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2-Keto acids based biosynthesis pathways for renewable fuels and chemicals

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Abstract Global energy and environmental concerns have driven the development of biological chemical production from renewable sources. Biological processes using microorganisms are efficient and have been traditionally utilized to convert biomass (i.e., glucose) to useful chemicals such as amino acids. To produce desired fuels and chemicals with high yield and rate, metabolic pathways have been enhanced and expanded with metabolic engineering and synthetic biology approaches. 2-Keto acids, which are key intermediates in amino acid biosynthesis, can be converted to a wide range of chemicals. 2-Keto acid pathways were engineered in previous research efforts and these studies demonstrated that 2-keto acid pathways have high potential for novel metabolic routes with high productivity. In this review, we discuss recently developed 2-keto acid-based pathways.

Keywords Metabolic engineering · 2-Keto acid · Alcohol · Aldehyde · Acid · Ester

Introduction

Affordable energy and advanced chemical products have played a major role in the increase in standard of living in the last century. The majority of fuels and chemicals (rubbers, plastics, solvents, etc.) are derived from fossil fuels. The global consumption rate of fossil fuels is increasing every year. However, this resource is finite and rational analysis has predicted that crude oil supply will peak around 2040 and decrease sharply thereafter [63, 68]. In addition, the combustion of fossil fuels is the greatest contribution to the increase of greenhouse gases and global warming. These economic and environmental reasons are driving the development of renewable replacements for petroleum-based fuels and chemicals. Biomass sources, such as sugar, have garnered much interest because they represent recycled carbon dioxide emitted from the atmosphere via photosynthesis.

Microorganisms, such as *Escherichia coli* and the yeast *Saccharomyces cerevisiae*, have been utilized for efficient conversion of biomass to other chemicals. Metabolic pathways have been enhanced and expanded in these microorganisms using metabolic engineering and synthetic biology approaches to generate diverse products, including advanced biofuels, bulk chemicals, and pharmaceutical drugs [20, 38, 43, 55, 56, 71, 78]. The isobutanol pathway is being utilized for industrial-scale production. Gevo, Inc. was founded aimed at commercializing production of isobutanol as a drop-in biofuel. Gevo broke ground on its first plant on May 31, 2011, in Luverne, Minnesota. Butamax Advanced Biofuels LLC, a joint venture between British Petroleum and DuPont, is also developing commercial microbial production of isobutanol. Techno-economic analysis has been performed to assess the feasibility for cellulosic isobutanol [72]. These results indicate that cellulosic isobutanol has high potential compared to cellulosic ethanol, a biofuel widely considered as most feasible.

Amino acid biosynthesis pathways are some of the most naturally active metabolic pathways and are universally conserved among cells. In amino acid pathways, 2-keto acids are a key metabolic intermediate. 2-Keto acids can be converted to a wide range of products by combination of decarboxylation, reduction, oxidation, chain elongation, and condensation. Additionally, 2-keto acid-based

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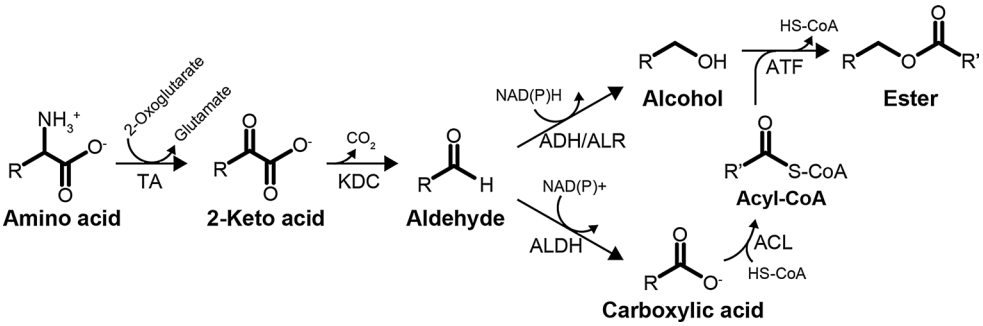

Fig. 1 Ehrlich amino acid degradation pathway and its derivatives *TA* transaminase, *KDC* 2-keto acid decarboxylase, *ADH* alcohol dehydrogenase, *ALR* aldehyde reductase, *ALDH* aldehyde dehydrogenase, *ATF* alcohol *O*-acyltransferase, *ACL* acyl-CoA ligase

Table 1 Products from 2-keto acid and their production cost

Product	Carbon number	Theoretical carbon yield	Required ATP	Required redox	Ref
1-Propanol	3	100	2	3	[65]
		50[a]	−2[a]	−3[a]	[6]
1-Butanol	4	67	1	0	[65]
		44[a]	−3[a]	−6[a]	[6]
Isobutanol	4	67	−2	0	[4]
Isobutyraldehyde	4	67	−2	−1	[58]
Isobutyric acid	4	67	−2	−2	[82]
1-Pentanol	5	56	0	−3	[51]
(S)-2-Methyl-1-butanol	5	83	1	3	[14]
3-Methyl-1-butanol	5	56	−3	−3	[18]
2-Methylbutyric acid	5	83	1	1	[23]
Isovaleric acid	5	56	−3	−5	[77]
Valeric acid	5	56	0	−5	[23]
1-Hexanol	6	50	−1	−6	[51]
4-Methyl-1-pentanol	6	50	−4	−6	[81]
(S)-3-methyl-1-pentanol	6	67	0	0	[81]
Isobutyl acetate	6	67	−3	−2	[60]
Isocaproic acid	6	50	−4	−8	[77]
1-Heptanol	7	47	−2	−9	[51]
(S)-4-Methyl-1-hexanol	7	58	−1	−3	[81]
1-Octanol	8	44	−3	−12	[51]
(S)-5-Methyl-1-heptanol	8	53	−2	−6	[81]
Isobutyl isobutyrate	8	67	−4	−2	[60]
Phenylethanol	8	67[b]	2[b]	−4[b]	[51]
Phenylpropanol	9	60[b]	1[b]	−7[b]	[51]

[a] Citramalate pathway

[b] Pentose phosphate pathway

pathways maintain high activity in various conditions (i.e., aerobic or anaerobic, minimal media). For these reasons, various metabolic pathways based on 2-keto acids have been designed and constructed to produce fuels and chemicals (Fig. 1;Table 1). Here, we review 2-keto acid-based pathways for the production of alcohols, aldehydes, acids, and esters, which are useful as fuels and chemical feedstocks.

Branched-chain amino acid biosynthesis and the Ehrlich pathway

The branched-chain amino acids (L-leucine, L-isoleucine, and L-valine) are formed primarily from the 2-keto acid, pyruvate, which is further converted to larger 2-keto acid precursors (Fig. 2). The 2-keto acids (2-ketobutyrate (2-KB), 2-ketovalerate (2-KV), and phenylpyruvate) are

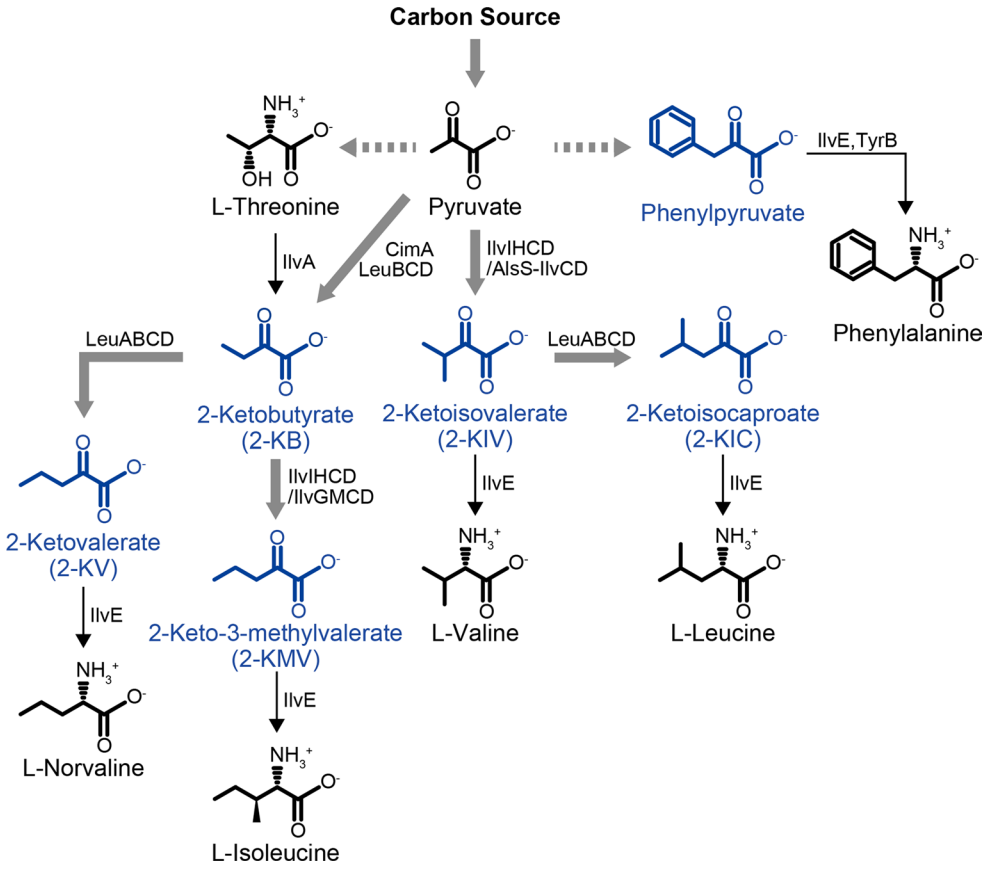

Fig. 2 Branched-chain amino acid biosynthesis IlvIH, acetolactate synthase large and small subunit; *IlvC* 2-hydroxy-3-ketol-acid reductoisomerase, *IlvD* dihydroxy-acid hydratase, *AlsS* acetolactate synthase, *IlvGM* acetolactate synthase II large and small subunit, *LeuA* 2-isopropylmalate synthase; *LeuB* 3-propylmalate dehydrogenase, *LeuCD* isopropylmalate isomerase, *CimA* citramalate synthase, *IlvE* branched-chain amino acid aminotransferase, *TyrB* tyrosine aminotransferase

also formed in the degradation of L-threonine and the biosynthesis of phenylalanine (Fig. 2). These 2-keto acids are then transaminated to form the corresponding amino acids [75]. The Ehrlich pathway [28] is the basis for the production of various fuel/chemical molecules from branched-chain amino acid biosynthesis (Fig. 1). In some bacteria and fungi, amino acids are degraded through the Ehrlich pathway, whereby amino acids are deaminated to the corresponding 2-keto acids, then the decarboxylated to an aldehyde by a 2-keto acid decarboxylase (KDC), and finally either reduced to an alcohol by an alcohol dehydrogenase/aldehyde reductase (ADH/ALR) or oxidized to an acid by an aldehyde dehydrogenase (ALDH). The alcohols and acids can also be condensed to synthesize esters.

2-Keto acid-based pathways maintain relatively high activity in various conditions (i.e., oxygen concentration, media condition, and so on). In almost every case, lab-scale production is performed in M9 minimal media containing yeast extract (~0.5 %w/v) to increase expression level of enzymes in the host under micro-aerobic condition. In micro-aerobic condition, oxygen concentration is less than that of aerobic conditions, allowing maintenance of high protein expression levels and reduced carbon loss by TCA cycle [3]. For these reasons, micro-aerobic condition is utilized for demonstration.

2-Keto acid-based alcohols

Atsumi et al. [4] evaluated a variety of alcohols produced from *E. coli* utilizing 2-keto acid precursors in branched-chain amino acid biosynthesis (Fig. 3). In these pathways, 2-keto acids are converted to their corresponding aldehydes using a KDC and then reduced to the alcohols with an ADH. The amino acid precursors 2-KB, 2-ketoisovalerate (2-KIV), 2-ketoisocaproate (2-KIC), 2-keto-3-methylvalerate (2-KMV), and phenylpyruvate can be converted to 1-propanol, isobutanol, 3-methyl-1-butanol, 2-methyl-1-butanol, and 2-phenylethanol, respectively (Fig. 3). Additionally, 2-KV, which comes from a side reaction in L-leucine biosynthesis (Fig. 2), can be used to produce 1-butanol. To demonstrate the viability of these pathways in *E. coli*, five KDCs along with Adh2 from *S. cerevisiae* were expressed and tested for alcohol production. The results showed that strains expressing the KDC (Kivd from *L. lactis* or Aro10 from *S. cerevisiae*) produced all five of the expected alcohols.

Isobutanol

Optimizations were then performed to produce isobutanol in higher productivity. Isobutanol is a particularly desirable

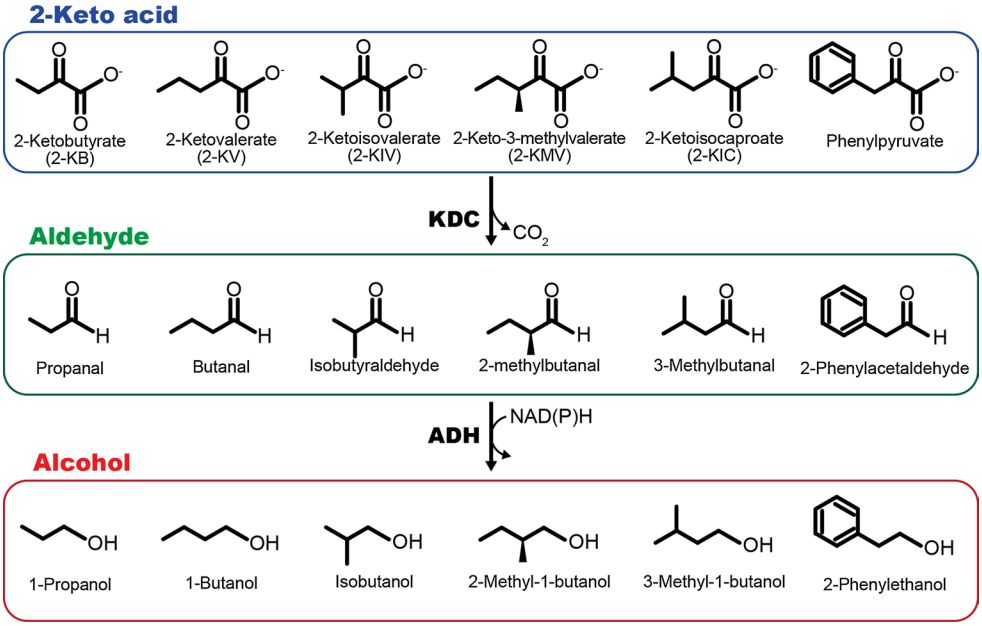

Fig. 3 Conversion of 2-keto acids to higher chain alcohols. *KDC* 2-keto acid decarboxylase, *ADH* alcohol dehydrogenase

product due to its similar energy density (29 MJ/L) to gasoline (32 MJ/L) [44]. The genes *ilvIHCD*, *kivd*, and *ADH2* coding for the entire isobutanol pathway from pyruvate (Figs. 2, 3) were expressed in *E. coli*. The strain produced 1.7 g/L isobutanol, a fivefold increase over the strain not overexpressing *ilvIHCD*. To further increase the production, genes for pathways that compete for pyruvate were deleted (*adhE*, *ldhA*, *frdAB*, *fnr*, and *pta*), leading to the production of 2.2 g/L of isobutanol. Next, *ilvIH* was replaced by *alsS* from *Bacillus subtilis* due to its higher affinity for pyruvate [31]. This increased the production 1.7-fold to 3.7 g/L. Finally, *pflB* was deleted to further decrease competition for pyruvate. The combined genetic manipulations led to a maximum titer of 22 g/L of isobutanol. Yields reached 0.35 g isobutanol per glucose, representing 86 % of the theoretical maximum [4]. The high titer and yield of isobutanol achieved in this work demonstrated the potential of these 2-keto acid-based pathways for useful biofuels.

In a later study, Atsumi et al. [7] evaluated three ADHs (YqhD from *E. coli*, Adh2 from *S. cerevisiae*, and AdhA from *L. lactis*) for their ability to produce isobutanol in *E. coli*. It was discovered that a strain lacking overexpression of exogenous ADH was still able to produce the same amount of isobutanol as a strain expressing *ADH2*. It was determined that the native YqhD in *E. coli* was responsible for the ADH activity. When *yqhD* was deleted from the genome, isobutanol production decreased by ~80 %. The activities of YqhD and AdhA were very similar, whereas Adh2 was determined to not favor isobutyraldehyde. AdhA utilizes NADH, a cofactor more advantageous under anaerobic conditions compared to the NADPH-dependent YqhD. Anaerobic conditions are preferred for large-scale production due to lower operation cost and higher carbon yield. In anaerobic conditions, a cofactor imbalance in engineered metabolic pathway is important to maintain high yielding productions. Bastin et al. [10] achieved 100 % of theoretical carbon yield from glucose by constructing totally NADH-dependent isobutanol pathway.

Following the initial success of isobutanol production in *E. coli*, Baez et al.[9] evaluated the optimized isobutanol strain in a 1-L bioreactor combined with a gas-stripping system . The optimized strain produced 51 g/L isobutanol after 72 h at 30 °C. Production at 37 °C was also tested, but led to lower titers in all strains. To determine if the enzymes were less active at 37 °C, each enzyme in the pathway was assayed for their activity at both temperatures. All showed similar activities at 30 and 37 °C except for the last enzyme AdhA, which could be the reason for lower production at 37 °C.

To further increase production efficiency of isobutanol in cost, direct conversion systems of cellulose or CO_2 have been developed. For cellulosic isobutanol, novel pathways have been designed in single cells [22, 34] and in microbial consortia [52]. The isobutanol pathway was also successfully introduced into the cyanobacterium *Synechococcus elongatus* PCC7942 [5].

3-Methyl-1-butanol

Another 2-keto acid-based biofuel is 3-methyl-1-butanol. This pentanol isomer is suitable as a gasoline supplement or replacement since its energy density (30.5 MJ/L)

approaches that of gasoline (32 MJ/L) [44]. 3-methyl-1-butanol is naturally occurring in yeast and fungi, though only in very small quantities [1, 24, 62].

Connor and Liao reported the engineering of *E. coli* for the production of 3-methyl-1-butanol [18]. Utilizing the 2-keto acid-based pathway from L-leucine biosynthesis, 3-methyl-1-butanol was produced from 2-KIC, an intermediate in L-leucine biosynthesis (Figs. 2, 3). With *ilvIHCD* from *E. coli*, *kivd* from *L. lactis*, and *ADH2* from *S. cerevisiae* overexpressed, the strain produced 56 mg/L of 3-methyl-1-butanol after 18 h. To increase the production, *ilvIH* was replaced by *alsS* from *B. subtilis*. This replacement increased production to 67 mg/L. Overexpression of *leuABCD* lead to a further increase in production. Next, competing pathways were deleted to increase flux toward 3-methyl-1-butanol. The genes *adhE*, *frdBC*, *ldhA*, *pta*, *fnr*, and *pflB* were previously reported to increase the production of isobutanol in *E. coli* [4]. However, these deletions yielded only 76 mg/L of 3-methyl-1-butanol. To analyze the bottlenecks in this pathway, production of the 2-keto acid precursors, 2-KIV and 2-KIC, was evaluated. With *leuABCD* and *alsS-ilvCD* overexpressed, 0.3 g/L 2-KIV and less than 5 mg/L 2-KIC were produced. This limitation of the 3-methyl-1-butanol precursor, and abundance of the isobutanol precursor explain the differences in the production of isobutanol and 3-methyl-1-butanol. To increase the 2-KIC pool, the ribosomal binding site of *leuA* was changed to a consensus sequence [50]. This led to 0.2 g/L 2-KIC, but also 0.4 g/L 2-KIV. This suggested that 2-KIV and 2-KIC were not competitive and low activity of LeuA was responsible for low 2-KIC production. To increase the activity of this step, the feedback-resistant mutant $LeuA^{FBR}$ [32] was overexpressed. This led to 1.6 g/L 2-KIC and just 0.2 g/L 2-KIV, a dramatic shift in the 2-keto acid products. A second separate strategy was also employed, which was to delete the final enzymes in the L-leucine pathway (coded by *ilvE* and *tyrB*) to prevent feedback inhibition of LeuA by L-leucine. This also increased 2-KIC, to 1.2 g/L. A combined strain with both $leuA^{FBR}$ and Δ*ilvE*, produced 2.3 g/L 2-KIC. With these optimized strains, 3-methyl-1-butanol titers reached 0.8 g/L with minimal isobutanol formation. Increasing the concentration of glucose in the media led to a further increase to 1.3 g/L 3-methyl-1-butanol. The best estimated yield was determined to be 0.13 g/g glucose.

Following this work, Connor and Liao further improved 3-methyl-1-butanol production in *E. coli* by employing a mutagenesis strategy previously used to develop amino acid biosynthesis [17]. In this work, random mutagenesis was carried out with the mutagen N-methyl-N′-nitrosoguanidine (NTG) and mutants were grown in the presence of the amino acid analog 4-aza-D, L-leucine (AZL) [17]. The leucine analog is readily incorporated into proteins during translation, rendering the proteins non-functional, and is thus toxic to *E. coli*. However, mutants that are able to increase production of L-leucine will be able to better survive an AZL challenge by having higher levels of L-leucine to compete with AZL. The most productive strain after the second AZL challenge round produced 1.5 g/L of 3-methyl-1-butanol. Subsequent attempts at mutagenesis failed to yield better mutants. Growth experiments revealed that 3-methyl-1-butanol inhibited *E. coli* growth at 1 g/L with more severe effects at >3 g/L. To relieve the toxicity of 3-methyl-1-butanol during production, a two-phase fermentation was employed to extract product into a non-aqueous phase. To accomplish this, oleyl alcohol was added as a second phase to the culture flask. With the two-phase fermentation, production of 3-methyl-1-butanol increased to 9.5 g/L after 60 h. Total alcohol production reached 12.5 g/L.

2-Methyl-1-butanol

Another pentanol isomer derived from amino acid biosynthesis is 2-methyl-1-butanol. Cann and Liao produced 2-methyl-1-butanol in *E. coli* from 2-KMV, the precursor to isoleucine (Figs. 2, 3) [14]. From the evaluation of several acetohydroxy acid synthase (AHAS) enzymes from various sources, it was determined that AHAS II from *Salmonella typhimurium* showed the highest 2-methyl-1-butanol production. Carbon flux was then increased by expression of threonine deaminase to increase levels of 2-KB precursor in the cell. It was determined that IlvA from *Corynebacterium glutamicum* showed the best threonine deaminase activity. The final strain overexpressed *ilvGM* from *S. typhymurium*, *ilvCD* from *E. coli*, *ilvA* from *C. glutamicum*, and *thrABC* from *E. coli* with deletion of *metA* and *tdh*, produced 1.3 g/L after 24 h with a yield of 0.17 g/g glucose.

1-Butanol and 1-propanol

1-Butanol and 1-propanol were produced using the same scheme as for 2-methyl-1-butanol (Figs. 2, 3) [4]. 1-Butanol can be produced from 2-KV. However, 2-KV is the product of a side reaction from leucine biosynthesis (Fig. 2), wherein LeuABCD elongates 2-KB–2-KV [12, 40]. More 2-KB can be generated through the threonine degradation pathway (encoded by *ilvA*). Therefore, a strain overexpressing *ilvA* and *leuABCD* was constructed. This strain produced 44 mg/L 1-butanol.

Shen and Liao further improved 1-butanol and 1-propanol production [65]. In this work, the conversion of 2-KB–2-KV was increased by overexpressing *ilvA* and *leuABCD* genes in *E. coli* and *kivd* and *ADH2* were overexpressed to convert 2-KV to 1-butanol. This led to a fivefold

increase in 1-butanol (30 mg/L) and 1-propanol (60 mg/L) compared to the strain without overexpressing *ilvA* and *leuABCD*. To explore the potential feedback inhibition of ThrA in the threonine pathway, the feedback-resistant ThrA mutant [67] was expressed. This led to a three to fourfold increase in both alcohols. A feedback-resistant mutant of LeuABCD [32] was also evaluated, but no increase of either alcohol was observed with that enzyme, suggesting that L-leucine within the cells was below inhibitory levels. To further increase production, elimination of competing pathways was carried out. Here, homoserine *O*-succinytransferase and threonine dehydrogenase encoded by *metA* and *tdh*, respectively, were deleted to minimize loss of precursors. These deletions increased production to 1.2 g/L, mainly from an increase in 1-propanol production. To increase 1-butanol levels, the acetyl hydroxy acid synthase isozymes that compete for acetyl-CoA and 2-KB in L-valine, L-leucine, and L-isoleucine biosynthesis pathways were also deleted. This resulted in a twofold increase in 1-butanol production, while 1-propanol remained the same, and nearly all side products such as isobutanol, 2-methyl-1-butanol, and 3-methyl-1-butanol were abolished. However, this created auxotrophy of L-valine, L-leucine, and L-isoleucine. Finally, to reduce ethanol production, *adhE* was deleted and resulted in a 2.5-fold reduction in ethanol from 0.25 g/L to 0.1 g/L. This final strain produced a 1:1 ratio of 1-propanol and 1-butanol.

In a different strategy, Atsumi and Liao improved on 1-propanol and 1-butanol production in *E. coli* by utilizing the citramalate pathway to produce higher levels of 2-KB [6]. Some organisms, such as *Methanococcus jannaschii*, contain a more direct route to produce 2-KB by combining pyruvate and acetyl-CoA using citramalate synthase (coded by *cimA*) [35]. In this work, CimA of *M. jannaschii* was evolved for better activity in *E. coli*. Because 2-KB is an essential precursor in the biosynthesis of L-isoleucine, an *E. coli* strain deficient in *ilvA* and *tdcB* cannot synthesize 2-KB and becomes auxotrophic for L-isoleucine. Thus, CimA with increased activity would allow for sufficient biosynthesis of 2-KB, and therefore L-isoleucine, to rescue the Δ*ilvA*Δ*tdcB* strain of *E. coli*. Six rounds of evolution were carried out. The best CimA mutant had five amino acid substitutions as well as a frame-shift at base pair 1117. The frame-shift created a stop codon, giving the gene a ~350 bp non-coding region. The best variant produced 2.7 g/L 1-propanol and 0.4 g/L 1-butanol, representing a roughly ninefold and 22-fold increase, respectively, compared to the parent strain.

These two pathways (the native threonine pathway and the citramalate pathway) leading to the synthesis of 1-propanol and 1-butanol differ in cofactor preference and ATP requirement (Table 1). As shown in the isobutanol pathway, cofactor balance has a high impact on the production. In

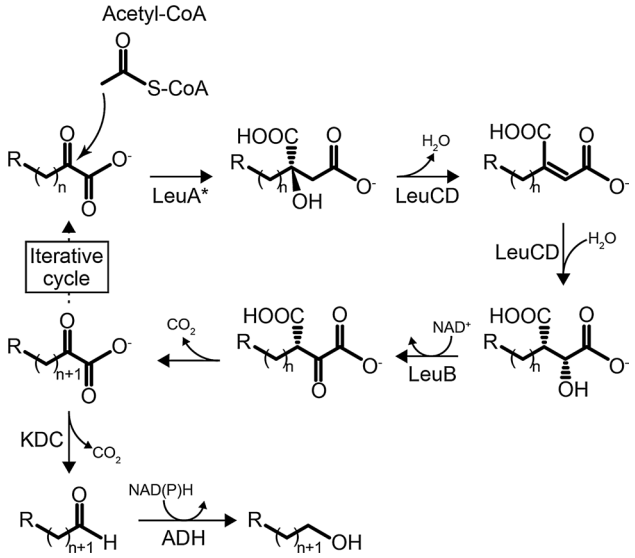

Fig. 4 Engineered LeuABCD "+1" biosynthetic pathway for alcohol production. *LeuA** 2-isopropylmalate synthase mutant, *LeuCD* isopropylmalate isomerase complex, *LeuB* isopropylmalate dehydrogenase, *KDC* 2-keto acid decarboxylase, *ADH* alcohol dehydrogenase

1-propanol production, ATP and redox cofactors are not evenly balanced in each pathway. The threonine pathway requires three NAD(P)H. On the other hand, the citramalate pathway generates three NAD(P)H. Shen and Liao coupled these pathways in *E. coli* [66] allowing the redox cofactors to remain balanced. The yield of 1-propanol (0.15 g/g of glucose) achieved higher than individual ones alone: the threonine pathway (0.09 g/g of glucose) or the citramalate pathway (0.11 g/g of glucose).

Expanding 2-keto acid pathways for higher alcohols

Zhang et al. engineered a non-natural metabolic pathway to produce C6 alcohols (1-Hexanol, 4-methyl-1-pentanol, and (S)-3-methyl-1-pentanol) by expanding the 2-keto acid-based pathways [51, 81]. The 3-step elongation performed by LeuABCD was utilized to elongate the L-isoleucine precursor, 2-KMV (Figs. 2, 4). The elongation product, 2-keto-4-methylhexanoate, was converted to the corresponding aldehyde by KDC and then reduced to the C6 alcohol (S)-3-methyl-1-pentanol by ADH. With the 14 relevant genes overexpressed in a threonine hyper producing *E. coli* strain, 6.5 mg/L of the C6 alcohol was produced. Using a leucine feedback-insensitive mutant of LeuA increased C6 alcohol production to 41 mg/L. To reduce the formation of other alcohols by KDC, a more selective KDC mutant was designed. A F381L/V461A mutant showed the best production with titers of C6 alcohol at 384 mg/L. An attempt to expand the binding pocket of LeuA to better incorporate

2-keto-4-methylhexanoate led to the LeuA mutant G462D/S139G that doubled C6 alcohol production to 794 mg/L. A LeuA quadruple mutant G462D/S139G/H97A/N167A produced small amounts of non-natural C7 and C8 alcohol ((S)-4-methyl-1-hexanol and (S)-5-methyl-1-heptanol), indicating that the LeuA mutant was able to incorporate even larger 2-keto acids as a result the larger binding pocket.

Marcheschi et al. [51] expanded the substrate range of this "+1" pathway from branched-chain 2-keto acids to linear-chain and aromatic-chain 2-keto acids. This strategy has enabled C5-C8 n-alcohols and aromatic alcohols to be produced. In the elongation process by LeuABCD, LeuA acts as the gate-keeper by catalyzing the condensation of acetyl-CoA and 2-keto acid (Fig. 4). If the selectivity of LeuA is altered, it could be a very useful elongation pathway for biosynthesis of non-natural 2-keto acids and possibly non-natural aldehydes and alcohols. Marcheschi et al. analyzed the reaction mechanism of LeuA from *E. coli* by using a combination of quantum mechanical modeling and protein engineering. This modeling predicted an intrinsic steric barrier to the use of long alkyl chain or aromatic 2-keto acids by LeuA. To increase the size of the substrate-binding pocket of LeuA, the residues in the pocket of the LeuA G462D mutant (H97, S139, N167, and P169) were mutated to smaller amino acids such as alanine or glycine. A series of LeuA mutants were made and screened by computational analysis [27] and in vitro assays, including, LeuA* (H97A/S139G/N167G/P169A/G462D) was created. Wild-type LeuA cannot accept 2-ketoheptanoate or bulkier 2-keto acids. On the other hand, the LeuA* mutant showed activity for 2-ketoheptanoate, 2-ketooctanoate, phenylpyruvate, and homophenylpyruvate. Finally, the LeuABCD elongation pathway with LeuA* (LeuA*BCD) was incorporated into an *E. coli* strain engineered for the overproduction of either threonine or a phenylalanine pathway. In the threonine-hyperproduction strain, the LeuA*BCD catalyzed the recursive elongation of 2-KB over 5 cycles to 2-ketooctanoate, and 80 mg/L 1-heptanol and 2 mg/L 1-octanol were produced. In the phenylalanine-overproducing *E. coli* strain, LeuA*BCD elongated phenylpyruvate into homophenylpyruvate. The strain produced 4 mg/L phenylpropanol.

2-Keto acid-based aldehyde

Aldehydes are the precursors to alcohols in the 2-keto acid-based pathways. Aldehydes serve as chemical feedstocks in the synthesis of larger and more complex molecules [57]. Aldehydes have been difficult to produce microbially due to numerous promiscuous ALRs in *E. coli*.

Rodriguez and Atsumi engineered *E. coli* for the production of isobutyraldehyde, the precursor to isobutanol [58]. With the isobutyraldehyde pathway (*alsS, ilvCD,* and *kivd*) expressed in a strain of *E. coli* lacking all fermentative pathways (*adhE, ldh, frd, pflB, pta,* and *fnr*). The strain converted all the isobutyraldehyde to isobutanol with endogenous ALRs. However, by deleting six ALR candidate genes (*yqhD, adhP, eutG, yiaY, yjgB, betA,* and *fucO*) from the genome, the engineered *E. coli* strain was able to produce high amounts of isobutyraldehyde and low isobutanol. Individual deletions of the candidate ALRs did not reduce isobutanol formation, indicating that the endogenous ALR activity was the result of multiple enzymes in *E. coli*. Through a series of in vivo and in vitro characterizations, it was revealed that AdhP, EutG, YjgB, and FucO exhibited isobutyraldehyde reductase activity. Long-term production was carried out with the optimized *E. coli* strain, using a gas-stripping system to collect the isobutyraldehyde product outside the production flask. The strain was able to produce 35 g/L isobutyraldehyde after 5 days with a final yield of 45 %. However, the strain produced ~10 g/L isobutanol as well, indicating further ALR activity still remainedin the engineered *E. coli*.

In follow-up work, Rodriguez and Atsumi further elucidated ALRs in *E. coli* by performing a comprehensive screening of oxidoreductases [59]. By expressing candidate ALRs in the optimized isobutyraldehyde strain, AL626, active ALRs would produce significant isobutanol titers, while non-active enzymes would produce isobutyraldehyde. With this screening, 44 candidate ALRs were tested and five ALRs were confirmed (YahK, DkgA, YbbO, GldA, and YghA). The five enzymes were characterized in vitro. YahK and DkgA were found to be the most active and promiscuous of the five. Finally, the five ALRs were deleted from the genome of *E. coli* strain AL626, and the new strain was characterized for its ability to convert various aldehydes to alcohols. This strain showed little to no conversion of branched-chain aldehydes and was also unable to convert acetaldehyde to ethanol, or longer chain aldehydes to alcohols such as decanal and dodecanal. This work detailed the majority of ALRs that are found in *E. coli* and may pave the way to produce a wide range of aldehydes and alkane compounds in *E. coli*.

2-Keto acid-based acids

A 2-keto acid-based alcohol pathway can be converted into an acid pathway by using ALDH instead of ADH (Fig. 1). Organic acids are currently produced from petroleum, widely used as additives for food or beverages andare an important feedstock for chemical industries. Thus producing organic acids from renewable sources is desirable.

Isobutyric acid

Isobutyric acid is a carboxylic acid with a four-carbon chain. Various useful derivatives from isobutyric acid are known [82]. Isobutyric acid can be chemically produced from propene, carbon monoxide, and water in the presence of strong acids [11]. In nature, the butyric acid pathway has been identified in bacteria [48]. However, no natural organism has been identified that produces significant amounts of isobutyric acid.

Zhang et al. [82] constructed an isobutyric acid synthesis pathway in *E. coli*. The pathway design closely resembles that of the isobutanol pathway, with the exception that the ALDH PadA is used instead of an ADH at the end of the pathway (Fig. 1). ALDH oxidizes isobutyraldehyde to isobutyric acid. Isobutyraldehyde is produced via 2-KIV. To increase carbon flux from glucose to isobutyric acid, four genes (*alsS* from *B. subtilis*, *ilvD* from *E. coli*, *kivd* from *L. lactis*, and *padA* from *E. coli*) were introduced into the *E. coli* strain BW25113. This strain produced 4.8 g/L of isobutyric acid and also 4.8 g/L isobutanol as a byproduct. Since YqhD competes for the aldehyde with PadA, *yqhD* was deleted from the strain. The Δ*yqhD* mutant showed decreased isobutanol production (0.8 g/L) and increased the isobutyrate production (12 g/L) from 40 g/L glucose. The yield was 0.29 g/g glucose which represents 59 % of the theoretical maximum.

Lang et al. [42] engineered *Pseudomonas* sp. strain VLB120 as a host for isobutyric acid production. *Pseudomonas* species have high potential for bioremediation because they possess a rich pool of pathways for the degradation of a variety of non-natural toxic compounds such as aromatic organics, and they have high tolerance for organic chemicals [33, 69, 76]. In addition, carbon is utilized in *Pseudomonas* with little production of byproducts such as acetate, succinate, and lactate [29] improving carbon yields and simplifying downstream processing in comparison to other organisms. The genes in the isobutyric acid synthesis pathway (*alsS* from *B. subtilis*, *ilvCD* from strain VLB120, *kivd* from *L. lactis*, and *ALDH* from*Pseudomonas* sp. strain VLB120) were expressed. The genes involved in isobutyric acid degradation and competing pathways for 2-KIV were deleted. The production of isobutyric acid using the engineered strain reached 2.4 g/L from 20 g/L glucose. The yield was 0.12 g/g glucose, which is about 25 % of the theoretical maximum.

Isovaleric acid and isocaproic acid

Isovaleric acid is naturally produced by some plants [79]. Isovalerate esters have pleasing scents and are used widely as fragrances and flavors. Isovaleric acid is also valuable for industry. For example, methyl isobutyl ketone (MIBK) and diisobutyl ketone (DIBK) can be synthesized from isovaleric acid by condensation with acetic acid and self-condensation, respectively [77]. The annual production of these ketones was more than 1 billion pounds [54], making it among the top ten most widely used organic solvents in industry. Although isovaleric acid is naturally produced as a minor metabolite in lactobacillus and yeast [41], no microorganism has been discovered to accumulate a significant quantity of isovaleric acid.

To biosynthesize isovaleric acids, Xiong et al. [77] reported a synthetic metabolic pathway in *E. coli*. The pathway is similar to the 3-methyl-1-butanol pathway described above (Figs. 2, 3). In this pathway, 2-KIC is converted to an aldehyde by KDC and then oxidized to isovaleric acid by ALDH. The combination of indolepyruvate decarboxylase (IPDC) from *S. typhimurium* and PadA from *E. coli* produced 8.9 g/L isovalerate from 40 g/L glucose, which represents a yield of 0.22 g/g glucose which is 58 % of the theoretical maximum.

Isocaproic acid can be converted to methyl isoamyl ketone (MIAK) using acetic acid. The market volume is roughly 80 million pound per year. By modifying the isovaleric acid pathway, Xiong et al. [77] also developed an isocaproic acid biosynthesis pathway in *E. coli* by modifying the isovaleric acid pathway. LeuA was replaced with a LeuA mutant (G462D/S139G/H97L) that catalyzes further elongation of ketoleucine to ketohomoleucine [81]. KCD and ALDH were screened for isocaproic acid production. The combination of IPDC with an L544A mutation and α-ketoglutaric semialdehyde dehydrogenase from *Burkholderia ambifaria* (KDH_{ba}) produced the highest titer in this study, reaching 5 g/L isocaproic acid.

2-Methylbutyric acid and valeric acid

2-methylbutyric acid and valeric acid are important feedstocks because these chemicals serve as intermediates for a variety of application such as plasticizers, lubricants, and pharmaceuticals. The total U.S. consumption of 2-methylbutyric acid and valeric acid was approximately 14,000 metric tons in 2005 [25]. Estersderived from valeric acid as valeric biofuels are compatible as transportation fuels infrastructure and so can be used as alternatives for gasoline and diesel. 2-methylbutyric acid and valeric acid are produced by oxidizing 2-methyl butyraldehyde and valeraldehyde in the Oxo process (also known as hydroformylation) in which 2-butene and 1-butene are reacted with synthesis gas [25].

2-methylbutyric acid and valeric acid biosynthesis pathways have been identified in nature. 2-methylbutyric acid has been reported as the main product of anaerobic metabolism of parasites such as *Ascaris lumbricoides* and *Ascaris*

suum [13, 15]. Propionate and acetate are the precursors for 2-methylbutyric acid in these organisms. Fang et al. [30] reported valeric acid accumulation during metabolism of mixed rumen microorganisms as well as during the hydrogen production. However, the C5 acid production levels were very low.

Dhande et al. [23] expanded the leucine and isoleucine biosynthesis pathways in *E. coli* by expressing KDC and ALDH. The 2-methylbutyric acid and valeric acid pathways share the metabolic route from glucose toward 2-KB via threonine. The carbon flux toward 2-KB was enhanced by overexpressing *thrABC* and *ilvA*.

For 2-methylbutyric acid production, 2-KB is converted into 2-KMV by IlvGMCD (Fig. 2). KDC and ALDH then convert the 2-KMV to 2-methylbutyric acid. For valeric acid production, 2-KB is elongated through two cycles of "+1" carbon chain elongation (Fig. 4) to make 2-ketocaproate (2-KC). The 2-KC can then be decarboxylated and subsequently oxidized to valeric acid. The optimal combinations of the enzymes enabled production of 2.6 g/L 2-methylbutyric acid and 2.6 g/L for valeric acid. These values correspond to yields of 17 % and 22 % of theoretical maximum for 2-methylbutyric acid and valeric acid, respectively.

2-Keto acid-based esters

Esters are a class of compounds that are synthesized by the condensation of an alcohol and an organic acid. Larger esters are fatty acid esters of glycerol and commonly occur as fats, oils, and waxes. Fatty Acid Ethyl/Methyl Esters (FAEEs/FAMEs) are known as biodiesels and are typically generated from the transesterification of acylglycerols with ethanol/methanol [19]. FAEE/FAMEs are highly desirable as fuels and compatible with the current diesel infrastructure [2]. Kalscheuer et al. [39] previously developed a metabolic pathway to a microbially produced biodiesel ester, called 'microdiesel,' by combining a wax ester synthase/acyl-CoA:diacylglycerol acyltransferase from *Acinetobacter baylyi* with enzymes derived from *Zymomonas mobilis* to overproduce ethanol. This strategy to produce biodiesel was successful and further developed by altering enzymes, deleting competing genes, and incorporating a regulation [70, 80].

In nature, small volatile esters (C4-C12) produced in many ripening fruits and flowers serve an important role in attracting animals and for protection against pathogens. Some fungi such as yeast also generate such esters during fermentation processes (i.e., wine and beer). Such small esters are utilized as flavors and fragrances in a global market worth $16.6 billion in 2012 [36]. Small esters are also used in industry for solvents, coatings, and paints [37].

Low-molecular weight esters are commonly produced by acid-catalyzed esterification synthesis of an alcohol and an organic acid [37, 49]. In water, the reaction is thermodynamically favored toward hydrolysis of the ester at ambient temperatures. In cells, acyl-CoA units are commonly utilized as the acid component for ester formation. Thus, the release of free CoA upon esterification of the acyl group with an alcohol provides the free energy needed to facilitate ester formation in water at ambient temperatures.

Efficient alcohol production systems have been developed as described above. Various esters can be produced by combination of these alcohols and acyl-CoAs. Small volatile ester formation by several alcohol *O*-acetyltransferases (ATFs) by *S. cerevisiae* during beer and wine fermentation has been well characterized [46, 74].

Rodriguez et al. [60] reported the production of various esters using the 2-keto acid-based alcohol pathways and an ATF (Fig. 1). First, to test the ability of the pathway, they evaluated the production of various acetate esters in *E. coli* using any one of several 2-keto acid-based alcohols (ethanol, isopropanol, isobutanol, 3-methyl-1-butanol, 2-methyl-1-butanol, and 2-phenylethanol). Fed 3 g/L of each 2-keto acid (2-KB, 2-KIV, 2-KMV, 2-KMV, and Phenylpyruvate), the engineered *E. coli* produced 0.5-2 g/L of each acetate ester after 24 h of incubation. The conversion rate of branched-chain 2-keto acids to acetate esters was relatively high (>80 %) in these experiments suggesting that both KDC and ATF are relatively promiscuous. As such, the profile of esters produced can be determined by the spectrum of 2-keto acids in the cell.

Isobutyl acetate

Isobutyl acetate has a fruity flavor and is used as a flavoring agent for foods and beverages and as a common solvent in industry. Isobutyl acetate can be synthesized from isobutanol and acetyl-CoA by using ATF [74]. Isobutanol production from glucose has been well established [4, 7], and acetyl-CoA is the most abundant acyl-CoA in cell.

Rodriguez et al. introduced codon-optimized ATF1 from *S. cerevisiae* into *E. coli* strain JCL260 (Δ*adhE,* Δ*frd,* Δ*ldhA,* Δ*pta,* Δ*pflB,* and Δ*fnr*) harboring the isobutanol pathway. This strain produced 2.7 g/L of isobutyl acetate at 24 h. However, from 24–48 h, the titer only marginally increased to ~3 g/L. It was determined that *E. coli* was not unable to grow in the presence of 3 g/L isobutyl acetate. To alleviate isobutyl acetate toxicity and further increase titer and yield, they incorporated an in situ removal system for isobutyl acetate. Hexadecane was chosen as an extraction layer because it does not contain hydrogen-bonding elements, is nontoxic to *E. coli*, has low water miscibility, and cannot be degraded by *E. coli*. The use of a hexadecane

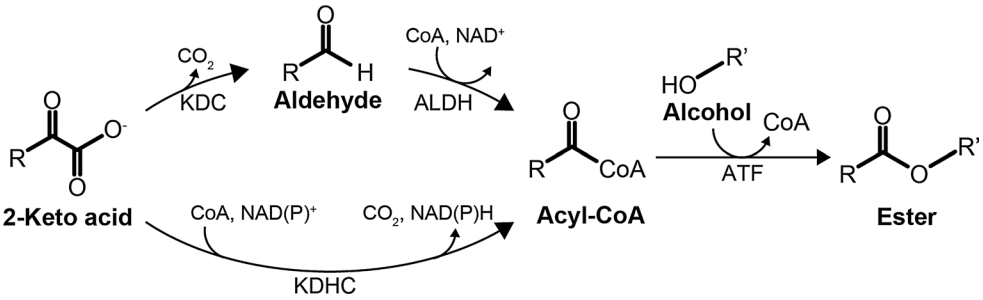

Fig. 5 Biosynthesis pathway for higher chain esters. *KDC* 2-keto acid decarboxylase, *ALDH* aldehyde dehydrogenase 2, *KDHC* branched-chain keto acid dehydrogenase complex, *ATF* alcohol *O*-acyltransferase

layer enabled the strain to produce 17.2 g/L of isobutyl acetate at 72 h. The yield was 0.3 g/g glucose, which is 80 % of the theoretical maximum.

Higher chain esters

Branched-chain esters are commonly used as solvents for industry and have similar features and energy density as fuels [16]. This ester synthesis chemistry can be further extended to using larger acyl-CoA units, such as isobutyryl-CoA and butyryl-CoA. Some organisms naturally metabolize 2-keto acids to form branched-chain CoAs by branched-chain keto acid dehydrogenase complex (KDHC) (Fig. 5) [53].

E. coli is unable to generate significant amounts of branched-chain acyl-CoAs. Rodriguez et al. [60] reported two different types of branched-chain acyl-CoA pathways in *E. coli* (Fig. 5). Their most successful pathway utilized KDHC from *Pseudomonas putida* [60]. In the *E. coli* strain expressing this KDHC, various 2-keto acids (2-KV, 2-KIV, 2-KMV, and 2-KIC) were converted to corresponding acyl-CoAs. Expression of KDHC along with a suitable ATF allowed production of several isobutyrate esters and butyrate esters with a suitable ATF. Isobutyl isobutyrate can be efficiently produced from glucose in *E. coli*. These pathways require further improvement to achieve increased branched-chain acyl-CoA flux and more active ATFs for these acyl-CoAs. However, these higher ester pathways are now possible with adequate flux of alcohol and acyl-CoA molecules.

Conclusions

Production processes from biomass have attracted a lot of attention, and various metabolic pathways have been engineered in microorganisms. Engineered 2-keto acid-based metabolic pathways are relatively successful in productivity and yield for several reasons. The enzymes in 2-keto acid synthesis are highly active. Selective pressure may have actuated a high flux toward amino acids. Additionally, the irreversible decarboxylation reaction to form 2-keto acids plays a role as a driving force [64]. Furthermore, the accumulated knowledge of amino acid biosynthesis pathways enables scientists to carry out efficient engineering of 2-keto acid-based pathways.

Challenges, however, still remain for industrial production. One of the biggest challenges in using the 2-keto acid pathways is product specificity. In most of cases, Kivd from *L. lactis* is used for decarboxylation due to its high activity. In previous reports, several strategies (using protein engineering, protein scaffold [26], dynamic controller [73], and so on) are applied for improved product specificity. These strategies may compensate for the drawback of 2-keto acid-based pathways. Another limit is the tolerance of the host toward the product. In general, tolerance is one of the biggest limiting factors determining the final titer. To overcome this issue, host strain engineering and/or in situ removal system have been applied [8, 17, 60].

2-Keto acid-based pathways also have further potential. As described, the final product from 2-keto acid-based pathways can be changed by altering a single enzyme. Recently, Schirmer et al. [61] identified aldehyde deformylating oxygenase (ADO). Because hydrocarbons can be produced from renewable source via aldehyde, ADOs are under intense investigation. If it is possible to engineer the specificity and the activity of ADO, a "2-keto acid-based alkane pathway with high flux" could be constructed in the future.

The other key advantage of 2-keto acid pathways is that it is applicable for various hosts. For instance, the isobutanol pathway has been successfully introduced into a thermophilic cellulolytic bacterium [47], chemolithotrophs [45], and autotrophs [5, 21]. These engineered microorganisms enable direct conversion of cellulosic biomass or CO_2 to isobutanol. Genetic manipulation tools and gene regulation systems for these microorganisms have been and continue to be developed for even more sophisticated metabolic engineering strategies. With these efforts, biological production will develop into an ever more mature process for renewable fuels and chemicals.

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