium with 0.2 M glucose plus 0.1 mM IPTG at 30 °C for 40 h. These products were identified by GC–MS and quantified by GC–FID (see Methods). ND, not detectable.

Table 2 | Alcohol production with the supply of 2-keto acids

Product (μM)	2-Ketobutyrate	2-Keto-isovalerate	2-Ketovalerate	2-Keto-3-methyl-valerate	2-Keto-4-methyl-pentanoate	Phenylpyruvate
1-Propanol	2,138	ND	ND	ND	ND	8
Isobutanol	98	10,016	ND	ND	ND	64
1-Butanol	492	ND	3,926	ND	ND	23
2-Methyl-1-butanol	1,315	ND	ND	5,284	ND	ND
3-Methyl-1-butanol	ND	ND	52	ND	3,756	105
2-Phenylethanol	26	109	66	ND	ND	7,269

Strains and culture conditions are the same as described in Table 1. A total of 8 g l^{-1} of 2-keto acids was added, except for 2-ketovalerate, where 1 g l^{-1} was added because of its toxicity. ND, not detectable.

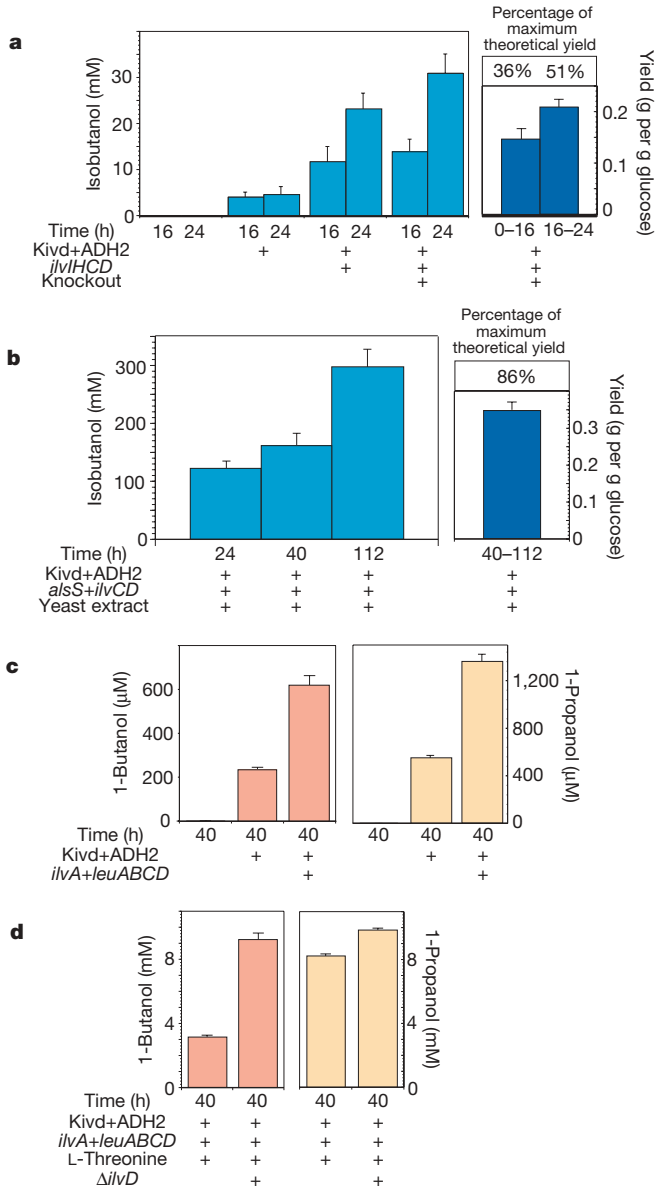

Figure 2 | Summary of results for isobutanol and 1-butanol production in *E. coli*. The cells were grown in M9 medium containing 36 g l^{-1} glucose in shake flasks at 30 °C with or without other nutrients indicated, and induced with 0.1 mM IPTG. Overexpressed genes and nutrient supplementation are indicated below the axis. Error bars indicate s.d. **a**, Left panel, isobutanol production; right panel, isobutanol yield per g of glucose. The theoretical maximum yield of isobutanol is 0.41 g g^{-1}. Knockout, Δ*adh*, Δ*ldh*, Δ*frd*, Δ*fnr* and Δ*pta*. **b**, Isobutanol production with *B. subtilis alsS* and yeast extract (5 g l^{-1}) supplementation to increase cell density. The host is JCL260. Detailed results are shown in Supplementary Fig. 2. **c**, **d**, Left panel, 1-butanol production; right panel, 1-propanol production in the same strain. The host strain is JCL16, with or without Δ*ilvD*. L-Threonine, L-threonine (8 g l^{-1}) supplementation.

1-butanol fermentative pathway, the cell produced a small amount of 1-butanol (Table 1) from glucose in a non-fermentative growth, indicating the existence of a corresponding 2-keto acid precursor, 2-ketovalerate. Unfortunately, 2-ketovalerate is not a common metabolite in *E. coli*. To increase the amount of synthesized 2-ketovalerate, we took advantage of the broad substrate specificity of the *leuABCD* pathway, the natural substrate of which is 2-ketoisovalerate (Fig. 1b). By using a smaller substrate, 2-ketobutyrate, which has one less methyl group than 2-ketoisovalerate (Fig. 1a), we attempted to synthesize 2-ketovalerate in a manner similar to the steps used in leucine biosynthesis[23]. 2-Ketobutyrate can be generated from L-threonine by the threonine dehydratase, encoded by the *ilvA* gene[24], or from an alternative pathway identified in *Leptospira interrogans*[25] and *Methanocaldococcus jannaschii*[26]. In the latter pathway, 2-ketobutyrate is generated from citramalate by the enzymes isopropylmalate isomerase (LeuCD) and β-isopropylmalate dehydrogenase (LeuB)[27].

Therefore, to produce 1-butanol, the operon encoding the *ilvA–leuABCD* pathway under the control of $P_L$lacO1 (ref. 20) was constructed. It was found that the strain with the *ilvA–leuABCD* pathway produced 0.6 mM 1-butanol, which is a ~3-fold increase compared with the strain without overexpression of this pathway (Fig. 2c and Supplementary Table 4). When the media was supplemented with 8 g l^{-1} L-threonine, a marked increase of 1-butanol production to 3.2 mM was observed, suggesting that 2-ketovalerate could be produced from L-threonine by means of an IlvA-mediated reaction (Fig. 2d).

To improve 1-butanol production further, the *ilvD* gene was deleted. This gene encodes dihydroxy-acid dehydratase[28], an enzyme that produces both 2-ketoisovalerate (a precursor for leucine and valine) and 2-keto-3-methyl-valerate (a precursor for isoleucine). This deletion could be beneficial for two reasons. First, the deletion of *ilvD* eliminates the native substrate, 2-ketoisovalerate, for the *leuABCD* pathway, thus reducing inhibition by the competitive substrate. Second, the deletion of *ilvD* eliminates competing substrates for Kivd: 2-keto-3-methyl-valerate and 2-keto-4-methyl-pentanoate. As expected, deletion of *ilvD* improved 1-butanol production (Fig. 2d).

Because strains of *E. coli* that hyperproduce L-threonine have been developed[29] for commercial production, it would be straightforward to modify a threonine producing strain with the above strategy for 1-butanol production. For further improvement, it would be necessary to increase the activity of the *leuABCD* pathway towards the non-native substrate, 2-ketobutyrate, and to raise the specificity of Kivd for 2-ketovalerate. Because 2-ketobutyrate is also the substrate for 1-propanol (Fig. 1b), increasing 2-ketobutyrate availability also enhances the production of 1-propanol (Fig. 2c, d, right). Therefore, increasing the LeuABCD activity and the specificity of KDC would be crucial for high-efficiency 1-butanol production.

Non-native hosts such as *E. coli* lack tolerance to high alcohols. Isobutanol is slightly less toxic to microorganisms than 1-butanol. The native 1-butanol producers can tolerate concentrations of 1-butanol up to about 2% (w/v) (ref. 1). To show the potential for improving tolerance, we conducted serial transfer of cultures to enrich for isobutanol-tolerant strains. We found that a wild-type *E. coli* strain (JCL16) was inhibited by 1.5% (w/v) isobutanol. However, after only five rounds of culture transfer with increasing isobutanol

concentrations, mutants were found to grow in the presence of 2% (w/v) isobutanol (Supplementary Fig. 4). This level of solvent tolerance is comparable or better than native producers of 1-butanol[1], suggesting that *E. coli* can adapt to high concentrations of long-chain alcohols. Other strategies such as global transcription machinery engineering can be used for further improvement of tolerance[30].

The strategy described above opens up an unexplored frontier for biofuels production, both in *E. coli* and in other microorganisms. This strategy takes advantage of the well-developed amino acid production technology, and channels the amino acid intermediates to the 2-keto acid degradation pathway for alcohol production. The strategy avoids CoA-mediated chemistry, which is commonly used in alcohol production in native organisms, and enables the synthesis of other higher and complex alcohols on large scales. Specific strategies for producing other alcohols can be readily devised based on the synthetic pathways and metabolic physiology. These strategies can also be implemented in yeast or other industrial microorganisms. In the case of isobutanol production, the complete pathway is CoA-independent and requires only pyruvate as a precursor. This feature avoids the mitochondria compartmentalization issue of acetyl-CoA when implementing the strategy in yeast.

METHODS SUMMARY

Strains and plasmids. The JCL16 strain is BW25113 ($rrnB_{T14}$ Δ$lacZ_{WJ16}$ *hsdR514* Δ$araBAD_{AH33}$ Δ$rhaBAD_{LD78}$) with F′ transduced from XL-1 blue to supply $lacI^q$. JCL88 is JCL16 with Δ*adh*, Δ*ldh*, Δ*frd*, Δ*fnr* and Δ*pta*. JCL260 is the same as JCL88 but with Δ*pflB*. A list of the strains used is given in Supplementary Table 1. Construction of plasmids is described in Methods, and the primers used are listed in Supplementary Table 2.

Medium and cultivation. Unless stated otherwise, M9 medium containing 0.2 M glucose and 1,000th dilution of Trace Metal Mix A5 (2.86 g H_3BO_3, 1.81 g $MnCl_2{\cdot}4H_2O$, 0.222 g $ZnSO_4{\cdot}7H_2O$, 0.39 g $Na_2MoO_4{\cdot}2H_2O$, 0.079 g $CuSO_4{\cdot}5H_2O$, 49.4 mg $Co(NO_3)_2{\cdot}6H_2O$ per litre water) was used for cell growth. Ampicillin (100 μg ml^{-1}) and kanamycin (30 μg ml^{-1}) were added as appropriate. L-Valine (35 μg ml^{-1}), L-isoleucine (39.5 μg ml^{-1}) and L-leucine (39.5 μg ml^{-1}) were used to culture strains with Δ*ilvD*. Pre-culture in test tubes containing 3 ml of medium was performed at 37 °C overnight on a rotary shaker (250 r.p.m.). Overnight culture was diluted 1:100 into 20 ml of fresh medium in a 250-ml conical flask. For Fig. 2b, 250-ml screw-cap conical flasks were used. Cells were grown to an optical density at 600 nm of 0.8 at 37 °C, followed by adding 0.1 mM isopropyl-β-D-thiogalactoside (IPTG). For 1-butanol production (Fig. 2c, d), 8 g l^{-1} L-threonine was added together with IPTG. Cultivation was performed at 30 °C on a rotary shaker (250 r.p.m.). Gas chromatography–mass spectrometry (GC–MS) and gas chromatography–flame ionization detector (GC–FID) analyses are described in Methods.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions S.A. and J.C.L. designed experiments; S.A. and T.H. performed the experiments; S.A. and J.C.L. analysed the data; and S.A. and J.C.L. wrote the paper.

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METHODS

Reagents. Restriction enzymes, Klenow fragment and Antarctic phosphatase were from New England Biolabs. Rapid DNA ligation kit was from Roche. KOD DNA polymerase was from EMD Chemicals. 2-Ketobutyrate, 2-ketoisovalerate, 2-ketovalerate, 2-keto-3-methyl-valerate, 2-keto-4-methyl-pentanoate, phenylpyruvate and glucose assay reagent were from Sigma. Oligonucleotides were from Invitrogen.

KDC and ADH plasmid construction. A list of the oligonucleotides used is given in Supplementary Table 2. To clone *PDC6*, we used genomic DNA of *Saccharomyces cerevisiae* (ATCC) as a PCR template with a pair of primers A65 and A66. PCR products were digested with *Acc*65I and *Sph*I and cloned into pZE12-luc (ref. 20) cut with the same enzyme, creating pSA46.

To clone *ADH2*, genomic DNA of *S. cerevisiae* (ATCC) was used as a PCR template with a pair of primers A67 and A68. PCR products were digested with *Sph*I and *Xba*I and cloned into pSA46 cut with the same enzyme, creating pSA49.

To clone *kivd*, genomic DNA of *Lactococcus lactis* (ATCC) was used as a PCR template with a pair of primers A96 and A97. PCR products were digested with *Acc*65I and *Sph*I and cloned into pSA49 cut with the same enzyme, creating pSA55.

To clone *ARO10*, we used genomic DNA of *S. cerevisiae* (ATCC) as a PCR template with a pair of primers A98 and A99. PCR products were digested with *Acc*65I and *Sph*I and cloned into pSA49 cut with the same enzyme, creating pSA56.

To clone *THI3*, we used genomic DNA of *S. cerevisiae* (ATCC) as a PCR template with a pair of primers A100 and A101. PCR products were digested with *Acc*65I. pSA49 was digested with *Sph*I and blunted with Klenow fragment, followed by digestion with *Acc*65I. This backbone was ligated with PCR products, creating pSA57.

To clone the *pdc* gene of *Clostridium acetobutylicum*, we used genomic DNA of *C. acetobutylicum* (ATCC) as a PCR template with a pair of primers A102 and A103. PCR products were digested with *Acc*65I and *Sph*I and cloned into pSA49 cut with the same enzyme, creating pSA58.

***ilvIHCD* plasmid construction.** To replace $P_L$tetO1 of pZE21-MCS1 (ref. 20) with $P_L$lacO1, pZE12-luc was digested with *Aat*II and *Acc*65I. The shorter fragment was purified and cloned into plasmid pZE21-MCS1 cut with the same enzymes, creating pSA40.

To clone *ilvC*, genomic DNA of *E. coli* MG1655 was used as a PCR template with a pair of primers A71 and A72. PCR products were digested with *Sal*I and *Xma*I and cloned into pSA40 cut with the same enzyme, creating pSA45.

To clone *ilvD*, genomic DNA of *E. coli* MG1655 was used as a PCR template with a pair of primers A74 and A84. PCR products were digested with *Bsp*EI and *Mlu*I and cloned into pSA45 cut with *Sal*I and *Mlu*I, creating pSA47.

To clone *ilvI* and *ilvH*, genomic DNA of *E. coli* MG1655 was used as a PCR template with a pair of primers A70 and A83. PCR products were digested with *Bsa*I and *Sal*I and cloned into pSA40 cut with *Acc*65I and *Sal*I, creating pSA51.

To clone *ilvC* and *ilvD* downstream of *ilvH*, pSA47 was digested with *Sal*I and *Mlu*I. The shorter fragment was purified and cloned into plasmid pSA51 cut with the same enzymes, creating pSA52.

To replace replication origin with p15A, pZA31-luc (ref. 20) was digested with *Sac*I and *Avr*II. The shorter fragment was purified and cloned into plasmid pSA52 cut with the same enzymes, creating pSA54.

***alsS–ilvCD* plasmid construction.** pSA66 includes the 3′ fragment of an *alsS* sequence. The *alsS* sequence was obtained using the genomic DNA of *Bacillus subtilis* as a PCR template with a pair of primers A123 and A124. PCR products were digested with *Acc*65I and *Sal*I and cloned into pSA40 cut with the same enzyme.

pSA67 includes *alsS* sequence. The 5′ fragment of the *alsS* sequence was obtained using the genomic DNA of *B. subtilis* as a PCR template with a pair of primers A125 and A126. PCR products were digested with *Bsr*GI and *Xba*I and cloned into pSA66 cut with *Acc*65I and *Xba*I.

pSA68 includes *ilvC* and *ilvD* sequence downstream of *alsS*. pSA47 was digested with *Sal*I and *Mlu*I. The shorter fragment was purified and cloned into plasmid pSA67 cut with the same enzymes.

pSA69 was created by transferring the p15A replication origin from pZA31-luc, digested with *Sac*I and *Avr*II, to plasmid pSA68.

***ilvA–leuABCD* plasmid construction.** To clone *leuABCD*, genomic DNA of *E. coli* MG1655 was used as a PCR template with a pair of primers A106 and A109. PCR products were digested with *Sal*I and *Bgl*II and cloned into pSA40 cut with *Sal*I and *Bam*HI, creating pSA59.

To clone *ilvA*, genomic DNA of *E. coli* MG1655 was used as a PCR template with a pair of primers A104 and A105. PCR products were digested with *Acc*65I and *Xho*I and cloned into pSA59 cut with *Acc*65I and *Sal*I, creating pSA60.

To replace replication origin with p15A, pZA31-luc (ref. 20) was digested with *Sac*I and *Avr*II. The shorter fragment was purified and cloned into plasmid pSA60 cut with the same enzymes, creating pSA62.

GC–MS analysis. Alcohol compounds produced by our strains were identified by GC–MS. The system consisted of model 6890N network GC system (Agilent Technologies), a model 7883B injector and autosampler (Agilent Technologies) and a model 5973 network mass selective detector (Agilent Technologies). A DB-5ms capillary column (30 m, 0.25-mm internal diameter, 0.25-μm film thickness; Agilent Technologies) was used, with helium (1 ml min^{-1}) as the carrier gas. An oven temperature was programmed from 75 °C (2.6 min) to 200 °C at 30 °C min^{-1}. The injector and detector were maintained at 250 °C. Alcohol compounds were isolated by solvent extraction. Three-hundred microlitres of supernatant of culture broth after centrifugation was extracted with 150 μl GC standard grade toluene (Fluka). A 1 μl sample was injected in split injection mode with a 30:1 split ratio.

GC–FID analysis. The produced alcohol compounds were quantified by a gas chromatograph equipped with flame ionization detector. The system consisted of a model 5890A gas chromatograph (Hewlett Packard) and a model 7673A automatic injector, sampler and controller (Hewlett Packard). The separation of alcohol compounds was carried out by A DB-FFAP capillary column (30 m, 0.32-mm internal diameter, 0.25-μm film thickness; Agilent Technologies). GC oven temperature was initially held at 40 °C for 2 min and raised with a gradient of 5 °C min^{-1} until 45 °C and held for 4 min. And then it was raised with a gradient 15 °C min^{-1} until 230 °C and held for 4 min. Helium was used as the carrier gas with 14 p.s.i. inlet pressure. The injector and detector were maintained at 225 °C. A 0.5-μl sample was injected in splitless injection mode. Methanol was used as the internal standard.