Metabolic engineering of Corynebacterium crenatium for enhancing production of higher alcohols

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Biosynthesis approaches for the production of higher alcohols as a source of alternative fossil fuels have garnered increasing interest recently. However, there is little information available in the literature about using undirected whole-cell mutagenesis (UWCM) in vivo to improve higher alcohols production. In this study, for the first time, we approached this question from two aspects: first preferentially improving the capacity of expression host, and subsequently optimizing metabolic pathways using multiple genetic mutations to shift metabolic flux toward the biosynthetic pathway of target products to convert intermediate 2-keto acid compounds into diversified C4–C5 higher alcohols using UWCM in vivo, with the aim of improving the production. The results demonstrated the production of higher alcohols including isobutanol, 2-methyl-1-butanol, 3-methyl-1-butanol from glucose and duckweed under simultaneous saccharification and fermentation (SSF) scheme were higher based on the two aspects compared with only the use of wild-type stain as expression host. These findings showed that the improvement via UWCM in vivo in the two aspects for expression host and metabolic flux can facilitate the increase of higher alcohols production before using gene editing technology. Our work demonstrates that a multi-faceted approach for the engineering of novel synthetic pathways in microorganisms for improving biofuel production is feasible.

Currently, one of the most promising approaches for replacing fossil fuels is the biological production of biofuels via genetically-modified microorganisms. Some examples, such as n-butanol and isobutanol, have been synthesized using genetically-engineered bacteria1–5. Of particular interest are longer chain hydrocarbons, such as C4–C5 higher alcohols including 3-methyl-butanol, 2-methyl-butanol, and isopentanol, which have been successfully synthesized through the use of non-synthetic E. coli and Corynebacterium based on their endogenous α-keto acid pathway6–9. These alcohols have a higher energy density close to that of gasoline, exhibit lower hygroscopicity compared to ethanol, and are currently regarded as very promising potential substitutes for fossil fuels.

One of the first challenges in the replacement of fossil fuels with biofuels derived via a biosynthesis pathway is the inefficient conversion of lignocellulosic substrate into biofuels by bacteria. To biosynthesize these C4-C5 higher alcohols by engineering bacteria such as E. coli, Bacillus subtilis, and Corynebacterium glutamicum, the fermentation substrates traditionally were pure sugar or starch without heavy metals and other impurities8–12. However, limited research has been reported using bioengineered strains to produce C4–C5 higher alcohols via fermenting hydrolysates of lignocellulose. Therefore, it is essential to investigate the capacity of bioengineered strains to directly ferment hydrolysates of lignocellulose as a more effective method of biofuel generation.

There is a growing trend to develop new, renewable, non-food plant sources as feedstock for biofuel production. Duckweed has received increasing attention as a potentially inexpensive and sustainable lignocellulose source of non-food plant biomass for producing biofuels such as ethanol and biobutanol, and has demonstrated great potential as a candidate for bioenergy feedstock8,13,14. However, there are no studies to date regarding the efficacy of bioengineered strains of Corynebacterium in producing other C4–C5 higher alcohols, especially

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3-methyl-butanol and 2-methyl-butanol using hydrolysates of lignocellulose as a fermentation substrate under SSF scheme. Therefore, we selected duckweed as a fermentation substrate to investigate the ability of bioengineered strains of a Corynebacterium, *C. crenatium*, to produce C4–C5 higher alcohols via SSF.

Secondly, in addition to the need to overcome the inefficiency of utilizing lignocellulosic substrate to convert into biofuels, it is also essential that improvement of the expression host’s catalytic activity via introducing novel metabolic pathways with high-activity enzymes in order to maximize higher alcohols production as much as possible. One major challenge in the construction of metabolic pathways is the identification and selection of enzymes with high activity. Protein-directed engineering *in vitro* such as error-prone PCR and DNA shuffling provides a foundation for screening interesting mutant genes that are favorable for improving higher alcohols. However, there may be undesirable effects that may lead to a metabolic flux imbalance when overexpressing these mutant heterologous genes from other species via *in vitro* mutation experiment, and thus ultimately decrease the yield of higher alcohols. Unfortunately, using these methods to obtain mutant genes with high enzymatic activity while maintaining metabolic flux balance in an expression host is not always achievable.

Mutant genes with high enzymatic activity can also be obtained using UWCM *in vivo*. For example, some high yield auxotrophs, such as amino acid-producing Corynebacterium, and *Saccharomyces cerevisiae* which produces 3-methyl-1-butanol, have been obtained via perturbing whole genome using the UWCM method15–20. Using this method, multiple mutant genes are identified that have metabolic balance favorable for optimization of metabolic pathways in these auxotrophic strains. There is a dynamic equilibrium of metabolic pathways between these mutant genes and auxotrophic strains, and thus improved production. However, until now, improving higher alcohols production via overexpressing exogenous genes from the UWCM approach *in vivo* into a expression host has not been shown. Therefore, we proposed to use the method to obtain multiple gene mutations in an auxotrophic strain, and utilize those mutant genes to construct novel metabolic pathways, and then introduce those novel metabolic pathways into another expression host reach to improve production of C4–C5 higher alcohols.

Thirdly, the capability of host itself is also an important consideration for improving higher alcohols production in addition to the introduction of metabolic pathways with highly activated genes. In general, the expression hosts used to produce higher alcohols were typically wild-type strains prior to the use of gene editing techniques. Using improved auxotrophic strains from UWCM instead of wild-type strain as expression host to increase yield of specific products prior to using gene editing techniques, followed by the use of gene editing techniques to further reform the host may maximize product yield. However, this has not yet been reported in the literature.

Therefore, in this study, we aimed to improve higher alcohols production using two aspects. First, improved metabolic capability of *C. crenatium* and *S. cerevisiae* via UWCM, whereby the improved mutant *C. crenatium* was used as expression host and the mutant *S. cerevisiae* was used to extract multiple mutant genes. Additionally, these newly identified mutant genes involving in the biosynthesis of C4–C5 higher alcohols from mutant *S. cerevisiae* were used to alter the metabolic flux of the improved mutant *C. crenatium* by constructing novel metabolic pathways to improve C4–C5 higher alcohol production. We assessed the fermentation efficiency of bioengineered strains for producing higher alcohols using glucose and duckweed substrate under SSF.

**Results and Discussion**

**Source of mutant genes.** It is well-known that the formation of higher alcohols by yeast is closely related to the metabolism of amino acids including the Ehrlich metabolic pathway21 and the biological synthesis pathway22,23. The metabolic flux distribution and relevant genes involved in the formation of higher alcohols are shown in Fig. 1. Previous studies have shown that metabolic perturbation derived from mutation of genes associated with the biosynthesis and degradation pathways of leucine, isoleucine, and valine in yeast can improve C4–C5 higher alcohol yield based on these two pathways14–20. Mutating acetohydroxyacid synthase (AHAS) gene responsible for transforming pyruvic acid into α-acetolactic acid can reduce the generation of the diacetyl, thereby improving the production of higher alcohols. Some reports have demonstrated the mutant strains with increased higher alcohols production can be obtained using some resistance marker such as streptomycin (SM)27–30, the arginine analogue canavanine31–33, and 2-thiazoyl-DL-alanine34. These studies shows that acetohydroxy acid synthase (AHAS) and α-isopropylmalate synthase (IPMS) are key genes in the generation of higher alcohols, and that feedback inhibition takes place between the two genes and their respective metabolite production15–37 (Fig. 2). Therefore, through controlling the enzymatic processes between them to block feedback inhibition, it is possible to screen out mutant leucine-deficient strains that generate high 3-methyl-1-butanol production. Auxotrophic strain can improve the production of higher alcohols as a result of the mutation of genes related to branched chain amino acid biosynthesis. For example, mutation of ILV2 gene in valine biosynthesis can lead to improved yields of higher alcohols16–17.

Learning from these previous studies, here, we used the leucine analogue 4-Aza-dl-leucine dihydrochloride (AZL) as a resistance screening compound to obtain feedback inhibition of AHAS or IPMS mutants with high 3-methyl-1-butanol production (Fig. 2). AZL has been used previously in *Bacillus subtilis, Escherichia coli*, and *Salmonella typhimurium* to identify resistant strains in which three enzymes of the leucine, isoleucine, valine metabolic pathway (isopropylmalate isomerase, isopropylmalate dehydrogenase (IPMDH), and isopropylmalate synthase (IPMS)) were inhibited38,39. These studies showed that perturbing the metabolic pathway involving AHAS regulation to obtain auxotrophic strains with some mutant genes is an effective method of generating a high yield of amino acids. And further investigated the ability of mutant strains via fermenting the hydrolysate of duckweed to identify the desirable strain with the highest 3-methyl-1-butanol. A total of 35 colonies grew after three days of incubation. The fermentative results were shown for the investigation of the capability of each colony to produce higher alcohols (Fig. 3). First, to verify the fermentative stability, these auxotrophic strains were cultured consecutively for 10 generations, and then given glucose as substrate to determine the capacity of mutant strains with stability for producing higher alcohols (Fig. 3b). Secondly, these positive auxotrophic strains with...
stability were used as reinspected strains to assess their capacity for fermenting duckweed hydrolysate (Fig. 3a). Compared with the original strain SC (S. cerevisiae AH109), there were 12 mutant strains possessed fermentative stability, and had significant higher titers of 3-methyl-1-butanol. Therein, the mutant strain NC-11 produced the highest production, approximately 25-fold as compared to the original strain. Additionally, using both glucose as well as hydrolysate demonstrated impressive capacity of the auxotrophic strain NC-11. Therefore, we selected the auxotrophic strain NC-11 as the candidate strain for the next step to survey which possible mutant genes are responsible for the increased biosynthesis of 3-methyl-1-butanol production. We hypothesized that these mutant genes were highly involved in Ehrlich metabolic pathway and/or the biological synthesis pathway, and would contribute to the improvement of higher alcohols in subsequent experiments. Thus supporting the extraction of these mutant genes in order to construct novel metabolic pathways.

**Improvement of expression host.** The production of higher alcohols in synthetic microbes relies on exogenous metabolic pathways such as the decarboxylation reduction pathway that converts intermediate metabolites of the amino acid synthesis pathway or Ehrlich metabolic pathway into alcohols. Various α-keto acid intermediate compounds are converted into corresponding fatty alcohols. Therefore, increasing amino acid production can result in the accumulation of precursors, thereby providing more substrate pool for subsequent conversion, and thus improving the final total production of higher alcohols.

Here, the representative auxotrophic strain with the highest yield of amino acid was selected from a total of 35 mutant strains after UWCM via batch fermentation using glucose. The fermentation results of each strain are shown in Fig. 4. The auxotrophic strain C. crenatium MA11C generated much higher levels of the amino acids isoleucine, leucine, and valine (approximate 2-fold) compared to the original strain C. crenatium CICC 20135 (Fig 4a,b). In particular, the representative auxotrophic strain C. crenatium MA11C produced the highest isoleucine (9.54 g/L), leucine (4.96 g/L), and valine (4.51 g/L) in the least amount of time (96 h). The substrate glucose was not completely consumed by C. crenatium MA11C, but its consumption levels were the lowest for all investigated mutant strains. For C. crenatium MA11C, the residual amount of glucose was up to 5.72 g/L, compared to the C. crenatium CICC 20135, for which the residual glucose reached 8.52 g/L (Fig. 4a,b). The changes in pH during fermentation are shown in Fig. 4(a,b). The largest pH change observed for all investigated mutant strains during fermentation was from C. crenatium MA11C (from 4.42 to 7.0; Fig. 4b). At the end of fermentation, the pH values were above 4.42. The pH during fermentation by the original strain C. crenatium CICC 20135 stayed above 5.3 (Fig. 4a).
These results demonstrate that the residual amount of glucose for the mutant strain was lower than that of the original strain, which suggests that the mutant strains have a higher uptake of glucose. This also helps to explain the improved amino acid production from the auxotrophic strain compared to the original strain. The changes in pH values also reflect the total amino acid titer; when the final pH was lower, the amino acid titer should be higher. These results indicate that high amino acid titer is likely the result of increased precursors (α-ketoacids), and that the efficiency with which the precursor was converted into higher alcohols by *C. crenatium* MA11C may be higher than that of the original strain *C. crenatium* CICC 20135. Furthermore, the increased amino acid production of *C. crenatium* MA11C could be due to mutation of some genes in the amino acid synthesis pathway. The results via batch fermentation repeatedly demonstrated that fermentation of the auxotrophic strain is stable after being sequentially cultured for 10 generations, thus *C. crenatium* MA11C is suitable for expressing heterologous genes as expression host.

**Extraction of mutant genes.** As above description, the improvement of higher alcohol production by mutant yeast strains is likely the result of mutation of genes associated with the metabolic pathways of higher alcohol intermediates, resulting in perturbation of metabolic flux via UWCM. The auxotrophic strain NC-11 was found to produce the highest yield of 3-methyl-1-butanol and has fermentation stability. Therefore, the auxotrophic strain was used for subsequent identification of potential genetic mutations. Genes related to the Ehrlich pathway and amino acid biosynthetic and degradation pathways have been implicated in the production of higher alcohols (Fig. 1), and our results showed that genes *ILV2*, *ILV5*, *BAT2*, *LEU1*, *AR010*, and *ADH6* acquired mutations (Fig. 5).

According to this results, mutant genes *ILV2*, *ILV5*, and *LEU1* are responsible for isoleucine, leucine, and valine biosynthetic metabolic pathways. This indicates that the metabolic flux generated a change in the synthesis of 2,3-Dihydroxy-3-methylbutanoate and 2,3-Dihydroxy-3-Methylpentanoate, which are the precursors of 2-oxoisovalerate and 3-Methyl-2-oxopentanoate, respectively (Figs 5 and 6). The *LEU1* also gave rise to the metabolic flux from 2-Isopropylmalate to 3-Isopropylmalate. Mutant gene *BAT2* is involved in the deamination reaction pathway, indicating that metabolic flux from valine, isobutanol, and leucine to 2-oxoisovalerate, 3-Methyl-2-oxopentanoate and 4-Methyl-2-oxopentanoate, respectively, had changed (Figs 5

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**Figure 2.** Metabolic mechanism of screening auxotrophic mutants of yeast using amino acid analogue via controlling the key enzymes based on the relationship between the formation of higher alcohols and the metabolism of amino acids. AHAS: acetohydroxyacid synthase, IPMS: α-isopropylmalate synthase.
and 6). These mutant genes are likely to increase accumulation of intermediate metabolites and precursors of higher alcohols such as the intermediates including isopropylmalate, 2-oxobutanoate, 2-oxoisocaproate, 2-isopropyl-2-oxosuccinate, and precursors including 3-methyl-2-oxopentanoate of 2-methyl-1-butanol, 4-methyl-2-oxopentanoate of 3-methyl-1-butanol and 2-oxoisovalerate of isobutanol. These results indicate that this genes mutation led to the abnormal metabolic flux of amino acid biosynthesis and Ehrlich pathway. Therefore, this increase in amino acid production also account for increase of 3-methyl-1-butanol production in auxotrophic strain NC-11. In addition, the gene dihydrolipoyl dehydrogenase, which is responsible for the degradation of valine, isoleucine, and leucine, and the genes ARO10 and ADH6 involved in the decarboxylation reduction pathway, were also investigated (Figs 1 and 5). It was concluded that the reason that the strain NC-11 can produce high yields of 3-methyl-1-butanol may be due to the change of metabolic flux from these mutant genes working synergistically. These mutant genes were extracted and used to construct novel metabolic pathways.

Higher alcohols from bioengineered strains. Engineering C. crenatium to express native genes from wild-type S. cerevisiae to produce higher alcohols have been investigated. Although the yield was low, they have demonstrated the potential capability of this host to be useful in improving production of higher alcohols. In order to further improve the fermentation efficiency of C. crenatium, compatibility of two expression vectors in a host was first demonstrated. The gene pBL1 from expression vector pXMJ19 was inserted into the expression vector pSVT29 to construct a new expression plasmid pTVpBL. The vectors pTVpBL and PEC-XK99E were compatible and stable in the expression host. Secondly, novel metabolic pathways were constructed using mutant genes. Thirdly, the improved mutant C. crenatium was used as expression host harboring novel metabolic pathways. A schematic representation of the novel metabolic pathways is shown in Fig. 7.

In order to guarantee the metabolic capacity of these engineered strains for producing higher alcohols, we verified whether these mutant genes involved branched-chain amino acid synthesis were overexpressed in C. crenatium MA11C. Expression levels of all mutant genes were confirmed by RT-PCR, and the results were further analyzed with a semi-quantitative method (Fig. 8). Our results demonstrated that the induced genes corresponding to respective metabolic pathways have been successfully expressed and that the expression level of these recombinant genes remained relatively different under similar cultivation conditions.

Fermentation was achieved for all novel metabolic pathways using pure glucose (Fig. 9) and duckweed via the SSF procedure (Fig. 10). To construct and overexpress two metabolic pathways Mep I and Mep VII, the highest yield of 2-methyl-1-butanol at 3476.52 mg/L was obtained using glucose as the fermentation substrate.

Figure 3. Fermentation experiments to determine the ability of each colony to produce higher alcohols. (Figure 5B) In order to verify the genetic stability of the mutant strains, glucose was used as a fermentation substrate. (Figure 5A) These positive mutant strains were then used as reinspected strains to assay the capacity for fermentation of duckweed hydrolysate. SC: Saccharomyces cerevisiae.
In addition, the by-products isobutanol at 735.18 mg/L and 3-methey-1-butanol at 403.53 mg/L were obtained in 96 h (Fig. 9, Table 1). Similarly, overexpressing the two metabolic pathways simultaneously led to the highest yield of 2-methyl-1-butanol (3071.5 mg/L) and byproducts of isobutanol (605.17 mg/L) and 3-methey-1-butanol (313.73 mg/L) using the duckweed via SSF. Our results showed that a higher titer can be obtained using pure glucose compared to using duckweed. The parameters of total alcohols, productivity, and total yield were higher using glucose than those using duckweed (Table 1). However, to overexpress other metabolic pathways Mep Ia + Mep VII and Mep Ib + Mep VII, the yield was clearly lower than that of Mep I and Mep VII when using either glucose or duckweed (Figs 9 and 10). Only 288.65 mg/L 2-methyl-1-butanol, 248.31 mg/L isobutanol, and 54.208 mg/L 3-methey-1-butanol were obtained from Mep Ia + Mep VII using glucose and approximately 1588.07 mg/L 2-methyl-1-butanol, 397.46 mg/L isobutanol, and 144.4 mg/L 3-methey-1-butanol were produced from Mep Ib + Mep VII using duckweed via SSF.

Maximum isobutanol titer 6207.15 mg/L, and productivity 76.06 mg/L/h were achieved with glucose. Using duckweed, the resulting isobutanol level was 5607.15 mg/L and productivity was 76.06 mg/L/h, which resulted from synergistically overexpressing two metabolic pathways Mep II + Mep VII. In addition, other byproducts such as 2-methyl-1-butanol (735.46 and 705.4 mg/L), and 3-methey-1-butanol (359.6 and 289.16 mg/L), can also be obtained from glucose and duckweed, respectively, and thus lead to the highest total alcohols solution 7302.21 and 6601.7 mg/L (Figs 9 and 10, Table 1), with a productivity of 76.06, 68.77 mg/L/h and total yield of 121.7 and 66.01 mg/g for glucose and duckweed, respectively (Table 1). In addition, the other metabolic pathways Mep IIa + Mep VII and Mep IIb + Mep VII can produce 773.68 and 2881.92 mg/L isobutanol, 240.8 and 393.88 mg/L 2-methyl-1-butanol, and 139.77 and 316.45 mg/L 3-methyl-1-butanol using glucose.

To summarize the results of above two novel metabolic pathways, owing to the genes ilv2* and ilv5* are closely related to the biosynthesis of isoleucine (Figs 1 and 5), it showed that the two genes catalyze not only the conversion of ketobutyrate and pyruvate, but also that of pyruvate and pyruvate to make ketomethyl valerate and ketoiso valerate, respectively (Fig. 1). The results showed the improvement of higher alcohols can be confirmed using the mutant genes ilv2* and ilv5* to construct novel metabolic pathways to constitute Mep I and Mep II. We demonstrated that increasing the pathway flux of valine allows for the accumulation of leucine, and thus improves 2-methyl-1-butanol reached to the highest yield 3476.52 mg/L. The results also showed that reinforcing the flux of pyruvate by redirecting the carbon flux via expressing ilv2*, ilv5*, and ilv3 (Mep II) in the L-valine biosynthesis pathway can lead to significant improvement of isobutanol, reaching an increased yield 6207.15 mg/L compared to previous studies which did not utilize gene editing techniques.

Isobutanol yields were also improved by introduction of the mutant gene BAT2* via constructing metabolic pathway Mep III + Mep VII, reaching 462.76 and 412.26 mg/L isobutanol, 236.08 and 212.18 mg/L 2-methyl-1-butanol and 135.02 and 99.12 mg/L from glucose and duckweed via SSF, respectively (Figs 9 and 10, Table 1). Furthermore, overexpressing the mutant gene BAT2* resulted in a higher relative proportion of C4 isobutanol.
than the other two C5 higher alcohols analyzed (Fig. 5). We conclude that the mutant BAT2* obtained by UWCM elicits an effect on the three branched-chain amino acids, and ultimately improved higher alcohols production. Our results revealed that mutant BAT2* supplied increased levels of precursors to valine, leucine, and isoleucine. Although BAT2 is the only transaminase of relevance in the transamination step within the biosynthesis of the three branched-chain amino acids valine, leucine and isoleucine, the results also demonstrated a greater impact on isobutanol yield than on yields of two other alcohols. Therefore we infer that the mutant gene BAT2* specifically plays an important role in the production of isobutanol.

In a similar manner, co-expression of other metabolic pathways Mep IV + Mep VII and Mep V + Mep VII, yielded different results as presented in Table 5. Other information can be seen in Fig. 9. For example, 385.39 and 491.57 mg/L isobutanol, 176.96 and 326.4 mg/L 2-methyl-1-butanol, and 727.52 and 817.97 mg/L 3-methyl-1-butanol can be generated from Mep IVa + Mep VII and Mep Va + Mep VII respectively using glucose (Fig. 9).

The two metabolic pathways Mep IV and Mep V have the same mutant gene LEU1*. We noted changes in the production of higher alcohols resulting from the two metabolic pathways. The yields of all higher alcohols assayed were significantly improved by Mep V as compared to those of Mep IV. The results revealed that overexpression of the LEU2 gene can produce much higher alcohols than overexpression of LEU4. This suggests that LEU2 has stronger catalytic activity for regulating metabolic flux to enhance 3-methyl-1-butanol production when simultaneously overexpressed with related genes via metabolic engineering.

To construct the metabolic pathways Mep VI + Mep VII, the highest yields of 582.02 and 502.92 mg/L isobutanol, 478.47 and 503.37 mg/L 2-methyl-1-butanol, and 1115.34 and 1415.73 mg/L 3-methyl-1-butanol were obtained from pure glucose and duckweed, respectively (Fig. 10, Table 1). Similarly, 432.58 and 660.67 mg/L isobutanol, 216.29 and 452.24 mg/L 2-methyl-1-butanol, and 530.89 and 1014.6 mg/L 3-methyl-1-butanol were generated from two metabolic pathways Mep VIa + Mep VII and Mep Vb + Mep VII using pure glucose. The increased outputs from glucose metabolism led to increases in other outcomes, including, higher total alcohol solution 2501.12 vs 2096.7 mg/L, productivity 26.05 vs 21.84 (mg/L/h), total yield 41.68 vs 20.97 (mg/g) from

Figure 5. Regarding mutant genes involved in the formation of higher alcohols. (Figure 7A): the genes with red show that genes are mutated determined by our results. (Figure 7B): Sequence alignment of amino acids of mutant genes involved in biosynthesis process of higher alcohols. The gene with an asterisk indicates the gene produced mutation. The gene without asterisk indicates that the gene is native gene.
Figure 6. The flowsheet of experiments following the methodology illustrated for screening exogenous mutant enzymes and expression host via undirected whole-cell mutagenesis (UWCM) in vivo and fermentation processes.

Figure 7. The schematic plot of designing novel metabolic pathways responsible for the improvement of higher alcohol production. Different colors rectangular frames represent different metabolic pathways. The gene with an asterisk indicates the gene produced mutation. The gene without asterisk indicates the gene is native gene. The novel metabolic pathways for accumulating precursor products is illustrated with the following pathways, labeled Mep I (LEU2-ILV2*-ILV5*), MepII (ILV2*-ILV5*-ILV3), MepIII (BAT2*), MepIV (LEU4-LEU1*), MepV (LEU1*-LEU2) and MepVI (BAT2*-LEU4-LEU1*), Metabolic pathway I, Ia, Ib including LEU2, ILV2*, ILV5*: Metabolic pathway II, Ila, Ib including ILV2*, ILV5*, ILV3; Metabolic pathway III including BAT2*; Metabolic pathway IV, Iva including LEU4, LEU1*; Metabolic pathway V, Va including LEU1*, LEU2; Metabolic pathway VI, Vla, Vlb including BAT2*, LEU4, LEU1*. Another metabolic pathway responsible for the decarboxylation reduction reaction, and MepDRVII (kivd-ADH6*) Metabolic pathway VII including kivd, ADH6*: LEU2: 3-isopropylmalate dehydrogenase, ILV1: L-serine/L-threonine ammonia-lyase, ILV2*: mutant acetolactate synthase, ILV5*: mutant ketol-acid reductoisomerase, ILV3: dihydroxy-acid dehydratase, BAT2*: branched-chain amino acid aminotransferase, LEU4: 2-isopropylmalate synthase, LEU1*: mutant 3-isopropylmalate dehydratase, kivd: alpha-ketoisovalerate decarboxylase, ADH6*: mutant alcohol dehydrogenase 6.
glucose and duckweed, respectively (Table 1). Out results showed that the yield can be improved by adding two genes LEU4 and LEU1*.

The genes LEU4 and LEU2 and mutant LEU1* are involved in leucine synthesis. Specifically, LEU4 and LEU1* are responsible for chain elongation from 2-Oxoisovalerate of the final step of L-valine synthesis to the initial step of leucine formation (Fig. 1). Therefore the catalytic activity of the LEU1* gene was confirmed by constructing MepI and Mep, resulting in increasing leucine accumulation. The results revealed an increased enzyme level for the mutant LEU1*, which also increased metabolic flux to improve 3-methyl-1-butanol production.

These results for surveying novel metabolic pathways demonstrated that the production of higher alcohols can be greatly improved when multiple mutant genes from other species are introduced via UWCM in vivo, compared to the use of only native genes. The highest higher alcohols production from our experiment is higher than
Figure 9. Fermentation experiment to produce higher alcohols for all constructed metabolic pathways using pure glucose. Labeled MEpIa (LEU2), MEpIb (LEU2-ILV2*), MEpI (LEU2-ILV2*-ILV5*); MepIa(ILV2*), MepIb(ILV2*-ILV5*), MepII (ILV2*-ILV5*-ILV3); MepIII (BAT2*); MepIVa(LEU4), MepIV (LEU4-LEU1*); MepV (LEU1*), MepV (LEU1*-LEU2); MepVla ((BAT2*), MepVlb (BAT2*-LEU4); MepVI (BAT2*-LEU4-LEU1*); MepVII (kivd-ADH6*).

Figure 10. Fermentation experiment to produce higher alcohols using duckweed via SSF procedure for the most efficient mutant strain. Labeled Mep I (LEU2-ILV2*-ILV5*), MepII (ILV2*-ILV5*-ILV3), MepIII (BAT2*), MepIV (LEU4-LEU1*), MepV (LEU1*-LEU2) and MepVII(BAT2*-LEU4-LEU1*), MepVII(kivd-ADH6*).
The highest productivity of alcohol production from duckweed was shown to be 146 mg/L. This was achieved through a process involving the use of a engineered strain of duckweed. The strain was engineered through a series of gene knockouts, which allowed for the redirection of metabolic flux. This resulted in an increase in the yield of the desired alcohols.

Table 1. The highest production and parameters corresponding to respective metabolic pathway. a: the highest yield from pure glucose; b: the highest yield from duckweed via SSF approach. c: the parameter was obtained based on pure glucose; d: the parameter was obtained based on duckweed substrate. Total yield (mg/g): total alcohols production divided by gross weight of duckweed. Productivity (mg/L·h): Total alcohols solvents divided by fermentation time.

| Parameters | Mep I + Mep VII | Mep II + Mep VII | Mep III + Mep VII | Mep IV + Mep VII | Mep V + Mep VII | Mep VI + Mep VII | Mep VII |
|------------|-----------------|-----------------|-----------------|-----------------|----------------|-----------------|--------|
| The highest isobutanol (mg/L) | 735.16, a/615.03, b | 6207.15, a/5607.15, b | 462.76, a/412.26, b | 503.37, a/453.27, b | 798.31, a/728.31, b | 582.02, a/502.92, b | 296.9, a/189.94, b |
| The highest 2-methyl-1-butanol (mg/L) | 3476.32, a/2974.52, a | 735.46, a/705.4, a | 236.08, a/212.18, b | 346.06, a/316.26, b | 491.57, a/411.57, b | 503.37, a/478.47, b | 36.02, a/27.05, b |
| The highest 3-methyl-1-butanol (mg/L) | 403.53, a/3113.73, a | 359.6, a/289.16, a | 135.02, a/99.12, a | 1034.26, a/897.26, b | 1573.03, a/1534.13, b | 1415.73, a/1115.3, b | 14.28, a/9.18, b |
| Total alcohols (mg/L) | 4615.23, a/3903.3 | 7302.01, a/6601.7, a | 833.86, a/723.56, a | 1883.69, a/1666.8, a | 2853.91, a/2483.0, a | 2501.12, a/2096.7, a | 347.2, a/226.2, a |
| Productivity (mg/L·h) | 48.07, a/40.66 | 76.06, a/68.77 | 8.69, a/7.53, a | 19.62, a/17.36, a | 29.72, a/25.86 | 26.05, a/21.84 | 3.62, a/2.36 |
| Total yield (mg/g) | 76.92, a/39.03 | 121.7, a/66.01 | 13.39, a/7.23 | 31.39, a/16.67 | 47.56, a/24.83 | 41.68, a/20.97 | 5.79, a/2.26 |
| Fermentation time (hours) | 96 | 96 | 96 | 96 | 96 | 96 | 96 |

previous reports. For example, the highest isobutanol 151 mg/L can be obtained using S. cerevisiae as expression host via overexpression of 2-ketoisovalerate decarboxylase and valine biosynthetic enzymes40–42. The highest isobutanol, 2-methyl-1-butanol, 3-methyl-1-butanol from C. crenatum without improvement in cell properties and harbouring mutant genes only reach to 1264.63 mg/L, 1026.61 mg/L, 748.35 mg/L43. Furthermore, we showed that production of different higher alcohols can be successfully achieved via the construction of novel metabolic pathways using mutant genes from two metabolic pathways: the Ehrlich metabolic pathway and the biological synthesis pathway. In addition, these bioengineered strains of C. crenatum can successfully ferment duckweed via the SSF scheme to produce higher alcohols. Taking a comprehensive view of biofuel generation, such a holistic integration across multiple metabolic pathways to generate bioengineered strains that increased yields of higher alcohols is a useful approach for enhancing biofuel production using lignocellulose.

The above fermentative results indicate that the improvement of higher alcohols production is because, on the one hand, the metabolic capability of expression host itself has been improved via UWCM when used improved auxotrophic strain as expression host instead of wild-type strain. Regulating metabolic mechanisms by introducing heterogenous pathways produce generally limited production in the native expression host. The synthesizing capacity of the expression host is important but native hosts are usually not fully developed before using gene knockout methods. The improved mutant strain C. crenatum MA11C demonstrated a relatively high yield of precursor molecules, leading to increased higher alcohols production. The results can be indirectly demonstrated using amino acid yield because the higher alcohol intermediates and amino acids share the same precur-
by-products more than native genes, suggesting that the conditions in which abundant intermediate metabolites are available allow for the increase in desired end product. The results showed that a proper integration of multiple mutant genes is an attractive proposition for improving higher alcohols produce.

In conclusion, the above two aspects should be taken fully into account together to produce a high-efficiency strategy capable of improving production of higher alcohols. To overcome the obstacles to improve higher alcohols production, a synthetic multifactorial strategy is necessary that incorporates the exploitation of the synthetic capacity of the expression host with a high rate of accumulation for precursors of higher alcohols, as well as balancing the changes in metabolic flux which result from harboring multiple exogenous mutant genes. UWCM in vivo may be an alternative solution. We conclude that this combinatorial synthetic approach for improving upon existing pathways is feasible and will facilitate further development of processes to produce high-yield compounds from renewable resources through engineering strategies of genes editing biotechnology.

Materials and Methods

Experimental methodology. In this study, we sought to demonstrate the feasibility for improving C4–C5 higher biofuels production via engineering *C. crenatum* overproduction of amino acid. The methodology is illustrated in the flowchart of the experimental process (Fig. 6) including the processes of mutating wild-type *C. crenatum* and *S. cerevisiae*, identifying mutant genes, constructing novel metabolic pathways47,48, and fermentation experiment. First, we conducted the UWCM in vivo using the wild-type strains of *S. cerevisiae* and *C. crenatum* to generate auxotrophic strains with high metabolic capacity. Next, we identified mutant genes from auxotrophic strain of *S. cerevisiae*, and optimize metabolic flux of bioengineered strains of auxotrophic *C. crenatum* via importing a series of metabolic pathways using mutant genes. Finally, fermentation experiment were conducted using duckweed substrate under SSF. Each experiment was conducted in triplicate in a 150-mL triangular flask, unless otherwise indicated.

Rejuvenation and propagation of microorganisms. Culture conditions for the growth, rescue, rejuvenation and seeding of the original strains are as follows. The original bacterium *C. crenatum* CICC 20135 was purchased from the China Center of Industrial Culture Collection (CICC, Beijing, China), and rejuvenated and propagated in a liquid nutrition gravy medium (LMGM) containing 5.0 g peptone, 3.0 g beef extract, 5.0 g NaCl, 20 g glucose, 1 L ddH2O, pH 7.0 at 30°C in a rotary shaker for 72 h. Cultures of *Lactococcus lactis cremoris* CICC 1605 were obtained from the CICC and cultivated in liquid MRS medium containing 10 g peptone, 3.0 g beef extract, 3.0 g yeast extract, 2 g K2HPO4, 2 g citric acid diamine, 2 g sodium acetate, 20 g glucose, 0.6 g MgSO4·7H2O, 0.25 g MnSO4·4H2O, 1 L ddH2O, pH 6.2 at 32°C in a rotary shaker for 72 h. Cultures of original *S. cerevisiae* AH109 were obtained from Clontech Laboratories, Inc. (Beijing, China) and propagated at 30°C in liquid TGY medium in a rotary shaker for 72 h.

Analysis of duckweed on basic composition. The wild duckweed (*Lanodilota punctata*) was randomly gathered from the surface of wild ponds that were left uncultivated for many years in Huilong Town located in Chengdu, China. It is necessary to determine the composition of duckweed in order to provide a reference value to compare with other studies. First, fresh plants were dried at 60°C in a drying oven (DH29050A, Shanghai, China), then pulverized into dried powder with a pulverizer (FS100S-3, Guangzhou, China). Duckweed powder was further hydrolyzed using H2SO4 at 1% (v/v) concentration. The starch content was assessed roughly according to the total sugar content (starch content = glucose content × 0.91)49,50. The total crude protein content of duckweed was measured using the Kjeldahl Method (CP = K, N × 6.25)51,52. Cellulose content was assessed roughly using spectrophotometry with the absorbance at 620 nm53–55. Processing and testing of resulting materials were performed as previously described. The content of lignin was determined using acetyl bromide according to standard methods55,57. Trace elements in duckweed were determined as follows: samples were washed with deionized water, dried at 80°C, milled to powder, and finally digested using a wet digestion method58,59. The elemental composition in the digested solution was analyzed using atomic absorption spectrometry (Z-2300, Hitachi, Japan). The resulting components of duckweed are presented in Table 2.

Acquisition and analysis of mutant genes. Mutagenesis yeast using UWCM. The original strain *S. cerevisiae* AH109 was propagated in YPDA medium after inoculation with seeding liquid in a constant temperature oscillation incubator at 30°C, 200 rpm, until the growth solution concentration reached OD600 = 1.5. Growth solution was separated into 4 portions with equal distribution (each sample was 15 mL), and then centrifuged independently (3000 rpm for 1 minute). After centrifugation the supernatant was discarded and the cultured yeast cells were collect. Each sample was then pretreated as follows: Treatment 1 (NJ): 1.5% (v/v) methanol solution was added and kept for 10 min to shock cells; Treatment 2 (NS): cells were soaked in a solution containing 5% methanol and 0.2% polysorbate 80 for 15 min; Treatment 3 (NC): 10% sorbitol was added and incubated for 20 mins; Treatment 4 (PS), yeast cells were washed with 5 mL distilled water, then centrifuged at 3000 rpm for 5 mins, and collected. Yeast cells were resuspended in 5 mL solution containing 5% (v/v) glycine and 10% (v/v) dimethyl sulfoxide (DMSO), and preserved in a −70°C freezer overnight for subsequent use for further mutagenicity experiment.

Yeast cells were thawed and the pH was adjusted to 6.0 with phosphate buffer solution. The chemical mutagen 1-methyl-3-nitro-1-nitroso-guanidine (NTG) was dissolved in 1 mL aceton and added to each sample (5 mL). The final NTG concentration was adjusted to 500 μg/mL, and then cultured in a constant temperature incubator shaker at 30°C for 15 min. Mutated cells were then collected by centrifugation and washed three times with distilled. 200 μL of resuspended mutated cells was plated on Yeast Nitrogen Base (YNB) solid medium containing 20 mg/mL 4-Aza-dl-leucine dihydrochloride (AZL) (Sigma Aldrich, USA), and then placed in 30°C constant temperature incubator to culture approximately three days until the appearance of colonies. Finally, a
PCR products were linked to a pEASY-T3 Cloning Vector (TransGen Biotech Company) with T4 DNA ligase Kit including high fidelity polymerase (CB111-01) and TransStart FastPfu DNA Polymerase (AP221-11). The evisiae ing to confirm cloning products was performed by Quintara Biosciences.

Genes were amplified using their respective primers (Table 3) from the template of the mutant strain of AH109 that demonstrated the highest yielding alcohol production using the pEASY-Blunt Simple Cloning Kit including high fidelity polymerase (CB111-01) and TransStart FastPfu DNA Polymerase (AP221-11). The PCR products were linked to a pEASY-T3 Cloning Vector (TransGen Biotech Company) with T<sub>e</sub> DNA ligase (TransGen Biotech Company), and the sequences were detected by BGI Tech. Company (Beijing, China).

Acquisition of the expression host. Mutagenesis C. crenatum using UWC. C. crenatum was grown up to exponential phase, the cells were centrifuged and collected at 4°C, placed on an ice bath for 30 min, cleaned with sterile water, supplemented with 20% glycerin, and then frozen overnight. The preparation of competent cells for freezing C. crenatum was conducted according to the protocols mentioned in the Handbook.

| Metallic element composition of duckweed (μg/g) | Composition | Concentration | Composition | Concentration | Composition | Concentration |
|-----------------------------------------------|-------------|---------------|-------------|---------------|-------------|---------------|
| Mg                                            | 5.24 ± 0.45 | Cr            | 3.07 ± 0.53 | Zn            | 301.11 ± 5.25 |
| P                                             | 343.03 ± 3.23 | Mn           | 501.13 ± 5.47 | Pb            | 31.27 ± 1.74 |
| K                                             | 1167.89 ± 4.32 | Cd           | 5.11 ± 0.38 | Al            | 401.32 ± 6.13 |
| Ca                                            | 154.25 ± 3.13 | Fe            | 802.52 ± 4.27 |

Table 2. The main components of wild duckweed *Ludoltia punctate* (before fermentation: 0 h). *Content (g) of various main composition of duckweed measured in 10 g pretreatment samples.*
of Corynebacterium glutamicum\(^{46}\). Wild-type C. crenatum competent cells were collected by centrifugation at 6000 \(\times\) g for 10 min at 4°C. The supernatant was discarded, and the competent cell pellet was resuspended in 20 mL fresh LMGM medium by gentle pipetting. NTG was then added at a final concentration of 200 \(\mu\)g/mL. The mixture was incubated for 20 min after moderate mixing, followed by centrifugation to collect the cells. The NTG-treated cells were washed three times with PB buffer to remove residual NTG, then resuspended in 10 mL fresh LMGM medium, and incubated at 28°C for 24 h. The NTG-treated cell suspension was diluted and plated onto LMGM solid medium. After colonies had appeared on the plate, mutants were selected to identify which auxotrophic strain had the highest isoleucine yield via fermentation experiments.

**Identifying and obtaining the expression host.** The expression host was determined to be the auxotrophic strain with the highest isoleucine titer identified via batch fermentation experiments. To prepare the fermentation inoculum for batch cultivation of mutant strains, we prepared a 100 mL TGYM medium containing 20 g/L glucose, 5 g/L yeast extract, 3 g/L ammonium acetate, 1 g/L sodium chloride, 1 g/L KH2PO4, 0.2 g/L MgSO4, 0.02 g/L MnSO4·7H2O, and 0.02 g/L FeSO4·7H2O and autoclaved it at 115°C for 20 min and cooled to 30°C. Mutant monoclonal colonies that appeared on the plate were selected out using sterile toothpicks, transferred to sterile bottles containing TGYM medium, and cultured in a constant temperature oscillation incubator at 30°C until the optical density (OD 600) reached 1.6.

Batch cultures were carried out for isoleucine production tests. The pH of the fermentation substrates was adjusted to 6.8 with 1% NaOH following batch culturing, which was conducted without pH control. The substrate concentration for each batch culture example was 6 g/L glucose, 5 g/L yeast extract, and 10 g/L peptone in a 250-mL glass anaerobic bottle (Haimen Huakai, Haimen, China) sealed with parafilm. The substrate solutions were sterilized at 115°C for 20 min and cooled to room temperature. Subsequently, 5 mL mixture solution containing a combination of 50 g/L KH2PO4, 50 g/L K2HPO4, and 220 g/L CH3COONH4, trace elements (0.1 g/L thiamin, and 0.001 g/L biotin), and minerals (20 g/L MgSO4·7H2O, 1 g/L MnSO4·H2O, 1 g/L FeSO4·7H2O, and 1 g/L NaCl) was filtered for sterilization (0.22-μm Millipore filter) and added to each bottle. The bottles were then inoculated with 0.5 mL fermentation inoculum (OD600 = 1.6), and fermented in a constant temperature oscillation incubator at 30°C for 3 days.

**Construction of metabolic pathways and engineering C. crenatum.** The auxotrophic strain of C. crenatum CICC 20135 with the highest isoleucine titers was selected as expression host strain. All strains and plasmids used in the study are listed in Table 4. The plasmids are physically available from Addgene (http://www.addgene.org). The genes were amplified from the template of mutant strain of S. cerevisiae AH109 for constructing metabolic pathways using their respective primers (Table 5).

All restriction endonucleases were purchased from NEB (Shanghai, China) and T4 DNA ligase (EL0334) was supplied by MBI Fermentas (Chengdu, Beijing). All plasmids were propagated using Trans-T1 Phage Resistant Chemically Competent Cells (TransGen Biotech Company, CD501-01, Beijing, China). The Trans-T1 bacterial strains were cultivated in LB medium, and grown at 37°C in a rotary shaker for 4 h. All cultures of the bioengineered strains of E. coli and mutant strains of C. crenatum CICC 20135 were then induced with 2 mM isopropyl-\(\beta\)-D-thiogalactoside (IPTG) and grown at 30°C for 18 h. Antibiotics (ampicillin, 100 μg/mL; chloramphenicol, 35 μg/mL; kanamycin, 50 μg/mL) were also added when needed. The bioengineered strains were reproduced in a rotary shaker under the following conditions: E. coli in LB medium at 37°C for 12 h, and C. crenatum in a nutrition gravey medium at 30°C for 48 h, and moved into 4°C to terminate the reaction.

We constructed new metabolic pathways responsible for the accumulation of higher alcohol intermediates (Fig. 7). They are designated metabolic pathway I, Ia, Ib including gene LEU2, mutant ILV2(2ILV2*), and mutant ILV5(2ILV5*); metabolic pathway II, Iia, Iib including gene mutant ILV2*, mutant ILV5*, and ILV3; metabolic pathway III including mutant gene BAT2(BAT2*); metabolic pathway IV, Iva including genes LEU4, and mutant LEU1(LEU1*); and metabolic pathway V, Va including gene mutant LEU1* and LEU2; metabolic pathway VI, Vla and Vlb including gene mutant BAT2*, LEU4*, and LEU1*, which were constructed using genes from auxotrophic strain S. cerevisiae AH109. The metabolic pathway VII responsible for oxidation-reduction reactions including genes Kivd and mutant ADH6(ADH6*) was constructed using the Kivd gene from L. lactis cremoris CIC1605 and the ADH6* gene from auxotrophic strain S. cerevisiae AH109.

The genes LEU2, ILV2*, and ILV5* of metabolic pathway I, Ia, and Ib were amplified with the primer pairs A1L2, A2I2, and A3I5, respectively. The genes ILV2*, ILV5* and ILV3 of metabolic pathway IIa, Iib, and Iib were amplified with the primer pairs B1L2, B2I2, and B3I3, respectively. The gene BAT2* of metabolic pathway III was amplified with the primer pair CBA. The genes LEU4 and LEU1* of metabolic pathway IVa and IVb were amplified with the primer pairs D1L4 and D2L1. The genes LEU1* and LEU2 of metabolic pathway V and Va were amplified with the primer pair E1L1 and E2L2. The genes BAT2*, LEU4*, and LEU1* of metabolic pathway VIa, Vla, and Vlb were amplified with the primer pairs F1BA, F2LE4, and F2LE1 and finally the gene pBL1 was amplified with the primer pair pBLAG1. The genes Kivd and ADH6* of metabolic pathway VII were amplified with the primer pairs G1K1 and ADH1, respectively.

The plasmids PEC-XK99e and pSTV29 were prepared as the standard expression vectors for constructing metabolic pathways. To construct the following expression vectors pTVPBL, PEC-KA6, pTVpBLAL, pTVpBLAILI, pTVpBLBII, pTVpBLBIII, pTVpBLECB, pTVpBLBLL, pTVpBLEEL, pTVpBLEB, pTVpBLFLB, and pTVpBFLFLB, the construction process was manipulated using the following instructions. First, the gene pBL1 from the standard expression vector pXM119 was inserted into the standard expression vector pSVT29 using restriction enzymes SacI and ClaI to construct a new expression plasmid pTVPBL. The in-series genes Kivd and ADH6* of metabolic pathway VII were inserted into standard expression vector PEC-XK99e using restriction enzymes PstI, XbaI, and KpnI to construct expression plasmid PEC-KA6.
The in-series genes LEU2-ILV2*-ILV5* of metabolic pathway I, Ia, and Ib were inserted into the new vector pTVpBL using restriction enzymes SphI, SalI, BamHI, SacI, and T4 ligase to construct plasmids pTVpBLAL, pTVpBLALI, and pTVpBLALII including different expression genes. The in-series genes ILV2*-ILV5*-ILV3 of metabolic pathway II, IIa, and IIb were inserted into vector pTVpBL using restriction enzymes PstI, SalI, BamHI, SacI and T4 ligase to construct expression plasmids pTVpBLBI, pTVpBLBII, and pTVpBLBIII responsible for expressing different genes, respectively. The gene BAT2* of metabolic pathway III was inserted into the vector pTVpBL using restriction enzymes SbfI, BamHI, and T4 ligase to construct plasmid pTVpBLCB. The in-series genes LEU4-LEU1* of metabolic pathway IV and IVa were inserted into the vector pTVpBL using restriction enzymes SbfI, XmaI, SacI, and T4 ligase to construct plasmids pTVpBLDL and pTVpBLDLL in charge of different

| Genes to be detected | Primer names | Base sequence (5’ to 3’) |
|----------------------|--------------|--------------------------|
| LEU2                | leu2-1       | ATGTCTGCCCCTAAGAAGAT     |
|                     | leu2-2       | TTAAGCAAGGATTTTCTTAAA    |
| ILV1                | ilv1-1       | ATGTCAGCTACCTCTACTAA     |
|                     | ilv1-2       | GCGGCTTAATTTTCAAGA       |
| ILV2                | ilv2-1       | ATGATCAGACAATCTACGCTAA   |
|                     | ilv2-2       | TCAGTGCTACCGCTCTGATC     |
| ILV5                | ilv5-1       | ATGTTGAGAATCTAAGGCCG     |
|                     | ilv5-2       | TTATTGGTTTTCTTCTGACAC    |
| ILV3                | ilv3-1       | ATGGGCTTTGTTAAGGAAGAT    |
|                     | ilv3-2       | TCAGCAAGGATTTTCTTAA      |
| BAT1                | BAT1-1       | ATGTTGCAAGACATCCCTTT    |
|                     | BAT1-2       | TTATTCGAAGGCAAGACAG     |
| BAT2                | BAT2-1       | ATGACCTTGGCACCCTAGAC     |
|                     | BAT2-2       | TCAGTTCAAAATCTAGTAAAC    |
| LEU4                | LEU4-1       | ATGTTAAGAGAGATATTATG     |
|                     | LEU4-2       | TTATGCAGAGGCGAGGGG       |
| LEU1                | LEU1-1       | ATGTTTACACTCCATCACA      |
|                     | LEU1-2       | CTACCAATCTGGTTCGACTT     |
| LEU2                | LEU2-1       | ATGTTGCGCCCTAAGAAGAT     |
|                     | LEU2-2       | TTAAGCAAGGATTTTCTTAA     |
| IRC15               | IRC15-1      | ATGGGAGGTGAAAGACGAGAAT   |
|                     | IRC15-2      | CATATTCCCGGACATGAGCC     |
| LPD1                | LPD1-1       | ATGTTAAGATCAGATCTCC      |
|                     | LPD1-2       | TCAACAAATGGAAGCTTTATC    |
| CAN1                | CAN1-1       | ATGCAAAATCCAAGAAGAGCGCC  |
|                     | CAN1-2       | CATAGCTACAACATCCTCAAATTTG |
| ARO10               | ARO10-1      | ATGTCACCGTGTAACATGAAAA   |
|                     | ARO10-2      | ATATTGTTCATGCTTCCGAGG    |
| PDC1                | PDC1-1       | ATGACTGAAATCATTCTGCGG    |
|                     | PDC1-2       | TTATGTGTCTAGCTTGGTAG     |
| PDC5                | PDC5-1       | ATGTCGAAAATACCTTACGG     |
|                     | PDC5-2       | TTATGTGTCTAGCTTGGGAC     |
| PDC6                | PDC6-1       | ATGTCGAAATGCACTCTTG      |
|                     | PDC6-2       | TTATGTGTCTGGAATCTTATG    |
| ADH1                | ADH1-1       | ATGTTACATCCAGAAAATCTCA   |
|                     | ADH1-2       | TTATTAAGAGTGCTCAAAACG    |
| ADH2                | ADH2-1       | ATGTTATCCAGAAAATCTCA     |
|                     | ADH2-2       | TTATTAAGAGTGCTCAAAACG    |
| ADH6                | ADH6-1       | ATGTTACATCCAGAAAATCTCA   |
|                     | ADH6-2       | CTATTCCCGGCAATCTTCCGAGG  |
| SFA1                | SFA1-1       | ATGTCGCCGCTGCTGTTG       |
|                     | SFA1-2       | CTATTCCCGGCAATCTTCCGAGG  |
| AAD6                | AAD6-1       | ATGTCGATTTATTTTATACCTCC  |
|                     | AAD6-2       | TCAACAGGTTCCATTTTACCT    |
| AAD10               | AAD10-1      | ATGCGATCAAAGAAGACAGGG    |
|                     | AAD10-2      | CTAAAATCTGGGGAATGCTT     |

Table 3. Detected genes and primers used in this study. “1”: Sense strand; “2”: Antisense strand.
calculated using integral optical density (IOD).

C. crenatum provided by the Acetic Acid Fermentation Institute (TransGen Biotech, Beijing, China). For semi-quantitative RT-PCR analysis, expression profiles of all exogenous genes were formed according to the operation manual of TransScript First-Strand cDNA Synthesis SuperMix kit of the manufacturer (TransGen Biotech, Beijing, China), and the calculated relative expression values for all exogenous genes were obtained.

0.01 g/L MnSO₄, 0.01 g/L biotin), and minerals (20 g/L (NH₄)₂SO₄, 1.5 g/L KH₂PO₄, 0.3 g/L MgSO₄·7H₂O, 0.05 g/L FeSO₄·7H₂O, 0.01 g/L MnSO₄·H₂O, 1.0 g/L CH₃COONH₄, and 1 g/L NaCl) were filter sterilized (Millipore filter; 0.22 μm) and added to fermentation solutions in bottle. In addition, 2 mM IPTG and antibiotics (ampicillin, 100 μg/mL; chloramphenicol, 35 μg/mL; kanamycin, 50 μg/mL) were also added. The bottles were then inoculated with 0.5 mL fermentation inoculum (OD₆₀₀ = 1.6), and fermented in constant temperature oscillation incubator at 30 °C for 3 days.

catalytic reactions. The in-series genes LEU¹⁺-LEU₂ of metabolic pathway V and Va were inserted into the vector pTVpBL using restriction enzymes SbfI, BamHI, SphI, and T4 ligase to construct plasmids pTVpBLELL and pTVpBLEL. The in-series genes BAT²⁺, LEU₄, and LEU¹⁺ of metabolic pathway VI, VIa, and VIb were inserted into the vector pTVpBL using restriction enzymes SbfI, BamHI, XmaI, SacI, SphI, and T4 ligase to construct plasmids pTVpBLFB, pTVpBLFBL, and pTVpBLBFL, which are responsible for different metabolic reactions. The ribosome binding site (RBS) sequence was inserted into 6 nucleotides upstream of each structural gene to facilitate mRNA translation.

Competent cells of mutant strain C. crenatum were prepared as previously described in the Handbook of C. glutamicum[60]. All construction plasmids were introduced into mutant strains of C. crenatum using electroporation[61,62]. Electroporation was conducted according to the following manipulative conditions 100 Ω, 50 μF, 2.2 kV, and 8 ms using the Gene Pulser Xcell Microbial System165–2662 (BIO-RAD, Chengdu).

**Gene expression analysis.** Gene expression of all recombinant genes for each metabolic pathway in the production host was analyzed using semi-quantitative RT-PCR. RNA extraction and cDNA synthesis were performed according to the operation manual of TransScript First-Strand cDNA Synthesis SuperMix kit of the manufacturer (TransGen Biotech, Beijing, China). For semi-quantitative RT-PCR analysis, expression profiles of all recombinant genes were evaluated based on semi-quantified analysis using Gel-Pro analyzer 4.0 software (Media Cybernetics, Silver Spring, MD, USA), and the calculated relative expression values for all exogenous genes were calculated using integral optical density (IOD).

**Batch fermentation using bioengineered strains.** Fermentation using glucose. Glucose (60 g/L) was used as a fermentation substrate. Glucose was sterilized at 115 °C for 20 h, then cooled to room temperature for use in fermentation assays. Batch fermentation cultures were carried out under aerobic conditions, and the pH of glucose before fermentation was automatically maintained at 6.5 by a pH controller for 24 h (PHC-220; Abele, Tokyo, Japan). The fermentation processes were conducted in 250 mL glass anaerobic bottles (Haimen Huakai experiment glass instrument Co., Ltd, Haimen, China) sealed with sealing film. After addition of 1 g yeast extract and 2 g peptone to each bottle, 5 mL of a combination of P2 trace elements mixture solution (50 g/L KH₂PO₄, 50 g/L K₂HPO₄, and 220 g/L CH₃COONH₄), vitamins (0.1 g/L para-aminobenzoic acid, 0.1 g/L thiamin, and 0.001 g/L biotin), and minerals (20 g/L (NH₄)₂SO₄, 1.5 g/L KH₂PO₄, 0.3 g/L MgSO₄·7H₂O, 0.05 g/L FeSO₄·7H₂O, 0.01 g/L MnSO₄·H₂O, 1.0 g/L CH₃COONH₄ and 1 g/L NaCl) were filter sterilized (Millipore filter, 0.22 μm) and added to fermentation solutions in bottle. In addition, 2 mM IPTG and antibiotics (ampicillin, 100 μg/mL; chloramphenicol, 35 μg/mL; kanamycin, 50 μg/mL) were also added. The bottles were then inoculated with 0.5 mL fermentation inoculum (OD₆₀₀ = 1.6), and fermented in constant temperature oscillation incubator at 30 °C for 3 days.

**Table 4. The bacterial strains and vectors used in the bioengineering of bacteria to produce higher alcohols.** C. cerevisiae NC-11.
Fermentation using duckweed under SSF. For the enzymatic hydrolysis method, the fermentation substrate in duckweed trials was hydrolysate in 20 mL modified M9 medium (2 g (NH₄)₂PO₄, 2 g KH₂PO₄, 1 g K₂HPO₄, 1 g NH₄Cl, 0.5 g NaCl, 0.5 mM MgSO₄, 1 mM CaCl₂, 20 mg vitamin B₁, and 2 mg biotin per L of water) containing 5 g/L yeast extract. The pH of the slurry of duckweed was adjusted to 6.0 with 1% H₃PO₄, and 0.2 mg/g α-amylase (120 KUN/g) was added, then hydrolyzed at 50 °C for 6 h. Subsequently, 0.2 mg/g β-amylase was added, and saccharified at 45 °C for 10 h. After that, 0.2 mg/g cellulase (500000 U/g, where the enzyme activity (U/g) is defined as follows: CMCA = 1 g enzyme powder decomposes the substrate CMC-Na to produce 1 mg glucose with treatment at 50 °C and pH 4.8 for 1 h; Thinkly, China) and 0.2 mg/g Optimash BG (containing 5.4 U β-glucosidase activity and 1.9 U β-xylosidase activity; Genencor, USA) were added to the solution. The reaction mixture was then buffered with 50 mM phosphate buffer at pH 5.0 and incubated on a rotary shaker (HZQ-X500; Yiheng, Shanghai, China) at 220 rpm for 12 h at 50 °C.

For the SSF procedure, the fermentation procedure was simultaneously conducted alongside enzymatic hydrolysis in a 150-mL triangular flask. Supplementary Trace Metals Mix solution (2 g H₃BO₃, 2.1 g MnCl₂·4H₂O, 0.3 g ZnSO₄·7H₂O, 0.002 g MnSO₄, 2.5 g Na₂MoO₄·2H₂O, 0.05 g CuSO₄·5H₂O, 21.2 mg Co(NO₃)₂·6H₂O, and 0.05 g FeSO₄ per L of water) and the substrates were fermented under aerobic conditions. 2 mL rejuvenated seed-ing solution of bioengineered strains from liquid medium was inoculated into 50 mL of the fermentation substrate. All cultures were induced with 2 mM IPTG, kanamycin, and chloramphenicol. The pH of the fermentation substrate after enzymatic hydrolysis was automatically maintained at 6.5, with continued fermentation at 30 °C with shaking at 200 rpm for 96 h in a constant-temperature oscillation incubator.

Analysis production and data statistics. Alcohol compounds were measured with a model 6890 gas chromatograph (GC) equipped with a flame ionization detector (Agilent Technologies, Santa Clara, CA, USA).
with a model 7673 A automatic injector, sampler, and controller (Hewlett-Packard). Alcohol compounds were separated out using a ZB-WAX capillary column (30 m, 0.25 mm inside diameter, 0.25 μm film thickness; Phenomenex Inc., PA, USA). The GC oven temperature was held initially at 40 °C for 5 min, then raised stepwise, by 15 °C/min, until it reached 150 °C. It was then raised by 5 °C/min up to 250 °C, and held for 4 min. Helium was used as carrier gas, with an inlet pressure of 9.3 lb/in². The injector and detector were maintained at 220 °C. A 1-μL volume of supernatant from the culture broth was injected in split-injection mode at a 1:30 split ratio. For other secreted metabolites, the constituent compounds (20 μL) were detected with an Agilent 1100 high-performance liquid chromatography system equipped with an auto-sampler and a Bio-Rad (Hercules, CA: carbohydrate analysis column Aminex HPX-87P Column 300 × 7.8 mm catalog 125-0098 serial 426070) (5 mM H₂SO₄, 0.6 mL/min; column temperature at 65 °C). Glucose was detected with an ELSD 2000 CSC detector, while organic acids were detected using a photodiode array detector at 210 nm. Concentrations were determined using extrapolation from standard curves. Amino acid and other organic acid production were determined with a DIONEX UltiMate 3000 liquid chromatograph in a column packed with Aminex HPX-87H and 0.05 mM H₃PO₄ on Chromosorb WAW. Chromatography was conducted at an injector temperature of 175 °C, detector temperature of 180 °C, and oven temperature of 125 °C.

For each experiment, all results were repeated for three times, we calculated the mean response variables and their standard deviations (SD), unless otherwise indicated. Comparisons of variable(s) were made with Student's t-test; values of P < 0.05 were considered to indicate statistically significant differences. Tukey's honest significant difference test was used when the null hypothesis was rejected (P < 0.05). Statistical analyses were conducted using the software program SPSS 21.0 (IBM, USA).

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