Trends in Biotechnology

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Opinion

Strategies for optimizing acetyl-CoA formation from glucose in bacteria

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Acetyl CoA is an important precursor for various chemicals. We provide a metabolic engineering guideline for the production of acetyl-CoA and other end products from a bacterial chassis. Among 13 pathways that produce acetyl-CoA from glucose, 11 lose carbon in the process, and two do not. The first 11 use the Embden–Meyerhof–Parnas (EMP) pathway to produce redox cofactors and gain or lose ATP. The other two pathways function via phosphoketolase with net consumption of ATP, so they must therefore be combined with one of the 11 glycolytic pathways or auxiliary pathways. Optimization of these pathways can maximize the theoretical acetyl-CoA yield, thereby minimizing the overall cost of subsequent acetyl-CoA-derived molecules. Other strategies for generating hyper-producer strains are also addressed.

Highlights

Industrial production of acetyl-CoA derivatives, especially commercial chemicals, depends on low feedstock costs.

Enhancing acetyl-CoA synthesis from feedstocks such as glucose is essential for reducing the production costs of these end products.

Reasonable use of carbon-saving pathways can maximize the production yield and rate of acetyl-CoA while lowering CO_2 emission and oxygen dependency.

The balance between reducing equivalents and energy must be well designed, especially for anaerobic processes.

Fine-tuning the carbon flux between carbon-saving pathways and other pathways via metabolic engineering strategies is not only essential for redox and power balancing but also impels cells to fully utilize the feedstock to maximize the yield of acetyl-CoA and other end products.

Challenges in metabolic engineering of acetyl-CoA formation from glucose in bacteria

Acetyl-CoA derived from glucose is the sole precursor for a variety of commercially valuable compounds such as fatty acids, *n*-butanol, isopropanol, acetone, butyric acid, hexanoic acid, polyhydroxyalkanoates, and polyketides. Acetyl-CoA also serves as a partial precursor for L-glutamate (L-Glu) and its derivatives such as L-arginine, L-proline, and L-glutamine, as well as an alternative precursor for ethanol, succinate, and isoprenoids (Figure 1A).

Therefore, one strategy to efficiently produce these **commodity chemicals** (see Glossary) by fermentation at large scale and low cost is to optimize the efficiency of acetyl-CoA biosynthesis from glucose using metabolic engineering. This is done through rewiring central carbon metabolism [1] via recombinant DNA technologies to improve the acetyl-CoA **yield** and **productivity** with reduced net loss of carbon and oxygen dependency. There have been many studies specifically on acetyl-CoA formation with regards to metabolic engineering. Krivoruchko and colleagues reviewed the current state of metabolic engineering for acetyl-CoA formation in *Saccharomyces cerevisiae* and *Escherichia coli* [2]. Van Rossum and coworkers reviewed the metabolic engineering of acetyl-CoA formation from glucose in *S. cerevisiae* and provided strategies for the subsequent synthesis of four bulk chemicals [3]. Finally, Ku and colleagues reviewed strategies for increasing acetyl-CoA flux by metabolic engineering [4]. Generally, the optimal strategies to engineer acetyl-CoA production for different bioproducts are distinct and depend greatly on the requirements for acetyl-CoA, redox cofactors, and ATP under either aerobic or anaerobic conditions. This Opinion article attempts to find a generally optimal strategy for maximum acetyl-CoA production from glucose. The proposed solutions emerge from analyses of various acetyl-CoA synthetic pathways and the latest advances in metabolic engineering.

Analysis of the 13 pathways of acetyl-CoA synthesis from glucose in bacteria

Six enzymes are responsible for catalyzing the last reaction of the acetyl-CoA synthesis pathways in prokaryotes (Table 1 and Figure 1B). Of these six enzymes, the **pyruvate dehydrogenase**

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complex (PDHc) [5], pyruvate-formate lyase (PFL) [6], and pyruvate ferredoxin/flavodoxin oxidoreductase (PFOR) [7] directly convert pyruvate to acetyl-CoA. The other three enzymes – acetylating acetaldehyde dehydrogenase (A-ALD) [8], Acetyl-CoA synthetase (ACS) [9], and phosphotransacetylase (PTA) [10] – catalyze the conversion of intermediate metabolites (acetaldehyde, acetate, and acetyl phosphate) into acetyl-CoA. In addition, routes relying on PFL, ACS, and PTA can be combined with formate dehydrogenase (FDH) [11], four pathways that supply acetate [12,13], and four pathways that supply acetyl phosphate [14–16] to generate nine different acetyl-CoA synthesis pathways, making a total of 13 acetyl-CoA synthesis pathways that are available in bacteria (Table 1 and Figure 1B).

The **PDHc pathway** naturally only works under aerobic conditions because its E3 component, one of three components in the PDH complex, is sensitive to NADH levels [17,18]. The low level of intracellular NADH/NAD^+ (~0.03) under aerobic conditions allows PDHc to remain active [19]. Under anaerobic conditions, inhibition of E3 by high levels of intracellular NADH/NAD^+ (~0.7) [19] results in loss of PDHc function [18]. This issue can be resolved by increasing the expression of PDHc [20,21] or by mutating E3 to lower its NADH sensitivity [22–24]. Thus, the PDHc pathway can technically be used under both aerobic and anaerobic conditions. On the other hand, the **three pyruvate decarboxylase (PDC) pathways** [25] (PDC-A [26], PDC-B [12,27], and PDC-C [1]) work under both aerobic and anaerobic conditions because the enzymes involved can function with or without oxygen. The **PFOR pathway** and **two PFL pathways** (PFL and PFL-FDH) were thought to be only functional anaerobically because an oxygen-sensitive radical intermediate is formed during the reaction [6,7]. **Four pyruvate oxidase (POX)-catalyzed routes** (POX-A, POX-B, POX-C and POX-D) require oxygen in the reaction to produce the precursor of acetyl-CoA [14]. The other two pathways (the **PK-A pathway** and the **PK-B pathway**) are based on phosphoketolase (PK) [28], leading to net hydrolysis of ATP, and they must therefore be combined with other pathways to work [10].

Eleven of these routes decompose one molecule of glucose into two molecules of acetyl-CoA with two carbon atoms being lost through the EMP pathway, whereas the other two pathways based on PK split one molecule of glucose into three molecules of acetyl-CoA without losing any carbon atoms [4] (Table 1). The two pathways based on PK are more efficient in terms of carbon use than the other 11 routes. Taking into consideration the yield of the reducing equivalents and ATP from glucose among the 11 pathways that lose carbon, the PDHc, PFL-FDH, PDC-A, and PFOR pathways are the most preferred, followed by the PDC-B and PDC-C pathways, with the PFL and POX pathways being the least preferred (Table 1).

Among the 11 pathways that lose carbon, the PDHc pathway is optimal for metabolic engineering owing to its ability to directly convert pyruvate to acetyl-CoA without using a complex electron acceptor, the absence of side reactions, and its functionality in bacteria under both aerobic and anaerobic conditions. The PFL-FDH pathway is suboptimal but remains useful because PFL is common in facultative and obligate anaerobic bacteria. It can therefore replace the PDHc pathway when high levels of NADH/NAD^+ inhibit PDHc activity under anaerobic conditions. The remaining pathways have special requirements that limit their applications in metabolic engineering. The PFOR pathway is also widely distributed in bacteria and is generally coupled with other pathways forming H_2, H_2S, or N_2 (through fixation) [7], but few studies on the metabolic engineering of this pathway have been reported [29]. POX pathways have a low level of redox formation and special requirements. The hydrogen peroxide-producing POX pathway requires cofactors FAD and thiamine pyrophosphate (TPP) to function [14], and the acetate-producing POX pathway requires membrane-embedded coenzyme Q8 [30], menaquinone [31], or quinone [32] as a direct electron acceptor. There are no current reports on the use of PDC

pathways for acetyl-CoA synthesis in bacteria, even though PDC-A has the same stoichiometric relations as the PDHc pathway.

Comparing the two pathways that save carbon, the PK-A pathway consumes less ATP (2) than PK-B, which is preferred because it improves the theoretical product yield [15], but it does not generate ATP or redox cofactors. Therefore, the PK-A pathway can be used in combination with the 11 pathways (especially PDHc, PFL-FDH, PDC-A, and PFOR) that generate the highest amount of ATP and redox cofactors from glucose, or with other pathways that also generate ATP and redox cofactors.

General strategies for bacterial metabolic engineering of acetyl-CoA formation from glucose

The general strategy to maximize the theoretical yield of acetyl-CoA is to combine the PK-A pathway with the PDHc, PFL-FDH, PDC-A, or PFOR pathways to generate acetyl-CoA, redox cofactors, ATP, and/or other products irrespective of biomass formation. In addition, processes should preferably be anaerobic to maximize product yields and eliminate the cost of oxygenation in large reactors [33].

Based on the strategies mentioned previously, the most appropriate strategies for the production of different end products by coupling PK-A and other pathways under anaerobic and aerobic conditions are summarized in Table 2 and Figure 1D.

Appropriate strategies under anaerobic conditions

The PK-A pathway, combined with the PDHc, PFL-FDH or PROR pathway, is appropriate for products that consume <2 reducing equivalents per acetyl-CoA molecule. Products that can be generated via these pathways include ethyl acetate [34], butyric acid [35,36], hexanoic acid [37,38], caprylate [39], and caproate [40,41] (Table 2 and Figure 1D). For example, producing 1 butyric acid requires 2 acetyl-CoA and 2 NADH with the net formation of 1 ATP [36]. Combining the PK-A pathway with the PDHc, PFL-FDH, or PFOR pathway has a metabolic flux ratio of 2:1 and produces a maximum butyric acid yield of 1.2 mol/mol glucose (0.586 g/g glucose), which is 20% higher than the amount produced by the PFL pathway alone (1.0 mol/mol glucose; 0.489 g/g glucose). Hexanoic acid, a **medium-chain carboxylic acid** that has two more carbons than butyric acid, is synthesized from 3 acetyl-CoA and 4 NADH via reverse β-oxidation [37]. When coupled with PK-A the PDHc/PFL-FDH/PFOR flux ratio is 1:3, and the maximum hexanoic acid yield reaches 0.75 mol/mol glucose (0.484 g/g glucose), which is 12.6% higher than the amount obtained via only the PDHc or PFL-FDH pathway (0.666 mol/mol glucose; 0.429 g/g glucose). Other **even-numbered medium-chain carboxylic acids** such as caprylate and caproate [40] have 2 and 4 more carbons, respectively, than hexanoic acid. Combining the PK-A pathway with the PDHc, PFL-FDH, or PFOR pathway could increase the **theoretical maximum yield** (TMY) of caprylate and caproate on glucose, but the degree of improvement decreases as the chain length increases (Figure 1D).

Under some circumstances, the PK-A pathway cannot be configured to achieve a higher TMY of the end products from glucose (Table 2 and Figure 1D). In situations where two redox cofactors are required for each acetyl-CoA molecule consumed, such as in ethanol and butanol production [42], the PDHc, PFL-FDH, or PFOR pathway is preferred. In situations where two or more redox cofactors are required for each acetyl-CoA consumed, as in the production of **odd-numbered carboxylic acids** such as valerate [40], heptylate, or nonanoate [43]. These compounds require glucose via the PDHc, PFL-FDH, or PFOR pathway to provide acetyl-CoA molecules and a portion of the redox cofactors, as well as the pentose phosphate pathway (PPP) pathway to

Glossary

Commodity chemicals: these play a key role in supporting diverse industries as essential basic materials.
Even-numbered medium-chain carboxylic acids: carboxylic acids that have a straight chain of even-numbered carbon atoms.
Four pyruvate oxidase (POX)-catalyzed routes: these use the EMP pathway, H_2O_2-forming or acetic-acid-forming pyruvate oxidase, combined with phosphotransacetylase or acetate kinase and acetyl-CoA synthetase, respectively, to produce acetyl-CoA from glucose.
Medium-chain carboxylic acids: carboxylic acids with an aliphatic straight carbon chain of 6–12 carbon atoms.
Odd-numbered carboxylic acids: carboxylic acids that have a straight chain of odd-numbered carbon atoms.
PDHc pathway: this pathway uses the Embden–Meyerhof–Parnas (EMP) pathway and the pyruvate dehydrogenase complex (PDHc) to split 1 glucose to produce 2 acetyl-CoA with the emission of 2 CO_2, producing 4 NADH and 2 ATP.
PFOR pathway: this pathway uses the EMP pathway and pyruvate ferredoxin/flavodoxin oxidoreductase (PFOR) to produce acetyl-CoA from glucose.
PK-A pathway: this uses phosphoketolase (PK) and other enzymes to cycle catalyze fructose-6-phosphate to acetyl phosphate from glucose, which is then converted to acetyl-CoA via phosphotransacetylase.
PK-B pathway: the acetyl phosphate produced can also converted to acetyl-CoA by acetate kinase and acetyl-CoA synthetase.
Productivity: the amount of product generated from 1 l volume of reaction system per hour.
Pyruvate dehydrogenase complex (PDHc): the complex is composed of pyruvate dehydrogenase (E1), dihydrolipoamide acetyltransferase (E2), and dihydrolipoamide dehydrogenase (E3). E1 catalyzes the decarboxylation of pyruvate to CO_2 with the formation of C2-hydroxyethylidene thiamin diphosphate (ThDP) intermediate and the reductive acetylation of the lipoyl groups covalently attached to the E2. The E2 transfers an acetyl moiety to CoA to form acetyl-CoA. E3 transfers electrons from the dihydrolipoyl moieties of E2 to FAD and then to NAD.

supply the other required redox cofactors. For products formed from acetyl-CoA that do not require redox cofactors and ATP, such as acetone [44,45], the PFL pathway combined with pathways that produce hydrogen is preferred.

Appropriate strategies under aerobic conditions

For products that require <2 reducing equivalents per acetyl-CoA molecule, such as isoprenoids [46], lipids [47], poly(3-hydroxybutyrate) (PHB) [48,49], and glycolate [50], or products that do not require reducing equivalents and ATP, such as acetone [51], the PK-A pathway can be combined with the PDHc or PDC-A pathway (Table 2 and Figure 1D).Taking farnesene as an example, synthesizing 1 farnesene requires 9 acetyl-CoA, 6 reducing equivalents, and 9 ATP [52]. Using only the pathway that loses carbon, 4.5 glucose molecules are required for the synthesis of 1 farnesene molecule, and its maximum yield from glucose is 0.222 mol/mol glucose (0.252 g/g glucose). When adapting the combined PK-A and PDHc (or PDC-A) pathways with a metabolic flux ratio of 1.8:2.25, the yield increases by nearly 22% to 0.271 mol/mol glucose (0.308 g/g glucose). Applying a similar strategy can also increase the TMY of isoprene [53], amorphadiene [54], lycopene [55,56], β-carotene [57], and astaxanthin [58]. For lipids, using stearic acid as an example, the synthesis of 1 stearic acid requires 9 acetyl-CoA molecules, 16 reducing equivalents, and 8 ATP [47]. Using only the PDHc or PDC-A pathway, two reducing equivalents remain, excluding the byproduct pathway used for balancing the reducing equivalents, and the TMY of stearic acid can reach 0.222 mol/mol glucose (0.351 g/g glucose). For the combined PK-A and PDHc (or PDC-A) pathway with a metabolic flux ratio of 4.026:0.315, the theoretical yield can be further increased by 3.6 % to 0.23 mol/mol glucose (0.363 g/g glucose).

As mentioned previously, the PK-A pathway is not appropriate for the production of acetone by anaerobic fermentation but is advantageous in aerobic fermentation [44], where 1 molecule of glucose produces 1.5 molecules of acetone [51], coupled with the consumption of 1/38 of a glucose molecule to supplement the net ATP consumption [59]. In addition, autotrophic microorganisms can also assimilate H_2 to supplement ATP [60], such that the yield can reach 1.5 mol/mol glucose (Table 2 and Figure 1D).

For products that require >2 molecules of reducing equivalents per acetyl-CoA molecule consumed, such as the unsaturated fatty acids docosahexaenoic acid (DHA) [61], eicosapentaenoic acid (EPA) [62], and polyketides, it is suitable to combine the PDHc or PDC-A pathway with other pathways that have an excess of reducing equivalents, such as the PPP pathway. Under these conditions it is difficult to utilize the PK-A pathway to improve the TMY of acetyl-CoA (Table 2 and Figure 1D).

For L-Glu [63] and its derived amino acids that require acetyl-CoA and oxaloacetate as their precursors, the PK-A pathway combined with glycolysis can increase the TMY on glucose. For example, 1 acetyl-CoA required for synthesizing 1 L-Glu is produced by 0.4 glucose molecules via the PK-A pathway, and 1 oxaloacetate and 1 NADH molecule are supplied by 0.6 glucose molecules through the EMP pathway and pyruvate carboxylase [64]. As a result, the maximum yield of L-Glu from glucose can reach 1.2 mol/mol glucose (0.98 g/g glucose), which is 20% higher than that of the traditional PDHc pathway, yielding 1.0 mol/mol glucose (0.82 g/g glucose) [63,64] (Table 2 and Figure 1D).

In general, the less redox cofactors are required per acetyl-CoA for a given product's synthesis, the more the TMY could be improved utilizing the PK pathway. However, for products that require two or more redox-cofactors per acetyl-CoA (e.g., ethanol and valerate) cannot have its TMY improved using the PK pathway (Table 2 and Figure 1D).

Reductive carbon sources: these are used to produce acetyl-CoA coupled with a net production of redox cofactor.
Theoretical maximum yield (TMY): when the substrate is totally converted to the product, the yield of the product on that substrate is the theoretical maximum.
Three pyruvate decarboxylase (PDC) pathways: the PDC-A pathway uses the EMP pathway, PDC, and acetylating acetaldehyde dehydrogenase to produce acetyl-CoA from glucose. PDC-B uses the EMP pathway, PDC, acetaldehyde dehydrogenase, and acetyl-CoA synthetase to produce acetyl-CoA from glucose. PDC-C uses the EMP pathway, PDC, acetate kinase, and phosphotransacetylase to produce acetyl-CoA from glucose.
Two PFL pathways: the PFL pathway combines the EMP pathway with pyruvate-formate lyase (PFL) to split one glucose to produce 2 acetyl-CoA with the formation of 2 formate, 2 NADH, and 2 ATP. The PFL-FDH pathway combines the PFL pathway with formate dehydrogenase (FDH) to convert formate acids into CO_2, H_2O, and redox cofactors.
Yield: mole or weight conversion rate from feedstock such as glucose to products.

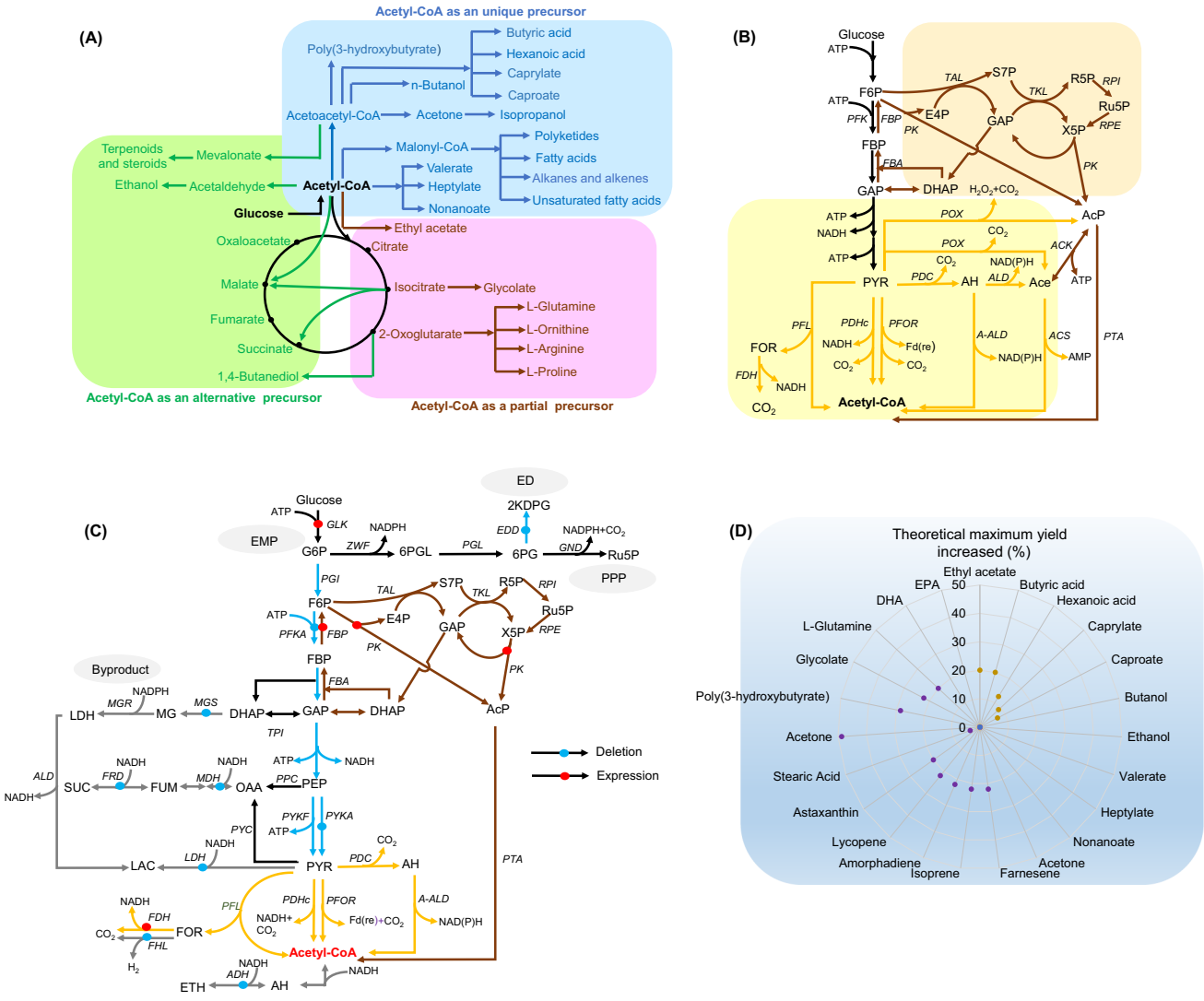

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Figure 1. Synthesis of acetyl-CoA and onward conversion to other products. (A) Acetyl-CoA is the sole (blue), partial (pink) or alternative (green) biosynthetic precursor of various chemicals. (B) The 13 acetyl-CoA synthesis pathways: 11 lose carbon in the process (yellow) and two do not (dark red). (i) The PDHc pathway (uses the EMP pathway and PDHc to split 1 glucose molecule, forming 2 acetyl-CoA molecules with 2 CO_2 molecules emitted, producing 4 NADH and 2 ATP). (ii) Two PFL pathways use the EMP pathway and pyruvate-formate lyase to split one glucose, forming 2 acetyl-CoA. The PFL pathway forms 2 formates, 2 NADH, and 2 ATP, whereas the PFL-PDH pathway converts formate into CO_2, H_2O, and redox cofactors using formate dehydrogenase. (iii) The PFOR pathway uses the EMP pathway and pyruvate ferredoxin/flavodoxin oxidoreductase to produce acetyl-CoA from glucose. (iv) Three PDC pathways: PDC-A uses the EMP pathway, pyruvate decarboxylase, and acetylating acetaldehyde dehydrogenase to produce acetyl-CoA from glucose. PDC-B uses the EMP pathway, pyruvate decarboxylase, acetaldehyde dehydrogenase, and acetyl-CoA synthetase to produce acetyl-CoA from glucose. PDC-C uses the EMP pathway, pyruvate decarboxylase, acetate kinase, and phosphotransacetylase to produce acetyl-CoA from glucose. (v) Four POX-catalyzed routes: these use the EMP pathway, H_2O_2-forming or acetic-acid-forming pyruvate oxidase, combined with phosphotransacetylase or acetate kinase and acetyl-CoA synthetase, respectively, to produce acetyl-CoA from glucose. (vi) The PK-A pathway uses PK and other enzymes to convert glucose to fructose-6-phosphate and then to acetyl phosphate, which is then converted to acetyl-CoA via phosphotransacetylase. In the PK-B pathway acetyl phosphate is converted to acetyl-CoA by acetate kinase and acetyl-CoA synthetase. (C) Blocking the formation of side-products of acetyl-CoA and its precursors, such as lactate, ethanol, succinate, acetate, and formate (grey line). The introduction and enhancement of the PK-A pathway (dark red line), downregulation of the EMP pathway, and disruption of the ED pathways (blue line) diverts the carbon flux to the PK-A pathway. (D) Improvements (%) in the theoretical maximum yield of the products after optimizing the acetyl-CoA supply under anaerobic conditions (orange dots) or aerobic conditions (purple dots). Abbreviations for metabolites: AcP, acetyl phosphate; AH, acetaldehyde; DHAP, dihydroxyacetone phosphate; E4P, erythrose-4-phosphate; ETH, ethanol; F6P, fructose-6-phosphate; FBP, fructose-1,6-bisphosphate; FOR, formate acid; FUM, fumaric acid; G6P, glucose-6-phosphate; GAP, glyceraldehyde-

(Figure legend continued at the bottom of the next page.)

Introducing the PK-A pathway into strains that include the Entner–Doudoroff (ED) pathway but not the EMP pathway (such as *Zymomonas mobilis* [65]) could theoretically increase the yield of acetyl-CoA per mol glucose. However, because the ED pathway produces 50% less ATP than the EMP pathway, the increase in the theoretical yield of acetyl-CoA is less than that from the PK-A pathway combined with the EMP pathway. If the theoretical yield of acetone using the ED pathway is 1 mol/mol glucose, its yield under the adapted PK-A pathway would reach 1.437 mol/mol glucose, which is 0.05 mol/mol glucose less than a combination of the EMP, PDHc, and PK-A pathways.

Adding other carbon sources to increase carbon yield

The metabolic engineering strategies mentioned previously use glucose as a sole carbon source. Alternative **reductive carbon sources** such as fatty acids [66], glycerol [67], ethanol [68], and methanol [69], as well as non-reducing carbon sources such as formaldehyde [70], can be selected to further improve the TMY. However, the cost of these sources in terms of atom economy is higher than that of glucose (Table 3). The cost per mole carbon of feedstocks such as acetate and ethanol will only be lower than that of glucose if both the raw materials (e.g., lignocellulose and blast furnace gas) and the conversion process are inexpensive [71,72].

Discussions on the practicality of these strategies

Acetyl-CoA is a key molecule in sugar metabolism, and its intracellular flux and concentration are strictly regulated [4]. Therefore, optimization of acetyl-CoA formation requires extensive engineering work, such as blocking side reactions, optimizing synthesis pathways, and introducing new pathways to maximize its yield and rate of formation [4].

Blocking side reactions of acetyl-CoA and its precursors

Several endogenous reactions in *E. coli* compete for acetyl-CoA and its precursors and convert them to five undesired products: lactate, ethanol, succinate, acetate, and formate. These reactions are well studied and the key genes to knockout for their removal are clear (Figure 1C). Knockout of *idhA* [73] and *mgsA* [21], two key genes in each of the two pathways that synthesize lactate [74], removes this side product. Deletion of *adhE* eliminates ethanol [11,75]. Disruption of *mdh* and the *frdABCD* operon prevents production of succinate. Because acetate has many pathways of synthesis, knocking out *ackA* and *poxB* is another option to remove this product. Under some circumstances (e.g., when the matching pathway that loses carbon is not the PFL pathway), formate produced by the PFL pathway is technically also a side product, and it can be removed by deletion of *pflA* or *pflB* [76]. When acetate is the target product, *idhA*, *adhE*, and *frdBC* are all knocked out to increase its yield from 0.4 to 1.1 mol/mol xylose. Knocking out *pflB* further increases the yield to 2.2 mol/mol xylose [76].

3-phosphate; 2KDPG, 2-keto-3-deoxy-7-phosphoglucoheptonic acid; LAC, lactate; LDH, lactaldehyde; MG, methylglyoxal; OAA, oxaloacetic acid; PEP, phosphenolpyruvate; 6PG, 6-phosphogluconate; PGL, 6-phosphoglucono 1,5-lactone; PYR, pyruvate; R5P, ribose-5-phosphate; Ru5P, ribulose-5-phosphate; S7P, sedoheptulose-7-phosphate; SUC, succinic acid; X5P, xylulose-5-phosphate, Abbreviations for enzymes: A-ALD, acetylating acetaldehyde dehydrogenase; ACK, acetate kinase; ACS, acetyl-CoA synthetase; ADH, alcohol dehydrogenase; ALD, acetaldehyde dehydrogenase; EDD, 6-phosphogluconate dehydratase; FBA, fructose-1,6-bisphosphate aldolase; FBP, fructose-1,6-bisphosphatase; FDH, formate dehydrogenase; FHL, formate hydrogen lyase; FRD, fumarate reductase; GLK, glucokinase; GND, 6-phosphogluconate dehydrogenase; LDH, lactate dehydrogenase; MDH, malate dehydrogenase; MGR, methylglyoxal reductase; MGS, methylglyoxal synthase; PDC, pyruvate decarboxylase; PDHc, pyruvate dehydrogenase complex; PFK, 6-phosphofructokinase; PFL, pyruvate-formate lyase; PFOR, pyruvate ferredoxin oxidoreductase; PGI, glucose-6-phosphate isomerase; PGL, 6-phosphogluconolactonase; PK, phosphoketolase; POX, pyruvate oxidase; PPC, PEP carboxylase; PTA, phosphotransacetylase; PYC, pyruvate carboxylase; RPE, ribose-5-phosphate isomerase; RPI, ribulose-5-phosphate epimerase; TAL, transaldolase; TKL, transketolase; TPI, triosephosphate isomerase; ZWF, glucose-6-phosphate 1-dehydrogenase. Other abbreviations: ED, Entner–Doudoroff pathway; EMP, Embden–Meyerhof–Parnas pathway; PPP, pentose phosphate pathway.

Table 1. Thirteen acetyl-CoA synthesis pathways

Pathway	Enzymes[a]	EC number	Glucose to acetyl-CoA				Refs
			Reaction stoichiometry[b]	Acetyl-CoA	NAD(P)H	ATP	
PDHc	PDHc	1.2.1.1/2.3.1.12/1.8.1.4	1glucose + $4NAD^+$ + 2ADP + 2Pi + 2CoA → 2acetyl-CoA + $4(NADH + H^+)$ + 2ATP + $2CO_2$ + $2H_2O$	2	4	2	[21]
PDC-A	PDC	4.1.1.1		2	4	2	[3]
	A-ALD	1.2.1.10					
PDC-B	PDC	4.1.1.1	1glucose + $4NAD(P)^+$ + 2ATP + 2CoA + H_2O → 2acetyl-CoA + $4(NAD(P)H + H^+)$ + $2(ADP + Pi)$ + $2CO_2$	2	4	–2	[3]
	ALD	1.2.1.3/1.2.1.4					
	ACS	6.2.1.1					
PDC-C	PDC	4.1.1.1	1glucose + $4NAD(P)^+$ + 2CoA + H_2O → 2acetyl-CoA + $4(NAD(P)H + H^+)$ + $2CO_2$	2	4	0	[1]
	ALD	1.2.1.3/1.2.1.4					
	ACK	2.7.2.1					
	PTA	2.3.1.8					
PFL	PFL	2.3.1.54	1glucose + $2NAD^+$ + 2ADP + 2Pi + 2CoA → 2acetyl-CoA + $2(NADH + H^+)$ + 2ATP + $2CO_2$ + $2H_2O$	2	2	2	[73]
PFL-FDH	PFL	2.3.1.54	1glucose + $4NAD^+$ + 2ADP + 2Pi + 2CoA → 2acetyl-CoA + $4(NADH + H^+)$ + 2ATP + $2CO_2$ + $2H_2O$	2	4	2	[75]
	FDH	1.17.1.9					
PFOR	PFOR	1.2.7.1	1glucose + 4Fd(ox) + 2ADP + 2Pi + 2CoA → 2acetyl-CoA + 4Fd(re) + 2ATP + $2CO_2$ + $2H_2O$[c]	2	4	2	[83]
POX-A	POX/CAT	1.2.3.3/1.11.1.6	1glucose + $2NAD(P)^+$ + $2(ADP + Pi)$ + 2CoA → 2acetyl-CoA + $2(NAD(P)H + H^+)$ + 2ATP + $2CO_2$	2	2	2	[14]
	PTA	2.3.1.8					
POX-B	POX/CAT	1.2.3.3/1.11.1.6	1glucose + $2NAD(P)^+$ + 2CoA → 2acetyl-CoA + $2(NAD(P)H + H^+)$ + $2CO_2$	2	2	0	[3]
	ACK	2.7.2.1					
	ACS	6.2.1.1					
POX-C	POX	1.2.5.1	1glucose + $2NAD^+$ + 2ubiquinone + H_2O + 2CoA → 2acetyl-CoA + $2(NADH + H^+)$ + $2CO_2$ + 2ubiquinol	2	2	–2	[93]
	ACS	6.2.1.1					
POX-D	POX	1.2.5.1	1glucose + $2NAD^+$ + 2ubiquinone + H_2O + 2CoA → 2acetyl-CoA + $2(NADH + H^+)$ + $2CO_2$ + 2ubiquinol	2	2	0	[9]
	ACK	2.7.2.1					
	PTA	2.3.1.8					
PK-A[b]	PK	4.1.2.9/4.1.2.22	1glucose + ATP + 3CoA → 3acetyl-CoA + $(ADP + Pi)$ + $2H_2O$	3	0	–1	[15]
	PTA	2.3.1.8					
PK-B[b]	PK	4.1.2.9/4.1.2.22	1glucose + 4ATP + 3CoA → 3acetyl-CoA + $4(ADP + Pi)$ + H_2O[b]	3	0	–4	[94]
	ACK	2.7.2.1					
	ACS	6.2.1.1					

[a]Enzyme abbreviations: A-ALD, acetylating acetaldehyde dehydrogenase; ACK, acetate kinase; ACS, acetyl-CoA synthetase; ALD, acetaldehyde dehydrogenase; FDH, formate dehydrogenase; PDC, pyruvate decarboxylase; PDC, pyruvate decarboxylase; PDHc, pyruvate dehydrogenase complex; PFL, pyruvate-formate lyase; PFOR, pyruvate ferredoxin oxidoreductase; PK, phosphoketolase; POX/CAT, pyruvate oxidase/catalase; PTA, phosphotransacetylase.
[b]The reaction stoichiometry from glucose to pyruvate: glucose + 2Pi + 2ADP + $2NAD^+$ → 2pyruvate + 2ATP + 2NADH + $2H^+$ + H_2O; the reaction stoichiometry from glucose to fructose-6-phosphate: glucose + ATP → fructose-6-phosphate + ADP + Pi.
[c]fd(ox), ferredoxin/flavodoxin (oxidized); fd(re), ferredoxin/flavodoxin (reduced).

Channeling the catabolic flux from glucose to acetyl-CoA

Of the 13 pathways that synthesize acetyl-CoA, 11 lose carbon. Of these 11 pathways, the PDHc, PFL-FDH, PDC-A, and PFOR pathways conserve the most ATP and reducing equivalents. The engineering of these four pathways is summarized in the following text. Strategies to strengthen the pathway from glucose to pyruvate can be found in a review by Jojima and colleagues [77] and will not be explained in detail here.

Table 2. Strategies for producing different products[a]

Product	ACA:RE:ATP:OR[b]	Flux ratio[c]	Reaction stoichiometry	TMY			CO_2	Refs[d]
				mol/mol	g/g	Increased %	Reduced %	
Production strategies under anaerobic conditions								
Ethyl acetate	1:0:0:1 Ethanol	PFL	1glucose → 1ethyl acetate + 2formate + 2ATP	1	0.489	20	40	[73]
		PK-A/PDHc	glucose → 1.2ethyl acetate + 1.2CO_2 + 0.4ATP	1.2	0.587			
		04:06						
Butyric acid	2:2:–1:0	PFL	1glucose → 1butyric acid + 2formate + 3ATP	1	0.489	20	83.33	[36]
		PK-A/PDHc	0.83glucose → 1butyric acid + 0.28ATP + CO_2	1.2	0.586			
		02:01						
Hexanoic acid	3:4:–1:0	PDHc	1.5glucose → 1hexanoic acid + 2NADH + 3ATP + 3CO_2	0.666	0.429	12.6	62.5	[37]
		PK-A/PDHc	0.75glucose → 1hexanoic acid + 2ATP + 1.125CO_2	0.75	0.484			
		01:03						
Caprylate	4:6:–1:0	PDHc	2glucose → 1caprylate + 2NADH + 5ATP + 4CO_2	0.5	0.4	9	25	[95]
		PK-A/PDHc	1.83glucose → 1caprylate + 1.83ATP + 3CO_2	0.545	0.436			
		02:09						
Caproate	5:8:–1:0	PDHc	2.5glucose → 1caproate + 2NADH + 5ATP + 5CO_2	0.4	0.382	7	20	[40]
		PK-A/PDHc	2.33glucose → 1caprate + 11.6ATP + 4CO_2	0.428	0.409			
		01:06						
Butanol	2:4:0:0	PDHc	1glucose → 1butanol + 2CO_2 + 2ATP	1	0.411	0	0	[96]
		PK-A/PDHc	1glucose → 1butanol + 2CO_2 + 2ATP	1	0.411			
		00:01						
Ethanol	1:2:0:0	PDHc	1glucose → 2ethanol + 2CO_2 + 2ATP	2	0.511	0	0	[96]
		PK-A/PDHc	1glucose → 2ethanol + 2CO_2 + 2ATP	2	0.511			
		00:01						
Valerate	1:4:–1:1pyr	PDHc/PPP	1.08glucose → 1valerate + 3ATP + 1.5CO_2	0.857	0.485	0	0	[97]
		06:01						
		PDHc/PPP	1.08glucose → 1valerate + 3ATP + 1.5CO_2	0.857	0.485			
		06:01						
Heptylate	2:6:–1:1pyr	PDHc/PPP	1.58glucose → 1heptylate + 4ATP + 2.5CO_2	0.631	0.455	0	0	[97]
		18:01						
		PDHc/PPP	1.58glucose → 1heptylate + 4ATP + 2.5CO_2	0.631	0.455			
		18:01						
Nonanoate	3:8:-1:1pyr	PDHc/PPP	2.08glucose → 1nonanoate + 5ATP + 3.5CO_2	0.48	0.421	0	0	[95]
		24:01:00						
		PDHc/PPP	2.08glucose → 1nonanoate + 5ATP + 3.5CO_2	0.48	0.421			
		24:01:00						
Acetone	2:0:0:0	PFL/other	1glucose → 1acetone + 2ATP + 2formate	1	0.322	0	0	[51]
		PK-A/other	1glucose → 1acetone + 1CO_2 + 1Ac	1	0.322			
		01:01						

Table 2. (continued)

Product	ACA:RE:ATP:OR[b]	Flux ratio[c]	Reaction stoichiometry	TMY			CO_2	Refs[d]
				mol/mol	g/g	Increased %	Reduced %	
Production strategies under aerobic conditions								
Farnesene	9:6:9:0	PDHc	4.5glucose → 1farnesene + 12NADH + $9CO_2$	0.222	0.252	22	16.7	[26]
		PK-A/PDHc	3.68glucose → 1farnesene + $7.5CO_2$	0.271	0.308			
		1.8:2.25						
Isoprene	3:2:3:0	PDHc	1.5glucose → 1isoprene + 4NADH + $3CO_2$	0.666	0.252	22	16.7	[53]
		PK-A/PDHc	1.23glucose → 1isoprene + $2.5CO_2$	0.813	0.308			
		1.8:2.25						
Amorphadiene	9:6:9:0	PDHc	4.5glucose → 1amorphadiene + 12NADH + $9CO_2$	0.222	0.252	22	16.7	[98]
		PK-A/PDHc	3.68glucose → 1amorphadiene + $7.5CO_2$	0.271	0.308			
		1.8:2.25						
Lycopene	24:16:24:0	PDHc	12glucose → 1lycopene + 32NADH + $24CO_2$	0.083	0.248	22	54.6	[98]
		PK-A/PDHc	9.82glucose → 1lycopene + $10.9CO_2$	0.101	0.308			
		0.4:0.5						
Astaxanthin	24:18:24:0	PDHc	12glucose → 1astaxanthin + 30NADH + $24CO_2$	0.083	0.276	20	51.2	[99]
		PK-A/PDHc	9.95glucose → 1astaxanthin + $11.72CO_2$	0.1	0.331			
		1.8:2.58						
Stearic Acid	9:16:8:0	PDHc	4.5glucose → 1stearic acid + 2NADH + 1ATP + $9CO_2$	0.222	0.351	3.6	10.8	[47]
		PK-A/PDHc	4.33glucose → 1stearic acid + $8.03CO_2$	0.23	0.363			
		0.79:10						
Acetone	2:0:0:0	POX-B	1glucose → 1acetone + $2CO_2$ + 2NADH	1	0.322	48.7	47	[51]
		PK-A/PDHc	0.67glucose → 1acetone + $1.06CO_2$	1.487	0.479			
		38:01:00						
PHB[e]	2:1:0:0	POX-B	1glucose → 1PHB + $2CO_2$ + 1NADH	1	0.578	28.5	74.5	[100]
		PK-A/PDHc	0.78glucose → 1PHB + $0.51CO_2$	1.285	0.742			
		02:01						
Glycolate	1:1:0:0	POX-B or POX-D	0.5glucose → 1glycolate + $1CO_2$	2	0.844	22.2	54	[50]
		PK-A/PDHc	0.41glucose → 1glycolate + $0.46CO_2$	2.444	1.031			
		04:05						
L-Glutamate	1:1:0:1OAA	PDHc	1glucose + NH_3 → 1Glu + 3NADH + $1CO_2$	0.5	0.817	20	116	[63]
		PK-A/EMP	1glucose + $0.2CO_2$ + NH_3 → 1.2Glu + 1.07NADH	1.2	0.98			
		04:06						
DHA	11:26:10:0	PDHc/PPP16.5:1	5.83glucose → 1DHA + 1ATP + $13CO_2$	0.171	0.312	0	0	[61]
		PDHc/PPP	5.83glucose → 1DHA + 1ATP + $13CO_2$	0.171	0.312			
		16.5:1						
EPA	10:23:9:0	PDHc/PPP15:0.25	5.25glucose → 1EPA + 1ATP + $11.5CO_2$	0.19	0.319	0	0	[101]
		PDHc/PPP	5.25glucose → 1EPA + 1ATP + $11.5CO_2$	0.19	0.319			
		15:00.3						

Table 3. Carbon cost for acetyl-CoA formation from different feedstocks

Pathway stoichiometry[a]	Feedstock price ($/kg)[b]	Carbon cost ($/mol carbon)[c]	Refs
1Glucose → 2acetyl-CoA + 4NADH + 2ATP + $2CO_2$[d]	0.247	0.012	[15]
1Glucose → 3acetyl-CoA-ATP[e]	0.247	0.007	[15]
2Glyceral → 2acetyl-CoA + 6NADH + 2ATP + $2CO_2$	0.617	0.028	[102]
6Methanol → 2acetyl-CoA + 6NADH + $2CO_2$	0.278	0.013	[69]
Palmitic acid → 3acetyl-CoA + NADH + FADH	0.772	0.012	[103]
3Ethanol → 3acetyl-CoA + 6NADH	0.849	0.02	[68]
6Formaldehyde → 3acetyl-CoA	0.185[f]	0.015	[70]
3Acetic acid → 3acetyl-CoA-3ATP	0.293	0.009	[59]

[a]Pathway stoichiometry was estimated from the cited references.
[b]The prices of these feedstocks were estimated from online data in December 2020 (https://b2b.baidu.com/); 1US Dollar ($) = 6.4822 Chinese Yuan Renminbi (¥) (2021-01-22).
[c]Mol carbon cost for producing acetyl-CoA was estimated from the prices of these feedstocks.
[d]The carbon-loss pathway.
[e]The carbon-save pathway.
[f]The concentration of formaldehyde was 37%.

High levels of NADH accumulate under anaerobic conditions and inhibit the activity of the E3 subunit [19]. This renders PDHc inactive in oxidizing pyruvate to acetyl-CoA for most bacteria [18]. This feedback inhibition was reported to be eliminated by replacing its native promoter with an anaerobic [20] or a constitutive promoter [21] to control the PDHc operon gene *aceEF.lpd*, by knocking out an anaerobic regulator, a ferredoxin-$NADP^+$ reductase that represses the expression of PDHc genes during anaerobic growth [78], or by placing *aceEF.lpd* on a high copy-number plasmid [21,79]. For instance, the amount of succinate produced was 98.8% of its TMY by expressing the entire *aceEF.lpd* operon under the control of a constitutive promoter [21]. An alternative and more effective solution is to obtain an E3 mutant that is active under anaerobic conditions, which can be achieved in three ways. Kim and colleagues selected a dihydrolipoamide dehydrogenase (LPD) mutant LPD^{E354K} [22] through chemical mutagenesis, and Zhu and coworkers obtained a mutant LPD^{A358V} through adaptive evolution [23], both under anaerobic conditions. Wang and colleagues obtained the mutants $LPD^{I350V/A351V/A358V}$ through protein engineering [24]. Among them, the PDHc using the E3 subunit with three amino acid substitutions maintained high activity even when the ratio of [NADH]/[NAD^+] reached 0.15 [24]. Its activity in pyruvate oxidation is sixfold higher than the mutant LPD^{A358V} and approximately 156-fold higher than that of the wild type at this ratio [24]. Therefore, expressing PDHc with such an LPD triple mutant under the control of the constitutive promoter mentioned previously may further enhance the synthesis efficiency from pyruvate to acetyl-CoA under anaerobic conditions.

The key to optimizing the PFL-FDH pathway is for the proton (produced from the catalytic synthesis of formate by PFL) to be transferred from FDH to NAD^+ instead of allowing its transformation into hydrogen gas by the formate hydrogen lyase complex (FHL). Shen and colleagues expressed an FDH from *Candida boidinii* (cbFDH) and the *n*-butanol titer subsequently reached 15 g/l, which is comparable to the levels achieved by *Clostridium* species at an industrial scale [75]. Dong and

Notes to Table 1:
[a]Abbrevations: Ac, acetate; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; Glu, L-glutamate; PHB, poly(3-hydroxybutyrate).
[b]The mole ratio of acetyl-CoA (ACA), redox cofactor, ATP and other precursors required for synthesis 1 molecule of product.
[c]The pathway and flux ratio before and after the use of each particular strategy is shown for each product. Under anaerobic conditions, the three carbon-loss pathways, PDHc, PFL-FDH, or PFOR, can be combined with the PK-A pathway. Under aerobic conditions, two carbon-loss pathways, PDHc or PDC-A, can be combined with the PK-A pathway.
[d]Because many products were generated by the strategies listed, we only list the latest relevant publications.
[e]PHB is based on the monomer 3-hydroxybutyric acid, whose molecular weight is 104.11.

coworkers screened for an *ydfZ* promoter to fine-tune the expression of cbFDH, and inactivated FHL activity, leading to a *n*-butanol titer of 20 g/l, which is more competitive than both natural and engineered *Clostridium* strains [11].

To debottleneck the PDC-A pathway, an A-ALD or its mutant of high activity in the synthesis of acetyl-CoA is needed and can be obtained by screening enzymes from various sources [26,80] or by using directed evolution [3,81,82].

The production of ethanol in anaerobic bacteria such as *C. thermocellum* can be enhanced by replacing its endogenous PFOR (CtPFOR) with the PFOR from *Thermoanaerobacterium saccharolyticum* [29]. The introduction of this PFOR increased the maximum titer of ethanol by 14% because, unlike CtPFOR, it is not subject to feedback inhibition by ethanol and NADH [83].

Introducing pathways that conserve carbon to increase the theoretical yield of acetyl-CoA

There are three different PK pathways: the synthesis of acetyl-CoA by the introduction of PK into *Corynebacterium glutamicum*, as reported by Ajinomoto [64], the nonoxidative glycolysis (NOG) pathway [76], and the 'EF-bifido' pathway [49]. The theoretical yield of acetyl-CoA for the NOG pathway is 3 acetyl-CoA molecules for each glucose and ATP molecule, where the first and third pathways produced 2.5 acetyl-CoA and 2 NAD(P)H molecules for each glucose and ATP molecule. Based on the analysis of the 13 pathways that synthesize acetyl-CoA, introducing the PK-A pathway (that does not lose carbon) on top of the PDHc, PFL-FDH, PFOR, or PDC-A pathway can improve the theoretical yield of acetyl-CoA. Applications of this combination have been reported in *C. glutamicum* [64], *E. coli* [76], *S. cerevisiae* [26], *Yarrowia lipolytica* [47], *Synechocystis* sp. [48] and *Methylomicrobium buryatense* [84] (Table 4).

To improve the yield of L-Glu in *C. glutamicum*, the PK from *Bifidobacterium animalis* was installed into L-Glu metabolic pathway of this strain. The yield of L-Glu on glucose subsequently increased from 60.8% to 69.4% (Box 1) [64].

To increase the yield of acetate from glucose in *E. coli*, the PK from *B. adolescentis* (BaFK) was introduced and bisphosphatase (FBP) was overexpressed to regenerate glyceraldehyde-3-phosphate (GAP). The resulting yield of acetate reached 0.4 mol/mol xylose. Blocking the formation of four byproducts (ethanol, lactate, succinate, and formate) formation ultimately directed the metabolic flux to the PK pathway for acetate production, increasing its yield to 2.2 mol/mol xylose – which exceeds the TMY of 1.67 mol/mol xylose by using only the PDHc pathway [76]. Zhao and colleagues adapted this combination strategy for acetone production. The yield of acetone produced by fermenting glucose increased up to 0.47 mol/mol glucose compared to 0.38 mol/mol glucose in non-adapted strains. Inactivating acetate kinase (ACK) or increasing ACS expression was not sufficient to divert all the carbon flux from acetate to acetone [51]. To channel glucose to products other than acetate, Wang and coworkers recently introduced BaFK into *E. coli* and coupled it with disruption of the 6-phosphofructose kinase gene *pfk* to downregulate the EMP pathway, as well as with deletion of the 6-phosphogluconate dehydratase gene *edd* to block the ED pathway. They obtained a strain that generated almost no acetate [49]. Further introduction of the PHB, fatty acid, or mevalonate synthesis pathways into the chassis of the cells obtained above significantly increased the yields of these products compared to the control in which the pathway was introduced but the genes *pfk* and *edd* were not disrupted and no exogenous PK gene was present (Table 4).

To increase the yield of β-farnesene in *S. cerevisiae*, Amyris introduced a PK-A pathway [composed of a PK from *Leuconostoc mesenteroides* (LmPK) and a heterologous PTA] and a PDC-A pathway

Table 4. Metabolic engineering studies using the phosphoketolase pathway

Host	Product	Strategies	Yield (increase)[e]	Refs
E.coli	Acetate	Introduce the PK-A pathway, disrupt the byproduct pathways generating lactate, ethanol, succinate, and formate	2.2 mol/mol xylose (100%)	[76]
E.coli	Acetone	Introduce the acetone synthesis pathway and the PK-A pathway	0.47 mol/mol[a] (23%)	[51]
E.coli	PHB	Introduce the PK-A pathway, downregulate the EMP pathway, disrupt the ED pathway and the byproduct pathways, and introduce the PHB pathway	0.637 mol/mol (145%)	[49]
	Fatty acid	Use the same strategies as above, enhance the fatty acid pathway	0.144 mol/mol (56%)	
	Mevalonate	Use the same strategies as above, introduce the mevalonate (MVA) pathway	0.643 mol/mol (48%)	
E.coli	Mevalonate	Use the same strategies as above, enhance the PPP pathway	0.687 mol/mol (30%)	[104]
C. glutamicum	L-Glutamate	Introduce the PK-A pathway	0.849 mol/mol (14%)	[64]
C. glutamicum	L-Glutamate	Introduce the PK-A pathway with the $PKT^{T2A/I6T/H260Y}$ mutant	0.453 mol/mol (18%)	[63]
C. acetobutylicum	Acetate	Enhance the native PK-A pathway	NA[b] mol/mol xylose (100%)	[105]
	Acetyl phosphate		NA mol/mol xylose (600%)	
Synechocystis sp.	PHB	Introduce the PK-B pathway	NA mol/mol CO_2 (364%)	[48]
M. buryatense	Lipid	Enhance the PK-A pathway	0.09 g/g CH_4[c] (100%)	[84]
S. cerevisiae	Fatty acid ethyl esters	Introduce both the PK-A and PK-B pathway	NA (50%)	[106]
S. cerevisiae	β-Farnesene	Enhance the MVA pathway, replace the PDC-B pathway with the PK-A combined PDC-A pathways, balance the redox-cofactor, downregulate the byproduct pathway, multiple rounds of mutagenesis	0.2 g/g (40%)	[26]
S. cerevisiae	3-Hydroxypropionic acid	Introduce and simplify the PK-A pathway	NA (109%[d])	[107]
S. cerevisiae	*p*-Coumaric acid	Introduce the PK-A pathway, downregulate the EMP pathway, enhance the *p*-coumaric acid pathway	0.17 mol /mol (NA)	[108]
Y. lipolytica	Lipid	Introduce the PK-A pathway coupled with NADPH regeneration	0.225g/g[c] (28%)	[47]

[a]Yield in mol/mol represents mol product per mol glucose.
[b]NA, data not available.
[c]The molar yield of lipid is not available because lipid consists of multiple components.
[d]Percent increased titer of 3-hydroxypropionic acid.
[e]Increase means the percentage increase in the product yield of the strains after adopting these strategies.

(created from heterologous A-ALD and endogenous PDC) to replace the endogenous PDC-B pathway which was inactivated by deletion of the gene encoding acetaldehyde dehydrogenase ALD6. However, unexpected hydrolysis of acetate phosphate was observed, which led to high acetate flux [26,85]. Further analysis identified two glycerol-3-phosphate phosphatases that were responsible for the hydrolysis, revealing a promiscuous function of these enzymes. Deletion of the gene encoding one glycerol-3-phosphate phosphatase dramatically reduced acetate formation, resulting in an increased β-farnesene yield from 0.143 to 0.173 g/g glucose. The engineered yeast is now commercially used for β-farnesene production in Brazil [26,86].

All PK enzymes characterized to date have dual-substrate activity with a k_{cat}/K_m between 0.17 and 11.9 s^{-1}/mM on fructose-6-phosphate (F6P) and 193.15-378.62 s^{-1}/mM on xylose-5-phosphate (X5P) [63,87]. In the case of farnesene production in *S. cerevisiae*, the PK was introduced from *L. mesenteroides* [26] with a k_{cat}/K_m of 0.96 s^{-1}/mM on F6P and 213.43 s^{-1}/mM on X5P instead of the PK from *Pseudomonas aeruginosa* that has a higher k_{cat}/K_m [87]. It is possible that LmPK performs better in *S. cerevisiae.* In addition, a system to select a highly active PK from the low-activity enzyme BaFK was constructed by coupling PK activity to cell growth [63]. The highest BaFK mutant k_{cat}/K_m on F6P obtained via this system was doubled to 0.32 s^{-1}/mM. This approach may be used to improve the activity of PK enzymes from other species that exhibit even higher activities.

Box 1. Application of the PK-A pathway for L-glutamate production in *C. glutamicum*

L-Glu is an industrially important amino acid for the production of the flavoring agent monosodium glutamate. In *C. glutamicum*, L-Glu is biosynthesized from glucose via the EMP, the oxidative branch of the tricarboxylic acid (TCA) cycle, and the action of glutamate dehydrogenase. During these reactions, 1 mol of CO_2 per mol of L-Glu produced is 'fixed' to pyruvate by pyruvate carboxylase, whereas 2 mol of CO_2 is released by pyruvate dehydrogenase and isocitrate dehydrogenase (Figure IA). PK can be introduced to bypass the CO_2-releasing pyruvate dehydrogenase reaction (Figure IB), thereby increasing the TMY of L-Glu on glucose from 81.7% to 98.0% by weight (1.0 up to 1.2 mol/mol glucose) (Figure IB). Ajinomoto cloned the *xfp* gene encoding PK from *B. animalis* and overexpressed it under the strong *cspB* promoter in *C. glutamicum* CGS01 [64]. For strain CGS01, ~10.76 g of L-Glu was produced from 17.7 g of glucose, a yield of 60.8% by weight, and about 5.51 g of CO_2 was generated. By comparison, strain CGS01 expressing Xfp yielded 69.4% L-Glu by weight, and the amount of CO_2 released was reduced to ~84% of that of the control strain. The carbon distribution from glucose suggested that a decrease in the amount of carbon generated as CO_2 was mainly responsible for the increased amount of L-Glu produced. In this instance, the preferred flux ratio of the PK and EMP pathways is 8:2 (Figure IB), whereas the adapted flux ratio is 4:6 when the NOG pathway is used, which recycles the glyceraldehyde-3-phosphate into acetyl phosphate (see Table 2 in main text).

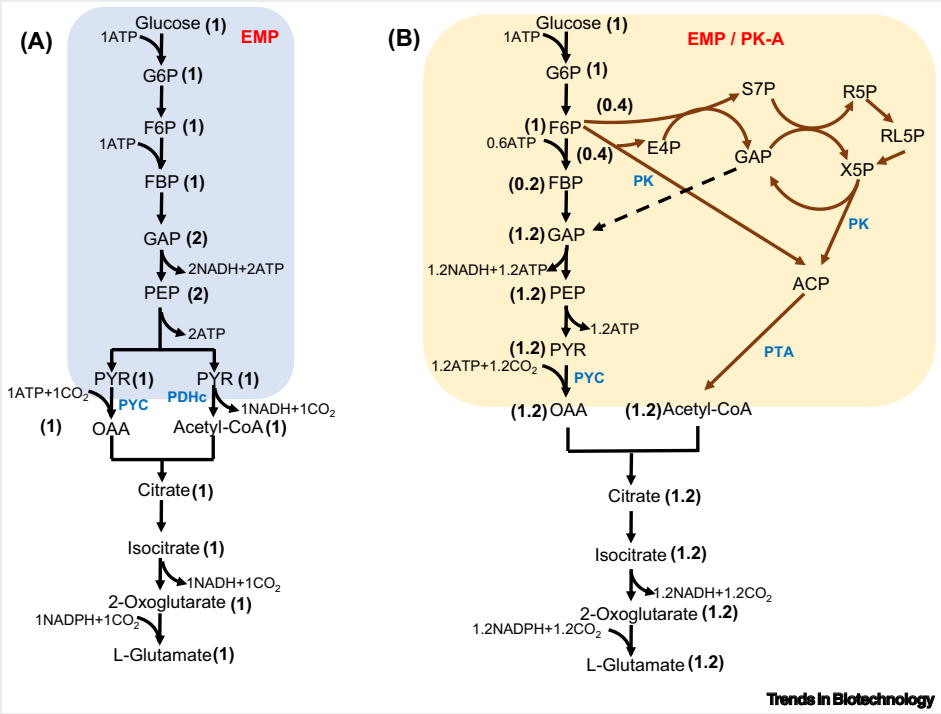

Figure I. L-Glu biosynthesis. (A) L-Glu is synthesized from glucose via the EMP pathway or (B) via the EMP pathway combined with the PK-A pathway. The numbers of compounds involved in the metabolic reactions under ideal conditions are shown in parentheses. Abbreviations for metabolites and enzymes are as in Figure 1 in main text.

Based on the previous text, practical strategies for the metabolic engineering of acetyl-CoA are summarized in the following text. First, the core biosynthetic pathways leading to the end products from acetyl-CoA need to be introduced or strengthened. In addition, key genes for the synthesis of acetate, ethanol, lactate, succinate, and even formate that compete with the synthesis of acetyl-CoA and its precursors must be disrupted [11], and the low activity of key enzymes in the main pathway (PDHc and A-ALD) needs to be improved [21,82].

Second, the PK-A pathway, which does not lose carbon, should be introduced when applicable. However, it must cooperate with the native pathway that synthesizes acetyl-CoA in the chassis cell. In addition, bacterial central metabolism should be adjusted such as by weakening the

EMP pathway and/or by knocking out key genes of the ED pathway [49], or even by identifying the key enzymes that generate unexpected products and knocking out the corresponding genes [26], so as to guide the metabolic flow towards the synthesis of acetyl-CoA.

The generation of reducing equivalents by the metabolic pathway from glucose to derivatives of acetyl-CoA must be balanced. This can be achieved by replacing enzymes having an NADPH-dependent cofactor with an enzyme that has an NADH-dependent cofactor. Thus, the NADH produced by the enzyme could match the NADH produced from the synthesis of acetyl-CoA from glucose. This method was successfully applied to the synthesis of farnesene from acetyl-CoA by editing an enzyme in the mevalonate pathway [26].

In addition, it is important to establish or optimize the transport system for the final product(s) to relieve feedback inhibition caused by their intracellular accumulation. Adjusting the efficiency of the respiratory chain to balance intracellular ATP and thereby promote the synthesis of the final product is also important. The production of acetyl-CoA from glucose, and the generation of the final product, must be regulated globally, and this can be achieved through random mutagenesis followed by high-throughput selection [26,86] or via manipulation of global regulatory factors to improve the efficiency of the synthesis pathways. Last but not least, there is a need to engineer stress-resistant strains to make cells more robust for production and to expand feedstock utilization to make the chassis cells adaptable to other carbon sources [88].

Concluding remarks

The aim of metabolic engineering of acetyl-CoA production is to maximize the yield of its derived biochemicals from glucose. Because acetyl-CoA is a node molecule, modifying its endogenous metabolic pathway and/or introducing a heterologous synthesis pathway faces strong regulation from the central metabolism of the chassis cell [4]. Therefore, entire cellular systems need to be optimized to achieve this purpose. This includes introducing/ enhancing the pathways of acetyl-CoA to the target product(s), channeling glucose into acetyl-CoA synthesis [11], introducing the PK-A pathway if necessary, balancing the reducing equivalents of the metabolic pathway from glucose to acetyl-CoA derivatives [26], establishing or optimizing the export system for the final product, ensuring the efficiency of ATP synthesis, engineering global regulation and cellular resistance, and expanding feedstock utilization [88]. In addition, mechanistic studies are required. In the case of β-farnesene production in *S. cerevisiae*, by genetic deletion of the glycerol-3-phosphatase with a promiscuous function in the PK-A pathway, and by using techniques including mutagenesis and high-throughput screening, Amyris successfully commercialized farnesene [26]. This example illustrates that acetyl-CoA metabolic engineering to successfully commercialize the target product requires thorough mechanistic research to solve key bottlenecks. In addition, random mutagenesis combined with high-throughput screening is necessary to resolve bottlenecks through an iterative design–build–test–learn cycle to achieve the ultimate goal of product commercialization.

However, many of these research developments have not yet gone through such a cycle (see Outstanding questions). This is because the industry of chemical production through fermentation has not changed significantly over the past 20 years. The task of production increasingly depends on emerging Chinese companies instead of on European, American, or Japanese companies. The newer Chinese companies do not yet have the financial resources to perform related research in view of the low profits resulting from fierce pricing wars, whereas older European, American, and Japanese companies stopped competing in this market before the relevant R&D was completed. Metabolic engineering of bulk biochemicals including acetyl-CoA will hopefully be continued by the manufacturers who survive in the market.

Outstanding questions

Can a more cost-effective feedstock than glucose be developed for acetyl-CoA synthesis? Could hydrogen be a promising co-substrate to increase the yield of acetyl-CoA and target molecules from glucose?

PDHc bypass routes for producing acetyl-CoA-based compounds are still in their early developmental stages. Can extensive enzyme and metabolic engineering make these pathways more efficient than the PK pathway? Can the PDHc mutants that are resistant to NADH inhibition be transferred from *E. coli* into other organisms such as *Lactobacillus* for anaerobic production of acetyl-CoA from pyruvate?

What is the outlook for the biotechnological production of acetyl-CoA from CO_2?

The PFL-FDH pathway is proved to be a suboptimal choice. Can the specific activity of formate dehydrogenase (FDH) from *Candida boidinii* or *Pseudomonas* be drastically improved? PFOR is widely distributed in anaerobes. Does this mean that PFOR in anaerobes does not need to be replaced with PDHc and PFL-FDH?

In addition to the aforementioned strategy to improve the theoretical yield of acetyl-CoA, several other pathways have also been reported. For products such as isoprenoids that use acetyl-CoA as an alternative precursor, the methyl-D-erythritol-4-phosphate (MEP) pathway can be utilized to further increase the TMY. For example, the synthesis of 1 isoprene molecule through the MEP pathway from 1 glucose requires 1 redox cofactor and 2 ATP molecules [53], which is less than the requirement of the combined PK-A/PDHc pathways. Combining the MEP pathway with the PK-A/PDHc pathways can further increase the glucose-to-isoprene conversion rate from 0.807 to 0.842 mol/mol. Pathways that increase the yield of acetyl-CoA through CO_2 fixation have also been reported. For example, the Gnd–Entner–Doudorof pathway (GED) fixes CO_2 through the reverse synthesis reaction of 6-phosphogluconate dehydrogenase in the PPP pathway to increase the yield of pyruvate [89], thereby compensating for carbon loss in the pyruvate oxidation reaction that forms acetyl-CoA. When the GED pathway is used for the synthesis of amino acids such as L-Glu, the TMY for glucose can be further increased from 1.2 to 1.3 mol/mol glucose [90].

Methods to bypass PDH to increase the yield of acetyl-CoA from sugar include the PK pathway [76], the reverse glyoxylate shunt (rGS) [91], and the maloyl-CoA glycerate pathway (MCG) [92]. The rGS pathway produces 3 molecules of acetyl-CoA, 1 NADH, and ATP molecule for each glucose molecule. The MCG pathway produces 3 molecules of acetyl-CoA and 1 molecule of ATP for each glucose molecule. In the case of butyric acid, when the metabolic flux ratio of rGS:PDHc/PFL-FDH/PFOR is 1:1, the yield is 1.25 mol/mol glucose, 0.05 higher than when combined with the PK-A pathway. Combining the MCG pathway with the PDHc/PFL-FDH/PFOR pathways does not change its yield compared to the PK-A pathway because the MCG pathway does not provide reducing equivalents. When external H_2 is supplied, the rGS and MCG pathways can achieve an acetyl-CoA yield of 4 acetyl-CoA per glucose molecule using carbon fixation, leading to a butyric acid yield of up to 2 mol/mol glucose. Nevertheless, the functional demonstration and applications of these pathways in producing compounds based on acetyl-CoA remain in their early developmental stages.

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Declaration of interests

The authors declare no conflicts of interest.

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