Biotechnology Advances 33 (2015) 830–841

Contents lists available at ScienceDirect

Biotechnology Advances

journal homepage: www.elsevier.com/locate/biotechadv

Research review paper

Metabolic engineering in the biotechnological production of organic acids in the tricarboxylic acid cycle of microorganisms: Advances and prospects

Xian Yin [a,b,c], Jianghua Li [a,c], Hyun-dong Shin [d], Guocheng Du [a,c], Long Liu [a,b,c,*], Jian Chen [a,e,**]

[a] Key Laboratory of Carbohydrate Chemistry and Biotechnology, Ministry of Education, Jiangnan University, Wuxi 214122, China
[b] State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, Shanghai, China
[c] Synergetic Innovation Center of Food Safety and Nutrition, Wuxi 214122, China
[d] School of Chemical and Biomolecular Engineering, GA Institute of Technology, Atlanta 30332, USA
[e] National Engineering of Laboratory for Cereal Fermentation Technology, Jiangnan University, Wuxi 214122, China

ARTICLE INFO

Article history:
Received 26 January 2015
Received in revised form 8 April 2015
Accepted 11 April 2015
Available online 19 April 2015

Keywords:
Metabolic engineering
Tricarboxylic acid (TCA) cycle
Citric acid
α-Ketoglutaric acid
Succinic acid
Fumaric acid
L-Malic acid
Oxaloacetate
C_4-dicarboxylic acid

ABSTRACT

Organic acids, which are chemically synthesized, are also natural intermediates in the metabolic pathways of microorganisms, among which the tricarboxylic acid (TCA) cycle is the most crucial route existing in almost all living organisms. Organic acids in the TCA cycle include citric acid, α-ketoglutaric acid, succinic acid, fumaric acid, L-malic acid, and oxaloacetate, which are building-block chemicals with wide applications and huge markets. In this review, we summarize the synthesis pathways of these organic acids and review recent advances in metabolic engineering strategies that enhance organic acid production. We also propose further improvements for the production of organic acids with systems and synthetic biology-guided metabolic engineering strategies.

Contents

* Correspondence to: L. Liu, Key Laboratory of Carbohydrate Chemistry and Biotechnology, Ministry of Education, Jiangnan University, Wuxi 214122, China. Tel.: +86 510 85918312; fax: +86 510 85918309.
** Correspondence to: J. Chen, Key Laboratory of Carbohydrate Chemistry and Biotechnology, Ministry of Education, Jiangnan University, Wuxi 214122, China. Tel.: +86 510 85913661; fax: +86 510 85910799.
E-mail addresses: longliu@jiangnan.edu.cn (L. Liu), jchen@jiangnan.edu.cn (J. Chen).

http://dx.doi.org/10.1016/j.biotechadv.2015.04.006
0734-9750/

1. Introduction

Organic acids are low-molecular-weight organic compounds with one or more acidic groups such as carboxyl, sulfonic, alcohol, and thiol groups. These functional groups make organic acids key building-block chemicals, which have huge markets and are commonly refined from petroleum. Because petroleum is an unsustainable resource and its use generates greenhouse gas emissions, petroleum-based chemical production must be shifted to biotechnological processes (Sauer et al., 2008). Most organic acids are intermediates in the metabolic pathways that occur naturally in microorganisms; among these pathways the tricarboxylic acid (TCA) cycle is the most crucial. The TCA cycle exists in almost all living organisms. Chemical compounds in the TCA cycle include citric acid (CA), α-ketoglutaric acid (KGA), succinic acid (SA), fumaric acid (FA), L-malic acid (MA), and oxaloacetate (OAA). The chemical structures of these compounds are listed in Fig. 1. These acids have key positions in the organic acid industry owing to their broad applications.

CA is a bulk product used mainly in food, pharmaceutical and other industrial applications. It is also used as a biocompatible polymeric material for nanomedicine (Naeini et al., 2010). KGA is used mainly as a dietary supplement but also has uses in diagnostic assays (Morgunov et al., 2013). SA, FA, MA, and OAA, which constitute the C_4-dicarboxylic acid family, have similar molecular structures and can be easily interconverted (Cao et al., 2011). The U.S. Department of Energy has proposed these acids as building-block chemicals that can be produced from biomass.

The potential market for these acids can be expanded if fermentation costs can be reduced. Strain improvement is crucial for fermentation and has occurred through random mutagenesis and screening processes, rational metabolic engineering, and currently, systems metabolic engineering. No review has summarized developments in the metabolic engineering of strains for the production of these organic acids. This review compares the existing synthesis pathways of these organic acids and summarizes the metabolic engineering strategies for bacteria, yeasts, and filamentous fungi implemented to improve performance. We also suggest strategies for combining systems biology, synthetic biology, and transporter engineering as efficient tools for improving the production and productivity of these organic acids in the future.

2. Citric acid

CA (2-hydroxy-propane-1,2,3-tricarboxylic acid) is used extensively in the food and pharmaceutical industries owing to its safety, pleasant acidic taste, high water solubility, and chelating and buffering properties. CA is also widely used in detergents, cosmetics, and a variety of other industrial applications (Soccol et al., 2006).

The work of Currie formed the basis of industrial CA fermentation in 1917 (Papagianni, 2007). Currently, 99% of the world's CA output is obtained through fermentation, and approximately 80% is obtained through submerged fermentation using *Aspergillus niger* (Dhillon et al., 2011). CA fermentation requires several critical nutrient conditions, including excessive concentrations of carbon source, low pH, sufficient dissolved oxygen, and suboptimal concentrations of Mn^{2+} and phosphate (Karaffa and Kubicek, 2003; Papagianni, 2007). The best strains produce CA at yields up to 0.95 g per gram of sugar. However, potential remains for a fivefold increase in CA productivity according to the model of Alvarez-Vasquez et al. (2000).

CA is formed mainly via the cytosolic glycolytic pathway and subsequent mitochondrial condensation of a C4 and a C2 moiety (Karaffa and Kubicek, 2003). Glycolysis catabolizes glucose to 2 mol pyruvate. One mole is transformed into the mitochondria and converted to acetyl-coenzyme A (acetyl-CoA) via the release of 1 mol CO_2, and the other is converted to OAA via the fixing of 1 mol CO_2. OAA is subsequently

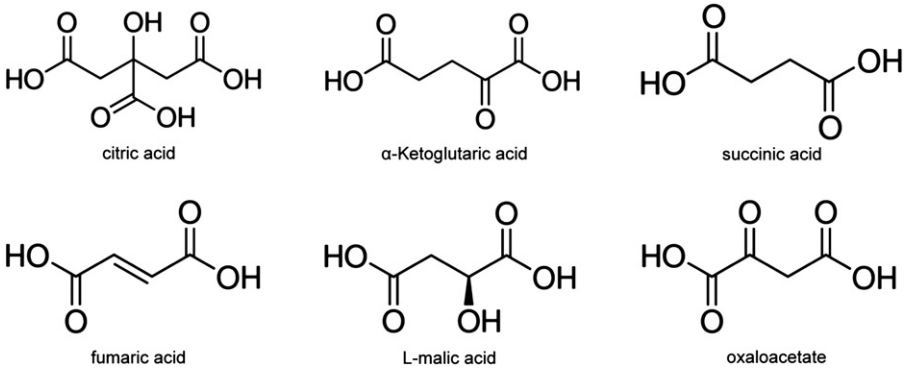

Fig. 1. Chemical structures of organic acids.

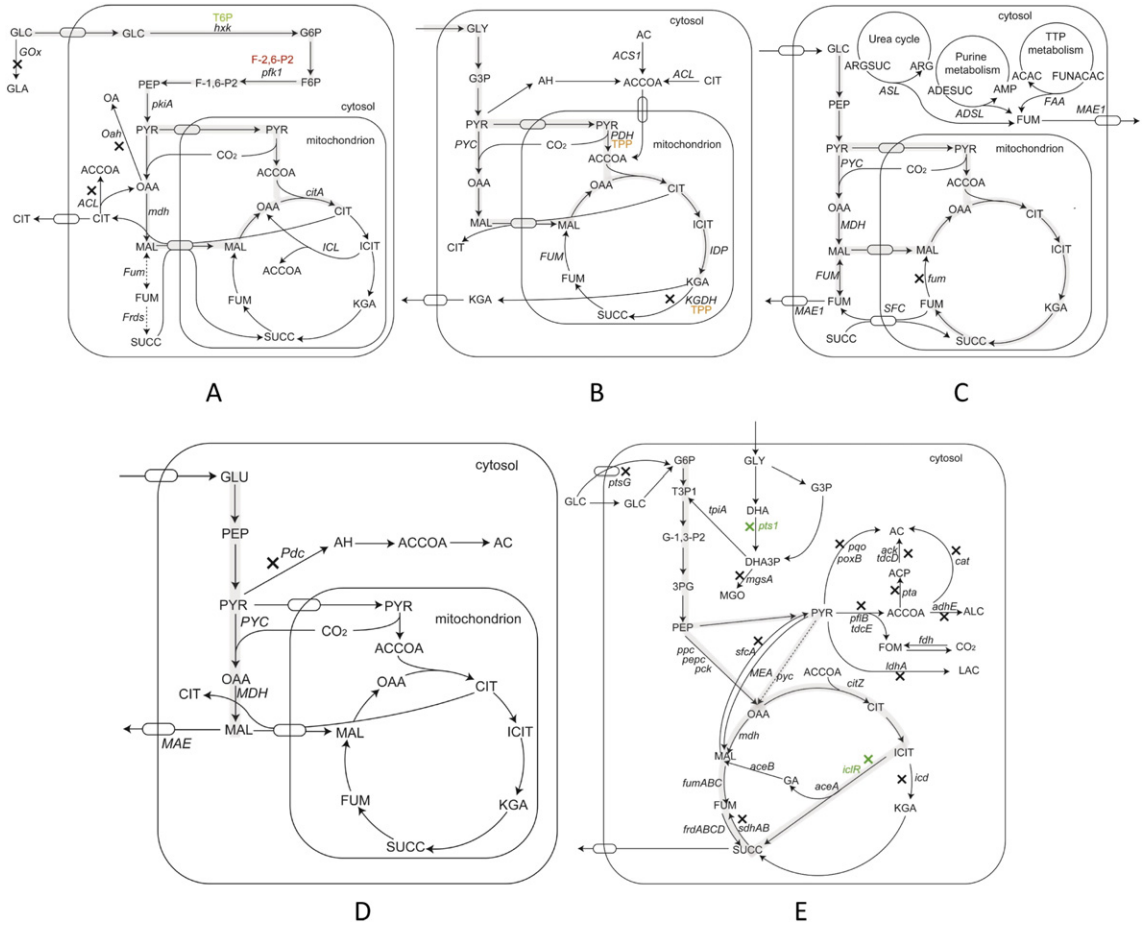

Fig. 2. Engineering strategies for enhancing organic acid production. Metabolic flux of (A) citric acid in *A. niger* and *Y. lipolytica*, (B) KGA in *Y. lipolytica*, (C) fumaric acid in *S. cerevisiae* and *T. glabrata*, (D) malic acid in *S. cerevisiae*, (E) succinic acid in *E. coli*. Gray line shows the main pathway of acid synthesis. The dotted line indicates exogenous pathway that does not exist in natural strain. Letters in green indicates inhibitor of the enzyme, while in red enhancer and in orange co-factor. The cross mark indicates the gene is knocked out. Abbreviations: 3PG, glycerate-3-phosphate; AC, acetate; ACAC, acetoacetate; ACCOA, Acetyl-CoA; ACP, acetyl phosphate; ADESUC, adenylosuccinate; AH, aldehyde; ALC, alcohol; AMP, adenosine monophosphate; ARG, arginine; ARGSUC, argininosuccinate; CIT, citrate; DHA, dihydroxyacetone; DHA3P, dihydroxyacetone 3-phosphate; G6P, glucose-6-phosphate; F-1,6-P2, fructose-1,6-bisphosphate; F-2,6-P2, fructose 2,6-bisphosphate; F6P, fructose-6-phosphate; FMA, formate; FOM, formate; FUM, fumarate; FUMACAC, fumarylacetoacetate; G-1,3-P2, glycerate-1, 3-biphosphate; G3P, glycerol-3-phosphate; GA, glyoxylate; GLA, gluconic acid; GLC, glucose; GLY, glycerol; G6P, glucose-6-phosphate; KGA, α-ketoglutarate; ICIT, isocitrate; LAC, lactic acid; MAL, malate; MGO, methylglyoxal; OA, oxalic acid; OAA, oxaloacetate; PEP, phosphoenolpyruvate; PYR, pyruvate; SUCC, succinate; T3P1, glyceraldehyde-3-phosphate; T6P, trehalose-6-phosphate; TPP, thiamine pyrophosphate; *aceA*, isocitrate lyase; *aceB*, malate synthase; *ackA*, acetate kinase; *ACL*, ATP-citrate lyase; *ADSL*, adenylosuccinate lyase; *ASL*, argininosuccinate lyase; *cat*, acetyl-CoA:CoA transferase; *cit*, citrate synthase; *eqo*, pyruvate:menaquinone oxidoreductase; *FAA*, fumarylacetoacetase; *fdh*, NAD^+-coupled formate dehydrogenase; *fum*, fumarase; *frd*, fumarate reductase; *gapA*, glyceraldehyde 3-phosphate dehydrogenase; *Gox*, glucose oxidase; *hxk*, hexokinase; *icd*, isocitrate dehydrogenase; *ICL*, isocitrate lyase; *ICLR*, Isocitrate lyase repressor; *IDP1*, isocitrate dehydrogenase; *KGDH*, alpha-ketoglutarate dehydrogenase; *ldhA*, lactate dehydrogenase; *MAE*, C_4-dicarboxylic acids transporter; *mdh*, malate dehydrogenase; *MEA*, malate dehydrogenase; *mgsA*, methylglyoxal synthase; *oah*, oxaloacetate acetylhydrolase; *pck*, phosphoenolpyruvate carboxykinase; *Pdc*, pyruvate decarboxylase; *PDHC*, pyruvate dehydrogenase complex; *pepc*, phosphoenolpyruvate carboxykinase; *pfk1*, phosphofructokinase; *pfl*, pyruvate:formate lyase; *pkiA*, phosphofructokinase; *poxB*, pyruvate oxidase; *ppc*, phosphoenolpyruvate carboxykinase; *pta*, phosphotransacetylase; *pyc*, pyruvate carboxylase; *sdhAB*, succinate dehydrogenase; *SFC*, succinate-fumarate transporter; *sfcA*, NAD^+-linked malic enzyme; *tdcD*, threonine decarboxylase; *tdcE*, 2-ketobutyrate formate-lyase.

reduced to MA and enters the mitochondria via a malate-citrate antiporter. Mitochondrial MA participates in the TCA cycle and forms CA (Fig. 2A). As a result, the maximum theoretical CA yield is 1 mol per mole glucose. The conversion of glucose to CA generates 1 mol ATP and 3 mol of nicotinamide adenine dinucleotide (NADH), which is redundant and must be turned over through an alternative respiratory pathway.

2.1. Engineering glycolysis and the TCA pathway for enhanced CA production

Metabolic engineering through the overexpression of rate-limiting enzymes involving glycolysis and the TCA cycle to increase CA productivity has had little success because of the stringent regulation of central carbon metabolism. For example, overexpression of pyruvate kinase and phosphofructokinase did not increase CA production; neither the intermediary metabolite levels nor the activities of other enzymes in the pathway changed. Although phosphofructokinase was overexpressed, enzyme activity decreased owing to a reduction of the level of fructose 2,6-bisphosphate, which was a positive allosteric effector. The constructed strain produced CA at a titer of 55 g/L, which was identical to the yield of 0.64 mol/mol in the wild-type strain (Ruijter et al., 1997). Similarly, increasing the expression level of citrate synthase (CS) did not improve CA production; the mutant strain produced approximately 46 g/L CA (Ruijter et al., 2000). When *ACO1*, which encodes aconitase, was expressed in *Yarrowia lipolytica*, the product proportion of isocitrate increased, but the total amount of acids produced was not influenced (Holz et al., 2009).

2.2. Engineering to eliminate inhibitor effect and by-product formation

Deletion of the gene encoding trehalose-6-phosphate synthase A decreased the level of trehalose-6-phosphate, an inhibitor of hexokinase. As a result, CA accumulation was initiated earlier (Arisan-Atac et al.,

1996). The gene *Brsa-25* is responsive to Mn^{2+}, the concentration of which influences the morphology of *A. niger* and CA production. Antisense expression of *Brsa-25* facilitates pelleted growth and increases CA production in the presence of Mn^{2+} (Dai et al., 2004).

Oxalic acid is produced by *A. niger* when the culture pH is above 3, and oxaloacetate acetylhydrolase drives oxalate synthesis. A recombinant strain, which lacked both glucose oxidase and oxaloacetate acetylhydrolase, tolerated a pH of 5 and the presence of Mn^{2+} to produce CA (Ruijter et al., 1999).

2.3. Engineering to enhance the supplement pathway

The influence of the cytosol reductive TCA (rTCA) cycle on CA production has been evaluated through individual or combined overexpression of genes including fumarases (*Fum1s* and *FumRs*), fumarate reductase (*Frds1*), and malate dehydrogenase (*mdh2*). Compared with the wild-type strain, all the engineered strains produced CA at higher yields and productivity. The finding that the *Mdh2*-overexpressing strain increased the CA production rate earlier in the initial phase supports the theory that the accumulation of cytosol MA triggers CA production. The overexpression of fumarase, which converts FA to MA, provided more substrate to the mitochondrial malate-citrate antiporter and increased CA secretion, but a large amount of by-product oxalate was generated. Higher CA productivity was obtained with the *Frds1*-overexpressing strain. *Frds1* catalyzed SA formation from FA. The relationship of cytosol SA synthesis and CA production suggests that SA is a potential substrate for the mitochondrial CA antiporter. The best result came from the strain co-expressing *fumRs* and *Frds1*, which created a cytosolic rTCA pathway from MA toward SA, which was then used to exchange mitochondrial CA (de Jongh and Nielsen, 2008).

2.4. Engineering to enhance diverse carbon absorption in Y. lipolytica

A CA-producing *Y. lipolytica* strain was constructed to express inulinase *INU1* from *Kluyveromyces marxianus*. The strain utilized inulin to synthesize CA at a titer of 68.9 g/L, with 4.1 g/L isocitric acid as a by-product (Liu et al., 2010). Deleting *ACL1*-encoding ATP-citrate lyase and expressing isocitrate lyase (*ICL1*) produced a final strain that yielded CA from 10% inulin at a titer of 84 g/L (Liu et al., 2013).

CA production was enhanced through the expression of invertase *SUC2* from *Saccharomyces cerevisiae* for the utilization of sucrose and *ICL1* in *Y. lipolytica*. The reconstructed strain H222-S4 (p67ICL1) T5 produced 140 g/L CA from sucrose, with a high productivity at 0.73 g/L per hour in a fed-batch culture (Forster et al., 2007).

3. α-Ketoglutaric acid

KGA (2-oxopentanedioic acid; 2-ketoglutaric acid) is used in dietary supplements as a direct precursor for glutamine and glutamate. It is also a building-block chemical for the synthesis of heterocycles, which are used as antitumor agents (Otto et al., 2011; Stottmeister et al., 2005). KGA is used as a substrate for 2-oxoglutarate dehydrogenase (EC 1.2.4.2), glutamate dehydrogenase (EC 1.4.1.2), aspartate transaminase (EC 2.6.1.1), alanine transaminase (EC 2.6.1.2), cysteine transaminase (EC 2.6.1.3), and others in diagnostic assays for a wide range of diseases (Morgunov et al., 2013).

KGA is crucial in the coordination of carbon and nitrogen utilization due to its role as a key intermediate in the Krebs cycle and amino acid metabolism (Doucette et al., 2011). It is mainly synthesized through the TCA cycle (Fig. 2B).

Using *Y. lipolytica* as a KGA producer yields 195 g/L KGA when n-paraffins are used as carbon sources, but the high price of paraffin inhibits the industrial application of this method. In a recent study, a *Y. lipolytica* strain utilized glycerol, ethanol, and vegetable oils as carbon sources (Yu et al., 2012b). The thiamine-auxotrophic *Y. lipolytica* is a robust KGA producer. A deficiency of thiamine, which is a cofactor of pyruvate dehydrogenase (PDH) and α-ketoglutarate dehydrogenase, inhibited KGA oxidation and caused the accumulation of KGA in broth (Kamzolova et al., 2012).

3.1. Cofactor engineering for enhanced KGA production

The effect of components of the pyruvate dehydrogenase complex on KGA production has been discussed by Guo et al. (2014). When the alpha or beta subunits of the PDH complex E1 were overexpressed, which is involved in the thiamine pyrophosphate binding, the recombinant strains accumulated pyruvate at low levels and were insensitive to exogenous thiamine concentration. This outcome indicated that the competition for the cofactor could be switched to ketoglutarate dehydrogenase. Among these mutants, *Y. lipolytica* T1, which expressed the PDH complex E1 component α subunit, yielded the highest production at 43.3 g/L (Guo et al., 2014).

In another study, cofactor metabolism was modulated by the overexpression of the acetyl-CoA synthetase gene *ACS1* from *S. cerevisiae* and the ATP citrate lyase gene *ACL* from *Mus musculus* to induce carbon flux flow from pyruvate to KGA. Both mutants increased acetyl-CoA formation and enhanced KGA production when either of these genes was expressed. The strain *Y. lipolytica*-ACL performed best, with a KGA titer of 56.5 g/L (Zhou et al., 2012).

3.2. By-product elimination and TCA cycle enhancement

A screened *Y. lipolytica* strain produced KGA at a titer of 39.2 g/L, with pyruvate as a by-product (Zhou et al., 2010). The pyruvate carboxylation pathway was enhanced by overexpressing pyruvate carboxylase genes with the aim of decreasing pyruvate production and replenishing the TCA cycle to increase KGA formation. The constructed strain increased KGA yield by 35.3% at a final titer of 62.5 g/L, with pyruvate yield decreased by 69.8% (Yin et al., 2012). The influence of cytosol pyruvate carboxylase (PYC) and mitochondrial fumarase (FUM) on KGA production was studied by overexpressing enzyme genes individually or simultaneously. These strains showed different product selectivities with identical KGA production of approximately 130 g/L (Otto et al., 2012).

The combination of TCA cycle enhancement and the elimination of pyruvate as a by-product improved KGA production. Dose-dependent gene overexpression was used to construct strains harboring multiple copies of the nicotinamide adenine dinucleotide phosphate ($NADP^+$)-dependent isocitrate dehydrogenase gene (*IDP1*) and PYC gene (*PYC1*). Reconstructed *Y. lipolytica* strains containing either of these genes increased KGA secretion. Overexpression of both genes further enhanced KGA production to 186 g/L (Yovkova et al., 2014).

4. Succinic acid

SA (1,2-ethanedicarboxylic acid) has broad applications in the food industry as an additive and flavoring agent and in pharmaceuticals as a supplement. It is also used as a surfactant, detergent extender, ion chelator, foaming agent, and aviation de-icer.

Anaerobiospirillum succiniciproducens and *Actinobacillus succinogenes* are natural SA producers, yielding SA as the dominant product at 50 g/L and 90 g/L, respectively, during anaerobic fermentation. However, these bacteria are unstable, tend to degenerate, and require strict anaerobic conditions for culture. These disadvantages disqualify them from commercial fermentation.

Three routes are used for SA formation (Cheng et al., 2013). The first route is the reductive TCA cycle under anaerobic conditions. OAA is synthesized from phosphoenolpyruvate (PEP) via CO_2 fixation and converted to MA, which subsequently forms FA and finally SA. The theoretical yield is 2 mol SA per mole glucose. Two moles of NADH are required for 1 mol SA formation from phosphoenolpyruvate. However, 1 mol glucose generates 2 mol NADH during the formation of 2 mol PEP, leading

to NADH limitation in SA production. The second pathway is a glyoxylate route under aerobic conditions. Two moles of acetyl-CoA are converted to 1 mol SA, with 1 mol NADH formation, thereby forming extra NADH. The third pathway is the oxidative TCA cycle. Two moles of pyruvate are converted to 1 mol SA, generating 3 mol NADH. The theoretical yield is 1 mol SA per mole glucose (Fig. 2E).

Escherichia coli has been used as a host to produce SA, and several engineered strains have shown high productivity and yield.

4.1. Engineering to block by-product formation

E. coli strains naturally synthesize large amount of acetate, lactate, and ethanol from pyruvate. As a result, *E. coli* engineering must first block by-product synthesis pathways. Genes involved in these pathway are the phosphotransacetylase (*pta*) plus acetate kinase (ackA), pyruvate:menaquinone oxidoreductase (*pqo*), acetyl-CoA:CoA transferase (*cat*), and L-lactate dehydrogenase (*ldhA*) genes.

Gene deletions and metabolic evolution resulted in strain KJ060, which lacked *ldhA*, *adhE*, *ackA*, *focA*, and *pflB*. This strain showed increased ATP production and produced SA at a titer of 86.6 g/L, with a yield of 1.41 mol/mol. Another strain, KJ071, in which *mgsA* was also deleted, produced MA instead of SA at a titer of 516 mM (Jantama et al., 2008a).

The additional deletion of *tdcD* encoding threonine decarboxylase and *tdcE* encoding 2-ketobutyrate formate-lyase in *E. coli* strain KJ091 (*ΔldhA*, *ΔadhE*, *ΔackA*, *ΔfocA-pflB*, *ΔmgsA*, *ΔpoxB*) inhibited acetate formation and increased SA yield by 10%. Individual deletion of *aspC* encoding aspartate amino-transferase and *sfcA* encoding nicotinamide adenine dinucleotide (NAD^+)-linked malic enzyme did not affect SA yield, but deleting them simultaneously increased SA yield to 1.5 mol/mol (near theoretical yields) at a titer of 700 mM (Jantama et al., 2008b).

A set of mutants was constructed to evaluate the influence of genes in the by-product pathway on SA production. The strains (*ΔadhE*, *Δpta*, *ΔpoxB*, *ΔldhA* [pZS-*pyc*] and *ΔpflB*, *ΔldhA*, *ΔpoxB*, *ΔadhE*, *ΔackA*, *Δpta* [pZS-*pyc*]) performed best, with yields of 60% and 54%, respectively (Blankschien et al., 2010).

The genes *ptsI*, which is involved in the phosphotransferase system, and *pflB*, which encodes pyruvate formate-lyase, were disrupted with the aim of eliminating formate by-product synthesis and using glycerol as a carbon source. The expression of *pck* was enhanced by mutating the promoter. The recombinant *E. coli* strain XZ721 produced SA at a yield of 0.8 mol/mol glycerol (Zhang et al., 2010).

4.2. Engineering to enhance flux for OAA generation

OAA is a core chemical in SA production as a precursor in both the TCA and rTCA cycles. Enzymes linked to OAA formation are PYC and phosphoenolpyruvate carboxylase (PEPC). Overexpression of the *pyc* gene in *E. coli* resulted in a low SA production of 1.77 g/L. Overexpressing the PEPC gene improved SA production to 10.7 g/L (Millard et al., 1996). *E. coli* strain AFP111 was derived from NZN111 through spontaneous chromosome mutation. The mutation site encoded phosphotransferase, which transported PEP as a co-substrate for glucose transport. The strain generated acetic acid and ethanol as well as SA, for which the yield was 0.7 g/g (Donnelly et al., 1998). When expressing the pyruvate carboxylase gene *pyc* from *Rhizobium etli*, an engineered strain AFP111/pTrc99A-*pyc* produced SA at a yield of 0.96 g/g (Vemuri et al., 2002a). When duel-phase fermentation was used in this strain, production increased to 99.2 g/L at a yield of 1.1 mol/mol glucose and a productivity of 1.3 g/L per hour (Vemuri et al., 2002b). Strain SBS110MG, an *adhE* and *ldhA* double mutant, was constructed to express *pyc* and achieved an SA production of 15.6 g/L at a yield of 1.3 mol/mol (Sanchez et al., 2005a). YBS132, an *ackA-pta* and *ldhA* mutant, was constructed to co-express *pepc* from *Sorghum vulgare* and *pyc* from *Lactococcus lactis*, improving SA production to 3.4 g/L (Lin et al., 2005e).

A recombinant *Corynebacterium glutamicum* strain was constructed to express *pyc*. The *ldhA* gene, encoding lactate dehydrogenase, was also knocked out in the strain. SA production by this strain reached 146 g/L within 46 h at a yield of 1.4 mol/mol glucose (Okino et al., 2008).

4.3. Engineering to enhance the TCA cycle, rTCA cycle, and glyoxylate flux

The strain *C. glutamicum* ZX1 (*ΔsdhCAB*, *ΔldhA*, *Δpqo*, *Δcat*, *Δpta*) was constructed to express *acsA* encoding acetyl-CoA synthase and *gltA* encoding CS to enhance the TCA cycle and increase the expression levels of *ppc* and *pyc* via promoter exchange. The strain produced 28.5 g/L SA at a yield of 0.63 mol/mol glucose (Zhu et al., 2013).

The deletion of the pyruvate:formate lyase gene *pfl* and lactate dehydrogenase gene *ldh*, resulted in a recombinant *E. coli* strain that synthesized abundant pyruvate. The introduction of an engineered malate dehydrogenase (MDH) gene, *MEA*, into the mutant to enhance the rTCA cycle yielded a final strain that produced SA at 7.07 g/L (Stols et al., 1997).

The glyoxylate pathway was reconstructed to produce SA. Five genes, including *pta-ackA*, *pqo*, *cat*, and *ldhA*, were first deleted. A recombinant *C. glutamicum* strain expressing the PYC gene *pyc*, PEPC gene *ppc*, ICL gene *aceA*, malate synthase gene *aceB*, CS gene *gltA*, and succinate exporter gene *SucE* produced 0.93 M SA, with a productivity of 9.4 mM/h and yield of 1.32 mol/mol glucose (Zhu et al., 2014).

4.4. Manipulation of reducing power balance and cofactor engineering

The NAD^+-coupled formate dehydrogenase gene (*fdh*) catalyzes NADH formation. The *fdh* and pyruvate carboxylase (pyc^{P458S}) genes were integrated into the chromosome of *C. glutamicum* BOL-1(*Δpta-ackA*, *Δpqo*, *Δcat*, *ΔldhA*), and the glyceraldehyde 3-phosphate dehydrogenase gene (*gapA*) was overexpressed from plasmid. The final strain produced SA at 133 g/L in 53 h with a yield of 1.67 mol/mol glucose (Litsanov et al., 2012). *E. coli* BA16 was derived from BA002, which lacked the *ldhA* and *pflB* genes and overexpressed *pncB*, which encodes a rate-limiting enzyme for NAD(H) synthesis and *pyc* from *L. lactis*. The strain produced 25 g/L SA under anaerobic fermentation in a 3-L bioreactor (Ma et al., 2013).

Pantothenate kinase was expressed in combination with PEPC or PYC in two strains to increase cytosol acetyl-CoA and CoA levels. Both strains showed improved SA production with a titer of approximately 20 mM (Lin et al., 2004).

4.5. Strain improvement with in silico modeling

The construction of *E. coli* strains must always use several engineering strategies to coordinate the carbon flux for SA production, by-product elimination with energy and reducing power balance to achieve the highest SA yield. A mutant strain, SBS550MG, lacking *adhE*, *ldhA*, *ack-pta*, and *iclR* was constructed to express *pyc*. The strain produced SA at a yield of 1.59 mol/mol. When the *adhE*, *ldhA*, and *ack-pta* gene mutant strain SBS990MG expressed *citZ*, SA fermentation yield reached 1.58 mol/mol (Sanchez et al., 2005b). Further expression of *fdh1*, encoding NAD^+-dependent formate dehydrogenase, and *pycA* from *L. lactis* improved SA productivity to 2 g/L per hour when formate was present in the fermentation culture owing to NADH and CO_2 supplementation through formate degradation (Balzer et al., 2013).

Three pathways were redesigned in *E. coli* GJT001 for SA production. The TCA cycle was blocked by deleting *sdhAB* and *icd*, the glyoxylate cycle was enhanced by disrupting the ICL depressor gene *iclR*, and the acetate pathway was inhibited via deletion of *poxB* and *ackA-pta*. The final strain, HL27615k, produced SA under aerobic conditions at a yield of 0.7 mol/mol glucose (Lin et al., 2005a). Strain HL27659k (*ΔsdhAB*, *ΔackA-pta*, *ΔpoxB*, *ΔiclR*, *ΔptsG*), expressing *pepc* from

S. vulgare, increased the SA yield to 0.94 mol/mol (Lin et al., 2005c). When the *pepc*^{S8D} mutant was expressed instead of natural *pepc*, the strain achieved an SA yield of 1.09 mol/mol (Lin et al., 2005b). Further study showed that strain HL27659k had higher CS, ICL, MDH, and malate synthase activities (Lin et al., 2005d).

According to the CAASOP (computational approach for strain optimization aiming at high productivity) analysis of *E. coli* ZJG13 (*ΔsdhA*, *ΔackA-pta*, *ΔpoxB*, *ΔmgsA*, *ΔiclR*), the strain was transformed with plasmid pT184*pyc* to overexpress pyruvate carboxylase. The SA production of the constructed strain was 36.1 g/L (Yang et al., 2014).

Mannheimia succiniciproducens MBEL55E, a capnophilic bacterium, natively accumulates SA as a major product at a yield of 0.68 g/g glucose (Lee et al., 2002). The genome sequence of the strain and in silico genome-scale metabolic network were reported (Hong et al., 2004). Based on this work, *ldhA*, *pflB*, *pta*, and *ackA* were disrupted to reduce by-product formation, and the final strain, *M. succiniciproducens* LPK7, produced SA at 52.4 g/L, with a productivity of 1.8 g/L per hour (Lee et al., 2006).

4.6. Engineering S. cerevisiae for SA production

Acid-tolerant *S. cerevisiae* was selected as an SA production host (Raab et al., 2010). Through deletion of the succinate dehydrogenase subunits *SDH1* and *SDH2*, isocitrate dehydrogenase subunit *IDH1*, and mitochondrial isocitrate dehydrogenase isoenzyme *IDP1*, the engineered strain enhanced the glyoxylate pathway and achieved an SA titer of 3.62 g/L at a yield of 0.11 mol/mol glucose.

With the help of a genome-scale metabolic model, two pathways involving three targets, including *Sdh3p* encoding succinate dehydrogenase complex and *Ser3p*/*Ser33p* encoding 3-phosphoglycerate dehydrogenase, were predicted to consume SA and needed to be deleted. The three-gene disrupted mutant was selected through directed evolution for faster growth. Finally, a plasmid-harbored isocitrate lyase gene, *icl1p*, was transformed into the strain. The constructed strain, 8D Evolved (pICL1), produced SA at a titer of 0.9 g/L (Otero et al., 2013).

5. Fumaric acid

FA (2-butenedioic acid trans; 1,2-ethylenedicarboxylic acid) is a valuable platform chemical used mainly in the food and beverage industries (Xu et al., 2012a,b,c). It is also used as a starting material for the synthesis of polymers and resins because it has a carbon–carbon double bond and two carboxylic acid groups (Roa Engel et al., 2008). FA is used to treat the skin condition psoriasis and is commonly added to cattle feed as an antibacterial factor and physiologically active agent (McGinn et al., 2004).

Two metabolic pathways can be used to produce FA (Xu et al., 2013). The first is the rTCA cycle, which provides a maximum theoretical yield of 2 mol FA per mole glucose through CO_2 fixation. However, the energy and reducing power are unbalanced, which causes cell maintenance and acid transport problems. The second pathway is the oxidative TCA cycle, which limits the maximum theoretical yield to 1 mol FA per mole glucose but provides a stable recombinant strain (Xu et al., 2012a,b,c). *Rhizopus* species such as *Rhizopus arrhizus*, *Rhizopus delemar*, and *Rhizopus oryzae*, can produce high levels of FA (Das and Brar, 2014; Wen et al., 2013; Zhou et al., 2011; Zhou et al., 2014).

5.1. Engineering to enhance metabolic flux toward the rTCA and TCA cycles

Enhancing FA production by yeast involves the modification of two pathways (Fig. 2C). One is the rTCA cycle, in which the maximum theoretical FA yield is 2 mol per mole glucose, and the other is the oxidative TCA cycle.

The rTCA cycle was introduced in *S. cerevisiae* by overexpressing genes encoding MDH and FUM from *R. oryzae* and endogenous PYC. Compared with the control, the recombinant strain expressing PYC and MDH produced a higher titer of FA. However, the production was low at 3.18 g/L due to energy imbalance (Xu et al., 2012a).

Simultaneous introduction of the oxidative and reductive routes to *S. cerevisiae* strain FMME003, a pyruvate-producing strain, yielded FA at a titer of 5.64 g/L. In this study, native *fum1* involved in the oxidative route was knocked out, and *RoMDH* and *RoFUM1* from *R. oryzae*, which are involved in the reductive route, were overexpressed; the expression of *RoPYC* showed extreme importance in the balancing of intermediate metabolites for final FA production (Xu et al., 2013).

5.2. Systems metabolic engineering

The oxidative TCA cycle has been enhanced to manage the problem of energy deficiency and improve FA production. Flux balance analysis with a genome-scale metabolic model predicted that mitochondrial FUM1 deletion, pyruvate carboxylase expression, and succinate-fumarate transporter SFC1 introduction in *S. cerevisiae* would enhance FA synthesis. The metabolic engineered strain improved FA production step by step when deletion/insertion was conducted in turn, achieving a final titer of 1.67 g/L (Xu et al., 2012b).

A pyruvate-producing strain of *Torulopsis glabrata* was engineered to produce FA. According to the genome-scale metabolic model, four cytosolic enzymes—argininosuccinate lyase in the urea cycle; adenylosuccinate lyase involved in purine metabolism; fumarylacetoacetase involved in tyrosine, tryptophan, and phenylalanine metabolism, and FUM1 in the TCA cycle—were overexpressed. The recombinant strain with high-level expression of argininosuccinate lyase and low-level expression of adenylosuccinate lyase showed high FA production of 5.62 g/L. Further expression of the *SpMAE1* gene, which encoded a C_4-dicarboxylic acid transporter, increased the FA titer to 8.83 g/L (Chen et al., 2015).

Gu et al. (2014) proved that the hypothetical protein AOX mediates the alternative respiration pathway of *R. oryzae*, which is required for FA production. The alternative respiration pathway was essential in replenishing redundant redox power to produce CA in *A. niger* (Papagianni, 2007). The role of the pathway for FA synthesis, which lacked reducing power for high production, was interesting. Systems biology will determine the mechanism in future studies.

5.3. Enhancement of the rTCA cycle in R. oryzae

A strategy of increasing precursor OAA has been used with the aim of enhancing FA production. Overexpressing exogenous PEPC in *R. oryzae* successfully increased FA yield by 26% at 1.21 mol per mole glucose, with a titer of 24 g/L. However, the endogenous pyruvate carboxylase-expressing mutants produced low levels of FA with poor growth due to ATP shortage and pyruvate flux imbalance (Zhang et al., 2012a). Endogenous fumarase was overexpressed in *R. oryzae* to eliminate by-product MA, but adversely, the mutant strain increased MA production at a yield of 0.38 g/g, which is twice that of the wild type (Zhang and Yang, 2012b).

5.4. Combined enhancement of the noncyclic glyoxylate route with the rTCA cycle in E. coli

The noncyclic glyoxylate route has been used to produce FA in *E. coli*. The isocitrate lyase repressor gene *iclR* was deleted to enhance the carbon flow into a glyoxylate shunt. *fumA*, *fumB*, and *fumC* were knocked out for FA accumulation. Endogenous *ppc* encoding PEPC was overexpressed to increase rTCA cycle flux. Additionally, *arcA*, *aspA*,

lacL, and *ptsG* were deleted. The FA production of the final strain was 28.2 g/L, with a productivity of 0.448 g/L per hour (Song et al., 2013).

6. Malic acid

MA (2-hydroxybutanedioic acid; 2-hydroxysuccinic acid) has wide applications in the food and beverage industries as an acidulant and flavor enhancer (Goldberg et al., 2006). Compared with CA, MA provides a softer taste. MA also has nonfood applications in metal cleaning and finishing, textile finishing, cosmetics, pharmaceuticals, hospital infusions, and paints.

Microbial fermentation of MA as a by-product of yeast fermentation dates back to 1924. As a key constituent of the TCA cycle, MA is generated by variety of microorganisms including bacteria, yeast, and fungi, among which *Aspergillus flavus* is a well-known producer, achieving 63% of the highest theoretical yield of MA on glucose (Battat et al., 1991). Nevertheless, *A. flavus* is disqualified for industrial MA production due to aflatoxin production. Other *Aspergillus* species and *Schizophyllum commune* are also candidate MA producers, but their yields and productivity are low (Kawagoe et al., 1997). Through random mutagenesis and screening processes, a mutant malate-producing strain that lacks alcohol dehydrogenase activity and belongs to *R. delemar* has been isolated. The final mutant, HF-121, produced 120 g/L MA, with a productivity of 2.03 g/L/h—the highest productivity reported to date (Li et al., 2014).

Four possible metabolic pathways exist for MA production (Goldberg et al., 2006; Zelle et al., 2008). The first is the rTCA pathway, in which pyruvate is converted to OAA by pyruvate carboxylase and subsequently transformed to MA by MDH. The pathway is ATP neutral and involves CO_2 fixation, leading to a maximum theoretical yield of 2 mol MA per mole glucose (Fig. 2D). The second pathway involves the classic TCA cycle. Because two CO_2 molecules are released via the oxidation of CA to MA, the theoretical yield is limited to 1 mol per mole glucose. The third route involves the glyoxylate cycle in which OAA is replenished by MA and the theoretical yield is 1 mol per mole glucose due to CO_2 release during the conversion of pyruvate to acetyl-CoA. The fourth route is a noncyclic glyoxylate shunt, in which OAA is supplied via pyruvate carboxylation leading to a theoretical yield of 1.33 mol/mol glucose. When yield and simplicity are considered, the cytosolic rTCA pathway is optimal and has been used for the metabolic design of MA production in *E. coli*, *S. cerevisiae*, and *Aspergillus oryzae*.

6.1. Combined enhancement of the rTCA cycle and transporter engineering

Isolated by directed evolution, a pyruvate decarboxylase-negative (Pdc^-) *S. cerevisiae* strain, which was alcohol-eliminated and produced high levels of pyruvate, was selected as a platform to introduce *PYC2* encoding the cytoplasmic pyruvate carboxylase gene *MDH3* encoding the MDH gene, in which the peroxisomal targeting sequence was modified for cytosol location, and Sp*MAE1* encoding the malate transporter gene from *Schizosaccharomyces pombe*. When these modifications were introduced simultaneously, the engineered strain improved MA production significantly to a titer of 59 g/L at a yield of 0.42 mol/mol glucose, both of which were the highest reported for *S. cerevisiae*. However, the strain generated large amounts of by-products including glycerol, pyruvate, SA, and FA (Zelle et al., 2008).

A. oryzae, which has generally recognized as safe (GRAS) status, is a basic MA producer. Despite large transforming and screening challenges, this fungus was used to overexpress a native C_4-dicarboxylate transporter, pyruvate carboxylase, and MDH. Microarray experiments that revealed the expression profiles of several candidate genes during the fermentation were coupled with protein localization prediction by WoLF PSORT software to identify the primary genes involved in the cytosolic rTCA pathway and MA transportation. Overexpression of the C_4-dicarboxylate transporter (C4T318) greatly improved MA production to 122 g/L. Additional expression of the native cytosolic pyruvate carboxylase and MDH yielded an additional 27% increase in the MA production rate. The final recombinant strain achieved the highest-ever MA titer of 154 g/L, with a high yield of 1.38 mol/mol on glucose (Brown et al., 2013). Transcription levels of the three genes—pyruvate carboxylase, MDH, and the MA transporter—were evaluated with quantitative polymerase chain reaction. The results indicated that the genes were integrated once, three times, and twice, respectively, into the genome. Thus, further expression of *pyc* might lead to an additional improvement in MA production. Because small amounts of SA and CA are produced as by-products, future strategies to improve MA production may be based on the formation mechanisms of SA and CA. The cytosol route of the rTCA branch from MA via FA to SA must be verified. One means of reducing CA formation may be to knock out the tricarboxylate transporter, which exchanges cytosolic MA for mitochondrial CA; alternatively, the export capacity of MA can be increased (Knuf et al., 2014).

6.2. Engineering of E. coli for MA production

When two additional genes, *poxB* and *mgsA*, were knocked out in *E. coli* KJ060 to generate strain KJ071, the mutant produced MA at a titer of 62.9 g/L, with a yield of 1.42 mol/mol. However, SA also accumulated in large amounts as a by-product (Jantama et al., 2008a). A new strategy for enhancing MA production in *E. coli* was reported by Moon et al. (2008). The results of metabolic flux analysis suggested that increased PEP carboxylation flux facilitates MA production. When energy neutrality was considered, PEP carboxykinase was chosen instead of PEP carboxylase to engineer the *E. coli* strain. The *pta* mutant strain WGS-10, which limited the conversion of acetyl-CoA to acetate, was constructed by transforming the PEP carboxykinase gene *pckA* from *M. succiniciproducens*. Although the final titer was low at 9.25 g/L, yield and productivity were considerable at 0.74 mol/mol and 0.75 g/L per hour, respectively.

Zhang et al. (2011) also developed a succinate-producing *E. coli* KJ060 strain for MA production. OAA was generated through PEP carboxylation by *pck* and subsequently reduced to MA, which was converted to FA and then SA. Deletion of fumarase isoenzymes *fumB* and *fumAC* and especially fumarate reductase (*frdBC*) redirected the carbon flow toward MA. By-products, including lactate, acetate, and ethanol, were inhibited by the deletion of eight additional genes. The final engineered strain, XZ658, produced MA in a high yield of 1.42 mol/mol glucose in a two-stage fermentation.

7. Oxaloacetate

OAA (2-oxosuccinic acid) is a four-carbon dicarboxylic acid-like material that is a starting material in the TCA cycle and a precursor of amino acid synthesis (Kwon et al., 2007). Attempts to improve OAA production have been achieved by engineering *E. coli* to overexpress codon-optimized PEPC genes from *Dunaliella salina* (Park et al., 2013) and *Photobacterium profundum* SS9 (Park et al., 2014). The strain produced OAA at a yield of approximately 5% mol/mol glucose and MA at a yield of 9.9% mol/mol (Park et al., 2013).

8. Conclusions and perspective

Bacteria, yeasts, and filamentous fungi are candidates for organic acid production through metabolic engineering (see Table 1). Bacteria such as *E. coli* are well-established industrial production hosts (Yu et al., 2011). They grow fast, their genetic backgrounds are clear, and many feasible genetic tools have been developed (Kirchner and Tauch, 2003). However, these prokaryotic cells require neutral pH growth conditions and consequently secrete the salt forms of organic acids, which require cost-intensive acidification and precipitation for conversion to the desired products. Yeasts—for instance, *S. cerevisiae*—are also good candidate biocatalysts for organic acid production. They are generally

Table 1
Production of organic acids in terms of performance and engineering strategies.

Strain	Engineering strategy	Culture method	Titer (g/L)	Yield (mol/mol)	Productivity (g/L/h)	By-product	References
Citric acid							
A. niger Δ1-3	*ΔggsA*	Aerobic, batch	115	ns	ns	ns	Arisan-Atac et al. (1996)
A. niger NW185	*ΔgoxC17*, *ΔprtF28*	Aerobic, batch	90	1.15[a]	0.375	No oxalate	Ruijter et al. (1999)
A. niger 50-2-12	*↑pfk*, *↑pki*	Aerobic, batch	55	0.64	0.33	ns	Ruijter et al. (1997)
A. niger 55-13	*↑citA*	Aerobic, batch	46	0.54	0.27	ns	Ruijter et al. (2000)
A. niger Frds(V)-FumRs	*↑Frds1*, *↑FumRs*	Aerobic, batch	20	0.43	0.10	Oxalate	de Jongh and Nielsen (2008)
Y. lipolytica H222-S4 (p67ICL1) T5	*↑SUC2*, *↑ICL1*	Aerobic, batch	140	1.46[a]	0.73	ns	Forster et al. (2007)
Y. lipolytica 30	*ΔACL1*, *↑ICL1*, *↑INU1*	Aerobic, batch	84	0.93[b]	0.39	Iso-citrate	Liu et al. (2013)
Y. lipolytica 87	*↑INU1*	Aerobic, batch	68.9	ns	0.22	Iso-citrate	Liu et al. (2010)
α-Ketoglutaric acid							
Y. lipolytica H355A (PYC1-IDP1) T5	*↑PYC1*, *↑IDP1*	Aerobic, pH control, fed-batch	186	0.23[c]	1.59	Pyruvate	Yovkova et al. (2014)
Y. lipolytica H355A (FUM1-PYC1) T4	*↑FUM1*, *↑PYC1*	Aerobic, pH control, fed-batch	138	ns	1.21	Malate, pyruvate	Otto et al. (2012)
Y. lipolytica-RoPYC2	Thiamine^{-}, *↑PYC2*	Two-stage pH control,	62.5	ns	0.434	Pyruvate	Yin et al. (2012)
Y. lipolytica-ACL	*↑ACL*	Two-stage pH control, batch	56.5	0.36[c]	0.39	Pyruvate	Zhou et al. (2012)
Y. lipolytica T1	*↑PDA1*	Aerobic, pH control, batch	42.5	ns	0.29	Pyruvate	Guo et al. (2014)
Succinic acid							
M. succiniciproducens LPK7	*ΔldhA*, *ΔpflB*, *Δpta*, *ΔackA*	Anaerobic, fed-batch	52.4	1.16	1.8	Pyruvate, malate	Lee et al. (2006)
C. glutamicum (ΔldhA-pCRA717)	*ΔldhA*, *↑pyc*	Micro-aerobic, fed-batch	146	1.4	3.17	Acetate	Okino et al. (2008)
C. glutamicum BOL-3	*Δcat*, *Δpqo*, *Δpta-ackA*, *ΔldhA*, *↑pyc*, *↑fdh*, *↑gapA*	Anaerobic, fed-batch	134	1.67	2.52	ns	Litsanov et al. (2012)
C. glutamicum SA5	*Δcat*, *Δpqo*, *Δpta-ackA*, *ΔldhA*, *↑pyc*, *↑ppc*, *↑aceA*, *↑aceB*, *↑gltA*, *↑sucE*	Anaerobic, fed-batch	109	1.32	1.11	ns	Zhu et al. (2014)
C. glutamicum ZX1 (pEacsAgltA)	*ΔsdhCAB*, *ΔldhA*, *Δpqo*, *Δcat*, *Δpta*, *acsA*, *gltA*, *↑ppc*, *↑pyc*	Aerobic, fed-batch	28.5	0.63	0.42	Pyruvate	Zhu et al. (2013)
E. coli AFP111/pTrc99A-*pyc*	*ΔptsG*, *Δpfl*, *Δldh*, *↑pyc*	Anaerobic, fed-batch, dual-phase	99.2	1.1	1.3	Ethanol	Vemuri et al. (2002b)
E. coli KJ060	*ΔldhA*, *ΔadhE*, *ΔackA*, *ΔfocA*, *ΔpflB*	Anaerobic, fed-batch	86.6	1.41	0.72	Acetate, malate	Jantama et al. (2008a)
E. coli KJ122	*ΔldhA*, *ΔadhE*, *ΔfocA-pflB*, *ΔmgsA*, *ΔpoxB*, *ΔtdcDE*, *ΔcitF*, *ΔaspC*, *ΔsfcA*	Anaerobic, fed-batch	82.7	1.5	0.9	ns	Jantama et al. (2008b)
E. coli HL27659k (pKK313)	*ΔsdhAB*, *ΔackA-pta*, *ΔpoxB*, *ΔiclR*, *ΔptsG*, *↑pepc*	Aerobic, fed-batch	58.3	0.85	1.08	Pyruvate, acetate	Lin et al. (2005c)
E. coli ZJG13 (pT184*pyc*)	*ΔsdhA*, *ΔackA-pta*, *ΔpoxB*, *ΔmgsA*, *ΔiclR*, *↑pyc*	Aerobic, fed-batch	36.1	0.72	0.694	Acetate, lactate, KGA	Yang et al. (2014)
E. coli BA016	*ΔldhA*, *ΔpflB*, *↑pncB*, *↑pyc*	Anaerobic, fed-batch	25	1.09	0.223	Pyruvate	Ma et al. (2013)
E. coli SBS550MG-Cms243 (pHL413KF1)	*ΔadhE*, *ΔldhA*, *Δack-pta*, *ΔiclR*, *↑fdh1*, *↑pycA*	Anaerobic, shake flask	20.3	1.74	ns	Formate, acetate	Balzer et al. (2013)
E. coli SBS550MG (pHL413)	*ΔadhE*, *ΔldhA*, *Δack-pta*, *ΔiclR*, *↑pyc*	Repeated feeding bioreactor	40	1.59	1.18	Formate, acetate	Sanchez et al. (2005b)
E. coli SBS990MG (pHL413 + pHL531)	*ΔadhE*, *ΔldhA*, *Δack-pta*, *↑pyc*, *↑citZ*	Anaerobic, batch	18.2	1.58	0.76	Formate, acetate	Sanchez et al. (2005b)
E. coli SBS110MG (pHL413)	*ΔldhA*, *ΔadhA*, *↑pyc*	Anaerobic, batch	15.6	1.27	0.65	Formate, acetate	Sanchez et al. (2005a)
E. coli AFP111	*Δpfl*, *Δldh*, *ΔptsG*	Anaerobic, batch	12.8	0.7	ns	Acetate, ethanol	Donnelly et al. (1998)
E. coli XZ721	*pck*ΔpflB*, *ΔptsI*	Anaerobic, batch	12	0.80[c]	0.083	Acetate, lactate,	Zhang et al. (2010)
E. coli JCL1208	*↑PEPC*	Anaerobic, batch	10.7	0.44	0.59	Ethanol, acetate, lactate, formate	Millard et al. (1996)
E. coli MG1655 ΔadhE, Δpta, ΔpoxB, ΔldhA (pZS-pyc)	*ΔadhE*, *Δpta*, *ΔpoxB*, *ΔldhA*, *↑pyc*	Microaerobic, batch	8.8	0.47[c]	0.183	Acetate, pyruvate	Blankschien et al. (2010)
E. coli AFP111/pTrc99A-*pyc*	*ΔptsG*, *Δpfl*, *Δldh*, *↑pyc*	Anaerobic, batch	8.0	1.46	0.286	Acetate	Vemuri et al. (2002a)

(continued on next page)

Table 1 (*continued*)

Strain	Engineering strategy	Culture method	Titer (g/L)	Yield (mol/mol)	Productivity (g/L/h)	By-product	References
E. coli NZN111 (pMEA1)	*Δpfl*, *Δldh*, ↑*MEA*	Anaerobic, batch	7.07	0.60	0.29	Ethanol, pyruvate	Stols et al. (1997)
E. coli HL51276k (pKK313)	*ΔsdhAB*, *ΔackA-pta*, *ΔpoxB*, *ΔiclR*, *ΔptsG*, ↑$pepc^{S8D}$	Aerobic, batch	6.72	1.09	0.14	Pyruvate, acetate	Lin et al. (2005b)
E. coli HL27615k	*ΔsdhAB*, *ΔackA-pta*, *ΔpoxB*, *Δicd*, *ΔiclR*	Aerobic batch	5.07	0.70	0.061	Acetate, pyruvate	Lin et al. (2005a)
E. coli YBS132 (pHL333, pHL413)	*Δack-pta*, ↑*PEPC*, ↑*PYC*	Anaerobic, batch	3.4	0.3	0.14	Acetate, ethanol	Lin et al. (2005e)
S. cerevisiae AH22ura3Δsdh1Δsdh2Δidh1Δidp1	*Δsdh1*, *Δsdh2*, *Δidh1*, *Δidp1*	Shake flask	3.62	0.11	0.022	Ethanol, glycerol, acetate	Raab et al. (2010)
S. cerevisiae 8D evolved (pICL1)	*Δsdh3*, *Δser3*, *Δser33*, ↑*icl*	Aerobic, batch	0.9	0.076	ns	ns	Otero et al. (2013)
Fumaric acid							
R. oryzae ppc	↑*pepc*	Batch	24	1.21	0.25	Malate	Zhang et al. (2012a)
R. oryzae fumR2	↑*fumR2*	Batch	21.5	1.00	0.22	Malate	Zhang and Yang (2012b)
S. cerevisiae FMME004-6	*Δfum1*, ↑*RoPYC*, ↑*RoMDH*, ↑*RoFUM1*	Shake flask	5.64	0.197	ns	Ethanol, glycerol, pyruvate	Xu et al. (2013)
S. cerevisiae FMME-001 ↑PYC2 + ↑RoMDH	↑*MDH*, ↑*PYC2*	Aerobic, batch	3.18	0.10	ns	Ethanol, glycerol, malate	Xu et al. (2012a)
S. cerevisiae FMME-002ΔFUM1 + ↑RoPYC + ↑SFC1	*Δfum1*, ↑*PYC*, ↑*SFC1*	Aerobic, batch	1.67	0.051	0.017	ns	Xu et al. (2012b)
E. coli CWF812	*ΔiclR*, *ΔfumC*, *ΔfumA*, *ΔfumB*, *ΔarcA*, *ΔptsG*, *ΔaspA*, *ΔlacI*, ↑*ppc*	Fed-batch	28.2	0.59	0.448	Acetic acid	Song et al. (2013)
T. glabrata T.G-$ASL_{(H)}$-$ADSL_{(L)}$-SpMAE1	↑*ASL*, ↑*ADSL*, ↑*SpMAE1*	Shake flask	8.83	0.23	0.12	Pyruvate	Chen et al. (2015)
Malic acid							
A. oryzae SaMF2103a-68	↑*pyc*, ↑*mdh*, ↑*C4T318*	Batch	154	1.38	0.94	Succinate	Brown et al. (2013)
S. cerevisiae RWB525↑PYC2 + ↑*MDH3ΔSKL* + ↑*SpMAE1*	*ΔPdc*, ↑*PYC2*, ↑*MDH3*, ↑*SpMAE1*	Shake flask	59	0.42	0.19	Glycerol, succinate, pyruvate, fumarate	Zelle et al. (2008)
E. coli KJ071	*ΔldhA*, *ΔadhE*, *ΔackA*, *ΔfocA*, *ΔpflB*, *ΔmgsA*	Anaerobic, fed-batch	69.2	1.4	0.72	Succinate, pyruvate, acetate	Jantama et al. (2008a)
E. coli XZ658	*ΔldhA*, *ΔackA*, *ΔadhE*, *ΔpflB*, *ΔmgsA*, *ΔpoxB*, *ΔfrdBC*, *ΔsfcA*, *ΔmaeB*, *ΔfumB*, *ΔfumAC*	Two-stage process	34	1.42	0.47	Lactate	Zhang et al. (2011)
E. coli WGS-10 (p104ManPck)	*ΔldhA*, *Δpta*, *Δadh*, ↑*pckA*	Batch	9.25	0.75	0.77	ns	Moon et al. (2008)
Oxaloacetate							
E. coli SGJS115	↑*DsPEPC*	Shake flask	ns	0.05	ns	Malate, succinate	Park et al. (2013)

ns, not specified.
[a] mol/mol sucrose.
[b] g/g inulin.
[c] mol/mol glycerol.

regarded as safe, robust, and tolerant to acid, and they grow both aerobically and anaerobically on diverse carbon sources and chemically defined media. Moreover, genetic engineering tools, many omics tools, and synthetic biology approaches are available (Yadav et al., 2012), and complimentary methods for directed evolution and selection are well established (Otero et al., 2013). Filamentous fungi can spontaneously produce high levels of diverse organic acids. However, the tremendous differences in genetic backgrounds complicate the design of metabolic engineering strategies. In addition, the available genetic tools for manipulating metabolic pathways are limited compared to those for bacteria and yeasts, and targeting genes to the right genome locus is laborious. Nevertheless, advances and improvements in genetic tools (Delmas et al., 2014) and the application of omics analysis tools make engineered fungi candidates for environmentally responsible and economically feasible fermentations processes for the production of organic acids.

As shown in Fig. 3, the strategies for enhancing organic acid production can be summarized as follows: (1) overexpression of rate-limiting enzymes or the introduction of new pathways to increase acid biosynthesis; (2) blocking of pathways for the synthesis of competing by-products; (3) overexpression of transporters to facilitate secretion; (4) coordination of sufficient energy, cofactor production, and supplement pathways for target product synthesis; and (5) elimination of inhibitor effects to enhance synthesis pathways. However, difficulties remain in the optimization of biological systems for designed tasks. First, optimizing the amount of carbon flux may require perfectly balanced enzymes expression levels, and the enzyme conversion direction in different environments and enzyme substrate specificity complicate the synthetic situation (Ma et al., 2014; Seo et al., 2013). Synthetic biology may provide approaches for optimizing a number of components, including genes from different sources and their mutants, devices, and novel pathways, in predictable and controllable manners to make biological production platforms more efficient (Seo et al., 2013).

Second, maintaining robustness and improving the product tolerance of microorganisms qualified for industrial production is critical. With various system-wide tools, including omics analyses (genome,

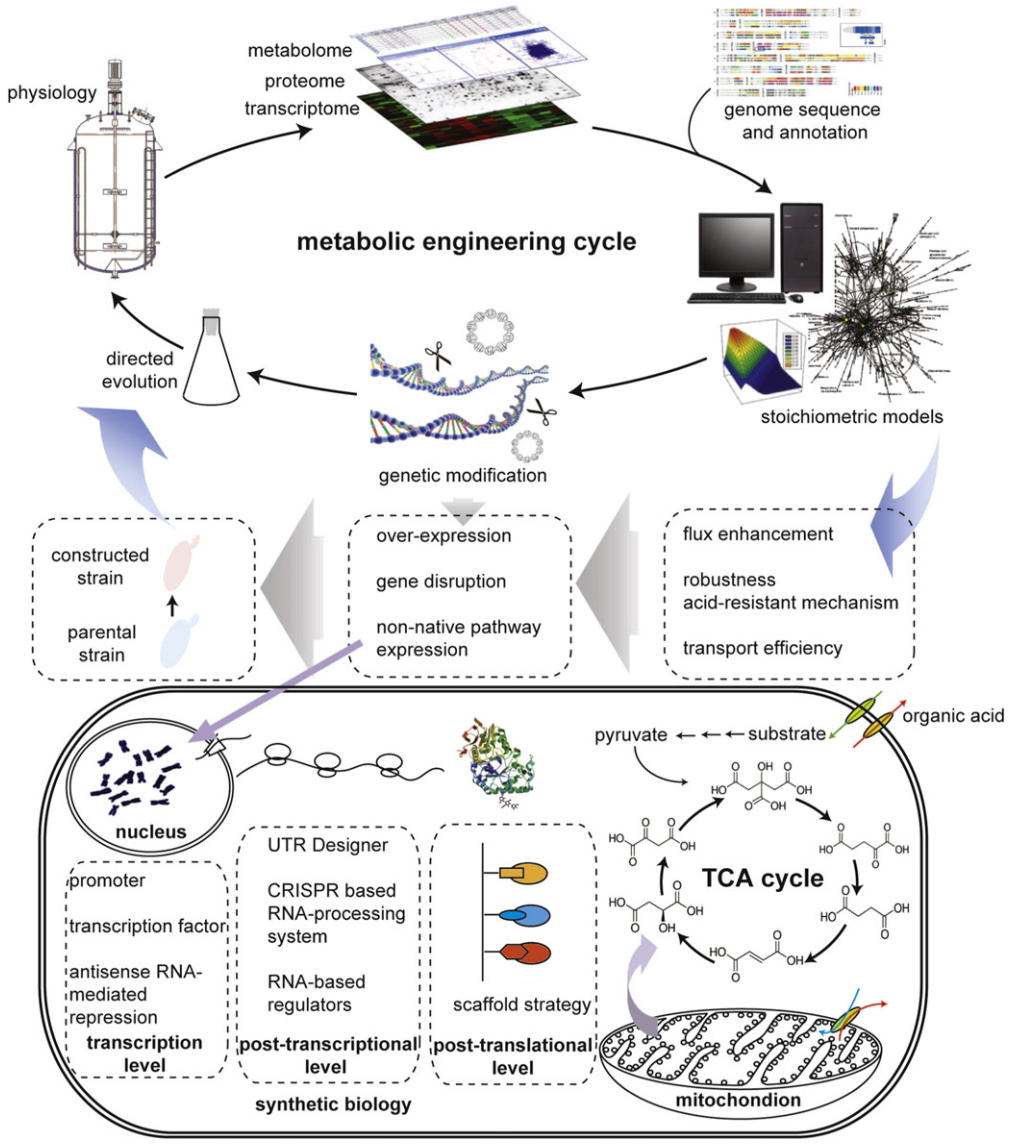

Fig. 3. Systems biology and synthetic biology guided metabolic engineering of microorganisms for organic acid production.

transcriptome, proteome, metabolome, and fluxome) and in silico modeling and simulation, metabolic engineering at the system level could help coordinate energy generation and cell growth with product synthesis (Chen et al., 2015; Cox et al., 2006; Kalinowski et al., 2003; Otero et al., 2013; Wang et al., 2013; Yu et al., 2012a). Systems biology may also detail stress responses to acids or salts, which can guide engineering to solve product resistance problems.

Third, the mechanisms of resource absorption, organic acid transportation from mitochondrion to cytosol, and secretion into the broth are rarely understood. With the help of omics technology, the putative pump candidates will be recognized and the mechanisms disclosed. Synthesis efficiency can be improved through transporter engineering that enhances substrate and product transportation.

Fourth, the compartmentalization of metabolic pathways in eukaryotes highlights the possibility of moving to the mitochondrion the syntheses of organic acids that usually take place in the cytosol. The benefits of compartmentalization are higher local concentrations of enzymes, substrates, and intermediates and weaker influence from competing pathways (Avalos et al., 2013).

Finally, it is important to pursue high yield and productivity as well as production to meet the economic needs of industry. Economic production depends on an optimized combination of substrate absorption and assimilation, metabolic pathways, and transportation of intermediates and products.

Acknowledgments

This work was financially supported by the 863 project (2014AA021201), the Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions, the 111 Project (111-2-06), and the Open Funding Project of the State Key Laboratory of Bioreactor Engineering.

References

Alvarez-Vasquez F, González-Alcón C, Torres NV. Metabolism of citric acid production by *Aspergillus niger*: model definition, steady-state analysis and constrained optimization of citric acid production rate. Biotechnol Bioeng 2000;70:82–108.

Arisan-Atac I, Wolschek MF, Kubicek CP. Trehalose-6-phosphate synthase A affects citrate accumulation by *Aspergillus niger* under conditions of high glycolytic flux. FEMS Microbiol Lett 1996;140:77–83.

Avalos JL, Fink GR, Stephanopoulos G. Compartmentalization of metabolic pathways in yeast mitochondria improves the production of branched-chain alcohols. Nat Biotechnol 2013;31:335–41.

Balzer GJ, Thakker C, Bennett GN, San KY. Metabolic engineering of *Escherichia coli* to minimize byproduct formate and improving succinate productivity through increasing NADH availability by heterologous expression of NAD^+-dependent formate dehydrogenase. Metab Eng 2013;20:1–8.

Battat E, Peleg Y, Bercovitz A, Rokem JS, Goldberg I. Optimization of L-malic acid production by *Aspergillus flavus* in a stirred fermentor. Biotechnol Bioeng 1991;37:1108–16.

Blankschien MD, Clomburg JM, Gonzalez R. Metabolic engineering of *Escherichia coli* for the production of succinate from glycerol. Metab Eng 2010;12:409–19.

Brown SH, Bashkirova L, Berka R, Chandler T, Doty T, McCall K, et al. Metabolic engineering of *Aspergillus oryzae* NRRL 3488 for increased production of L-malic acid. Appl Microbiol Biotechnol 2013;97:8903–12.

Cao YJ, Cao YG, Lin XZ. Metabolically engineered *Escherichia coli* for biotechnological production of four-carbon 1,4-dicarboxylic acids. J Ind Microbiol Biotechnol 2011;38:649–56.

Chen X, Wu J, Song W, Zhang L, Wang H, Liu L. Fumaric acid production by *Torulopsis glabrata*: engineering the urea cycle and the purine nucleotide cycle. Biotechnol Bioeng 2015;112:156–67.

Cheng KK, Wang GY, Zeng J, Zhang JA. Improved succinate production by metabolic engineering. Biomed Res Int. 2013;2013:12.

Cox SJ, Shalel Levanon S, Sanchez A, Lin H, Peercy B, Bennett GN, et al. Development of a metabolic network design and optimization framework incorporating implementation constraints: a succinate production case study. Metab Eng 2006;8:46–57.

Dai Z, Mao X, Magnuson JK, Lasure LL. Identification of genes associated with morphology in *Aspergillus niger* by using suppression subtractive hybridization. Appl Environ Microbiol 2004;70:2474–85.

Das RK, Brar SK. Enhanced fumaric acid production from brewery wastewater and insight into the morphology of *Rhizopus oryzae* 1526. Appl Biochem Biotechnol 2014;172:2974–88.

de Jongh WA, Nielsen J. Enhanced citrate production through gene insertion in *Aspergillus niger*. Metab Eng 2008;10:87–96.

Delmas S, Llanos A, Parrou JL, Kokolski M, Pullan ST, Shunburne L, et al. Development of an unmarked gene deletion system for the filamentous fungi *Aspergillus niger* and *Talaromyces versatilis*. Appl Environ Microbiol 2014;80:3484–7.

Dhillon GS, Brar SK, Verma M, Tyagi RD. Recent advances in citric acid bio-production and recovery. Food Bioprocess Technol 2011;4:505–29.

Donnelly MI, Millard CS, Clark DP, Chen MJ, Rathke JW. A novel fermentation pathway in an *Escherichia coli* mutant producing succinic acid, acetic acid, and ethanol. Appl Biochem Biotechnol 1998;70–72:187–98.

Doucette CD, Schwab DJ, Wingreen NS, Rabinowitz JD. alpha-ketoglutarate coordinates carbon and nitrogen utilization via enzyme I inhibition. Nat Chem Biol 2011;7:894–901.

Forster A, Aurich A, Mauersberger S, Barth G. Citric acid production from sucrose using a recombinant strain of the yeast *Yarrowia lipolytica*. Appl Microbiol Biotechnol 2007;75:1409–17.

Goldberg I, Rokem JS, Pines O. Organic acids: old metabolites, new themes. J Chem Technol Biotechnol 2006;81:1601–11.

Guo H, Madzak C, Du G, Zhou J, Chen J. Effects of pyruvate dehydrogenase subunits overexpression on the alpha-ketoglutarate production in *Yarrowia lipolytica* WSH-Z06. Appl Microbiol Biotechnol 2014;98:7003–12.

Gu S, Xu Q, Huang H, Li S. Alternative respiration and fumaric acid production of *Rhizopus oryzae*. Appl Microbiol Biotechnol 2014;98:5145–52.

Holz M, Forster A, Mauersberger S, Barth G. Aconitase overexpression changes the product ratio of citric acid production by *Yarrowia lipolytica*. Appl Microbiol Biotechnol 2009;81:1087–96.

Hong SH, Kim JS, Lee SY, In YH, Choi SS, Rih JK, et al. The genome sequence of the capnophilic rumen bacterium *Mannheimia succiniciproducens*. Nat Biotechnol 2004;22:1275–81.

Jantama K, Haupt MJ, Svoronos SA, Zhang XL, Moore JC, Shanmugam KT, et al. Combining metabolic engineering and metabolic evolution to develop nonrecombinant strains of *Escherichia coli* C that produce succinate and malate. Biotechnol Bioeng 2008a;99:1140–53.

Jantama K, Zhang X, Moore JC, Shanmugam KT, Svoronos SA, Ingram LO. Eliminating side products and increasing succinate yields in engineered strains of *Escherichia coli*. Biotechnol Bioeng 2008b;101:881–93.

Kalinowski J, Bathe B, Bartels D, Bischoff N, Bott M, Burkovski A, et al. The complete *Corynebacterium glutamicum* ATCC 13032 genome sequence and its impact on the production of L-aspartate-derived amino acids and vitamins. J Biotechnol 2003;104:5–25.

Kamzolova SV, Chiglintseva MN, Lunina JN, Morgunov IG. Alpha-ketoglutaric acid production by *Yarrowia lipolytica* and its regulation. Appl Microbiol Biotechnol 2012;96:783–91.

Karaffa L, Kubicek CP. *Aspergillus niger* citric acid accumulation: do we understand this well working black box? Appl Microbiol Biotechnol 2003;61:189–96.

Kawagoe M, Hyakumura K, Suye SI, Miki K, Naoe K. Application of bubble column fermenters to submerged culture of *Schizophyllum commune* for production of L-malic acid. J Ferment Bioeng 1997;84:333–6.

Kirchner O, Tauch A. Tools for genetic engineering in the amino acid-producing bacterium *Corynebacterium glutamicum*. J Biotechnol 2003;104:287–99.

Knuf C, Nookaew I, Remmers I, Khoomrung S, Brown S, Berry A, et al. Physiological characterization of the high malic acid-producing *Aspergillus oryzae* strain 2103a-68. Appl Microbiol Biotechnol 2014;98:3517–27.

Kwon YD, Kwon OH, Lee HS, Kim P. The effect of NADP-dependent malic enzyme expression and anaerobic C4 metabolism in *Escherichia coli* compared with other anaplerotic enzymes. J Appl Microbiol 2007;103:2340–5.

Lee PC, Lee SY, Hong SH, Chang HN. Isolation and characterization of a new succinic acid-producing bacterium, *Mannheimia succiniciproducens* MBEL55E, from bovine rumen. Appl Microbiol Biotechnol 2002;58:663–8.

Lee SJ, Song H, Lee SY. Genome-based metabolic engineering of *Mannheimia succiniciproducens* for succinic acid production. Appl Environ Microbiol 2006;72:1939–48.

Li XJ, Liu Y, Yang Y, Zhang H, Wang HL, Wu Y, et al. High levels of malic acid production by the bioconversion of corn straw hydrolyte using an isolated *Rhizopus delemar* strain. Biotechnol Bioprocess Eng 2014;19:478–92.

Lin H, Vadali RV, Bennett GN, San KY. Increasing the acetyl-CoA pool in the presence of overexpressed phosphoenolpyruvate carboxylase or pyruvate carboxylase enhances succinate production in *Escherichia coli*. Biotechnol Prog 2004;20:1599–604.

Lin H, Bennett GN, San KY. Genetic reconstruction of the aerobic central metabolism in *Escherichia coli* for the absolute aerobic production of succinate. Biotechnol Bioeng 2005a;89:148–56.

Lin H, Bennett GN, San KY. Metabolic engineering of aerobic succinate production systems in *Escherichia coli* to improve process productivity and achieve the maximum theoretical succinate yield. Metab Eng 2005b;7:116–27.

Lin H, Bennett GN, San KY. Fed-batch culture of a metabolically engineered *Escherichia coli* strain designed for high-level succinate production and yield under aerobic conditions. Biotechnol Bioeng 2005c;90:775–9.

Lin H, Bennett GN, San KY. Chemostat culture characterization of *Escherichia coli* mutant strains metabolically engineered for aerobic succinate production: a study of the modified metabolic network based on metabolite profile, enzyme activity, and gene expression profile. Metab Eng 2005d;7:337–52.

Lin H, San KY, Bennett GN. Effect of *Sorghum vulgare* phosphoenolpyruvate carboxylase and *Lactococcus lactis* pyruvate carboxylase coexpression on succinate production in mutant strains of *Escherichia coli*. Appl Microbiol Biotechnol 2005e;67:515–23.

Litsanov B, Brocker M, Bott M. Toward homosuccinate fermentation: metabolic engineering of *Corynebacterium glutamicum* for anaerobic production of succinate from glucose and formate. Appl Environ Microbiol 2012;78:3325–37.

Liu XY, Chi Z, Liu GL, Wang F, Madzak C, Chi ZM. Inulin hydrolysis and citric acid production from inulin using the surface-engineered *Y. lipolytica* displaying inulinase. Metab Eng 2010;12:469–76.

Liu XY, Chi Z, Liu GL, Madzak C, Chi ZM. Both decrease in *ACL1* gene expression and increase in *ICL1* gene expression in marine-derived yeast *Yarrowia lipolytica* expressing *INU1* gene enhance citric acid production from inulin. Mar Biotechnol 2013;15:26–36.

Ma JF, Gou DM, Liang LY, Liu RM, Chen X, Zhang CQ, et al. Enhancement of succinate production by metabolically engineered *Escherichia coli* with co-expression of nicotinic acid phosphoribosyltransferase and pyruvate carboxylase. Appl Microbiol Biotechnol 2013;97:6739–47.

Ma XH, Zhang XB, Wang BY, Mao YF, Wang ZW, Chen T, et al. Engineering microorganisms based on molecular evolutionary analysis: a succinate production case study. Evol Appl 2014;7:913–20.

McGinn SM, Beauchemin KA, Coates T, Colombatto D. Methane emissions from beef cattle: effects of monensin, sunflower oil, enzymes, yeast, and fumaric acid. J Anim Sci 2004;82:3346–56.

Millard CS, Chao YP, Liao JC, Donnelly MI. Enhanced production of succinic acid by overexpression of phosphoenolpyruvate carboxylase in *Escherichia coli*. Appl Environ Microbiol 1996;62:1808–10.

Moon SY, Hong SH, Kim TY, Lee SY. Metabolic engineering of *Escherichia coli* for the production of malic acid. Biochem Eng J 2008;40:312–20.

Morgunov IG, Kamzolova SV, Samoilenko VA. Enhanced alpha-ketoglutaric acid production and recovery in *Yarrowia lipolytica* yeast by effective pH controlling. Appl Microbiol Biotechnol 2013;97:8711–8.

Naeini AT, Adeli M, Vossoughi M. Poly(citric acid)–*block*–poly(ethylene glycol) copolymers—new biocompatible hybrid materials for nanomedicine. Nanomed-Nanotechnol Biol Med 2010;6:556–62.

Okino S, Noburyu R, Suda M, Jojima T, Inui M, Yukawa H. An efficient succinic acid production process in a metabolically engineered *Corynebacterium glutamicum* strain. Appl Microbiol Biotechnol 2008;81:459–64.

Otero JM, Cimini D, Patil KR, Poulsen SG, Olsson L, Nielsen J. Industrial systems biology of *Saccharomyces cerevisiae* enables novel succinic acid cell factory. PLoS One 2013;8.

Otto C, Yovkova V, Barth G. Overproduction and secretion of alpha-ketoglutaric acid by microorganisms. Appl Microbiol Biotechnol 2011;92:689–95.

Otto C, Yovkova V, Aurich A, Mauersberger S, Barth G. Variation of the by-product spectrum during alpha-ketoglutaric acid production from raw glycerol by overexpression of fumarase and pyruvate carboxylase genes in *Yarrowia lipolytica*. Appl Microbiol Biotechnol 2012;95:905–17.

Papagianni M. Advances in citric acid fermentation by *Aspergillus niger*: biochemical aspects, membrane transport and modeling. Biotechnol Adv 2007;25:244–63.

Park S, Chang KS, Jin E, Pack SP, Lee J. Oxaloacetate and malate production in engineered *Escherichia coli* by expression of codon-optimized phosphoenolpyruvate carboxylase gene from *Dunaliella salina*. Bioprocess Biosyst Eng 2013;36:127–31.

Park S, Hong S, Pack SP, Lee J. High activity and stability of codon-optimized phosphoenolpyruvate carboxylase from *Photobacterium profundum* SS9 at low temperatures and its application for in vitro production of oxaloacetate. Bioprocess Biosyst Eng 2014;37:331–5.

Raab AM, Gebhardt G, Bolotina N, Weuster-Botz D, Lang C. Metabolic engineering of *Saccharomyces cerevisiae* for the biotechnological production of succinic acid. Metab Eng 2010;12:518–25.

Roa Engel CA, Straathof AJ, Zijlmans TW, van Gulik WM, van der Wielen LA. Fumaric acid production by fermentation. Appl Microbiol Biotechnol 2008;78:379–89.

Ruijter G, Panneman H, Visser J. Overexpression of phosphofructokinase and pyruvate kinase in citric acid-producing *Aspergillus niger*. Biochim Biophys Acta (BBA)-Gen Subj 1997;1334:317–26.

Ruijter GJ, van de Vondervoort PJ, Visser J. Oxalic acid production by *Aspergillus niger*: an oxalate-non-producing mutant produces citric acid at pH 5 and in the presence of manganese. Microbiology 1999;145:2569–76.

Ruijter GJ, Panneman H, Xu DB, Visser J. Properties of *Aspergillus niger* citrate synthase and effects of *citA* overexpression on citric acid production. FEMS Microbiol Lett 2000;184:35–40.
Sanchez AM, Bennett GN, San KY. Efficient succinic acid production from glucose through overexpression of pyruvate carboxylase in an *Escherichia coli* alcohol dehydrogenase and lactate dehydrogenase mutant. Biotechnol Prog 2005a;21:358–65.
Sanchez AM, Bennett GN, San KY. Novel pathway engineering design of the anaerobic central metabolic pathway in *Escherichia coli* to increase succinate yield and productivity. Metab Eng 2005b;7:229–39.
Sauer M, Porro D, Mattanovich D, Branduardi P. Microbial production of organic acids: expanding the markets. Trends Biotechnol 2008;26:100–8.
Seo SW, Yang J, Min BE, Jang S, Lim JH, Lim HG, et al. Synthetic biology: tools to design microbes for the production of chemicals and fuels. Biotechnol Adv 2013;31:811–7.
Soccol CR, Vandenberghe LP, Rodrigues C, Pandey A. New perspectives for citric acid production and application. Food Technol Biotechnol 2006;44:141–9.
Song CW, Kim DI, Choi S, Jang JW, Lee SY. Metabolic engineering of *Escherichia coli* for the production of fumaric acid. Biotechnol Bioeng 2013;110:2025–34.
Stols L, Kulkarni G, Harris BG, Donnelly MI. Expression of *Ascaris suum* malic enzyme in a mutant *Escherichia coli* allows production of succinic acid from glucose. Appl Biochem Biotechnol 1997;63–65:153–8.
Stottmeister U, Aurich A, Wilde H, Andersch J, Schmidt S, Sicker D. White biotechnology for green chemistry: fermentative 2-oxocarboxylic acids as novel building blocks for subsequent chemical syntheses. J Ind Microbiol Biotechnol 2005;32:651–64.
Vemuri GN, Eiteman MA, Altman E. Effects of growth mode and pyruvate carboxylase on succinic acid production by metabolically engineered strains of *Escherichia coli*. Appl Environ Microbiol 2002a;68:1715–27.
Vemuri GN, Eiteman MA, Altman E. Succinate production in dual-phase *Escherichia coli* fermentations depends on the time of transition from aerobic to anaerobic conditions. J Ind Microbiol Biotechnol 2002b;28:325–32.
Wang G, Huang D, Qi H, Wen J, Jia X, Chen Y. Rational medium optimization based on comparative metabolic profiling analysis to improve fumaric acid production. Bioresour Technol 2013;137:1–8.
Wen S, Liu L, Nie KL, Deng L, Tan TW, Fang W. Enhanced fumaric acid production by fermentation of xylose using a modified strain of *Rhizopus arrhizus*. Bioresources 2013;8:2186–94.
Xu Q, Li S, Huang H, Wen JP. Key technologies for the industrial production of fumaric acid by fermentation. Biotechnol Adv 2012a;30:1685–96.
Xu GQ, Liu L, Chen J. Reconstruction of cytosolic fumaric acid biosynthetic pathways in *Saccharomyces cerevisiae*. Microb Cell Fact 2012b;11.
Xu G, Zou W, Chen X, Xu N, Liu L, Chen J. Fumaric acid production in *Saccharomyces cerevisiae* by in silico aided metabolic engineering. Plos One 2012c;7:e52086.
Xu G, Chen X, Liu L, Jiang L. Fumaric acid production in *Saccharomyces cerevisiae* by simultaneous use of oxidative and reductive routes. Bioresour Technol 2013;148:91–6.
Yadav VG, De Mey M, Lim CG, Ajikumar PK, Stephanopoulos G. The future of metabolic engineering and synthetic biology: towards a systematic practice. Metab Eng 2012;14:233–41.
Yang JG, Wang ZW, Zhu NQ, Wang BY, Chen T, Zhao XM. Metabolic engineering of *Escherichia coli* and *in silico* comparing of carboxylation pathways for high succinate productivity under aerobic conditions. Microbiol Res 2014;169:432–40.
Yin X, Madzak C, Du G, Zhou J, Chen J. Enhanced alpha-ketoglutaric acid production in *Yarrowia lipolytica* WSH-Z06 by regulation of the pyruvate carboxylation pathway. Appl Microbiol Biotechnol 2012;96:1527–37.
Yovkova V, Otto C, Aurich A, Mauersberger S, Barth G. Engineering the alpha-ketoglutarate overproduction from raw glycerol by overexpression of the genes encoding $NADP^+$-dependent isocitrate dehydrogenase and pyruvate carboxylase in *Yarrowia lipolytica*. Appl Microbiol Biotechnol 2014;98:2003–13.
Yu C, Cao YJ, Zou HB, Xian M. Metabolic engineering of *Escherichia coli* for biotechnological production of high-value organic acids and alcohols. Appl Microbiol Biotechnol 2011;89:573–83.
Yu SZ, Huang D, Wen JP, Li S, Chen YL, Jia XQ. Metabolic profiling of a *R. oryzae* fumaric acid production mutant generated by femtosecond laser irradiation. Bioresour Technol 2012a;114:610–5.
Yu Z, Du G, Zhou J, Chen J. Enhanced alpha-ketoglutaric acid production in *Yarrowia lipolytica* WSH-Z06 by an improved integrated fed-batch strategy. Bioresour Technol 2012b;114:597–602.
Zelle RM, de Hulster E, van Winden WA, de Waard P, Dijkema C, Winkler AA, et al. Malic acid production by *Saccharomyces cerevisiae*: engineering of pyruvate carboxylation, oxaloacetate reduction, and malate export. Appl Environ Microbiol 2008;74:2766–77.
Zhang BH, Yang ST. Metabolic engineering of *Rhizopus oryzae*: effects of overexpressing *fumR* gene on cell growth and fumaric acid biosynthesis from glucose. Process Biochem 2012b;47:2159–65.
Zhang X, Shanmugam KT, Ingram LO. Fermentation of glycerol to succinate by metabolically engineered strains of *Escherichia coli*. Appl Environ Microbiol 2010;76:2397–401.
Zhang X, Wang X, Shanmugam KT, Ingram LO. L-Malate production by metabolically engineered *Escherichia coli*. Appl Environ Microbiol 2011;77:427–34.
Zhang BH, Skory CD, Yang ST. Metabolic engineering of *Rhizopus oryzae*: effects of overexpressing *pyc* and *pepc* genes on fumaric acid biosynthesis from glucose. Metab Eng 2012a;14:512–20.
Zhou JW, Zhou HY, Du GC, Liu L, Chen J. Screening of a thiamine-auxotrophic yeast for alpha-ketoglutaric acid overproduction. Lett Appl Microbiol 2010;51:264–71.
Zhou Z, Du G, Hua Z, Zhou J, Chen J. Optimization of fumaric acid production by *Rhizopus delemar* based on the morphology formation. Bioresour Technol 2011;102:9345–9.
Zhou JW, Yin XX, Madzak C, Du GC, Chen J. Enhanced alpha-ketoglutarate production in *Yarrowia lipolytica* WSH-Z06 by alteration of the acetyl-CoA metabolism. J Biotechnol 2012;161:257–64.
Zhou Y, Nie K, Zhang X, Liu S, Wang M, Deng L, et al. Production of fumaric acid from biodiesel-derived crude glycerol by *Rhizopus arrhizus*. Bioresour Technol 2014;163:48–53.
Zhu NQ, Xia HH, Wang ZW, Zhao XM, Chen T. Engineering of acetate recycling and citrate synthase to improve aerobic succinate production in *Corynebacterium glutamicum*. Plos One 2013;8.
Zhu NQ, Xia HH, Yang JG, Zhao XM, Chen T. Improved succinate production in *Corynebacterium glutamicum* by engineering glyoxylate pathway and succinate export system. Biotechnol Lett 2014;36:553–60.