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REVIEW

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Metabolic engineering of carbon and redox flow in the production of small organic acids

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Abstract The review describes efforts toward metabolic engineering of production of organic acids. One aspect of the strategy involves the generation of an appropriate amount and type of reduced cofactor needed for the designed pathway. The ability to capture reducing power in the proper form, NADH or NADPH for the biosynthetic reactions leading to the organic acid, requires specific attention in designing the host and also depends on the feedstock used and cell energetic requirements for efficient metabolism during production. Recent work on the formation and commercial uses of a number of small mono- and diacids is discussed with redox differences, major biosynthetic precursors and engineering strategies outlined. Specific attention is given to those acids that are used in balancing cell redox or providing reduction equivalents for the cell, such as formate, which can be used in conjunction with metabolic engineering of other products to improve yields. Since a number of widely studied acids derived from oxaloacetate as an important precursor, several of these acids are covered with the general strategies and particular components summarized, including succinate, fumarate and malate. Since malate and fumarate are less reduced than succinate, the availability of reduction equivalents and level of aerobiosis are important parameters in optimizing production of these compounds in various hosts. Several other more oxidized acids are also discussed as in some cases, they may be desired products or their formation is minimized to afford higher yields of more reduced products. The placement and connections among acids in the typical central metabolic network are presented along with the use of a number of specific non-native enzymes to enhance routes to high production, where available alternative pathways and strategies are discussed. While many organic acids are derived from a few precursors within central metabolism, each organic acid has its own special requirements for high production and best compatibility with host physiology.

Keywords Oxidation–reduction · Redox · Succinate · Fatty acid · Formate · Propionate · Gene · Mutation · Metabolism · Pathway · Microbe

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Small monoacids

These acids include those of differing chain length and uses. The metabolic pathways producing various monoacids from glycolysis are connected; however, many specialized enzymes and reactions are used in their formation. As the carbon chain length increases the acids become more hydrophobic and while this factor can aid separation at some point when the chain length is sufficient to allow phase separation, the hydrophobic character of fatty acids

of intermediate length can also increase toxicity and limit cell growth [5, 10, 12]. The longer chain monoacids also require a high amount of reduction equivalents in their formation, which must be taken into account in devising efficient routes to these valuable molecules.

Formic acid

Formic acid formation and utilization have been reviewed [44]. Major industrial uses of formic acid are in leather and textile processing, cleaning and descaling, to maintain pH and as a deicer, salts are used in oil field operations for removal of carbonates and in desulfurization processing flues. The approximate general price for formate salts is $400 per MT, while the acid is more generally in the $600–$900 per MT depending on location and purity. Formate is also used as a counterion in pharmaceutical formulations but its interest here is its use as a redox carrier in enzyme or cellular processes where it can be used to generate reductant for specific reactions.

In *Escherichia coli* and other organisms that metabolize sugars by glycolysis, formate is produced by the pyruvate-formate-lyase enzyme, a glycyl radical enzyme [169, 172] that is subject to activation and inactivation mechanisms [168]. Formate can also be produced biologically through reduction of CO_2 using special formate dehydrogenases [5] and through the degradation of oxalate [181]. There have been significant efforts in chemical catalysis to produce formate from CO_2 and hydrogen using metal catalysts [61, 137] or through photo-catalyzed reactions [6, 23, 79, 194]. Production of formate through coupling formate dehydrogenases to electrodes has also generated recent interest [153, 175] and in the more general use of such technology in electro formation of useful chemicals [127].

Formate synthesis by a number of clostridium species, e.g. *C. sporogenes*, *C. thermocellum*, *C. phytofermentans* sp. nov, *C. thermoaceticum* (now *Moorella thermoacetica*) [126, 174, 200, 215], is known and the action of formate dehydrogenase to generate formate for further reduction and incorporation via an acetogenic pathway is also well established [185, 186, 196].

There has been little work on the production of formate as a final product via engineered microbes and reports have generally focused on the subsequent direct use of formate as a redox carrier for a reductive reaction to give a larger yield of a more desired compound. A number of these uses of formate and a NADH-coupled formate dehydrogenase such as the NAD^+-dependent formate dehydrogenase from *Candida boidinii* or other species that convert 1 mol of formate and NAD^+ into 1 mol of NADH and CO_2 [17–19, 162] have been reported including: contributing additional redox for improved succinate yield [10, 119], use of electrochemically formed formate in chemical production [111], use of formate to provide a redox driving force for fuel molecule production [170] and in mannitol formation [90].

Acetic, pyruvic and lactic acids

Acetate, pyruvate and lactate are small organic acids with close ties to the glycolytic pathway for the metabolism of glucose. The production of these compounds has been studied extensively from scientific and industrial points of view. Here, we will point out a few recent reviews for readers but do not have the space to cover these in detail. These molecules are often side products of microbes that are being engineered for production of other compounds so strains that minimize formation of these side products are a desirable feature. Acetate can be formed in many organisms by decarboxylation of pyruvate in a PoxB-type mechanism, typically being coupled to membrane redox processes or by first conversion to acetyl-CoA and then conversion through an acetyl-phosphate intermediate that produces ATP for cellular energy. If pyruvate is not metabolized at a rate consistent to its synthesis, it can build up and is excreted for later uptake and use. Lactate can serve as a final product in anaerobic conditions as it can recycle the reductant formed in glycolysis and pyruvate formation and can also be consumed later as a carbon and energy source when oxygen levels are high. Recent reviews on acetate formation via syngas [14] or traditional fermentations [30, 54, 86] have shown high yields from various feedstocks. Production of pyruvate in bacterial and fungal systems has been reviewed [135, 202, 204, 213]. Lactate production by various bacteria and yeasts [2, 80, 135, 159, 165, 202, 204] are well covered in the literature.

Propionic acid

The most common consumer item containing propionic acid (PA) is swiss style Emmental cheese, where the organic acid is formed by *Propionibacterium* during aging, giving the cheese a distinctive flavor. PA is widely used in the food industry as a preservative or as esters that have a fruity essence. It is also used in animal feed where it can improve feed utilization and health. Industrially, a major use of propionic acid is in the formation of cellulose acetate-based polymers. Propionic acid bulk prices are around $1,500–2,000 MT with calcium or sodium salts slightly less per ton with an overall annual volume of ~380,000 MT. In nature, propionic acid is formed by *Propionibacterium* and *Clostridium* species by two general routes (Fig. 1). *Propionibacterium freudenreichii* forms the three-carbon acid by conversion of succinyl-CoA to methylmalonyl-CoA by a methylmalonyl-CoA epimerase and mutase and then in a reaction with pyruvate gives propionic acid and oxaloacetate in a reaction catalyzed by transcarboxylase, called the Wood–Werkman cycle. In organisms like *Clostridium*

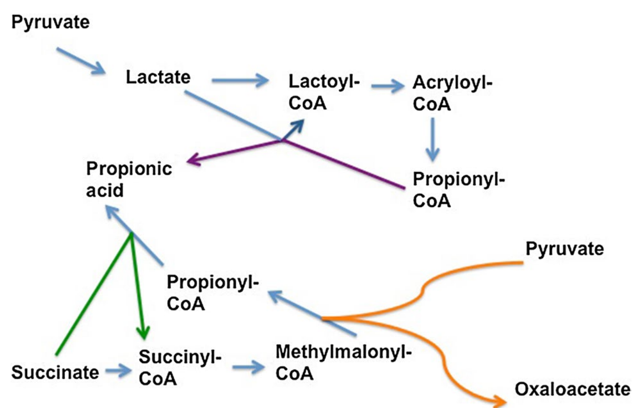

Fig. 1 The two routes to propionic acid found in *Propionibacterium freudenreichii* and *Clostridium propionicum*. The upper pathway from pyruvate and the coenzyme transferase in *blue* and *purple* shows the route found in Clostridium and the bottom route from succinate and using a transcarboxylase reaction in *orange* and a coenzyme transferase in *green* is found in *Propionibacterium*

propionicum or *Megasphaera elsdenii* the acrylate pathway is used. This pathway converts lactate directly to propionic acid via formation of lactoyl-CoA, its dehydration to form acryloyl-CoA and reduction by acryloyl-CoA reductase to generate propionyl-CoA. The propionyl-CoA can be exchanged with lactate to form the lactoyl-CoA and release propionic acid. A key enzyme is the acryloyl-CoA reductase enzyme, which appears like the bifurcating butyryl-CoA dehydrogenase enzyme of clostridium that is used in the formation of butyrate. However, this enzyme, although it also has electron-transferring flavoproteins as a component of the complex, seems capable of using NADH directly [72] even though acryloyl-CoA ($E^{\circ\prime} = +69$ mV) would be well suited to a bifurcation mechanism, considering that crotonyl-CoA ($E^{\circ\prime} = -10$ mV) is reduced by this mechanism. It has been proposed that the propionyl-CoA dehydrogenases/EtfBC complexes from *C. propionicum*, *C. homopropionicum* and *M. elsdenii* may have lost bifurcation function to better handle the highly reactive toxic acryloyl-CoA [25].

Since the formation of propionyl-CoA requires additional reduction compared to lactate, the biosynthesis from glucose will require oxidation of some of the lactate (1/3) to generate reductant for formation of propionic acid from the other 2/3 lactate or supply of reductant from another source, so overall it has a limit on the yield of ~1.3 mol of propionic acid from one molecule of glucose if formed via typical glucose to lactate EMP pathway. Since the Propionibacterium generates the acid from succinyl-CoA and pyruvate, its yield is dependent on the substrates used to form those key intermediates; from glucose it would be ~1.3 as well, but from a mixture of glucose and glycerol a higher carbon conversion is possible [124].

A number of articles have explored microbial production [57, 121, 226], different feedstocks especially with glycerol supplementation, and culture optimization of various Propionibacterium strains for high yield production under different protocols, immobilization or adaptation of cells [180, 207]. An example that includes an economic perspective is reported by Dishisha et al. [51]. The effect of redox potential was studied and could be optimized to give a very high proportion of propionic acid in the total acids (92 %) with high glycerol conversion (76 %) and the authors concluded that optimal control of redox potential during growth could provide a means to generate highly selective propionic acid production from glycerol [33].

A number of genetic studies have included efforts to overproduce or knock out key enzymes. Knock out of the acetate kinase led to a reduction of growth rate and a decrease in acetate and an increase in propionate yield by 13 % [179]. Overproduction of phosphoenolpyruvate carboxylase allowed the cells to grow faster, consume more glycerol, and more quickly form propionate to a higher final titer. The engineered strain also produced more propionate from glucose under conditions of high CO_2 [7]. In another study of production from glycerol, the glycerol dehydrogenase gene (*gldA*) from *Klebsiella pneumoniae* was expressed in *Propionibacterium jensenii* ATCC 4868. PA production was 28 g/L, a value 26 % higher than that of the corresponding culture of the wild-type parental strain [236].

Butyric acid

Butyric acid, found as an ester in butter, has a variety of industrial uses [53] including altering the consistency of cellulose acetate polymers, and there are many applications in the food industry of butyrate esters and butyrate as an animal feed additive. In nature, butyrate is formed in the intestinal tract and has a number of positive effects on gut development and health, and the use of butyrate additives has also been examined [43, 65, 67, 190, 191].

Bacterial production of butyric acid has been reviewed [228]. The current bulk prices of butyric acid or sodium butyrate are in the range $2,000–$4,000 per MT. A number of organisms produce butyric acid as a fermentation product from metabolism of sugars via glycolysis and acetyl-CoA. Solventogenic clostridial species make acetate and butyrate during the early growth phase, then re-uptake them and convert them into alcohols as the culture goes through stationary phase. Other species of clostridia make acids only and among those, *Clostridium tyrobacterium* has been the most well studied. The relative production of acetate vs butyrate by the organism is affected by the host's need for ATP which is formed when each mole of the acid is generated from

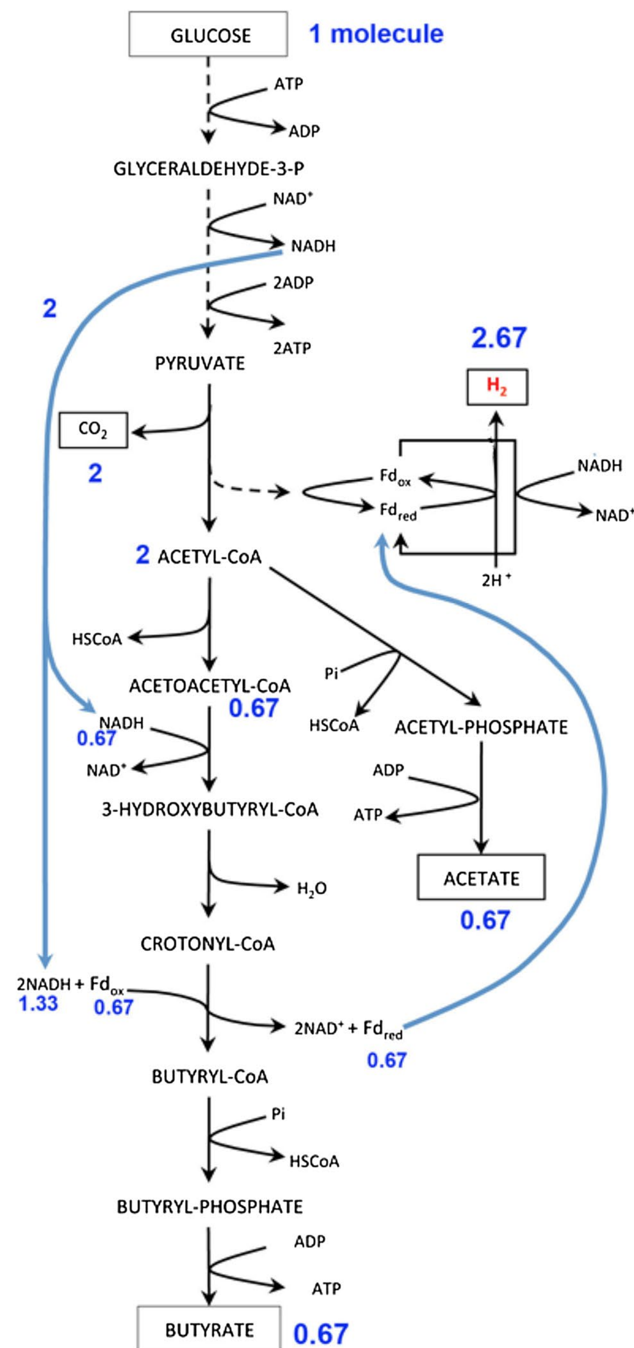

Fig. 2 Redox distribution in early acidic growth stage of *C. acetobutylicum*. Some of the NADH produced in glycolysis is used along with the reduced ferredoxin to form hydrogen and yield an approximately equal proportion of butyrate and acetate. When the route to hydrogen is limited more of the longer chain acid, butyrate is formed. The *numbers* along the enzyme reactions show the flux in that reaction in normal early stage growth

the CoA derivative, the added reductant available and required to form butyrate vs acetate and the competing pathways for use of reductant, e.g. in hydrogen or lactate formation (Fig. 2).

A number of workers have noticed that the addition of certain redox active dyes, such as neutral red or methylviologen, to the culture generates an altered pattern of products and in the solventogenic clostridia where butanol is the major desired product, a higher ratio of butanol is formed [151]. Less information about the role of such dyes on the butyrate vs acetate ratio has been discussed. Examination of literature and our experiments with these and other redox active molecules such as phenazines and TNT [26] has shown that increased ratios of the longer chain length acid, butyrate to acetate are formed in the treated cultures. The redox dyes can act to short circuit the e-transfer and provide an extra-route to coupling the redox needed for synthesis of butyrate. The two redox reactions involved in forming butyrate from acetyl-CoA are the reduction of acetoacetyl-CoA to hydroxybutyryl-CoA and then after dehydration, the reduction of crotonyl-CoA to butyryl-CoA. The midpoint redox potentials of these reactions are −240 and −10 mV [70] and the latter shows a large difference from that of NADH (−320 mV), the main redox carrier other than ferredoxin. The use of electrode and dye-mediated redox has been studied in various clostridial cultures, and electrodes coupled to neutral red (Em = −325 mV) have been used to form high levels (~55 g/L) of butyrate by *C. acetobutylicum* KCTC1037 [88] with low acetate in methyl viologen (Em = −440 mV) mediated cultures of *Clostridium tyrobutyricum* BAS 7 [40].

Shifts in the formation of metabolites are also observed upon inhibition of hydrogenase by CO, which then diverts the redox from low-potential reduced ferredoxin to the reactions involved in the formation of longer chain acids and alcohols [93]. Other studies using substrates of differing redox states such as glycerol and pyruvate also showed this shift toward a higher proportion of butyrate vs acetate [63, 146, 147, 192]. In contrast addition of iron oxide nanoparticles produced a higher acetate proportion [136]. The addition of anthrahydroquinone-2,6-disulfonate (AQDS, Em = −184 mV) was reported to increase hydrogen yield and reduce butyrate levels in cultures of *Clostridium beijerinckii* grown on xylose [219]. Butyrate production was also reported to be enhanced by reduced electron shuttles in growing cells [66, 69]. In *Clostridium* sp. BC1 AQDS, addition in a bicarbonate media showed no change in metabolite pattern while methylviologen shifted the metabolites toward butanol and ethanol [217].

The formation of butyrate by the bifurcating system is more complex in clostridium and less suitable for use in aerobic organisms although an oxygen-tolerant bifurcating butyryl-CoA dehydrogenase has been reported [3] than in organisms where the crotonyl-CoA is reduced by an enzyme directly using NADH [195]. The ter enzyme from *Treponema denticola* has been used to carry out this reaction in engineered *E. coli* [48, 170]. In engineered

E. coli where the butyrate pathway enzymes including ter were placed on a scaffold, butyrate production was improved [9]. The engineered strain carried mutations in *atoAD* and *pta*, elimination of *adhE*, *ldhA*, *frdABCD* pathways and replacement of the NADPH-dependent pathway for formation of crotonyl-CoA with theheterologous NADH-utilizing pathway by overexpressing *hbd* (encoding 3-hydroxybutyryl-CoAdehydrogenase) and *crt* (encoding crotonase) from *C. acetobutylicum*, *ter* (encoding *trans*-enoyl-CoAreductase) from *T. denticola*, and native acyl-CoA thioesterase *tesB* to form butyrate from butyryl-CoA. The strain was effective in converting low concentrations of glucose (11 g/L) to butyrate in high yield with a ratio of butyrate:acetate of 41 [116]. The absence of other routes to recycle NADH to NAD^+ favored the production of butyrate.

A pyrimidine nucleotide-based reduction reaction series has been used to form butyrate by reversal of the normal fatty acid degradation pathway in *E. coli* [42, 49]. This system involving engineered reversal of the β-oxidation cycle uses the following genes and activities: thiolase (*fadA* or *atoB*), hydroxyacyl-CoA dehydrogenase (*fadB*), enoyl-CoA hydratase (*fadB*), acyl-CoA dehydrogenase (*ydiO*, *fadE*, or *ter*) and a thioesterase to remove the butyrate from the CoA. The system has been adapted for the production of fatty acids and esters in an engineered yeast [114].

Another pathway for the formation of butyrate employs the ATP-requiring reaction to form malonyl-CoA from acetyl-CoA. The malonyl-CoA then serves as an addition substrate coupling to acetyl-CoA to form the acetoacetyl-CoA in a reaction similar to the enzymes of fatty acid synthesis or polyketide synthesis. The reaction catalyzed by NphT7 of *Streptomyces* sp. strain CL190 [143] is suitable for aerobic organisms and where there may be abundant ATP. The driving force can enable increased formation of butyryl-CoA-derived products [104].

In clostridium, there have been a number of genetic investigations motivated by increasing interest in biofuel butanol and the effort to generate more valuable longer chain length organic acids and alcohols for fuels and chemicals. Recently, the bifurcating nature of the crotonyl-CoA to butyryl-CoA reaction in clostridium has been determined where the reaction of 2NADH forms a reduced ferredoxin at the same time as the double bond reduction by an electron-transferring flavoprotein enzyme complex [25, 41]. The finding of different reactions for reduction of crotonyl-CoA and the development of genetic tools for clostridium has allowed more elaborate genetic experiments to be performed. Application of these methods has been used to examine and alter redox pathways related to the production of butyrate in clostridium.

The deletion of several genes (*pta*, *bukI*, *ctfB*, *adhE*1 and *hydA*) and addition of the *buk*II gene of *C. acetobutylicum* allowed high production of butyrate (32.5 g/L) and low acetate [82]. Studies of *C. acetobutylicum,* where the *adc*, *ctfA* and *pta* were knocked out, showed that acetate production was drastically reduced with increased butyrate [110]. The importance and broader specificity of acid forming were illustrated in the study of a phosphotransbutyrylase mutant of *C. tyrobutylicum* where the butyrate:acetate ratio was decreased but higher levels of both acids were formed [233]. An acetate kinase mutant of *C. tyrobutylicum* produced more butyrate and hydrogen than wild type on glucose, and on xylose at pH 5 produced butyrate (0.43 g/g xylose) rather than the acetate and lactate primarily formed by wild type [123]. The use of immobilized adapted *C. tyrobutyricum* in a fibrous-bed bioreactor gave a butyrate concentration in fed-batch culture of 86.9 g/L [89], and the method has been used with sugarcane bagasse hydrolysate [201].

Longer chain monofunctional acids

Free fatty acids which can be used as precursors for the production of fuels or chemicals have attracted significant attention in recent years [68, 108, 141, 158]. The pathways for fatty acid biosynthesis are detailed in Fig. 3. Briefly, the precursor for fatty acid biosynthesis is derived by acetyl-CoA and follows a sequence of condensation, reduction, dehydration and reduction reactions. In each cycle, two carbons are added from malonyl-ACP to a growing acyl chain and the resulting β-keto group is reduced to eventually yield a saturated C–C bond. The acyl-ACP thioesterase terminates fatty acyl group extension by hydrolyzing the acyl moiety from the acyl-ACP at the appropriate chain length, releasing free fatty acids [113, 160, 171, 187, 230]. Different acyl-ACP thioesterases have different degrees of chain length specificity [45], which can be varied from C8 to C18.

There are two reduction steps in each elongation cycle which are catalyzed by FabG and FabI, respectively. It is reported that FabI can use either NADH or NADPH as cofactor, while FabG only uses NADPH in *E. coli* [16]. To a growing fatty acid chain, every elongation cycle adds 2 carbon atoms and requires 2 redox equivalents, resulting in 14 NAD(P)H to form a 16-carbon fatty acid. There must be an efficient pathway to convert the NADH to NADPH. In *E. coli* cells, it can be achieved by transhydrogenases and NAD kinase. Depending on the redox state of cell, NADH can be converted to NADPH via proton-translocating transhydrogenase PntAB and a transhydrogenase UdhA [81, 164, 166]. The NAD^+ kinase encoded by nadK catalyzes the conversion of NAD^+ to $NADP^+$ through phosphorylation using ATP as the phosphoryl donor [91]. Several other strategies to increase intracellular NADPH availability, such as replacing native NAD-dependent GAPDH

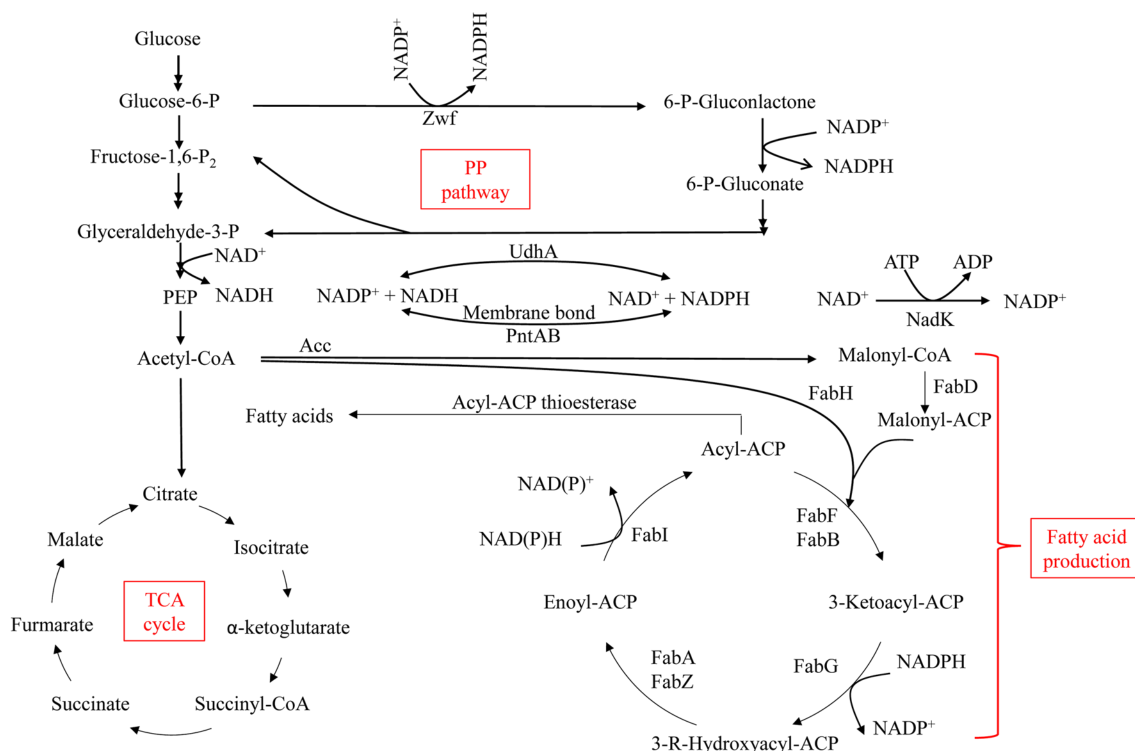

Fig. 3 Fatty acid biosynthesis pathway and involved cofactor NAD(P)H balance in *E. coli*

with NADPH-dependent variants, have been examined and reported [130, 197–199]. It has been shown that these NADPH manipulations significantly increase medium-chain fatty acid titers and yields [163, 208]. Another approach is to replace the native NADPH-dependent (FabG) to NADH-dependent 3-oxoacyl-ACP-reductase (FabG) with a NAD-dependent variant [87, 112] with a significant increase in both fatty acid titer and yield [112].

Diacids

The group of diacids includes oxalic acid, malonic acid and the longer 4- or 5-carbon acids primarily derived from reactions of oxaloacetate and these are covered in some depth below. The 2- and 3-carbon diacids are commonly found in some plants and fungi; however, there has been relatively little metabolic engineering for the production of these acids. As highly oxygenated carbon compounds, they are somewhat outside the scope of this review. Some recent reviews on oxalic acid production and metabolism [60, 62, 129] and malonic acid that emphasizes the role of malonyl-CoA in fatty acid and polyketide synthesis and the general inhibitory role of malonate [35, 98, 148] have been published and are referenced for readers as an introduction to those specialized small diacids.

Succinic acid

Succinic acid is a C4-dicarboxylic acid recognized by US Department of Energy as one of the top 12 biomass-derived building block chemicals having numerous applications in food, pharmaceutical, polymer, surfactants and detergents, flavors and fragrances, textile industries and fine chemicals. Various review articles have described advances made in last two decades of research towards biobased succinic acid production [28, 37, 83, 183]. While many organisms have been reported to produce succinate at low levels, some of the native and recombinant major succinate producers are *E. coli*, *Actinobacillus succinogenes*, *Anaerobiospirillum succiniciproducens*, *Mannheimia succiniciproducens*, *Corynebacterium glutamicum*, *Basfia succiniciproducens*, *Saccharomyces cerevisiae* and *Candida krusei*. The global production rate of succinic acid based on petrochemical processes is between 30,000 and 50,000 tons per year with a current market price of $6,000–9,000 per ton. Several companies and their joint ventures such as Myriant Technologies, BioAmber and Mitsui, Succinity (BASF/Corbion Purac) and Reverdia (DSM/Roquette) have been active in setting up demonstration or commercial biobased succinate production plants [83, 183].

Figure 4 shows metabolic routes for succinate production in *E. coli*. Fermentative production of succinate with

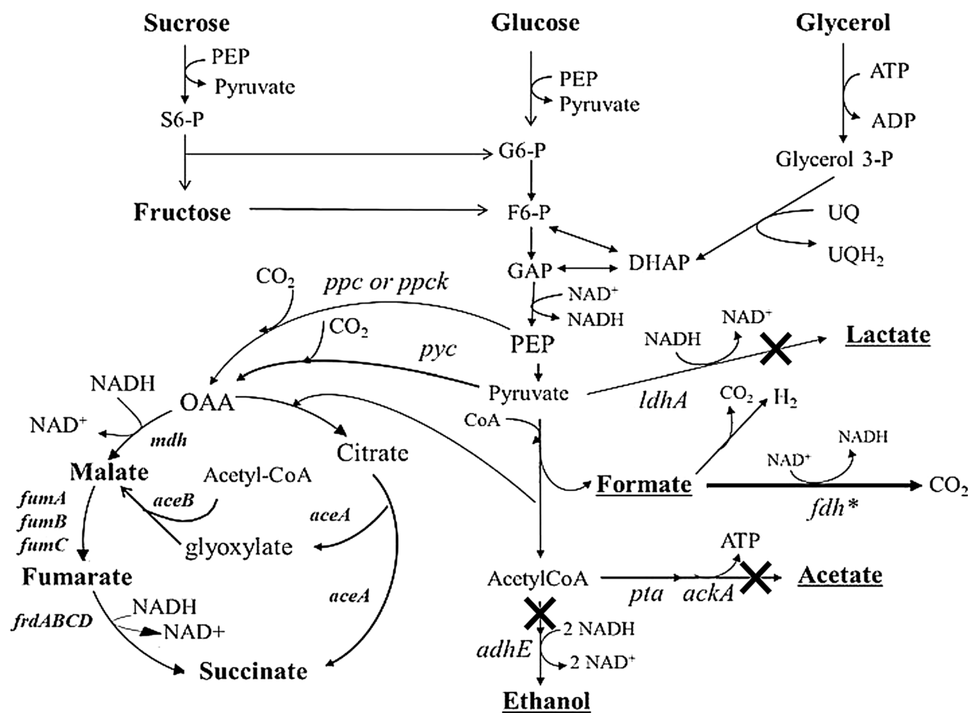

Fig. 4 Metabolic routes and involved cofactor for succinate production from glucose, glycerol and sucrose in *E. coli*. The *asterisk* on *fdh* indicates the NAD^+-dependent formate dehydrogenase from *Candida boidinii* that regenerates NADH from formate. *Black X* indicates deletion of corresponding genes for competing pathway

high yield and productivity under aerobic and anaerobic conditions using glucose and biomass sugars have been reported [4, 34, 184, 235]. The downstream processing of succinate has been described in several reports [38, 101, 144] and is critical to the cost of overall process economics. Various metabolic engineering strategies such as activation of glyoxylate pathway, improved glucose transport system, enhanced ATP supply, knockout of competing pathway genes, over expression of pyruvate-metabolizing enzymes and many others have been applied to improve the succinate production [37, 183]. Another important strategy relevant to this review is redox manipulation by providing additional reducing power to improve succinate yield. This has been demonstrated via use of more reduced carbohydrates (such as sorbitol) [32, 76], reducing agent dithiothreitol [128], hydrogen as electron donor in the head space gas [189] and increasing in vivo NADH availability [17].

Fermentative metabolites are greatly influenced by NADH availability as evidenced by previous studies in our laboratory using carbon sources with different oxidation states or genetic manipulations such as overexpressing an NADH-regenerating enzyme such as the NAD^+-dependent formate dehydrogenase (FDH; EC 1.2.1.2) from *Candida boidinii* that converts 1 mol of formate and NAD^+ into 1 mol of NADH and CO_2 [17–19, 162]. The native formate dehydrogenase converts formate to CO_2 and H_2 with no cofactor involvement. The newly introduced yeast FDH retains the reducing power that was otherwise lost by the release of formate or H_2 in the native pathway (Fig. 4). Recently, the application of *C. boidinii* FDH in high succinate-producing engineered *E. coli* SBS550MG(pHL413KF1) to retain the reducing power of formate as NADH and thereby minimizing byproduct formate production in succinate fermentation has been reported [10]. Increased in vivo availability of NADH resulted in twofold improvements in succinate productivity and about 80 % reduction in formate in fed-batch cultures of SBS550MG(pHL413KF1). Furthermore, external formate supplementation to cultures of SBS550MG(pHL413KF1) resulted in about 6 % increase in succinate yields indicating that the engineered strain is capable of handling increased redox availability. Another recent study has also utilized an NAD^+-coupled formate dehydrogenase from *Mycobacterium vaccae* in engineered *Corynebacterium glutamicum* BOL-3/pAN6-gap for anaerobic production of succinate by co-utilization of glucose and formate as an additional donor of reducing equivalents [119]. The engineered strains of *C. glutamicum* BOL-3, BOL-3/pAN6 and BOL-3/pAN6-gap showed a significant increase in the succinate yield in the presence of formate (1.3–1.4 mol/mol) compared to that its absence (1.0–1.1 mol/mol).

In addition to formate dehydrogenase, other enzymes improving NADH availability have been reported. Examples of these include enhancement of succinate production by regulating NADH pool and NADH/NAD ratio via nicotinic acid phosphoribosyltransferase (NAPRTase) encoded by the *pncB* gene, a rate-limiting enzyme of NAD(H) synthesis pathway [115, 128], and *E. coli* PntAB transhydrogenase that enhances the conversion of NADPH to NADH in *C. glutamicum* under microaerobic conditions, and the

increased NADH/NAD+ ratio results in increased succinic acid production [216].

Fumaric acid

Fumaric acid is an unsaturated four-carbon dicarboxylic acid naturally produced as an intermediate of the tricarboxylic acid cycle (TCA). It is 1.5× more acidic than citric acid and has been used as a food and beverages acidulant since the 1940s [214]. Fumaric acid serves as a starting material for L-malic and L-aspartic synthesis, which are also used in the food industry, e.g. in sweeteners and beverages. In addition, fumaric acid is currently used for the production of biodegradable polymers, plasticizers and polyesters resins [155], as well as a supplement in animal feed reducing the cattle methane emissions significantly [132]. More recently, some medical applications of fumaric acid derivatives have been discovered, such as the use of fumaric acid dimethyl ester to treat psoriasis and multiple sclerosis [92, 154]. Consequently, the number of applications is increasing; hence, the demand for fumaric acid and its derivatives is rising.

Fumaric acid production by fermentation has been primarily studied in filamentous fungi of the *Rhizopus* genus, such as *R. oryzae*, *R. nigricans* and *R. arrhizus* [178]. Several studies have shown that the carbon to nitrogen ratio in the culture medium is a key factor in fumaric acid accumulation in *Rhizopus* species, where nitrogen limitation favors malic acid conversion into fumaric acid [50, 155]. Despite the high product titer reached, up to 126 g/L, with *Rhizopus* species, their filamentous characteristic and ability to form cell aggregates make the scaling up of the process difficult, and is especially challenging to control the oxygen transfer in the implementation of a large-scale process [155, 214]. Moreover, the industrial-scale use of *Rhizopu*s species is questionable due to their potential pathogenicity [210]. However, a few nonspecific mutations (UV, chemical) have been performed and improved strains have been selected, although they are still far from a commercial use [214]. Only a few genetic tools are available for *Rhizopus* species genetic modifications, thus strain improvement for fumaric acid production has not been widely studied. Nevertheless, recently Zhang et al. [227] reported the construction of metabolic engineered *R. oryzae* strains for fumaric acid biosynthesis from glucose. The strains overexpressed endogenous pyruvate carboxylase (PYC) or exogenous phosphoenolpyruvate carboxylase (PEPC) from *E. coli* to increase carbon flux toward oxaloacetate and thus to fumarate. The results showed an increase of 26 % in fumaric acid for the PEPC-expressing strain compared to wild type, on the contrary the PYC-overexpressing strain showed significantly lower fumaric acid production than wild type. The last strain showed poor cell growth and the formation of large pellets. Fumaric acid yield decreased drastically while ethanol yield increased, presumably due to oxygen limitation caused by the increase in cell pellet size in this strain [227].

Since the industrial-scale use of *Rhizopu*s species is questionable, the use of GRAS (generally recognized as safe) strains such as *Saccharomyces cerevisiae* for fumaric acid production is becoming an attractive alternative. Although *S. cerevisiae* does not accumulate fumaric acid naturally, metabolic engineering tools are available for the construction of fumaric acid producing strains. Xu et al. [211, 212] reported fumaric acid production in a *S. cerevisiae* strain overexpressing the malate dehydrogenase from *R. oryzae* as well as overexpressing the native pyruvate carboxylase, leading to 3.18 g/L of fumaric acid from glucose, where the control strain did not produce detectable amounts of fumaric acid. Further strain improvement was reported [210] where pyruvate carboxylase, malate dehydrogenase and fumarase from *R. oryzae* were overexpressed in a pyruvate-producing strain background and when biotin was added to the culture medium titer reached 5.64 g/L of fumaric acid in nitrogen-limited culture. Despite the advances in fumarate production by metabolic engineered *S. cerevisiae*, fumarate production is still far from the levels reached with *Rhizopus* species.

Fumaric acid production has been recently explored in metabolic engineered *E. coli* strains. Figure 5 shows metabolic engineering approach for fumaric acid production in *E. coli*. Song et al. [173] reported the construction of several metabolic engineered *E. coli* strains for fumaric acid production. The highest producing strain had the *iclR* gene deleted to activate the glyoxylate shunt, fumarases genes *fumA*, *fumB*, *fumC* deleted to increase fumaric acid formation, native phosphoenolpyruvate carboxylase (PPC) overexpressed to increase carbon flux to oxaloacetate, *arcA* and *ptsG* genes deleted to enhance the TCA oxidative branch, the *aspA* gene deleted to avoid fumaric acid conversion into L-aspartic acid, *lacI* gene deleted to avoid inducer requirement and the native promoter of the *galP* gene was replaced by the strong *trc* promoter. This strain, named CWF812, produced 28.2 g/L of fumaric acid from glucose with a yield of 0.389 g fumaric acid/g glucose in a fed-batch fermentation in aerobic conditions [173].

On the other hand, significant amounts of fumaric acid are accumulated in some plants such as *Arabidopsis thaliana* and soybean. In *A. thaliana*, fumaric acid can accumulate in levels exceeding those of starch and soluble sugars, e.g. several milligrams per gram of fresh weight. Fumaric acid accumulation in this plant increases with age and light intensity in the leaves [39]. Fumaric acid is thought to be used as an alternative carbon sink to starch in the leaves specifically under rapid growth when high nitrogen is present and may contribute to maintain cellular pH [150]. To the extent of our knowledge no metabolic engineering has been performed yet to improve fumaric acid accumulation in plants.

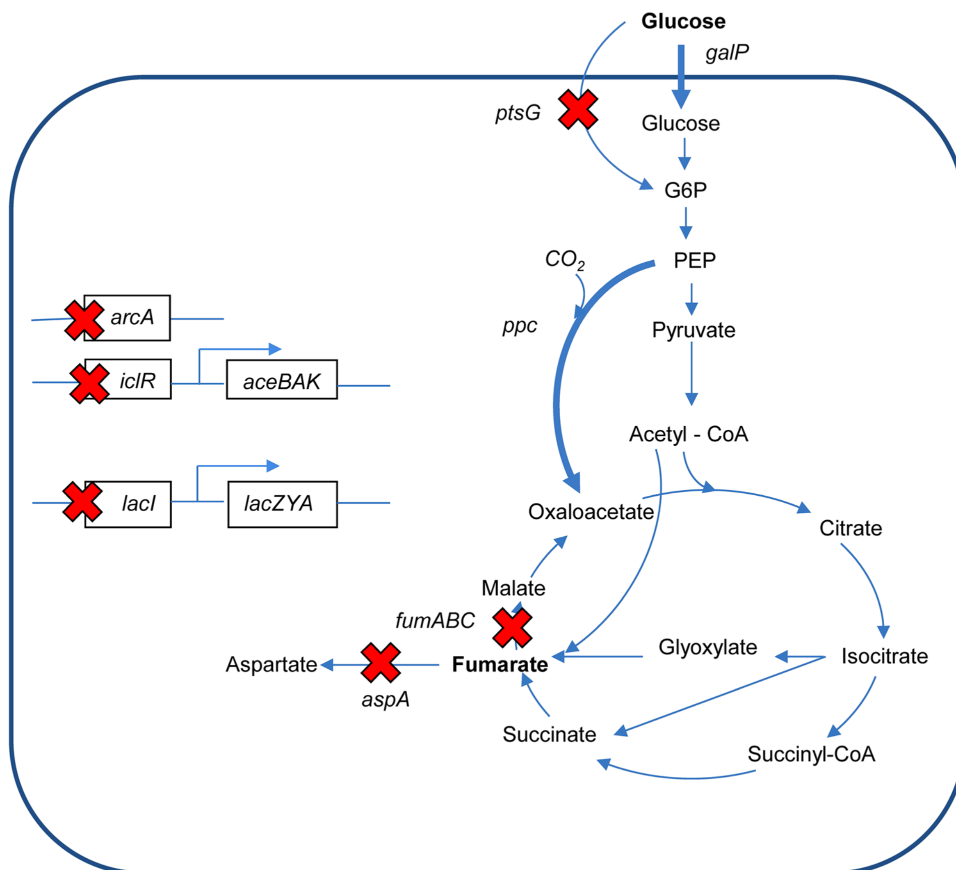

Fig. 5 *Escherichia coli* central metabolic pathways and metabolic engineering approach for fumaric acid production. *Red X* indicates corresponding gene deletion. *Thick arrows* indicate increased expression (adapted from [173])

Malic acid

As one of the family of 1,4-diacids, malic acid is a desirable chemical. The production of malic acid by engineered organisms has been recently reviewed in the context of other 4-carbon diacids [27, 122]. The review of the key metabolic node at phosphoenolpyruvate–pyruvate–oxaloacetate, and its importance in the formation of diacids and cell carbon flux served in subsequent development of metabolic engineering strategies [167]. The dehydrated form of this 4-carbon compound is produced and used in large quantity as maleic anhydride. The global production of maleic anhydride is around 1 million MT/yr. The chemical manufacture of maleic anhydride arises from oxidation of benzene, butane or butene and as a byproduct of phthalic acid production. It is widely used in the formation of alkyl and unsaturated polyester resins and coatings. The compound is converted to many derivatives, e.g. hydroxybutyrolactone. A compilation of chemical conversions of malic acid to many specialty chemicals is provided in a recent report from Huntsman Chemical [56]. Current bulk price of malic acid is ~$1,700/MT in China, and with US suppliers $2,000–3,000 MT. There also has been interest in the special properties of polymalate for medical applications such as in drug delivery systems [58, 97, 125]. Another major bulk use is in the food and feed industry, where malate is used as an acidulant and flavor enhancer, and additive–preservative. Calcium citrate malate is a widely used source of calcium that does not increase the risk of kidney stones while aiding bone strength [127]. While magnesium hydroxide is around $200 MT and calcium hydroxide is $120 MT, the price for Mg-malate is around $7,000 MT and calcium malate is $3,000 MT. L-malate as a precursor chemical in the pharmaceutical industry; these are high value but limited volume applications.

While malate is formed in plants to some extent it is not naturally a major metabolite released by bacteria; however, studies on metabolic engineering of malate production in bacteria have received attention [139]. The status of malate as requiring one reduction form oxaloacetate, in comparison to the two required for succinate, would seem to make the production easier as the reductant formed in glycolysis could be used in the reduction of oxaloacetate to malate. Efficient formation of oxaloacetate from the three carbon compounds formed by glycolysis with best energy efficiency would seem like an attractive route as is the case with succinate. Work has mainly been oriented toward this general principle. The malic enzyme has been used in a route to produce succinate via malate in *E. coli* [75, 102, 177]. Strains of *E. coli* derived from introducing known

mutations in *ldhA*, *adhE*, *ackA*, *focA*, *pflB*, *mgsA*, as well as evolving the strain for many generations under selective growth conditions, can form not only malate in considerable quantity (56 % of moles of product) but also succinate (26 % of moles of product) as well as acetate (7 % of moles of product) and pyruvate (6 % of moles of product) [85]. Strains with a mutation in *frdBC*, and deletion of *sfcA* and *maeB*, pyruvate kinase, *fumABC* and *ldhA*, *ackA*, *adhE*, *pflB* produced a titer of 34 g/L with a yield of 1.42 mole malate per mole of glucose [232].

The engineering of malate production in *Saccharomyces cerevisiae* [1] under conditions of calcium carbonate-supplemented cultures generated a titer of 59 g/L and a yield of 0.48 mole/mole under optimized conditions [225]. Engineering *Torulopsis glabrata* by overexpression of pyruvate carboxylase and malate dehydrogenase produced 8.5 g/L malate [36]. Efforts with *Aspergillus oryzae* NRRL 3488 have yielded superior strains. The best strain had an overexpressed C4-dicarboxylate transporter, cytosolic pyruvate carboxylase and malate dehydrogenase, and formed a titer of 154 g/L malate with 69 % of theoretical yield [24]. In another approach, an in vitro conversion of glucose to malate was achieved using thermophilic enzymes and a set of enzymes from a non-ATP-forming glycolytic pathway. The system produced malate at 60 % molar yield [218]. Current efforts toward production of this compound are focused on increasing yield while maintaining a high concentration of product.

Tartaric and Itaconic acids

While not requiring reduction of its precursor intermediate, these two compounds are derived from similar nodes and precursors as other diacids discussed in this review so they will be covered here briefly. In the case of these compounds the main metabolic engineering goal is to supply the key precursors for synthesis, i.e. oxaloacetate and acetyl-CoA, and afford recycling of NADH formed in earlier steps of the sugar-metabolizing pathways, allow energy for cell growth, and allowing high-concentration product accumulation and tolerance. Some aspects of the biochemical processes and limitations of particular pathways are also discussed.

Tartaric acid, a four-carbon diacid bearing two hydroxyl groups, exists as the L(+) form in nature, with D and meso forms also known as well as racemic mixtures of forms. It is found in many plants and is found in wine, providing some of the tartness. It is used in the food industry as an antioxidant and emulsifier, with soft drink, candy and baked products being major users. The diacetyl esters are used in baking. The chemical salts, potassium bitartrate (cream of tartar) and calcium tartrate, are well known and are used as food preservatives. Tartaric acid is also used as a finishing agent for fibers and in metal processing. The market for tartaric acid is around 28,000 MT with expected growth in food and other uses with a price of $6/kg, and the bulk price and composition of diacetyltartrate would agree with this value. The removal of tartrate from solutions during wine processing by calcium is well practiced [193] and high yield precipitation is obtained in the presence of excess calcium chloride.

Studies of biosynthesis of tartaric acid in plants [64] showed a pathway from ascorbate [46, 47] involving the reactions from 2-keto L-gluconic acid, L-idonic acid, 5-keto D-gluconic acid, and L-threo-tetruronate, and the enzyme catalyzing the step from 5-keto gluconic acid to idonic acid was identified and characterized; however, not all enzymes or genes encoding the pathway were identified in *Vitis vinifera* (grape). This pathway is interesting in that a corresponding enzyme exists in *E. coli* [11, 12] and further studies showed the presence of genes capable of reducing 2,5-diketogluconate to 5-keto gluconate [221]. The pathway starting from oxidation of glucose to gluconic acid by glucose dehydrogenase goes well and enzymes from Gluconobacter species are efficient in this catalysis. The formation of 5-keto gluconic acid has been described and the enzyme has been characterized [55, 71, 74, 133, 134]. An enzyme from *Gluconobacter suboxydans*, 5-ketogluconate dehydrogenase, forms 5-keto gluconic acid from gluconic acid and upon cloning into *E. coli*, the natural *E. coli* transketolases can form the semialdehyde precursor of tartaric acid from 5-keto gluconic acid which is oxidized to form some tartaric acid [161]. It has been shown that 5-keto gluconic acid can be converted in the presence of vanadate into tartaric acid [99, 131]. Studies of engineered *Gluconobacter oxydans* have shown conversion of glucose to 5-keto gluconic acid [133].

A second way to metabolize tartaric acid has been identified and operates in the utilization of tartaric acid for growth. This pathway involves the L-tartaric acid dehydratase genes of *E. coli* [152] and other organisms, and allows utilization of D-tartrate [156, 157] or L-tartrate [84, 206]. The D-tartrate family of enolases has been reviewed [220]. The regulation of the L-tartrate dehydratases in *E. coli* has been studied [94] and *ygiP* (*ttdR*) is a positive regulator [145]. Tartrate transport via a tartrate/succinate antiporter *tdtT* (*ygjE*) has been defined [95]. The general pathway of utilization of tartrate is through dehydration to oxaloacetate, and conversion to malate, which can either generate pyruvate (aerobically) or be converted to fumarate and reduced to succinate (anaerobically). If these reactions operated in the opposite direction and used oxaloacetate to form tartrate, this route might allow a high yield of tartrate to be obtained. The activity of fumarase A from *E. coli* in catalyzing the keto-enol isomerization of oxaloacetate has been reported [59].

There is little literature on the metabolic engineering of tartrate production. A potential design for L-tartrate

production could be based on previous work with oxaloacetate-derived diacids and the experience of workers interested in pyruvate formation [29, 222–224]. To obtain optimal production of tartrate, the pathway from oxaloacetate would seem preferable since it could generate two molecules of tartrate from one molecule of glucose with CO_2 fixation and would operate aerobically. The route from glucose via 5-keto gluconic acid which is oxidized to form some tartaric acid [161] would generate one molecule from glucose. The host strain for tartrate generation would need to have mutations blocking the pathway of pyruvate or PEP conversion to acetyl-CoA (*aceEF*, *pflB*) or PEP conversion to pyruvate (*pykF*, *pykA*). Other genes to be inactivated in the host chromosome would be *mdh* to remove the possible conversion of oxaloacetate to malate, and the *gltA* gene to remove possible reaction of oxaloacetate to produce citrate. If oxaloacetate was found to be degraded by decarboxylation, the gene *eda*, encoding oxaloacetate decarboxylase, could be inactivated. Methods to enhance conversion of phosphoenolpyruvate into oxaloacetate exist via either the feedback-resistant plant PEPC we previously used [117, 118] or an energetically favorable phosphoenolpyruvate carboxykinase derived from *E. coli* [229, 231], *A. succiniciproducens* [103] and *A. succinogenes* [96, 107]. The problem is that these reactions of forming tartrate from oxaloacetate are reversible and there is no direct driving force for formation of the product. The means by which tartrate can build up in plants should be better understood and the availability of suitable plant-derived pathways would stimulate further engineering research toward production of this useful compound.

Itaconic acid (IA) is an unsaturated dicarboxylic acid with one methylene group adjacent to one of the carboxylic acids. IA has wide use in agricultural, industrial, medical and pharmaceutical applications [142, 205]: its conversion to methacrylic acid [106] and use in polymers where its addition as a co-monomer gives special character to resins and latex. Its use in coatings, adhesives and textile industries makes it a valuable chemical. Polyitaconic acid can chelate calcium making it useful in water treatment (Itaconix). The highly acidic polymer containing itaconic acid is biodegradable and useful in detergents, water treatment and as a superabsorbent polymer, thickening agent, binding and sizing agent and emulsifier. These characteristics allow IA to serve in many versatile applications [142] including in oral drug-delivery [20] and dental glass-ionomer cements [138, 209]. IA has been categorized as an important renewable chemical by the United States Department of Energy [203]. Each year, more than eighty thousand tons of IA is produced [142]. The price of IA is now around \$2/kg [142] and the market has been continually growing. Chemical synthesis methods for IA have been developed, but none of these processes were practiced commercially due to high costs. Instead, the main route for production of IA was via fermentation of *Aspergillus terreus*. However, using the fungus confronts several disadvantages, as described for fumaric acid.

In the IA production pathway of *A. terreus*, glucose is degraded through glycolysis and forms citrate in the TCA cycle. Next, citrate is dehydrated by aconitase (Acn) to form *cis*-aconitate in mitochondria. The *cis*-aconitate is transferred to the cytoplasm and decarboxylated by *cis*-aconitate decarboxylase (CAD) to IA. In the pathway according to Fig. 6, two enzymes, cis-aconitate decarboxylase (CAD) and aconitase (Acn), are crucial for the biosynthesis of IA. Although CAD was discovered in the cell lysate of *A. terreus* [15], CAD was not isolated as a homogeneous protein until 2002 [52]. CAD is a 55-kDa protein and has optimal pH and temperature of 6.2 and 37 °C, respectively, with a Km of 2.45 mM for *cis*-aconitate. Moreover, IA production correlates with CAD activity, meaning that CAD is essential for IA production [52]. The ATEG_09971 gene (*CAD1*) from *A. terreus* NIH2624 was confirmed to code for CAD and the transformed *CAD1* gene has been expressed a functional protein in yeast. The other key enzyme is aconitase that catalyzes the reversible inter-conversion of citrate to isocitrate via *cis*-aconitate in the citric acid cycle, where a [4Fe-4S] cluster is required for the binding of these substrates at the catalytic site [13]. The proposed mechanism of aconitase activity involves citrate dehydration to form *cis*-aconitate, which is isomerized by rotation of 180° around the double bond; the isoform of aconitate is then hydrated to form isocitrate [105]. While most aconitases convert citrate to aconitate and then convert aconitate to isocitrate, the enzyme desired for aconitate conversion to itaconate would not have the isocitrate-forming function or be effectively competed by the decarboxylase. Beyond producing itaconic acid, a mutant strain of *A. terreus* has been patented for the production of *cis*-aconitic acid [73]. It is likely that the *CAD1* gene in this strain may have become nonfunctional. Consequently, this mutant of *A. terreus* accumulates the intermediate, *cis*-aconitate, without further degradation.

There has been considerable interest in the engineering of this pathway and a number of genetically engineered organisms were reviewed [100, 176]. Information of the past few years is briefly summarized and compared to enhancement of production using *Aspergillus* species. Improvement of *A. terreus* through genetic engineering showed an improvement of production of 9.4 and 5.1 % by overexpressing *cis*-aconitate decarboxylase and *mfsA* (major facilitator transporter for export of itaconate) [77] and the important role of transporters has been stressed [188]. Mitochondrial expression of cis-aconitate decarboxylase or aconitase was found to be superior to cytosolic expression in *A. niger* indicating that location of

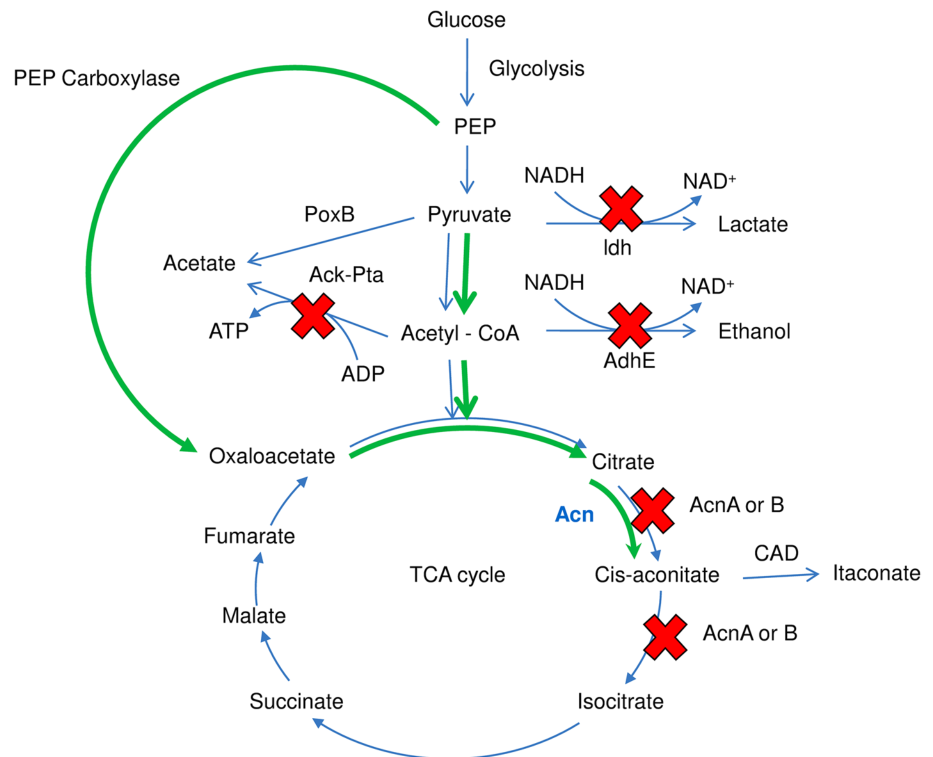

Fig. 6 Scheme for itaconate production in *E. coli*. *Red X* indicates genes or pathways to be inactivated and *green* pathways indicate those to be operating efficiently

the enzymes in this host is important [22]. The effect of enhanced glycolytic flux due to a shorter 6-phosphofructo-1-kinase that was resistant to citrate inhibition was tested by the insertion of the altered *A. niger pfkA* gene into *A. terreus*, and was found to increase productivity of itaconic acid [182]. The use of computational models to identify genes that could be altered to improve itaconate production has been employed in yeast and *A. terreus*. Engineering yeast to form itaconate achieved 168 mg/L upon identification of novel gene disruptions using in silico methods and overexpression of cis-aconitate decarboxylase. Among the proposed knockouts, mutations in *ade3*, *bna3* and *tes1* had a notable effect [21]. A genome-scale model of *A. terreus* was made and a set of pathway genes were identified for experimentation [120].

Conclusions and perspective

Metabolic engineering has been developed into a powerful enabling tool to create industrially relevant strains for the production of fuels and chemicals. Significant advances made in a number of areas, including software for metabolic pathway design, analytical techniques for metabolite analysis, high-throughput techniques for gene expression profiling, and synthetic biology for constructing genetic circuit/networks, have greatly increased the pace of the strain development process. While redirecting carbon flux to the desired product remains the major goal of these metabolic engineering efforts, and efforts to attain a high mass yield of product from the carbon source are of fundamental and economic importance; redox balance as discussed in this review can also play an important role in the strain development and optimization process. A number of articles have focused on the carbon pathways and minimizing the loss of carbon so as to achieve a high carbon yield of product from feedstock, and particularly, the approach of using information from various "omics" measurements to provide a global picture of the cell metabolic network and how it might be improved by such a system biotechnology perspective, where in silico models can suggest new genetic modifications to examine.

To provide the optimal amount of redox for the process, a number of strategies have been proposed and utilized. Here, we discuss a few general approaches. For enhancement of the total redox available for use in production of such highly valuable compounds as biofuel molecules or longer chain, largely hydrocarbon organic acids, it is important to limit the amount of redox (as NADH) that is oxidized by oxygen while maintaining active cell processes. There are culture processes that seek to use microaerobic culture conditions as a means to limit excessive conversion of the carbon feedstock to CO_2. Efforts to limit production of CO_2 through genetic means have also been reported [149, 234]. Additional strategies in this regard are those that seek to recapture redox before it is used in the formation of hydrogen

or other undesired compounds, or in the recycling of compounds formed by the cell, e.g. formate, to yield their redox so it can be used to drive reductive reactions in the cell and increase yield of redox-demanding compounds. Of course, efforts to add extra reducing power to the culture through adding compounds such as formate, hydrogen or reduced sugars while enabling the cell to effectively use those additional substrates at the same time it makes the desired highly reduced products, e.g. longer chain organic acids. The most appropriate strategy in terms of culture conditions, feedstock, host and the effect of any genetic manipulations on the cell's ability to robustly form product in an economic manner must be considered in the generation of an industrially useful process.

Another general consideration is providing the right reduced cofactor needed, NADH or NADPH, for the pathway or eliminating side reactions that consume the desired carbon molecule or reduced cofactor. One of these that has received attention is the imbalance of engineered yeast growing with xylose where the lack of the proper cofactor can lead to undesired low yield of ethanol from xylose and formation of a reduced sugar. The main approaches for limiting the imbalance are to change the specificity of the particular reaction by protein engineering so it will use the alternative cofactor, e.g. converting the NADPH specificity to NADH preferred. This strategy can work well and the desired pathway/network can be made more efficient to make better use of the available profile of reduced cofactor present in the cell under the desired culture conditions.

Another approach is to alter the host so it can provide the appropriate reduced cofactor availability by pathway alteration that will proportionate the flow of carbon through pathways that generate NADPH, for example, the pentose pathway, rather than so much through the regular glycolytic pathway [8, 140]. Other genetic approaches in making a host with higher availability have included replacing a normally NADH forming step with a NADPH forming step using an enzyme that has different specificities, from a different organism, a known mutant or a protein engineered version. Such a strategy has been reported with the GAPDH step of glycolysis [31, 78, 109, 130, 198]. The cell also contains enzymes that serve as transhydrogenases to interconvert NADH and NADPH, and these can also contribute and provide a higher availability of the desired cofactor in some circumstances.

In metabolic engineering, it is becoming more widely appreciated that redox balancing and overall meshing of the carbon and redox pathways need to be considered in generating an effective biocatalyst and that such redox contributions can have significant impact on the overall growth and efficiency of the production process. It is anticipated that more productive strains will be obtained by maintaining a carbon flux coupled with a proper redox and preferred cofactor balance, such that redox networks are as well engineered as the carbon flow networks in the engineered organism.

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