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REVIEW

Metabolically engineered *Escherichia coli* for biotechnological production of four-carbon 1,4-dicarboxylic acids

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Abstract Confronted with inescapable exhaustion of the earth's fossil energy resources, the bio-based process to produce industrial chemicals is receiving significant interest. Biotechnological production of four-carbon 1,4-dicarboxylic acids (C4 diacids) from renewable plant biomass is a promising and attractive alternative to conventional chemistry routes. Although the C4 diacids pathway is well characterized and microorganisms able to convert biomass to these acids have been isolated and described, much still has to be done to make this process economically feasible. Metabolically engineered *Escherichia coli* has been developed as a biocatalyst to provide new processes for the biosynthesis of many valuable chemicals. However, *E. coli* does not naturally produce C4 diacids in large quantities. Rational strain development by metabolic engineering based on efficient genetic tools and detailed knowledge of metabolic pathways are crucial to successful production of these compounds. This review summarizes recent efforts and experiences devoted to metabolic engineering of the industrial model bacteria *E. coli* that led to efficient recombinant biocatalysts for the production of C4 diacids, including succinate, fumarate, malate, oxaloacetate, and aspartate, as well as the key limitations and challenges. Continued advancements in metabolic engineering will help to improve the titers, yields, and productivities of the C4 diacids discussed here.

Keywords Biotechnological production · *Escherichia coli* · Four-carbon 1,4-dicarboxylic acids · Metabolic engineering

Introduction

Four-carbon 1,4-dicarboxylic acids (C4 diacids), including succinate, fumarate, malate, oxaloacetate, and aspartate (Fig. 1), are important cellular metabolic intermediates for bacteria. These C4 metabolites are of general interest due to their potential for commercial applications. For instance, succinate can be used as a surfactant, ion chelator, and as a precursor for valuable chemicals in the pharmaceutical and food industries [63]. Fumarate is commonly used as a food acidulant and beverage ingredient, and is also useful for making polyesters and other types of synthetic polymers [56]. Aspartate is an essential raw material for the manufacture of the most widely used nutritive sweetener known as aspartame [2]. In a report from the US Department of Energy, these C4 diacids, especially succinate and malate, are among the top 12 chemical building blocks manufactured from biomass [61]. Currently, all of the C4 diacids are mainly produced through the petrochemical synthesis routes and many of these products are expensive due to the complexity of downstream processing and purification. The trends toward "green" chemicals production require the development of innovative synthesis techniques that are both cost-effective and energy-saving. Biosynthesis of chemicals and biomaterials using microorganisms has arisen as a competitive alternative to traditional chemistry-based routes. In the face of rapidly rising petroleum prices

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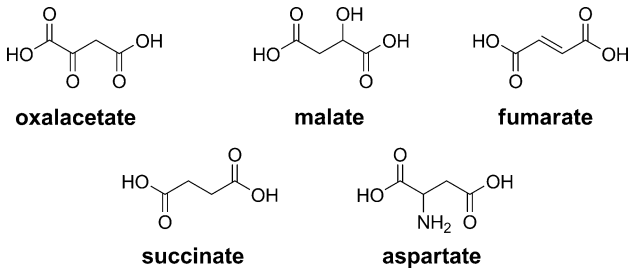

Fig. 1 Structures of the five kinds of four-carbon 1,4-dicarboxylic acids which are naturally produced by *E. coli*

and scarcity of fossil fuel supplies, the fermentative production of these compounds by using biocatalytic processes has received increasing attention [51].

Traditionally, the main biotechnological fermentation products were end products of high yields formed naturally by the respective microorganisms. However, a set of metabolic engineering approaches was recently developed to widen the biotechnological product spectrum [60]. Many metabolic intermediates, such as pyruvate [9, 67], citrate [15], and α-ketoglutarate [64], have been successfully produced by fermentative strategies. *Escherichia coli* has classically been considered to be a workhorse for the production of recombinant proteins and a variety of high value metabolites, including lactate, acetate, and ethanol. It has many advantages over other industrial microorganisms, e.g., clear genetic background, convenience with regard to genetic modification, and good growth properties with low nutrient requirements, and has the potential to produce bulk chemicals within the next few years [8]. Recently, metabolic engineering strategies were employed to create recombinant *E. coli* strains, which have enhanced productivity due to the reorientation of the metabolic fluxes [62]. Metabolically engineered high production systems are now playing a promising role in generating desired commercial products.

This review provides information on the biotechnological production of C4 diacids, which gives an alternative to conventional chemistry routes. We also briefly summarize the recent progresses in the metabolic engineering approaches for the production of these C4 diacids by recombinant *E. coli*, one of the most competitive C4 diacid producer microorganisms.

Anaerobic metabolic pathways to C4 diacids

The C4 diacids, except aspartate, are metabolites involved in the tricarboxylic acid (TCA) cycle. Under aerobic conditions, wild-type *E. coli* accumulates none of them. However, *E. coli* could convert sugars to a mixture of products by mixed-acid fermentation in the absence of alternative electron acceptors. The major soluble products are acetate, ethanol, lactate, and formate with smaller amounts of succinate [12]. The relative proportions of these fermentation products vary depending on the strain and growth condition employed [13]. The biosynthetic routes to these fermentation products are quite complex and regulated at different levels. To construct an efficient C4-diacid-producing strain, the metabolic pathways to these compounds must be elucidated in the first place. Here, we give an overview of the fermentative pathway for the production of C4 diacids from cost-effective glucose under anaerobic conditions (Fig. 2). The enzymes catalyzing these reactions are listed in Table 1.

The phosphoenolpyruvate (PEP)–pyruvate–oxaloacetate node acts as a highly relevant switch point for carbon flux distribution towards different intermediates within the central metabolism [50]. This node comprises a set of reactions that direct the carbon flux into appropriate directions. PEP occupies the most critical position during mixed-acid fermentation in *E. coli* [33]. Besides its metabolism to pyruvate catalyzed by pyruvate kinase, PEP is also the substrate for a carboxylase-catalyzed anaplerotic reaction and can be converted to oxaloacetate by two enzymes, PEP carboxylase and PEP carboxykinase. Pyruvate, the end product of glycolysis, has four destinations

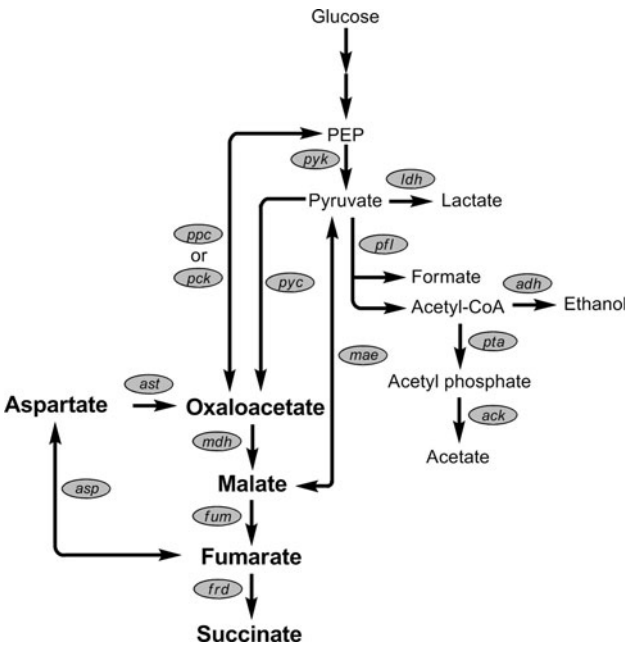

Fig. 2 Anaerobic metabolic pathways for the synthesis of C4 diacids in *E. coli*. Enzymes encoded by the genes shown are *ppc*, phosphoenolpyruvate (PEP) carboxylase; *pck*, PEP carboxykinase; *pyk*, pyruvate kinase; *pyc*, pyruvate carboxylase; *ldh*, lactate dehydrogenase; *pfl*, pyruvate-formate lyase; *adh*, alcohol/acetaldehyde dehydrogenase; *pta*, phosphotransacetylase; *ack*, acetate kinase; *mae*, malic enzyme; *mdh*, malate dehydrogenase; *fum*, fumarase; *frd*, fumarate reductase; *ast*, aspartate aminotransferase; *asp*, aspartase

Table 1 Key enzymes of C4 diacids metabolic pathway under anaerobic conditions in *E. coli*

Enzyme	EC number	Reaction	Co-substrate
Pyruvate kinase	EC 2.7.1.40	PEP + ADP ⇌ pyruvate + ATP	ATP
PEP carboxylase	EC 4.1.1.31	PEP + H_2O + CO_2 ⇌ oxaloacetate + Pi	
PEP carboxykinase	EC 4.1.1.49	PEP + CO_2 + ADP ⇌ oxaloacetate + ATP	ATP
Pyruvate carboxylase	EC 4.1.1.31	Pyruvate + CO_2 + ATP ⇌ oxaloacetate + ADP + Pi	ATP
Lactate dehydrogenase	EC 1.1.1.27	Pyruvate + NADH + H^+ ⇌ lactate + NAD^+	NADH
Pyruvate formate lyase	EC 2.3.1.54	Pyruvate + CoA ⇌ acetyl-CoA + formate	CoA
Alcohol dehydrogenases	EC 1.1.1.1	Acetyl-CoA + NADH + H^+ ⇌ acetaldehyde + CoA + NAD^+ Acetaldehyde + NADH + H^+ ⇌ ethanol + NAD^+	NADH
Phosphate acetyltransferase	EC 2.3.1.8	Acetyl-CoA + Pi ⇌ CoA + acetyl phosphate	CoA
Acetate kinase	EC 2.7.2.1	ATP + acetate ⇌ ADP + acetyl phosphate	ATP
Malic enzyme	EC 1.1.1.38(40)	Malate + $NAD(P)^+$ ⇌ pyruvate + CO_2 + NAD(P)H + H^+	NAD(P)H
Malate dehydrogenase	EC 1.1.1.37	Malate + NAD^+ ⇌ oxaloacetate + NADH + H^+	NADH
Fumarase	EC 4.2.1.2	Fumarate + H_2O ⇌ malate	
Fumarate reductase	EC 1.3.1.6	Fumarate + NADH + H^+ ⇌ succinate + NAD^+	NADH
Aspartase	EC 4.3.1.1	Aspartate ⇌ fumarate + NH_3	
Aspartate transaminase	EC 2.6.1.1	Oxaloacetate + glutamate ⇌ aspartate + α-ketoglutarate	

under anaerobic fermentation because the activity of pyruvate dehydrogenase is inhibited without any electron acceptor: reduction by lactate dehydrogenase, resulting in lactate; conversion to acetyl-CoA by pyruvate formate lysase, which is further converted to ethanol and acetate; incorporation with carbon dioxide by pyruvate carboxylase or malic enzyme, forming oxaloacetate or malate [49]. Both oxaloacetate and malate may be used as biosynthetic precursors or may be further reduced to succinate via fumarate. *E. coli* also contains high aspartase and aspartate transaminase activities at levels comparable to those of fumarase and malate dehydrogenase [14]. These two enzymatic activities form an alternative pathway for the formation of fumarate and serve as the initial step for the synthesis of several important amino acids [46].

Bioenergetics of the C4 diacids synthetic pathway

The PEP carboxylase catalyzed reaction is more preferable for the synthesis of C4 diacids in wild-type *E. coli* strains. On the basis of the stoichiometry of the reactions in this pathway, the overall fermentation equation from PEP to the end product of succinate is:

$$\mathrm{PEP} + \mathrm{CO_2} + 2\mathrm{NADH} + 2\mathrm{H^+} \rightarrow \mathrm{Succinate} + 2\mathrm{NAD^+} + \mathrm{Pi} + \mathrm{H_2O}.$$

In this scenario, for every mole of succinate to be formed, one mole of carbon dioxide and two moles of NADH are required. If the PEP carboxykinase or malic enzyme catalyzed pathway were employed, one net ATP could be generated with the fixation of one CO_2. According to the second law of thermodynamics, the changes in Gibbs free energy must be negative for individual enzyme reactions or overall pathways to proceed. Experimental thermodynamic data for the reactions involved in the C4 diacids production pathway are largely unavailable [41]. Thus, the present work uses an improved group contribution method [24] to study the thermodynamic properties of the reactions involved in the C4 diacids pathway. The free energy data are mainly obtained from an earlier genome-scale metabolic model of *E. coli* [19]. As shown in Fig. 3, the changes in Gibbs free energy of the four pathways from PEP to succinate (pathway 1 catalyzed by PEP carboxylase, pathway 2 catalyzed by PEP carboxykinase, pathway 3 catalyzed by pyruvate carboxylase, pathway 4 catalyzed by malic enzyme) are all negative. Among the four branches, pathway 1 and pathway 3 are more thermodynamically favorable. The cumulative free energy changes of these two pathways reach about 20 kcal/mol, and pathway 1 is the one generally used in common *E. coli* strains. Pathway 2 and pathway 4 could generate one more ATP and seem to be more energy-effective. Engineered strains harboring these pathways could provide additional energy for cell metabolism.

Genetic modification to improve C4 diacids production

Biosynthesis of C4 diacids from glucose requires carbon fluxes through the carboxylating anaplerotic pathways,

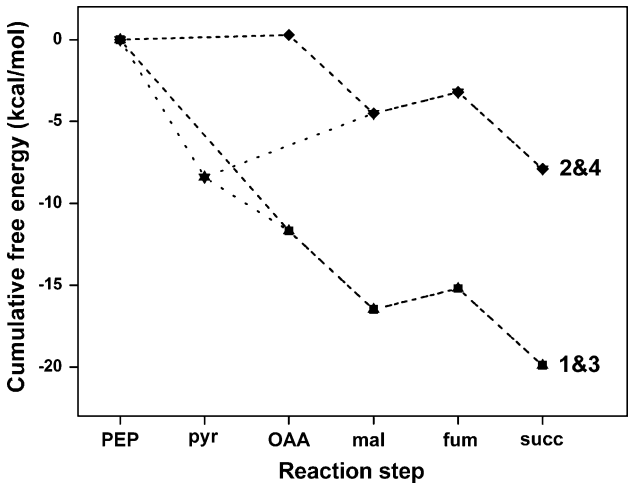

Fig. 3 Free energy landscape for the C4 diacids pathway. The cumulative free energy change of the pathway is given at each reaction step. Abbreviations for the metabolites are pyr, pyruvate; OAA, oxaloacetate; mal, L-malate; fum, fumarate; succ, succinate. The metabolic pathways shown are pathway 1, PEP → OAA → mal → fum → succ catalyzed by PEP carboxylase; pathway 2, PEP → OAA → mal → fum → succ catalyzed by PEP carboxykinase; pathway 3, PEP → pyr → OAA → mal → fum → succ catalyzed by pyruvate carboxylase; pathway 4, PEP → pyr → mal → fum → succ catalyzed by malic enzyme

converting the three-carbon intermediates of glycolysis into the desired four-carbon backbones [1]. All the C4 diacids employ the same arterial metabolic pathway in spite of several branches. Enhancement of an individual acid production always involves increasing metabolic flux through the other dicarboxylic acids. In the case of redox-neutral C4 diacids production, it would result in a maximum theoretical yield of two moles C4 diacids per mole glucose. During anaerobic fermentation, the acetate pathway and the lactate pathway are more competitive than the C4 diacids pathway for the excess carbon flux. Meanwhile, formation of C4 diacids through the reductive reactions of the TCA cycle arm also involves consumption of NADH and requires sufficient supplies of energy. With the aim of modifying *E. coli* to improve its ability to synthesize these C4 diacids, the following regulation points are of key importance:

1. Enhancement of carbon flux to the C4 diacids pathway from central metabolism and optimization of the primary metabolic pathway to the targeted product through modification of enzyme activities especially for the rate-limiting steps;
2. Genetic blockage or inactivation of the enzymes in competition pathways associated with by-products synthesis (e.g., lactate, acetate, ethanol);
3. Modification of secondary metabolic pathways as necessary to enhance energy metabolism (ATP) and availability of reducing power (NADH, NADPH).

Redirecting carbon flux to the C4 diacids branch

Given that C4 diacids are synthesized from the carboxylation of three-carbon metabolites, early efforts have been made to redirect carbon flux from PEP and pyruvate to the C4 diacids branch by overproducing PEP and pyruvate-carboxylating enzymes in *E. coli*, thereby enhancing C4 metabolism. Overexpression of *E. coli* native PEP carboxylase could increase succinate concentrations by 3.75 times [42]. *E. coli* strains heterologously expressing *Mannheimia succiniciproducens* PEP carboxykinase were able to produce malate more efficiently under anaerobic conditions [43]. Expression of pyruvate carboxylase from *Rhizobium etli* in *E. coli* also results in a significant increase in succinate production at the expense of lactate production [16, 17]. Malic enzyme, which catalyzes the reductive carboxylation of pyruvate to malate, provides a possible alternative route to C4 diacids from pyruvate instead of PEP, whereas the reversible reaction from pyruvate to malate is strongly shifted towards pyruvate [44]. When *E. coli* native malic enzyme (NAD^+-dependent) was induced under appropriate culture conditions in a mutant strain that was unable to accumulate pyruvate, fermentative metabolism was redirected to the C4 diacids arm and a significant amount of succinate and malate were accumulated during 120 h of anaerobic cultivation [54]. Further amplification of the fumarase activity in this strain could eliminate malate production [21]. Increasing expression of another malic enzyme ($NADP^+$-dependent) also enhanced the production of C4 metabolites by 2.4 times when compared with the wild-type strain in anaerobic glucose media with bicarbonate supplementation [31]. Additional genetic engineering could result in fumaric acid production [47]. Enzymes catalyzing the interconversion of the C4 diacids were also employed for the production of these acids. Immobilized cells of *E. coli* possessing aspartase activity have been used as biocatalysts for commercial production of aspartate by the amination of fumarate [26, 52]. Succinate was produced efficiently from fumarate by recombinant *E. coli* strains with amplified fumarate reductase activity [18, 59]. The conversion of fumarate to succinate could reach 93% under optimized conditions.

Inactivation of competition pathways

In order to further increase C4 diacids yield, knockout or inactivation of the enzymes in competition pathways was studied. Pyruvate is a key metabolite in central metabolism because it is the end product of glycolysis and most of the fermentative products are derived from it. Thus, metabolism at the pyruvate node is important from the metabolic engineering point of view as variation in the way pyruvate

is dissimilated would have significant impact on the overall cell metabolism and the product distribution. Under anaerobic conditions, the central intermediate pyruvate has two major alternative pathways in addition to the reductive TCA cycle arm. One simple pathway involves the direct conversion of pyruvate to lactate in a single step catalyzed by the fermentative lactate dehydrogenase [55]. This pathway can be inactivated by deletion of the *ldh* gene, encoding lactate dehydrogenase [40]. The more complex pathway involves splitting the pyruvate into acetyl-CoA and formate by means of pyruvate formate lyase (*pfl*). The acetyl-CoA is then converted to an approximately equal mixture of ethanol and acetate [53]. However, knockout of *pfl* gene could only result in enhanced lactate production while the C4 metabolite concentrations were even slightly lower in the *pfl*$^-$ mutants compared with the control strain [66]. Although the *ldh* and *pfl* double deletion strain could eliminate the by-products of lactate, acetate and ethanol, anaerobic growth of this strain on a wide range of sugars and derivatives was no longer possible, which hampered the utilization of this double mutant [7]. Chatterjee et al. [11] introduced another null mutation of the *ptsG* gene into the double mutant strain of *E. coli* to restore its ability to ferment glucose. The resulting strains produced more succinate, that is, 36 g/L in a batch reactor. The *ptsG* gene encodes a key enzyme for the PEP-dependent phosphotransferase system (PTS), the dominant transporter for glucose in *E. coli* [30]. Functional replacement of the PTS system by increased expression of the GalP permease and glucokinase could increase the pool of PEP available for redox balance and preserve an enhancement of succinate production [65]. In another approach, pyruvate formation was inhibited by deletion of *ptsG* and two pyruvate kinase (*pykF* and *pykA*) [32]. This triple mutation increased succinate production by more than sevenfold and the ratio of succinate to fermentation products by ninefold.

Enhancing energy metabolism and enzymatic cofactor supplies

Redox systems play a key role in the life processes of all living organisms, especially in various anaerobic fermentation processes. When manipulating metabolic fluxes for the production of metabolites, it is important to achieve a redox balance between the substrates and products [6]. The anaerobic fermentative pathway for C4 diacids converts oxaloacetate to malate, fumarate, and finally succinate, requiring two moles of NADH [34]. The reducing power generated in the formation of PEP through the glycolytic pathway—one NADH per PEP—is enough to convert all the PEP to malate, but insufficient to further form succinate. With the aim of producing the more valuable metabolite succinate, additional reductive reactions are needed. Although succinate production could be enhanced through adding more reduced carbon substrate (e.g., sorbitol) [22], this strategy seemed not to be economically efficient. By eliminating the IclR transcriptional repressor to activate the aerobic glyoxylate pathway in a mutant strain, the NADH insufficiency was resolved. This engineered strain was capable of achieving a succinate yield of 1.6 mol per mole glucose at very high rates [48]. As mentioned above, *E. coli* has four native carboxylation routes that redirect C3 metabolites (PEP and pyruvate) to the C4 diacids branch, among which, the energetically favorable carboxylation of PEP to oxaloacetate by PEP carboxylase is the primary fermentative pathway [27]. Energy contained in PEP is lost in this reaction with the release of inorganic phosphate. *E. coli* native PEP carboxykinase could not redirect carbon flux to the reductive reactions of the TCA cycle because this enzyme mainly functions in gluconeogenesis, catalyzing the decarboxylation and phosphorylation of oxaloacetate to yield PEP [10]. Expression of this gene is also feedback inhibited by glucose. In a recent study, the PEP carboxykinase was screened by spontaneous mutation to replace PEP carboxylase as the primary carboxylation enzyme and one more ATP was produced in the C4 diacids pathway [65]. Heterologous expression of *Actinobacillus succinogenes* PEP carboxykinase in an *E. coli* PEP carboxylase mutant strain also produced additional ATP and increased succinate production by 6.5-fold [28].

Aerobic production of C4 diacids: a new promising pathway

The various genetic improvements described above for C4 diacids production were done under anaerobic conditions. Unfortunately, anaerobic fermentation has inherent disadvantages that are difficult to surmount (e.g., long doubling time, slow carbon throughput, low product formation rates) [35]. The production of the most valuable C4 diacid, succinate, is also hampered by the limitation of NADH availability. In contrast, the aerobic strategy can produce much more ATP for cell growth and more NADH to balance the reducing power. One strategy to overcome these problems is to generate enough biomass under aerobic conditions, then switching to anaerobic conditions, which was called a ‘dual-phase’ fermentation system [57]. However, the dual-phase fermentation required strict control of the pH [38], CO_2 concentration [39], and transition between aerobic and anaerobic conditions [58], and it thus seems unsuitable for industrial applications.

An absolute aerobic pathway constructed by Lin et al. in recent years seemed to be a potential pathway for succinate and subsequent C4 diacids production. The metabolic

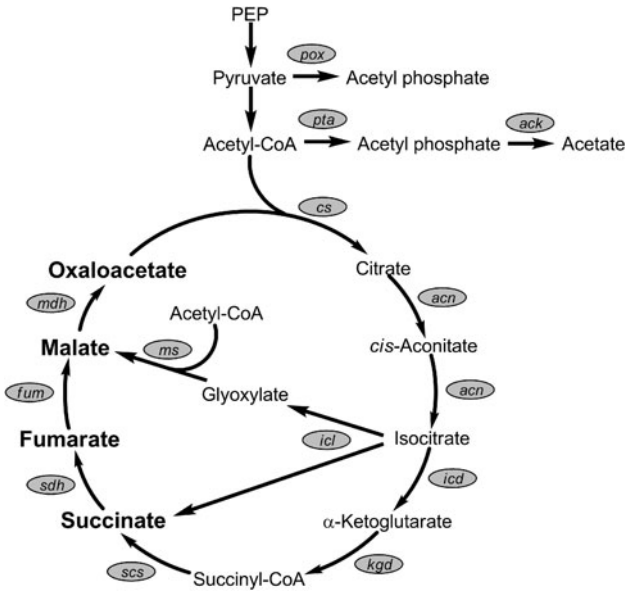

Fig. 4 Aerobic metabolic pathways for the synthesis of C4 diacids in *E. coli*. Enzymes encoded by the genes shown are *pox*, pyruvate oxidase; *pta*, phosphotransacetylase; *ack*, acetate kinase; *cs*, citrate synthase; *acn*, aconitase; *icd*, isocitrate dehydrogenase; *kgd*, α-ketoglutarate dehydrogenase; *scs*, succinyl-CoA synthetase; *sdh*, succinate dehydrogenase; *fum*, fumarase; *mdh*, malate dehydrogenase; *icl*, isocitrate lyase; *ms*, malate synthase

pathway for C4 diacids production through the aerobic glyoxylate cycle is shown in Fig. 4. Two alternative pathways are employed for C4 diacids formation—the TCA cycle and the glyoxylate cycle. These two diverse pathways separate at the isocitrate point. Metabolism of isocitrate to succinate through the TCA cycle needs to go through two intermediates, α-ketoglutarate and succinyl-CoA. For each acetyl-CoA that enters the oxidative TCA cycle arm, two carbon dioxide molecules would be lost. In the glyoxylate bypass, isocitrate undergoes cleavage into succinate and glyoxylate, which is catalyzed by isocitrate lyase. Then glyoxylate condenses with acetyl-CoA, yielding another C4 diacid, malate [29]. The glyoxylate cycle bypasses the steps in the TCA cycle where carbon is lost in the form of carbon dioxide. Therefore, this pathway seems to be more effective for C4 diacids production. The glyoxylate route can be easily activated because it is primarily controlled by a repressor protein, IclR [45]. Meanwhile, the TCA cycle can be blocked by deleting the isocitrate dehydrogenase gene (*icd*). The disruption of succinate dehydrogenase (*sdh*) in the TCA cycle is also a key regulatory point for succinate accumulation under aerobic conditions [3]. By knocking out the genes coding *iclR*, *sdh*, and *icd* in acetate pathways of *E. coli*, Lin et al. designed a mutant strain that produced a maximum of 43 mM succinate with a yield of 0.7 [36]. Fed-batch culture of this strain could produce 58.3 g/l of succinate in 59 h under completely aerobic conditions [35]. Further inactivation of the *ptsG* gene and overexpression of a mutant *Sorghum* PEP carboxylase in this production system showed that the maximum theoretical succinate yield was achieved (one mole succinate produced per mole glucose consumed) [37]. However, these constructed strains needed complex medium components such as tryptone and yeast extract for optimal growth as a result of the knockout of key enzymes in the TCA cycle, which make the system unsuitable for large-scale production [5].

Conclusion and future prospects

Metabolic engineering can be defined as redirecting cellular metabolic flux to desired metabolites by using recombinant DNA and other molecular biology tools [4]. Metabolic engineering has proven to be a rational alternative to traditional strain selection methods and provides important tools for engineering non-native organisms to produce a broad class of valuable platform chemicals. As the above examples show, metabolic engineering efforts have enabled *E. coli* to function as an efficient microbial whole-cell catalyst for the production of C4 diacids. Much higher product concentrations were obtained by using engineered strains when compared with the wild-type strain.

Despite the tremendous progress already made in metabolic engineering of *E. coli* for C4 diacids production, it is still necessary to increase rates, titers, and yields before industrial applications become economically feasible. Previous studies mainly focus on engineering the key enzymes in the C4 diacids pathway. In order to further improve the productivity, at least two points should be considered. First, the efflux of the C4 diacids seems to be of key importance. The exporter and transporter systems for C4 diacids have been well characterized by many former researchers [23]. Engineering of the transporter systems would be crucial to optimize C4 diacids production, but has not been applied in current studies. Second, product tolerance is an important issue affecting the application of microbes for production of organic acids [1]. The C4 diacids tolerance is the result of a several simultaneous mechanisms, thus making rational metabolic engineering aimed at implementation of tolerance strategies a daunting challenge.

The successes of metabolically engineered *E. coli* only open the door towards biotechnological production of C4 diacids. New advancements in this field such as computer-aided enzyme design [25] and genomic scale metabolic models [20] are rapidly expanding and will certainly improve the efficiency of metabolic engineering. By being fused with the development of system biology, metabolic engineering techniques can be expected to further advance

the performance of existing strains. As a host organism of industrial microbiology, *E. coli* will continue to be the approach of choice for commercially useful chemicals. The current achievements and the observed trends of metabolically engineered *E. coli* represent a promising path for the viable industrial production of bio-based chemicals.

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